

Sphingosine 1-Phosphate Receptors Mediate the Lipid-Induced cAMP Accumulation through Cyclooxygenase-2/Prostaglandin I₂ Pathway in Human Coronary Artery Smooth Muscle Cells

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ABSTRACT

Sphingosine 1-phosphate (S1P) has been shown to exert a variety of biological responses through extracellular specific receptors or intracellular mechanisms. In the present study, we characterized a signaling pathway of S1P-induced cAMP accumulation in human coronary artery smooth muscle cells (CASMCs). S1P induced biphasic cAMP accumulation composed of a short-term and transient response (a peak at 2.5 min) and a late and sustained response (~4–6 h). The late phase of cAMP accumulation was parallel to the increment of cyclooxygenase-2 protein expression and was inhibited by *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide (NS398), a cyclooxygenase-2-specific inhibitor. We were surprised to find that the cyclooxygenase-2 inhibitor also inhibited short-term cAMP accumulation even when cyclooxygenase-2 protein expression was not yet increased. More interestingly, the short-term cAMP accumulation was also completely

inhibited by pertussis toxin, an inhibitor of G_{i/o} proteins. JTE-013, a specific antagonist for S1P₂ receptors, inhibited the S1P-induced cAMP accumulation. Furthermore, small interfering RNAs targeted for S1P₂ receptors significantly inhibited the S1P-induced cAMP accumulation. The cAMP response was also inhibited by specific inhibitors for phospholipase C, extracellular signal-regulated kinase pathways, and cytosolic phospholipase A₂. S1P actually activated these enzyme activities and stimulated prostaglandin I₂ (PGI₂) synthesis. Finally, exogenously applied arachidonic acid and PGI₂ induced cAMP accumulation to a similar extent as S1P. In conclusion, S1P induced cAMP accumulation through S1P receptors, including S1P₂ receptor and G_{i/o} protein-mediated stimulation of intracellular signaling pathways involving cyclooxygenase-2-dependent PGI₂ synthesis.

Sphingosine 1-phosphate (S1P) has been shown to elicit diverse biological actions, including Ca²⁺ mobilization, change in cAMP accumulation, change in cell shape and motility in association with actin rearrangement, and proliferation in a variety of cell types (Hla et al., 2001; Tamama

and Okajima, 2002; Ishii et al., 2004). In vascular smooth muscle cells as well, S1P induces pleiotropic actions (i.e., contraction of the cells, stimulation of DNA synthesis, and inhibition of migration). These physiological actions are associated with the stimulation or inhibition of intracellular signaling pathways, including Ca²⁺ mobilization, cAMP accumulation, regulation of small molecular weight G proteins, and regulation of several protein kinases (Saba and Hla, 2004). Although most of the regulatory mechanisms of these signaling pathways are well characterized, there are still uncertainties with respect to the primary action sites for

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ABBREVIATIONS: S1P, sphingosine 1-phosphate; GPCR, G protein-coupled receptor; EDG, endothelial differentiation gene; CASMC, coronary artery smooth muscle cell; cPLA₂, cytosolic phospholipase A₂; AACOF₃, arachidonyltrifluoromethyl ketone; ERK, extracellular signal-regulated kinase; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; 6-keto-PGF_{1α}, 6-keto-prostaglandin F_{1α}; NS398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U-73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione; SW2871, 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole.

S1P. S1P was first recognized as a second messenger of some kinds of cytokines, including platelet-derived growth factor and tumor necrosis factor- α (Spiegel and Milstien, 2000). Later, S1P-specific cell-surface GPCRs, which are coupled to several intracellular signaling pathways, were identified; five subtypes of S1P receptors, i.e., EDG-1/S1P₁, EDG-5/S1P₂, EDG-3/S1P₃, EDG-6/S1P₄, and EDG-8/S1P₅, have been identified so far (Hla et al., 2001; Tamama and Okajima, 2002; Ishii et al., 2004). We have previously shown that S1P-induced Ca²⁺ mobilization and inhibition of migration are mediated by cell surface S1P receptors, especially EDG-5/S1P₂ in rat aortic smooth muscle cells (Tamama et al., 2001).

Bornfeldt et al. (1995) first reported that S1P increased cAMP accumulation, which seems to play a role in the inhibition of platelet-derived growth factor-induced migration of the cells (Bornfeldt et al., 1995). Cyclic AMP also acts as a second messenger for some vasodilators, such as prostaglandin I₂, and prostaglandin E₂, and may also be involved in the inhibition of contraction and proliferation (Weber et al., 1998). The mechanism by which S1P increased cAMP accumulation in vascular smooth muscle cells, however, remains uncharacterized. Proliferation or migration of vascular smooth muscle cells plays a pivotal role in the formation and progression of atherosclerotic lesions and restenotic lesions after angioplasty (Ross, 1999; Tamama and Okajima, 2002). Thus, the elucidation of the mechanism of cAMP accumulation as an inhibitory signal for cell proliferation and migration may be important for not only understanding the intracellular signaling networks but also developing therapeutic drugs for cardiovascular diseases, although cAMP signaling itself is a scientifically old subject. The regulation of adenylyl cyclase by GPCRs is well established; GPCRs couples to stimulatory (G_s) or inhibitory (G_i) G proteins and thereby stimulate or inhibit enzyme activity (Dessauer et al., 1996; Patel et al., 2001). In Chinese hamster ovary cells overexpressing the respective S1P receptor subtype, we demonstrated that S1P₂ and S1P₃ have the potential ability to couple to G_s proteins, resulting in cAMP accumulation (Kon et al., 1999). On the other hand, S1P may indirectly stimulate cAMP accumulation through prostaglandin synthesis (Davaile et al., 2000).

In the present study, we characterized S1P-induced cAMP accumulation in coronary artery smooth muscle cells (CASMCs) and found that there are several unique regulatory mechanisms of cAMP accumulation. For example, S1P induced early phase (~2.5 min) and late phase (~4–6 h) biphasic cAMP accumulation; both phases of cAMP accumulation involve cyclooxygenase-2. Furthermore, the early phase of cAMP accumulation was inhibited by pertussis toxin, suggesting G_{i/o} protein-mediated cAMP accumulation. Our results indicate that the early phase of cAMP accumulation is mediated through S1P receptors/G_{i/o} proteins/cytosolic phospholipase A₂ (cPLA₂)/cyclooxygenase-2/prostaglandin I₂ pathways and the late phase is mediated by cyclooxygenase-2 induction.

