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Sphingosine 1-Phosphate Stimulates Insulin Secretion in HIT-T 15 Cells and Mouse Islets

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Abstract. Sphingosine is involved in the regulation of cellular processes as a second messenger in various kinds of cells. Since the possible involvement of sphingosine has not been investigated in pancreatic β-cells, we determined the expression of putative sphingosine 1-phosphate (S1P) receptors and the effect of sphingosine on pancreatic β-cell function using a clonal Hamster β-cell line, HIT-T 15 cells and isolated mouse islets. We showed the expression of putative S1P receptors, Edg-3 and AGR16/H218 in HIT-T 15 cells. Ten and 20 μM S1P significantly stimulated insulin secretion for 10 minutes in HIT-T 15 cells. Ten μM S1P significantly increased insulin secretion from isolated mouse islets. Ten μM S1P obviously increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Fifty nM nifedipine did not affect the S1P stimulation of insulin secretion in HIT-T 15 cells. Two μM U73122 (phospholipase C inhibitor) completely deleted 10 μM S1P-induced stimulation of insulin secretion for 10 minutes, but U73343 (an inactive analogue of U73122) did not. S1P dose-dependently inhibited intracellular cyclic AMP levels. Pretreatment with 100 ng/ml pertussis toxin (PTX) partially, but significantly attenuated an increase of insulin secretion by 10 μM S1P. These data suggested that PTX-sensitive G-protein-dependent pathway may, at least in part, be involved in an increase of non-glucose stimulated insulin secretion by S1P through the activation of phospholipase C- Ca^{2+} system.

Key words: Sphingosine, Insulin, Ca^{2+}, Phospholipase C, G-Protein

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SPHINGOSINE is a metabolite of membrane sphingolipids. Sphingosine metabolism is associated with the changes caused by various bioactive substances. Sphingosine synthesis has been reported to increase by cytokines and stress [1, 2]. Tumor necrosis factor-α regulates thyroid-stimulating hormone-induced hydrogen peroxide production through a sphingomyelinase-ceramide pathway [3]. Intracellular content of sphingosine 1-phosphate (S1P), a phosphorylated product of sphingosine by sphingosine kinase, was accumulated in response to platelet-derived growth factor in Swiss 3T3 fibroblasts [4]. Furthermore, it was demonstrated that S1P is involved in the regulation of cellular processes including cell proliferation [5–9].

Recently, several lines of evidence have accumulated suggesting that sphingolipid metabolites may function as a new class of intracellular second messenger. S1P has been found to be a physiologically relevant ligand for Edg-1, Edg-3, and AGR16/H218 [10–12]. S1P increases cytoplasmic free Ca^{2+} con-

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centration and the production of inositol triphosphate (IP), and inhibits adenosine 3′,5′-cyclic monophosphate (AMP) accumulation in rat hepatocytes [13]. There remains a possibility that an increase of cytoplasmic free Ca^{2+} concentration occurs independent of the increased IP production [14]. Some of the S1P-induced modulation of intracellular signaling pathways are mediated by G-proteins [15–17]. However, the details of intracellular signal transduction mechanism of S1P have yet to be established.

Ceramide, another product of sphingomyelin hydrolysis, has recently been reported to affect secretory processes via post intracellular mobilization of Ca^{2+} from its storage sites in the exocrine pancreas [18]. In addition, extracellular sphingosylphosphorylcholine (SPC) can inhibit cell growth of human pancreatic cancer cells through regulation of the cell cycle process [19]. It is supposed that sphingosine may have some functions through its binding sites in pancreas. But, the possible involvement of sphingosine has not been investigated in pancreatic β-cells.

In the present study, therefore, we examined the possible expression of putative S1P receptor and determined the effect of S1P on insulin secretion by using a clonal hamster β-cell line, HIT-T 15 cells [20] and isolated mouse islets. We furthermore examined the possible involvement of phospholipase C- Ca^{2+} and adenylate cyclase systems in the S1P effect on insulin secretion in HIT-T 15 cells.

Materials and Methods

Chemicals

The incubation medium F-12 K was purchased from Flow Laboratories, Inc. (Irvine, Scotland). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, U.S.A.). Fura-2/AM was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Hanks' buffer was obtained from Nippon Suisan Co. (Tokyo, Japan). Other chemicals such as S1P, collagenase, pertussis toxin (PTX), dimethyl sulfoxide (DMSO), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture

Sixty two to 72 passage of HIT-T 15 cells were purchased from Flow Laboratories, Inc.. The cells were routinely cultured in the F-12 K medium containing 7 mM glucose supplemented with 10% fetal bovine serum. For the studies on insulin secretion, cells were passaged and seeded (10^5 cells per well) in 24-well multiple plates. On the day of the experiment, the culture medium was aspirated and washed by fresh medium. The medium was then supplemented with the chemicals described below.

Mouse Islet Isolation

Pancreatic islets were isolated from ICR mouse at age of 8 weeks, obtained from Imai Animal Laboratories (Saitama, Japan) by using collagenase technique in a modification of the method of Lacey and Kostianovsky [21]. Briefly, the pancreas was distended with a 6 mg/ml solution of collagenase in Hank's buffered saline solution. The pancreas was then taken out and moved into a plastic culture bottle and incubated for 35 minutes. After agitation, individual islets, free of attached acinar, vascular, and ductal tissue were selected and removed with a Pasteur pipette with the aid of a dissecting microscope. The hand-picked islets were cultured for 24 hours at 37°C in 95% O_2-5% CO_2 incubator. The islets were cultured in the F-12 K medium containing 10% fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 μg/ml) and D-glucose (7 mM). Following 24-hour preincubation, changes of insulin secretion for 10 minutes by 10 μM S1P were measured in 0.8 mM glucose-added F-12 K medium without FBS.

Northern blot analysis of putative S1P receptor

Total RNA was extracted from HIT-T 15 cells. The cDNAs for putative S1P receptors were cloned by RT-PCR as follows; Edg-1 [22] (1152 bp) from the obtained total RNA with 5′-gggaagcttCCACCATGGTGTCCTCCACCAGCATCCC-3′ and 5′-gggtctagaTTAAGAAGAAGAATTGACGTTTCC-3′, AGR16/H218 [23, 24] (1059 bp) with 5′-gggaagcttCCACCATGGGCGGTTTATACTCAGAGT-3′ and 5′-gggtctagaTCAGACCACTGTGTTGCCCTC-3′, and Edg-3 [25] (1137 bp) with 5′-ggggaattcCCA-

CCATGGCAACTGCCCTCCCGCCGCG-3′ and 5′-gggtctagaTCAGTTGCAGAAGATCCCATTCTG-3′. The 5′ primers contain a restriction enzyme site (Hind III 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), and the DNA sequence was checked.

Total RNA was electrophoresed through a 1% agarose gel containing 3.7% formaldehyde and 20 mM morpholinepropane sulfonic acid (MOPS) buffer and then blotted onto a nylon membrane (Hybond-N) with 20 × SSC. The probes were labeled with [α-^{32}P] dATP by random oligonucleotide priming. The procedures for hybridization were carried out at 65°C according to the method of Church and Gilbert [26]. Following hybridization, the blots were washed with 0.2 × SSC containing 0.1% SDS at 65°C. To normalize the amounts of total RNA present in the blots, GAPDH was used as described before [27].

Experimental protocols

After reaching confluent, cells were washed by fresh medium and 1 ml of F-12 K medium containing 0.8 mM glucose was added with 5, 10, 20 μM S1P in each well. Following 10-minute incubation at 37°C in 95% O_2-5% CO_2 incubator, the supernatant was collected and frozen at −20°C until insulin assay.