Materials and Methods

Materials. Human CASMCs were purchased from Cambrex Bio Science Rockland (Rockland, ME); S1P, AACOF₃, and NS398 were from Cayman Chemical (Ann Arbor, MI); arachidonic acid, prosta-

glandin D₂, prostaglandin I₂, prostaglandin E₂, and SW2871 were from Sigma-Aldrich (St. Louis, MO); fatty acid-free bovine serum albumin was from Calbiochem (San Diego, CA); pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA); fura 2/acetoxymethyl ester was from Dojindo (Tokyo, Japan); U73122 and U73343 were generously provided by Upjohn Co. (Kalamazoo, MI); cAMP radioimmunoassay kit was from Yamasa (Choshi, Japan); [³H]arachidonic acid was from American Radiolabeled Chemicals (St. Louis, MO); an ERK-specific antibody (K-23, against amino acids 305–327 of rat ERK1, which recognizes both ERK1 and ERK2) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and an antibody specific to phosphorylated forms of ERK (phospho-p44/42MAPK E10) was from Cell Signaling Technology Inc. (Beverly, MA). Rabbit antibody against synthetic carboxyl-terminal peptide (ASSSRGLD-DINPT, 581–594) of cyclooxygenase-2 was prepared by immunizing animals with peptide-KLH conjugate and specifically purified from antisera. JTE-013 was a gift from the Central Pharmaceutical Research Institute, Japan Tobacco Incorporation (Osaka, Japan). The sources of all other reagents were the same as described previously (Kon et al., 1999; Sato et al., 1999).

Cell Culture. CASMCs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 0.5 ng/ml human epidermal growth factor, 2 ng/ml human fibroblast growth factor-2, and 5 μ g/ml insulin in a humidified air/CO₂ (19:1) atmosphere. For cAMP assay, CASMCs were plated on rat-tail collagen (400 μ g/ml)-coated 12-multiplates or 10-cm dishes. Twenty-four hours before experiments, the medium was replaced with serum-free medium containing 0.1% (w/v) bovine serum albumin (fraction V). Where indicated, 100 ng/ml pertussis toxin was added to the culture medium 24 h before experiments.

cAMP Accumulation. CASMCs (passages 7–9) were washed once and preincubated for 20 min (PD98059, U0126, AACOF₃, indomethacin, and NS398) or for 10 min (JTE-013) at 37°C in HEPES-buffered medium. The HEPES-buffered medium was composed of 20 mM HEPES, pH 7.4, 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). After the preincubation, the cells were again washed once in the HEPES-buffered medium and then stimulated with S1P or other agonists for 2.5 min unless otherwise specified. In the experiments using U73122 and U73343, the cells were harvested from 10-cm dishes with trypsin (0.05% in phosphate-buffered saline containing 0.53 mM EDTA) and washed by sedimentation (250g for 5 min). The washing procedure was repeated, and the cells were finally resuspended in the same medium. The cells (about 5 \times 10⁶ cells) were preincubated for 2 min with 5 μ M U73122 or U73343 and further incubated for 2.5 min with S1P. The reaction was terminated by adding 100 μ l of 1 N HCl. The cAMP in the acid extract was measured using cAMP radioimmunoassay kit.

Measurement of [Ca²⁺]_i. The cells were harvested from 10-cm dishes with trypsin as described above, and then [Ca²⁺]_i was measured based on the change in fura-2 fluorescence as described previously (Kon et al., 1999).

Western Blot Analysis. The cells were washed once, preincubated for 10 min at 37°C in the HEPES-buffered medium and incubated with test agents for 2.5 min at 37°C. The incubation was terminated by washing twice with ice-cold phosphate-buffered saline 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 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin. 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 14,000g for 20 min. The supernatant was analyzed by Western blotting with the cyclooxygenase-2-specific or the ERK-specific antibodies. 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 (1:1300 dilution for cyclooxygenase-2, 1:1000 dilution for ERK) 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 tetrazolium/5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt system (Sato et al., 1999).

Quantitative RT-PCR Analysis. Total RNA was isolated using Tri Reagent (Sigma-Aldrich) according to the instructions from the manufacturer. After DNase I (Promega, Madison, WI) treatment to remove possible traces of genomic DNA contaminating in the RNA preparations, 5 μ g of the total RNA was reverse transcribed using random priming and Multiscribe reverse transcriptase according to the instructions from the manufacturer (Applied Biosystems). To evaluate the expression level of the cyclooxygenase-2, S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅ mRNAs, quantitative RT-PCR was performed using real-time TaqMan technology with a sequence detection system model 7700 (Applied Biosystems). The human cyclooxygenase-2, S1P₁, S1P₂, S1P₃, S1P₄, and S1P₅-specific probes were obtained from TaqMan gene expression assays (Applied Biosystems). The identification number of the products is Hs00153133 for cyclooxygenase-2, Hs00173499 for S1P₁, Hs00244677 for S1P₂, Hs00245464 for S1P₃, Hs00269446 for S1P₄, Hs00258220 for S1P₅, and Hs99999905 for GAPDH. The expression level of the target mRNA was normalized to the relative ratio of the expression of GAPDH mRNA. Each RT-PCR assay was performed at least three times, and the results are expressed as mean \pm S.E.

Transfection of siRNA. CASMCs were plated on 12-multiplates at 2.0×10^5 cells/well. Sixteen hours later, siRNAs (100 nM) were introduced into cells using RNAiFect reagent (QIAGEN, K.K.) according to the manufacturer's instructions. The cells were further cultured for 24 h. The S1P receptor mRNA level was measured using real-time TaqMan technology. Cyclic AMP response was performed 24 h after serum starvation as described above. The nonsilencing siRNA was obtained from QIAGEN, K.K. The siRNA targeted for S1P₂ was obtained from Dharmacon (Lafayette, CO). The identification number of S1P₂ is M-003952-00.

Release of Arachidonic Acid and Its Metabolites. The cells were cultured for 2 days in 12 multiplates, and the medium was replaced with serum-free medium containing 0.1% bovine serum albumin 24 h before experiments. [³H]Arachidonic acid (0.1 μ Ci/well) was then added to the medium 10 h before experiments. The cells were washed three times with HEPES-buffered medium and incubated with appropriate agents for 2.5 min. The supernatant was then collected, and its radioactivity ([³H]arachidonic acid and its metabolites) was measured.

Prostaglandin I₂ Measurement. Prostaglandin I₂ levels were determined using an enzyme immunoassay kit, according to the manufacturer's instructions (Cayman Chemical). The amounts of prostaglandin I₂ released in the supernatant were estimated from the levels of its stable metabolite 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}).