In the second experiment, cells were pretreated with 100 ng/ml PTX for 24 hours. After 24-hour pretreatment with PTX, 10 μM S1P was added and incubated for 10 minutes. The supernatant was collected and frozen until insulin assay.

In the third experiment, the cells were pretreated with 2 μM U73122 or U73343 [28–30] 2 minutes prior to the addition of S1P. Then, 10 μM S1P was added in each well and incubated at 37°C for 10 minutes. The medium was collected and the supernatant was stored following centrifugation.

In the fourth experiment, cells were pretreated with 50 nM nifedipine (dihydropyridine), L-type Ca^{2+} channel blocker [31], 10 minutes prior to the addition of S1P. Following the addition of 10 μM S1P, the cells were incubated for 10 minutes. The supernatant was collected and frozen until insulin assay.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

The $[Ca^{2+}]_i$ was measured according to the method described previously [32]. Briefly, HIT-T 15 cells were placed on round glass coverslip (diameter, 20 mm) 3 days before the experiment. The cells were incubated with Hanks' buffer containing 4 μM Fura-2/AM and 0.8 mM glucose for 10 minutes in a dark box at room temperature. After washing twice with Hanks' buffer, $[Ca^{2+}]_i$ dynamics were monitored by dual excitation microfluorometry (Model FC 300, Mitsubishi Kasei, Inc., Tokyo, Japan). Fluorescence from optically isolated single cell was monitored through a fixed square diaphragm before and after the addition of S1P. Photon counts at F340 and F360 were sampled with each second. From the ratio (F340/F360) of the fluorescence excited at the two wavelengths, from which background was subtracted, $[Ca^{2+}]_i$ was calculated and the peak value after the addition of S1P was compared with basal value just before S1P addition.

Measurement of intracellular cyclic AMP levels

HIT-T 15 cells were incubated for 10 minutes at 37°C in F-12 K medium containing 0.8 mM glucose and phosphodiesterase inhibitor, 0.5 mM IBMX, with and without the indicated dose of S1P. Medium was then aspirated and cyclic AMP content extracted from cells with 1 ml of 0.1 M HCl containing 0.1 mM $CaCl_2$ was measured by cyclic AMP enzyme immunoassay (EIA) kit (Amersham International plc., England).

Assays

Immunoreactive insulin (IRI) was assayed by radioimmunoassay using Phadeceph Insulin Kit (Pharmacia Japan, Tokyo, Japan).

Statistical analysis

All data were expressed as mean ± SE. The statistical analysis of the differences of the means was performed by the analysis of variance (ANOVA), followed by Duncan's multiple range test for the in-

dividual comparisons of the means.

Results

Fig. 1 shows the expressions of putative S1P receptors in HIT-T 15 cells, and 3T3 cells as positive control. The 3T3 cells expressed three kinds of putative S1P receptors, Edg-1, Edg-3 and AGR16/H218. In contrast, putative S1P receptors, Edg-3 and AGR16/H218 were expressed, but Edg-1 was not expressed in HIT-T 15 cells.

Fig. 2 demonstrated the effects of S1P on insulin secretion for 10 minutes in HIT-T 15 cells. Ten and 20 μM S1P significantly stimulated insulin secretion for 10 minutes. These results suggest that the effect on insulin secretion should be specific for S1P and the concentration of 10 μM was used in the following experiments. In mouse islet, 10 μM S1P significantly increased insulin secretion in the presence of 0.8 mM glucose (control (N=9): 11.65±1.29 ng/10 islets, 10 μM S1P (N=9): 20.88±2.91 ng/10 islets, $p<0.05$).

Changes of $[Ca^{2+}]_i$ induced by S1P in HIT-T 15 cells are presented in Fig. 3. As shown in Fig. 3a, 10 μM S1P increased $[Ca^{2+}]_i$. The effect of S1P on $[Ca^{2+}]_i$ was significant (Fig. 3b). In contrast, the effect of 50 nM nifedipine pretreatment on S1P-induced insulin secretion was investigated (Table 1). Nifedipine pretreatment failed to attenuate the effect of 10 μM S1P on insulin secretion for 10 minutes.

In addition, we examined the effects of U73122 (phospholipase C inhibitor) and U73343 (an inactive analogue of U73122) on S1P-induced stimulation of insulin secretion (Fig. 4). Two μM U73122 completely deleted 10 μM S1P-induced stimulation of insulin secretion for 10 minutes (Fig. 4a), but 2 μM

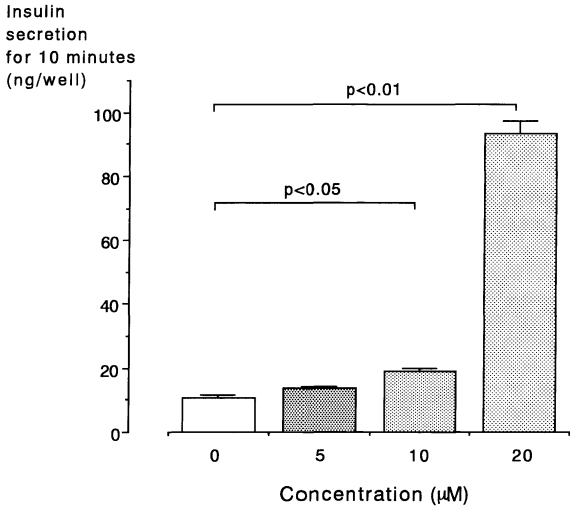

Fig. 2. Effect of 5, 10, 20 μM sphingosine 1-phosphate (S1P) on insulin secretion for 10 minutes in HIT-T 15 cells. Data are the mean±SE. N=6 in each group. *: $p<0.05$, **: $p<0.01$ vs. value of control group.

U73343 failed to affect 10 μM S1P-induced stimulation of insulin secretion for 10 minutes (Fig. 4b). These data indicate that the activation of phospholipase C, resulting in an increase of $[Ca^{2+}]_i$, should be important in the stimulation of insulin secretion by S1P. An increase of $[Ca^{2+}]_i$ by S1P is supposed to be attributable to the release from intracellular Ca^{2+} pool.

As shown in Fig. 5, S1P significantly inhibited intracellular cyclic AMP levels in a dose-dependent manner. This result may reflect the inhibition of adenylyl cyclase activity by S1P. Fig. 6 demonstrated the effect of pretreatment with 100 ng/ml PTX on S1P-induced stimulation of acute-phase insulin secretion. Pretreatment with 100 ng/ml PTX did not affect insulin secretion in the absence of S1P. In

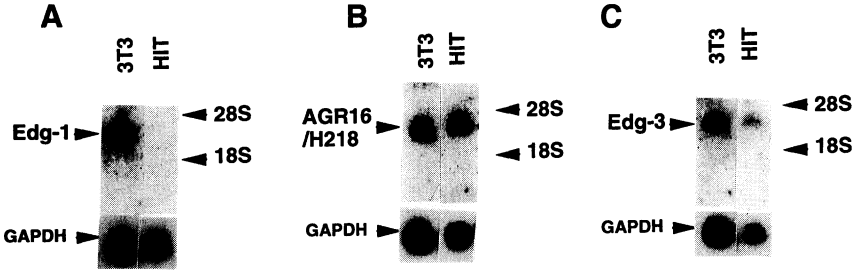

Fig. 1. Northern blot analysis of putative sphingosine 1-phosphate (S1P) receptors, Edg-1 (A), AGR16/H218 (B), and Edg-3 (C) in HIT-T 15 cells, and 3T3 cells.