Statistical Analysis. All experiments were performed in duplicate or triplicate, and the results of multiple observations were presented as means \pm S.E. of at least three independent experiments, unless otherwise stated. Statistical significance was assessed by Student's *t* test, and values were considered significant at *p* < 0.05 (*).

Results

Biphasic Accumulation of cAMP by S1P in CASMCs. The time-dependent effect of S1P on cAMP accumulation in CASMCs is shown in Fig. 1A. S1P rapidly increased the cAMP level 10- to 20-fold 2.5 min after the treatment, and its level then decreased. The decrease in the cAMP level may not be caused by a loss of S1P during incubation because an

additional supply of the same amount of S1P during the 30-min incubation did not change the magnitude and pattern of cAMP response (data not shown). The cAMP level, however, started to gradually increase again from 60 min to at least 6 h after the S1P treatment. Thus, S1P induced biphasic cAMP accumulation, (i.e., a short-term and transient response and a late and sustained response). As shown in Fig. 1B, we measured the expression of cyclooxygenase-2 protein by Western blotting and found that S1P markedly induced cyclooxygenase-2 protein expression. The time course of cyclooxygenase-2 expression was parallel to the late and sustained cAMP response to S1P. As expected, cAMP accumu-

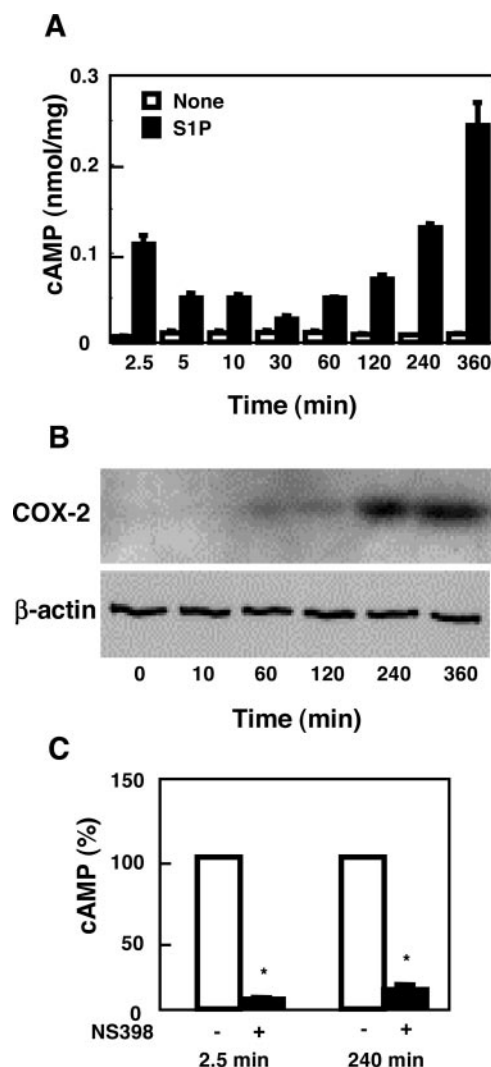


Fig. 1. Biphasic cAMP accumulation by S1P and the role of cyclooxygenase-2. **A**, cAMP accumulation at the indicated time was measured in the presence or absence of 1 μ M S1P in CASMCs. Results are expressed as cAMP accumulation (nanomoles) per milligram of cell proteins. **B**, time-dependent effects of S1P on cyclooxygenase-2 (COX-2) protein expression by Western blot analysis. Each lane was loaded with an equal amount of cell extract (30 μ g of protein), which was confirmed by β -actin expression. **C**, effect of NS398 on the cAMP response. The cells were preincubated for 20 min with or without 10 μ M NS398 and then stimulated by 1 μ M S1P for the indicated period. The cAMP accumulation is expressed as percentages of the value induced by 1 μ M of S1P in the absence of NS398. The basal value at 2.5 min was 0.0567 ± 0.0054 nmol/mg without NS398 and 0.0378 ± 0.0082 nmol/mg with NS398. These basal values were not significantly changed at 240 min. S1P-induced cAMP accumulations were 0.110 ± 0.010 nmol/mg for 2.5 min and 0.129 ± 0.005 nmol/mg for 240 min. Asterisk (*) indicates that the effect of NS398 was significant.

lation in the late phase (at 240 min) was attenuated by NS398, a cyclooxygenase-2-specific inhibitor (Fig. 1C). We were surprised to find, however, that cAMP accumulation at 2.5 min was also inhibited by NS398 (Fig. 1C). A significant cyclooxygenase-2 expression was not observed with our detection method at such an early phase (Fig. 1B). Thenceforth, we focused on the acute phase of cAMP accumulation and analyzed its signaling mechanisms.

Involvement of Pertussis Toxin-Sensitive $G_{i/o}$ Proteins in the S1P-Induced Acute Phase of cAMP Accumulation. As shown in Fig. 2, S1P induced the acute phase of cAMP accumulation with a half-maximal effective concentration of around 10 nM, in agreement with the reported K_d value of S1P receptors (Lee et al., 1998). If cyclooxygenase-2 is involved in S1P-induced action, prostaglandins may mediate cAMP accumulation. We examined the effects of prostaglandin I_2 , prostaglandin E_2 , and prostaglandin D_2 on cAMP accumulation and found that prostaglandin I_2 was the most potent stimulator (Fig. 2B). Treatment of the cells with pertussis toxin markedly inhibited the stimulatory effect of S1P (Fig. 2A) but not prostaglandin I_2 (Fig. 2B) on cAMP accumulation. Thus, the pertussis toxin effect was specific, and we postulated that S1P receptors that coupled to pertussis toxin-sensitive $G_{i/o}$ proteins may mediate the S1P-induced cAMP response.

S1P₂ Receptors Is Involved in S1P-Induced cAMP Accumulation. Fig. 3A shows the mRNA expression pattern of S1P receptor subtypes measured by the real-time TaqMan PCR method. All the receptor subtypes except for S1P₄ seemed to be expressed in CASCs; the rank order of expression of mRNA was S1P₂ > S1P₁ > S1P₃ > S1P₅. We therefore examined the possible involvement of S1P₂ receptors, which are expressed at the highest level. In Fig. 3B, we examined the effect of JTE-013, a specific antagonist of S1P₂ receptor, on the S1P-induced action (Arikawa et al., 2003; Ohmori et al., 2003). JTE-013 inhibited the S1P-induced cAMP accumulation in a competitive manner without any significant effect on the prostaglandin I_2 -induced cAMP accumulation, suggesting that the S1P₂ receptor is involved in the S1P action.

In the second line of experiments, we performed siRNA

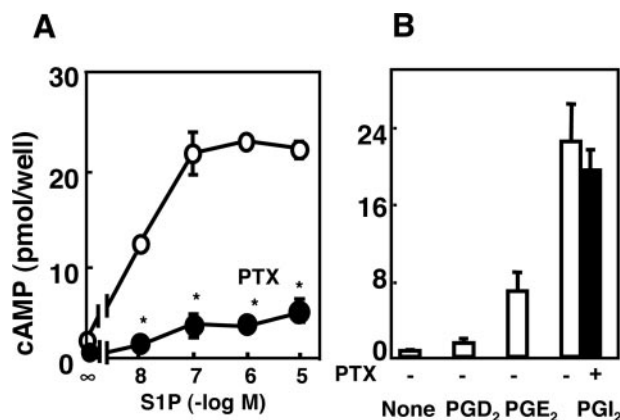


Fig. 2. Effect of pertussis toxin (PTX) on the S1P-induced cAMP accumulation. CASCs, which had been treated (● or closed column) or not treated (○ or open column) with PTX (100 ng/ml) for 24 h, were incubated for 2.5 min with the indicated concentrations of S1P in A or 1 μ M each of prostaglandin D_2 (PGD₂), prostaglandin E_2 (PGE₂), or prostaglandin I_2 (PGI₂) in B. The asterisk (*) indicates that the effect of PTX was significant.

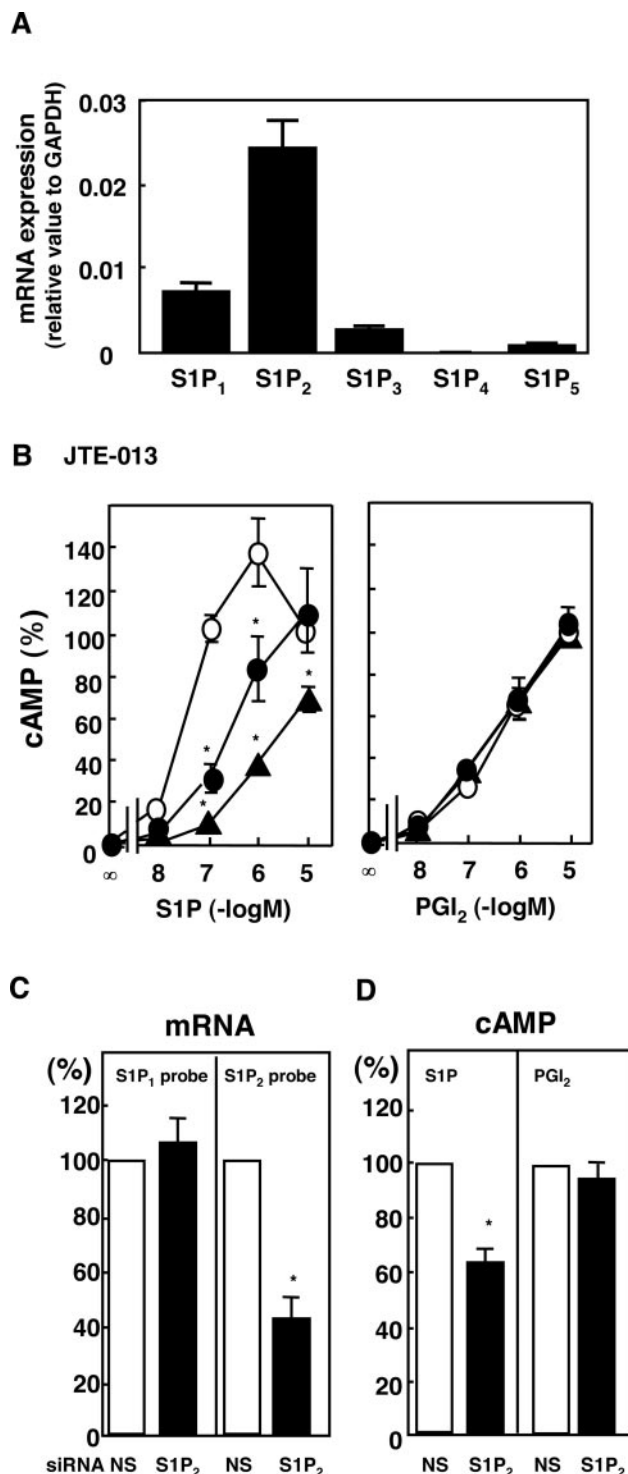


Fig. 3. Effects of JTE-013, an S1P₂ receptor antagonist, and siRNA targeted for S1P₂ receptor on cAMP response. A, mRNA expression of S1P receptor subtypes was assessed by real-time TaqMan PCR. Results are expressed as the relative ratios to GAPDH mRNA expression. B, cAMP response to the indicated concentrations of S1P (left) or prostaglandin I_2 (PGI₂) (right) for 2.5 min in the presence (●, 1 μ M; ▲, 10 μ M) or absence (○) of JTE-013 was measured. The 100% value was assigned to the activity obtained by 10 μ M S1P (17.0 \pm 3.15 pmol/well) or 10 μ M PGI₂ (38.6 \pm 3.54 pmol/well). The asterisk (*) indicates that the effect of the inhibitor was significant. C and D, CASCs were transfected with 100 nM each of nonsilencing RNA (NS; open column) or S1P₂ siRNA (closed column). S1P₁ and S1P₂ receptor mRNA expression (C) and cAMP response to 1 μ M S1P or 1 μ M prostaglandin I_2 (PGI₂) (D) were measured. The asterisk (*) indicates that the effect of siRNA was significant.

experiments. As shown in Fig. 3C, the siRNA specific to S1P₂ receptors decreased the S1P₂ receptor mRNA expression to 30 to 40% of the initial expression without any significant effect on S1P₁ receptor mRNA expression. Under these conditions, the siRNA inhibited about 40% of the S1P-induced cAMP accumulation (Fig. 3D, left) but hardly affected prostaglandin I₂-induced cAMP accumulation (Fig. 3D, right). These results indicate that the S1P₂ receptors, at least, may mediate S1P-induced cAMP accumulation in CASMCs.

Intracellular Signaling Pathways of the S1P-Induced Acute Phase of cAMP Accumulation. Because cAMP formation through GPCR systems that were not thought to stimulate it has often been reported (Brown and Rietow, 1981; Pyne et al., 1997), we next characterized the mechanism underlying the G_{i/o} protein-mediated cAMP formation in CASMCs. An involvement of cyclooxygenase-2 was postulated from the finding that the enzyme-specific inhibitor NS398 almost completely inhibited S1P-induced cAMP accumulation (Fig. 1). We examined the specificity of this observation. S1P-induced cAMP accumulation was also inhibited by indomethacin, a potent inhibitor for both cyclooxygenase-1 and cyclooxygenase-2 (Fig. 4A), but not by 100 nM moxifloxacin, an inhibitor of cyclooxygenase-1 (Goto et al., 1998) (data not shown). The inhibition of the cAMP response by NS398 and indomethacin was not caused by the nonspecific action of the drugs, as evidenced by the finding that prostaglandin I₂-induced cAMP accumulation was not affected by these inhibitors (Fig. 4A).