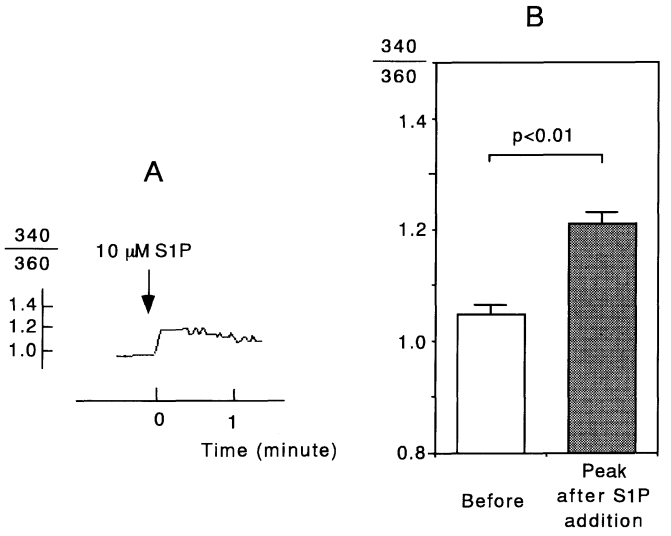

Fig. 3. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by the addition of 10 μM sphingosine 1-phosphate (S1P) in HIT-T 15 cells. a: chronological change of $[Ca^{2+}]_i$ after the addition of S1P. Arrow shows the time of the addition of 10 μM S1P. b: comparison of the value before and the peak value after the addition of 10 μM S1P. Data are the mean±SE. N=6 in each group.

Table 1. Effect of nifedipine addition on sphingosine 1-phosphate (S1P)-induced stimulation of insulin secretion in HIT-T 15 cells.

Nifedipine (nM)	S1P (μM)	N	Insulin secretion for 10 minutes (ng/well)	
0	0	6	16.3±0.8	p<0.01
	10	6	26.3±3.5	
50	0	6	17.8±0.4	p<0.05
	10	6	26.6±2.7	

N: number of each group.
Values represent mean±SE.

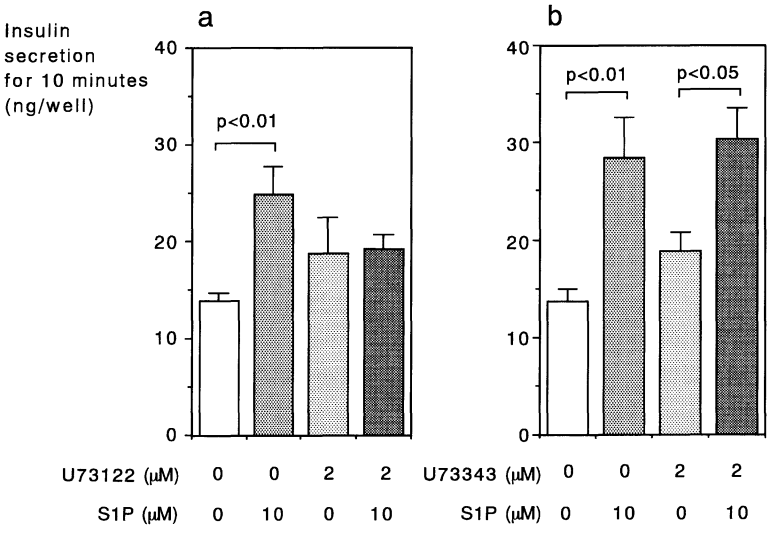

Fig. 4. Effect of 2 μM U73122 (a: *left panel*) or 2 μM U73343 (b: *right panel*) addition on 10 μM sphingosine 1-phosphate (S1P)-induced insulin secretion for 10 minutes in HIT-T 15 cells. Data are the mean±SE. N=6 in each group.

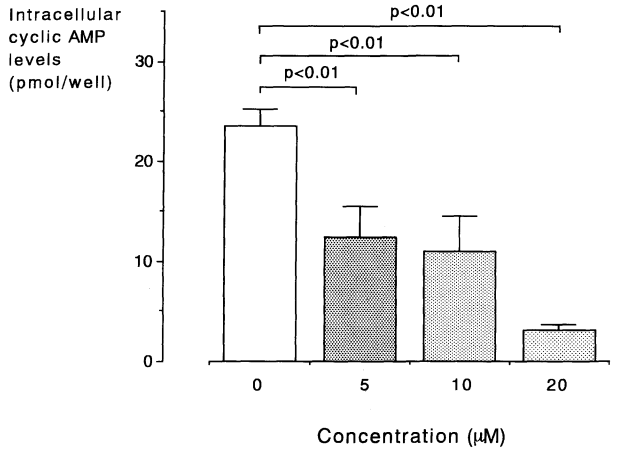

Fig. 5. Effect of 5, 10, and 20 μM sphingosine 1-phosphate (S1P) on intracellular cyclic AMP levels in HIT-T 15 cells. Data are the mean±SE. N=6 in each group.

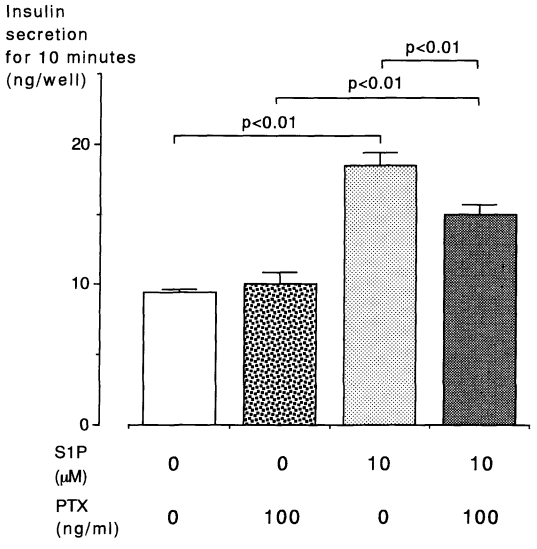

Fig. 6. Effect of 10 μM sphingosine 1-phosphate (S1P) on insulin secretion for 10 minutes in the presence or absence of 100 ng/ml pertussis toxin (PTX) pretreatment in HIT-T 15 cells. Data are the mean±SE. N=6 in each group.

contrast, PTX significantly attenuated an increase of insulin secretion by 10 μM S1P. But, S1P-induced stimulation of insulin secretion was still significant in PTX-pretreated cells. Therefore, it is supposed that both PTX-sensitive G-protein-dependent and -independent pathways may mediate S1P-induced stimulation of insulin secretion.

Discussion

The present study is the first to demonstrate the expression of putative S1P receptors in HIT-T 15 cells. We examined the effects of S1P on non-glucose stimulated insulin secretion. S1P stimulated acute-phase insulin secretion from HIT-T 15 cells without glucose stimulation. S1P also increases insulin secretion from isolated mouse pancreatic islets, raising the possibility that S1P may physiologically be involved in the regulation of non-glucose stimulated insulin secretion *in vivo*. Although the S1P levels in human serum are as low as about 0.5 nM [33], circulating platelets abundantly stores S1P and activated platelets may release S1P into the extracellular space in response to physiological agonists such as thrombin [34], resulting in a marked increase of local S1P concentrations. It is therefore possible that S1P, released from activated platelets, may modify basal insulin secretion *in vivo*, independent of glucose stimulation.

In the present study, putative S1P receptors, Edg-3 and AGR16/H218 expressed in HIT-T 15 cells. The Edg-3 and AGR16/H218 are involved in the stimulation of Ca^{2+} flux in *Xenopus* oocytes [10]. The expression of Edg-3 and AGR16/H218 in HIT-T 15 cells can explain an increase of $[Ca^{2+}]_i$ by S1P and these receptors could mediate the effects of S1P on insulin secretion. On the other hand, Edg-1 involves PTX-sensitive inhibition of adenylyl cyclase in Sf9 [11]. Pretreatment with PTX partially antagonized the effects of S1P on insulin secretion in HIT-T 15 cells, indicating that PTX-sensitive G-protein may partially be involved in a stimulation of acute-phase insulin secretory step by S1P. But, PTX failed to completely delete the S1P effects on insulin secretion. Edg-1 was not expressed in HIT-T 15 cells, but S1P decreased intracellular cyclic AMP levels. There may be another pathway which mediates these effects of S1P on insulin secretion and intracellular cyclic AMP levels through AGR16/H218 and/or Edg-3.