Because cyclooxygenase-2 protein expression was not yet increased by S1P under such an acute phase (Fig. 1), the supply of arachidonic acid, the substrate for the enzyme, was expected to be increased. We postulated an involvement of cPLA₂ as an enzyme for arachidonic acid production. We also assumed the participation of ERK and phospholipase C/[Ca²⁺]_i system because ERK has been reported to regulate cPLA₂ (Lin et al., 1993; Pyne et al., 1997) and phospholipase C/[Ca²⁺]_i signaling pathway has been shown to be involved in ERK activation (Agell et al., 2002). As shown in Fig. 4A, PD98059 and U0126, ERK kinase inhibitors, and AACOF₃, a cPLA₂ inhibitor, abolished S1P-induced cAMP accumulation. U73122, a phospholipase C inhibitor, but not U73343, an inactive form of U73122, also abolished cAMP accumulation (Fig. 4B). These results suggest the involvement of phospholipase C, ERK, and cPLA₂ for the S1P action.

The involvement of these signaling pathways was confirmed by the association of their activation by S1P. S1P increased [Ca²⁺]_i levels, which were inhibited by phospholipase C inhibitor U73122 but not by U73343, reflecting phospholipase C activation (Fig. 5A). The increase in [Ca²⁺]_i was inhibited by pertussis toxin pretreatment, indicating G_{i/o} proteins are also involved in this pathway like S1P-induced cAMP accumulation (Fig. 5B). S1P also induced ERK1/2 phosphorylation, reflecting the activation of the enzyme, in a manner sensitive to pertussis toxin and the ERK kinase inhibitor PD98059, whereas prostaglandin I₂ failed to activate the enzyme (Fig. 6A). The phospholipase C inhibitor U73122 but not U73343 also inhibited S1P-induced ERK1/2 phosphorylation, suggesting that phospholipase C/[Ca²⁺]_i signaling pathway may be located upstream in the ERK activation (Fig. 6B). Furthermore, S1P stimulated arachidonic acid release in a manner sensitive to U0126, another ERK kinase inhibitor (Fig. 7A), and exogenous arachidonic

acid induced cAMP accumulation to the same extent as S1P (Fig. 7B). Finally, we examined whether S1P actually induced prostaglandin synthesis in CASMCs. We measured 6-keto-prostaglandin F_{1α}, a stable metabolite of prostaglandin I₂, in the culture medium in the presence or absence of various inhibitors and found S1P markedly induced 6-keto-

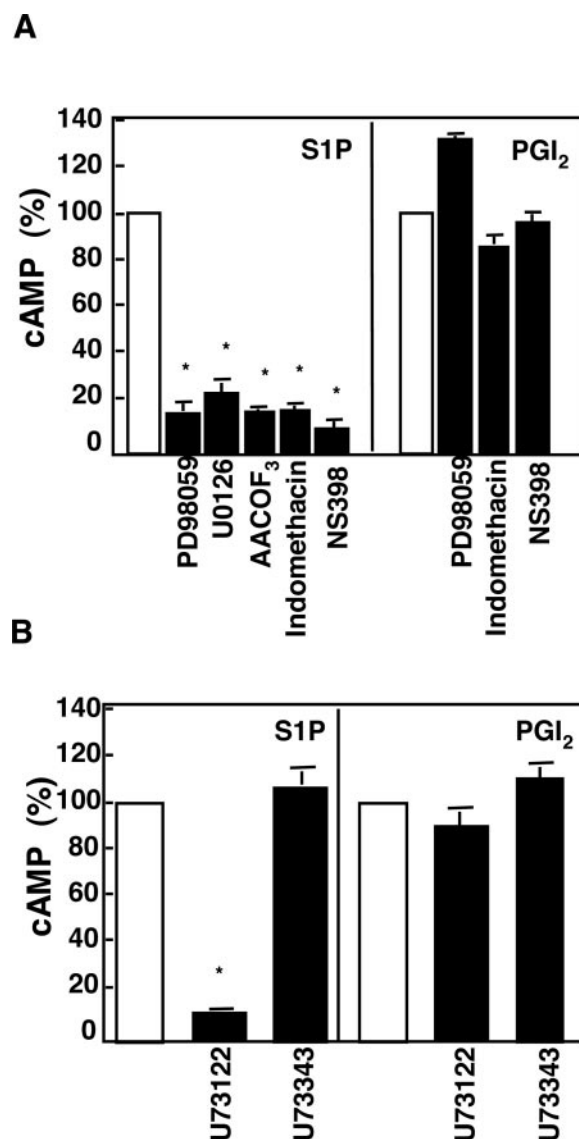


Fig. 4. Effects of various inhibitors for intracellular signaling pathways on cAMP response. A, CASMCs in 12-multiplates were pretreated with the indicated inhibitors (10 μ M each) for 20 min and then incubated for 2.5 min without, with 1 μ M S1P, or with 1 μ M prostaglandin I₂ (PGI₂) to measure cAMP accumulation. Results are expressed as percentages of the activity induced by S1P or PGI₂ in the absence of these inhibitors. The 100% value was evaluated by subtracting the basal activity (0.51 \pm 0.34 pmol/well) from the activity obtained by S1P (19.9 \pm 1.72 pmol/well) and by PGI₂ (25.6 \pm 3.3 pmol/well). The basal activity was not significantly changed by these inhibitors. B, CASMCs harvested from the 10-cm dish were incubated in suspension for 2 min with 5 μ M U73122, 5 μ M U73343, or vehicle (Me₂SO). The cells were then further incubated for 2.5 min without, with 1 μ M S1P, or with 1 μ M PGI₂ to measure cAMP accumulation. Results are expressed as percentages of the activity induced by S1P or PGI₂ in the absence of these inhibitors. The 100% value was evaluated by subtracting the basal activity (3.25 \pm 1.01 pmol/tube) from the activity obtained by S1P (34.3 \pm 2.06 pmol/tube) and by PGI₂ (52.7 \pm 2.19 pmol/tube). The basal activity was not significantly changed by these inhibitors. The asterisk (*) indicates that the effects of the inhibitors were significant.

prostaglandin $F_{1\alpha}$ production (Fig. 8A). As expected, S1P-induced 6-keto-prostaglandin $F_{1\alpha}$ production was almost completely inhibited by several inhibitors that were effective for the cAMP response (Fig. 8B).

Discussion

Numerous studies have shown that cyclooxygenase-2 is involved in a variety of biological functions induced by extracellular signaling molecules, including G protein-coupled receptor ligands and cytokines, through prostaglandin synthe-

sis (Robida et al., 2000; Martinez-Gonzalez et al., 2004). In many cases, the onset of cyclooxygenase-2-dependent extracellular signaling molecule-induced actions is rather slow because time for the induction of the cyclooxygenase-2 protein is necessary. In CSMCs as well, S1P induced the expression of cyclooxygenase-2 1 to 2 h after S1P treatment, accompanied by an increase in cAMP accumulation. A similar late phase of S1P-induced actions on prostaglandin synthesis depending on cyclooxygenase-2 induction has been shown in other cell types (Davaille et al., 2000; Kim et al., 2003; Pettus et al., 2003). The mechanisms by which S1P induces cyclooxygenase-2 expression, however, are controversial, especially with respect to its primary action site. S1P-induced prostaglandin synthesis seems to be mediated

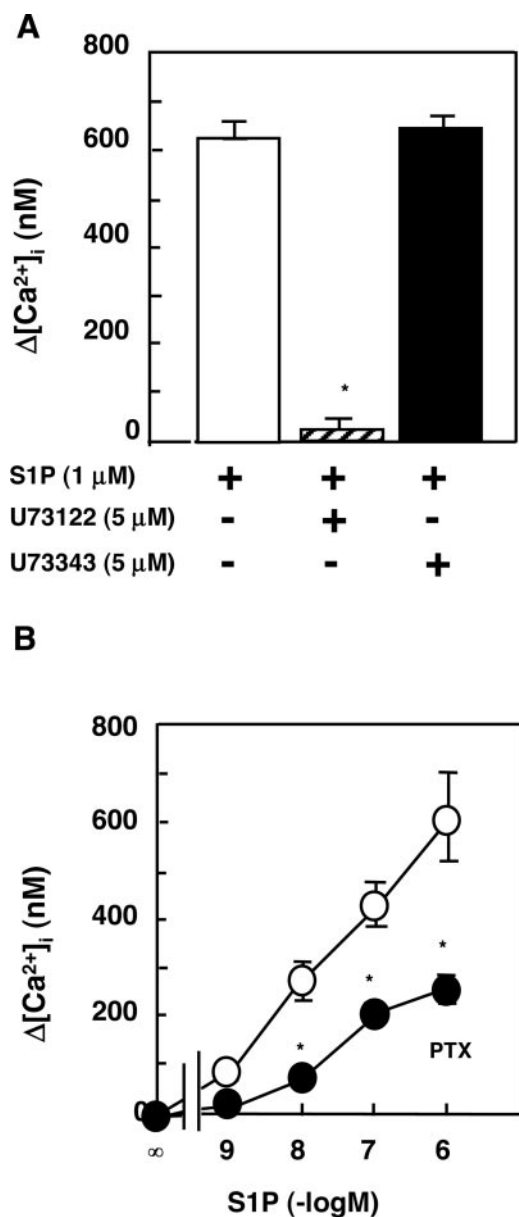


Fig. 5. Effects of phospholipase C inhibitor and pertussis toxin (PTX) on S1P-induced $[Ca^{2+}]_i$. A, CSMCs harvested from 10-cm dish were pretreated with 5 μ M U73122 (hatched column), U73343 (closed column), or Me_2SO (open column) for 2 min. The cells were then further incubated with 1 μ M S1P to monitor $[Ca^{2+}]_i$. B, CSMCs were pretreated with (●) or without (○) PTX (100 ng/ml) for 24 h and then harvested from 10-cm dish. The cells were incubated with the indicated concentration of S1P to monitor $[Ca^{2+}]_i$. The net $[Ca^{2+}]_i$ change (peak value-basal value) at around 15 s was calculated. Data are means \pm S.E. from four to five determinations of at least two separate experiments. The asterisk (*) indicates that the effect of U73122 or PTX was significant.

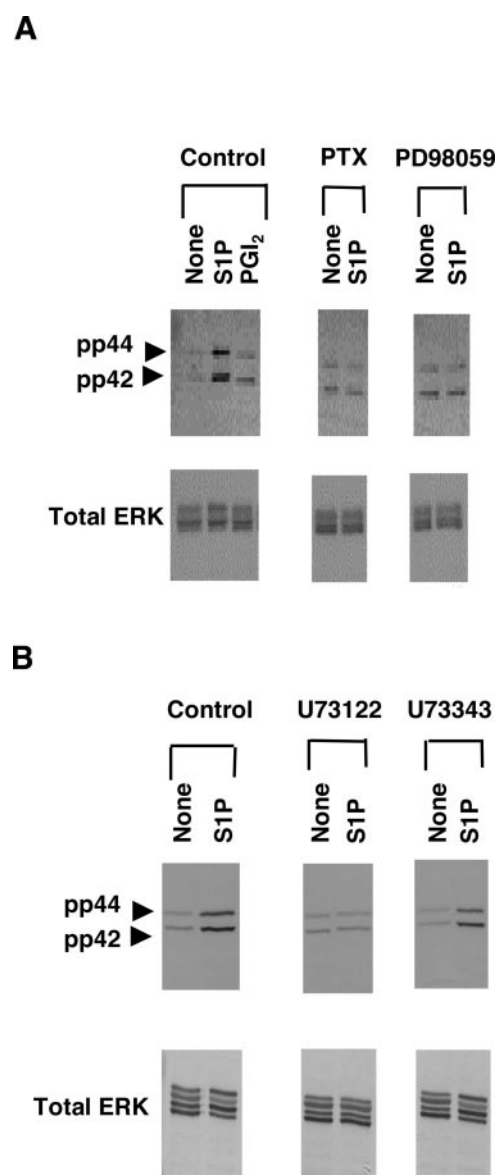


Fig. 6. Western blot analysis for phosphorylated ERK1/2. CSMCs, which had been pretreated without (control), with 100 ng/ml pertussis toxin (PTX) (for 24 h), or with 10 μ M PD98059 (for 20 min) in A, and without (control), with 5 μ M U73122, or with 5 μ M U73343 for 2 min in suspension in B, were incubated with 1 μ M each of S1P or prostaglandin I_2 (PGI₂) for 2.5 min. Top, phosphorylated forms of ERK1/2. Bottom, total ERK1/2 that indicates equal amount of protein (30 μ g) was loaded on each lane.

by the S1P receptors S1P₁ and S1P₃ in amnion-derived WISH cells (Kim et al., 2003); however, it seems to be mediated by intracellular mechanisms in hepatic myofibroblasts (Davaille et al., 2000), cultured fibroblasts, and lung adenocarcinoma cells (Pettus et al., 2003), although intracellular S1P targets still remain unidentified. Our preliminary experiments showed that S1P-induced cyclooxygenase-2 mRNA expression was attenuated by pertussis toxin pretreatment in CASMCs, suggesting an involvement of S1P receptors in the induction (data not shown).