S1P stimulated acute-phase insulin secretion accompanied by an increase of $[Ca^{2+}]_i$ in HIT-T 15 cells. It has been recently reported that internal S1P acts on the Ca^{2+} pool through the modulation of phospholipase C system in FRTL-5 thyroid cells and HL 60 leukemia cells [15, 16]. Himmel *et al.* have reported that sphingosylphosphorylcholine inhibited 30 mM KCl-induced increase in $[Ca^{2+}]_i$ without

affecting basal $[Ca^{2+}]_i$ and S1P had no effect even on the KCl-induced increase in $[Ca^{2+}]_i$ in RINm5F insulinoma cells [35]. The present study demonstrated that the addition of U73122, a potent phospholipase C inhibitor [28-30], completely antagonized S1P-induced insulin secretion, but U73343, an inactive analogue of U73122 [28–30], did not. These observations indicated that the activation of phospholipase C system by S1P should be involved in the stimulation of acute-phase insulin secretion through Ca^{2+} release from intracellular Ca^{2+} pool. Nifedipine did not attenuate the S1P effect on insulin secretion in 0.8 mM glucose. In this glucose concentration, the cells should be hyperpolarized and L-type Ca^{2+} channel are not activated. Thus, we should not exclude an involvement of increased activity of the L-type Ca^{2+} channel in the S1P-induced insulin secretion. Likewise, because the L-type Ca^{2+} channel must be closed by nifedipine in addition to the inactive state under these conditions, the stimulation of insulin secretion by S1P may be a consequence of the increase in $[Ca^{2+}]_i$ by the release from Ca^{2+} pool.

PTX partially antagonized S1P-induced stimulation of acute-phase insulin secretion. This result raised the possibility that insulin secretion stimulated by S1P may be mediated by two different pathways, a PTX-sensitive G-protein coupled pathway and a PTX-insensitive pathway. S1P has recently been suggested to activate phospholipase C and other effector systems through cell surface receptors [15, 36]. The present study demonstrated that some putative receptors for S1P exist in HIT-T 15 cells. It is proposed that the PTX-sensitive G-protein coupled pathway should be mediated by the putative S1P receptor on the surface of HIT-T 15 cells. On the other hand, there is a possibility that S1P may be able to permeate the cell membrane, and may act directly on the intracellular second messenger system such as phospholipase C system through the cell membrane. Another possibility is that the PTX-insensitive G-protein-mediated pathway may be involved in the S1P effects on insulin secretion.

In addition to phospholipase C system, S1P has recently been reported to influence the adenylyl cyclase system [7, 36]. In the present study, S1P dose-dependently inhibited intracellular cyclic AMP levels. However, it was demonstrated that an increase of intracellular cyclic AMP by forskolin stimulates insulin secretion [37]. ATP, which is a material of cyclic AMP, may be consumed for the insulin secretory step, and the production of cyclic AMP may decrease merely by deficiency of material. Therefore, the reduction of intracellular cyclic AMP levels by S1P appears to be a result from increased insulin secretory step by S1P, rather than to directly stimulate acute-phase insulin secretion.

In conclusion, exogenous S1P stimulates acute-phase insulin secretion in HIT-T 15 cells and mouse isolated pancreatic islets. The stimulation of insulin secretion by S1P may be mediated by phospholipase C-associated Ca^{2+} mobilization, partially through the PTX-sensitive G-protein coupled pathway. S1P also decreases intracellular cyclic AMP levels.

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