In addition to the late response, S1P also exerted short-term and transient cAMP accumulation in CASMCs; a 10- to 20-fold increase in cAMP accumulation was observed at 2.5 min after S1P treatment. We were surprised to find that this short-term and transient cAMP response was also completely inhibited by the cyclooxygenase-2-specific inhibitor NS398. At such an early time (2.5 min), of course, cyclooxygenase-2 protein induction was not stimulated by S1P. Thus, S1P induced biphasic cAMP accumulation composed of a short-term and transient response and a late and sustained response; both responses seem to depend on cyclooxygenase-2. Although cyclooxygenase-2 protein expression was too low to be detected with our detection method, the endogenous enzyme must have high enough activity to synthesize prostaglandins when its substrate arachidonic acid is supplied.

Four types of S1P receptors (i.e., S1P₁, S1P₂, S1P₃, and S1P₅) seem to be expressed in CASMCs (Fig. 3A). Pharmacological (JTE-013) and molecular biological (siRNA) experiments suggested that S1P-induced stimulation of cAMP accumulation may be mediated by at least S1P₂ receptors and pertussis toxin-sensitive G_{i/o} proteins (Fig. 3, B–D). Although S1P₂ receptor siRNA showed the specificity to the receptors, the inhibition of mRNA expression was partial. We cannot therefore exclude the possible involvement of S1P receptors other than S1P₂ receptors. In relation to this, our preliminary results showed that suramin, an antagonist for S1P₃

receptors (Ancellin and Hla, 1999), did not attenuate the S1P-induced cAMP accumulation. Furthermore, SW2871, a specific agonist for S1P₁ receptors (Sanna et al., 2004), alone did not increase cAMP accumulation but enhanced the S1P-induced cAMP accumulation (data not shown). These results suggest that S1P₂ receptors may play a major role in the S1P-induced cAMP accumulation; however, it would be possible that there is a cross-talk between S1P receptor subtype signalings. Further studies are necessary to evaluate the role of the respective S1P receptor subtype in the S1P actions. Nevertheless, our present results strongly suggest that the S1P-induced cAMP accumulation is mediated by G_{i/o} protein-coupled S1P receptors, at least S1P₂ receptors, but not by intracellular mechanisms.

These results were not necessarily expected because G_{i/o} proteins usually mediate the inhibition of cAMP accumulation and therefore may show that the S1P action is a secondary response through intracellular and/or intercellular signaling pathways but not a primary action through a typical adenylyl cyclase system in plasma membranes (Dessauer et al., 1996; Patel et al., 2001). The intracellular pathways involve cyclooxygenase-2 and prostaglandins, especially prostaglandin I₂ synthesis. Prostaglandin I₂ then activates adenylyl cyclase, in an autocrine manner, through a typical enzyme system composed of a stimulatory receptor (in this case, prostaglandin I₂ receptor) and G_s proteins.

Because the acute phase of prostaglandin I₂ synthesis was not associated with an increase in cyclooxygenase-2 protein expression, an increase in the supply of arachidonic acid, a substrate of the enzyme, was expected. Indeed, S1P induced

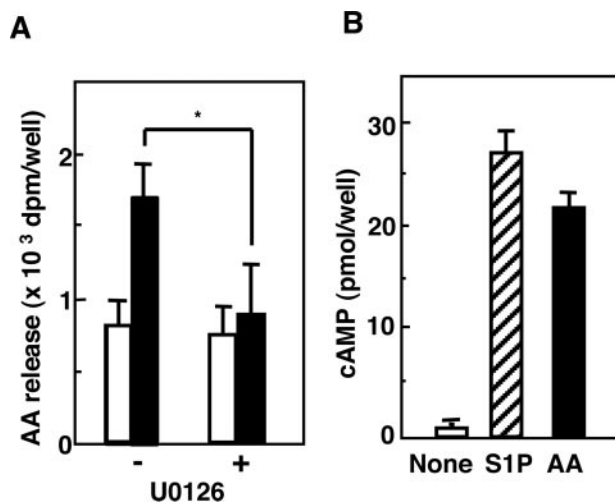


Fig. 7. Effect of arachidonic acid on cAMP response and change in arachidonic acid metabolism by S1P. A, CASMCs were pretreated with (+) or without (-) 10 μ M U0126 for 20 min and then incubated for 2.5 min in the presence (closed column) or absence of (open column) 1 μ M S1P to measure release of arachidonic acid and its metabolites. B, cAMP accumulation was measured in the absence (open column), presence of 1 μ M S1P (hatched column), or presence of 10 μ M arachidonic acid (AA; closed column) for 2.5 min. The results are expressed as cAMP accumulation (picomoles) per well. The asterisk (*) indicates that the effect of U0126 was significant.

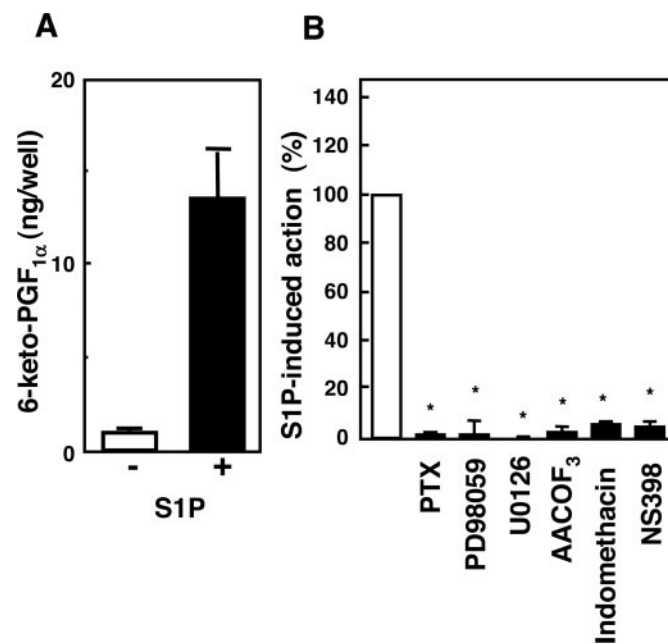


Fig. 8. Effects of various inhibitors for intracellular signaling pathways on S1P-induced prostaglandin I₂ (PGI₂) synthesis. A, CASMCs were incubated with or without 1 μ M S1P for 2.5 min to measure 6-keto-PGF_{1α}. B, cells were pretreated with the indicated inhibitors and then incubated with or without 1 μ M S1P for 2.5 min. The preincubation time was 24 h for pertussis toxin (PTX) and 20 min for other inhibitors. Amount of 6-keto-PGF_{1α} in the medium was measured. Results are expressed as percentages of the activity induced by 1 μ M S1P in the absence of these inhibitors. The basal activity was 0.85 \pm 0.26 ng/well, and this activity increased to 13.5 \pm 2.60 ng/well by S1P. The basal activity was not appreciably changed by treatment of these inhibitors. The asterisk (*) indicates that the effect of PTX or the inhibitor was significant.

an increase in arachidonic acid release, and the cPLA₂ inhibitor AACOF₃ inhibited S1P-induced prostaglandin I₂ synthesis and cAMP accumulation. The arachidonic acid release was completely inhibited by the ERK kinase inhibitor U0126, suggesting that cPLA₂ is regulated by ERK. Indeed, cPLA₂ has been reported to be activated by phosphorylation by ERK (Lin et al., 1993; Pyne et al., 1997). Phospholipase C inhibitor U73122 inhibited the S1P-induced phosphorylation of ERK, suggesting that phospholipase C/[Ca²⁺]_i signaling pathway may be located upstream in the ERK activation. Participation of phospholipase C/[Ca²⁺]_i signaling pathway in ERK activation has been shown (Agell et al., 2002). The S1P-induced [Ca²⁺]_i increase was inhibited by pertussis toxin, suggesting that G_{i/o} proteins mediate stimulation of phospholipase C/[Ca²⁺]_i signaling pathway. The intracellular signaling pathways of S1P receptor stimulation leading to prostaglandin I₂ synthesis and cAMP accumulation in CASMCs are illustrated in Fig. 9. This scheme may explain the acute phase of cAMP accumulation without a net increase in cyclooxygenase-2 induction. Although the late phase of cAMP accumulation was not analyzed in detail in the present study, prostaglandin I₂ synthesis may be synergistically stimulated by cyclooxygenase-2 induction (Fig. 1) and increase in the supply of arachidonic acid by cPLA₂ activation (Hamilton et al., 1999).

S1P has been shown to exert pleiotropic actions in a variety of cells and organs. In vascular cell systems, S1P induced both antiatherogenic and proatherogenic actions (Okajima, 2002; Tamama and Okajima, 2002). Thus, S1P stimulates proliferation, survival, migration, barrier integrity, nitric oxide synthesis, and other functions in endothelial cells and inhibits the migration of smooth muscle cells (Kimura et al., 2001, 2003; Tamama et al., 2001; Okajima, 2002; Saba and Hla, 2004). These actions seem to be antiatherogenic. On the other hand, S1P has been shown to increase the expression of adhesion molecules, such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, and thereby to accelerate monocyte interaction with endothelial cells and monocyte penetration into subendothelial space or the intima

of arterial walls (Xia et al., 1998; Auge et al., 2000). This proatherogenic action of S1P has been reported to be mediated by intracellular targets, although the molecular mechanisms are not fully characterized (Xia et al., 1998; Auge et al., 2000). The stimulation of the prostaglandin I₂/cAMP system by S1P, shown in the present study, may be considered an antiatherogenic aspect of S1P. In vascular smooth muscle cells, an increase in intracellular cAMP seems to be inhibitory for proliferation, migration, and contraction (Klemm et al., 2001). Moreover, prostaglandin I₂ has been shown to be a potent inhibitor for platelet aggregation (Fitzgerald et al., 1987). In the S1P-induced cAMP accumulation, phospholipase C/[Ca²⁺]_i signaling pathway seems to be involved. On the other hand, S1P has been shown to induce contraction of CASMCs through S1P₂ receptors (Ohmori et al., 2003). This contraction might be partly explained by the phospholipase C/[Ca²⁺]_i signaling pathway. Thus, S1P-induced phospholipase C/[Ca²⁺]_i signaling may be functioning in the CASMC contraction not only as a positive regulator but also as a negative regulator through cAMP accumulation, which might make possible a fine control of the contraction.

We have previously shown that S1P is accumulated in lipoprotein fractions, especially high-density lipoproteins (HDLs) (Murata et al., 2000; Kimura et al., 2001, 2003). The HDL-associated S1P mediates HDL-induced antiatherogenic actions, such as the stimulation of survival and the migration of endothelial cells (Murata et al., 2000; Kimura et al., 2001, 2003). HDL has also been shown to increase prostaglandin I₂ synthesis and cAMP accumulation in vascular smooth muscle cells (Vinals et al., 1999; Kothapalli et al., 2004) and endothelial cells (Fleisher et al., 1982). These results suggest that HDL-induced prostaglandin I₂ synthesis and cAMP accumulation may be partly mediated through S1P and S1P receptors. This is our next important subject of investigation.

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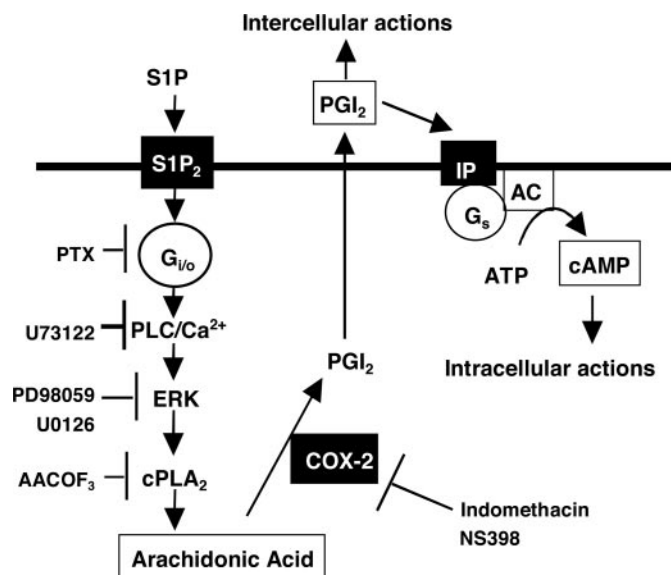


Fig. 9. A postulated pathway of S1P-induced short-term cAMP accumulation in CASMC. See Discussion for details. AC, adenylyl cyclase; IP, prostaglandin I₂ receptor.

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