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Involvement of pertussis toxin-sensitive GTP-binding proteins in sphingosine 1-phosphate-induced activation of phospholipase C–Ca^{2+} system in HL60 leukemia cells

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Abstract Exogenous sphingosine 1-phosphate (S1P) induced Ca^{2+} mobilization, in association with an increase in inositol polyphosphate production reflecting activation of phospholipase C in HL60 leukemia cells. The increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by S1P was inhibited by an appropriate treatment of the cells with pertussis toxin (PTX), U73122 (a phospholipase C inhibitor) or phorbol 12-myristate 13-acetate (PMA). In parallel with the Ca^{2+} response, these agents also inhibited inositol polyphosphate production. The S1P-induced Ca^{2+} response was also attenuated in the dibutyryl cAMP-induced differentiated cells, where GTP-binding protein-induced Ca^{2+} response is suggested to be enhanced. Lysophosphatidic acid (LPA) also increased $[Ca^{2+}]_i$ in the cells, but the maximal response was about half of that of S1P, and furthermore PTX and dibutyryl cAMP treatment hardly affected the LPA-induced Ca^{2+} mobilization. We conclude that exogenous S1P mobilizes Ca^{2+} through phospholipase C activation. The S1P-induced enzyme activation is at least partly mediated by PTX-sensitive GTP-binding protein-coupled receptors which may be different from LPA receptors.

Key words: Spingosine 1-phosphate; GTP-binding protein; Phospholipase C; HL60 cell

1. Introduction

Sphingolipids and lysosphingolipids have recently been shown to be involved in the regulation of a variety of cellular processes [1–3]. S1P, a phosphorylated product of sphingosine by sphingosine kinase, has been reported to act directly on the internal Ca^{2+} pool resulting in Ca^{2+} mobilization in a way similar to inositol 1,4,5-trisphosphate in permeabilized cells or purified endoplasmic reticulum [4,5]. S1P is accumulated in response to platelet-derived growth factor and serum in Swiss 3T3 fibroblasts; hence this lipid has been proposed as a second messenger of platelet-derived growth factor and serum on cell proliferation in Swiss 3T3 fibroblasts [6]. When intact fibroblasts were exposed to exogenous S1P, the lipid also induced Ca^{2+} mobilization [5]. Although the Ca^{2+} response is associated with an increase in IP_3 production probably reflecting the activation of phospholipase C, Ca^{2+} mobilization has been reported to be independent of the IP_3 accumulation [5]. On the contrary, a very recent study with the same Swiss 3T3 fibroblasts showed that PTX treatment of the cells inhibited both S1P-induced phospholipase C activation and Ca^{2+} mobilization, suggesting an involvement of PTX-sensitive GTP-binding proteins in the S1P signaling [7]. The parallelism of the toxin inhibition of the S1P-induced responses rather supports the idea that the enzyme activation is involved in the Ca^{2+} mobilization. Thus, the role of phospholipase C activation by exogenous S1P in the regulation of $[Ca^{2+}]_i$ still remains controversial.

In analogy with the role of PTX-sensitive G-proteins that communicate the signal of extracellular stimuli to effector enzymes through receptors, one can assume that S1P induces phospholipase C activation through G-protein-coupled receptors. However, the previous study failed to clarify whether the lipid activates G-proteins rather directly or indirectly through the putative receptors [7]. In relation to the putative receptors for S1P, it has also been proposed that S1P-induced responses are mediated through the recently identified LPA receptors [8,9].

Our preliminary study with HL60 leukemia cells showed that exogenous S1P induced both inositol phosphate production and Ca^{2+} mobilization in a manner sensitive to PTX. This prompted us to clarify the uncertain points as to the S1P signaling, especially the role of phospholipase C activation in the S1P-induced Ca^{2+} mobilization and the mechanism of the enzyme activation by the lipid. We found that, in HL60 cells, exogenous S1P-induced activation of phospholipase C is responsible for the increase in $[Ca^{2+}]_i$. Our results also suggest that S1P activates the enzyme at least partly through receptors coupling to PTX-sensitive G-proteins. The putative receptors for S1P may be different from the previously identified LPA receptors.

2. Materials and methods

2.1. Materials

Sphingosylphosphorylcholine (SPC), formyl-Met-Leu-Phe (fMLP), phorbol 12-myristate 13-acetate (PMA), adenosine deaminase, phospholipase D (from *Streptomyces chromofuscus*) and 1-oleoyl-*sn*-glycero-3-phosphate (lysophosphatidic acid; LPA) were purchased from Sigma; Fura 2/AM from Dojindo (Tokyo); and myo-[2-^{3}H]inositol (23.0 Ci/mmol) from Du Pont-New England Nuclear. Pertussis toxin (PTX), and U73122 and U73343 were generously provided by Dr. M. Ui of the Institute of Physical and Chemical Research (Wako, Japan), and Upjohn Co. (Kalamazoo, MI), respectively. Sphingosine 1-phos-

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Abbreviations: S1P, sphingosine 1-phosphate; PTX, pertussis toxin; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; G-protein, GTP-binding regulatory protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMA, phorbol 12-myristate 13-acetate; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; fMLP, formyl-Met-Leu-Phe.

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phate (S1P) was prepared by treatment of sphingosylphosphorylcholine with phospholipase D as described previously [10,11]. The sources of all other reagents were the same as described previously [12].

2.2. Cell cultures

In preliminary experiments, we found that passage of HL60 cells is critical for S1P-induced responses; the cells with a large passage number showed rather higher response to the lipid. We therefore used them after about 50 to 100 passages of the initial stocked cells given by Dr. S. Hoshino of Tokyo University (Tokyo, Japan) in the present study. The cells were routinely cultured in a RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (GIBCO) and maintained in a humidified atmosphere of 95% air and 5% CO_2. In some experiments in Fig. 5, the cells were cultured for 5 days in a medium containing 500 μM dibutyryl cyclic AMP to differentiate into neutrophil-like cells. Two days before the experiments, the cells were sedimented (250 × *g* for 5 min) and transferred to fresh medium for $[Ca^{2+}]_i$ measurement and to an inositol-free RPMI 1640 medium containing 10% fetal calf serum and myo-[2-^{3}H]inositol (4 μCi/ml) for inositol phosphate response. PTX treatment of the cells was performed by adding the toxin (100 ng/ml) to the medium 6 h before the experiments.

2.3. Measurement of [^{3}H]inositol phosphate production

It was performed as described previously [12]. The radioactivity of the trichloroacetic acid (5%)-insoluble fraction was measured as the total radioactivity incorporated into the cellular inositol lipids. Where indicated, the results were normalized to 10^5 cpm of the total radioactivity.

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

$[Ca^{2+}]_i$ was measured by the fluorescence change of Fura 2-loaded cells as described previously [12].

2.5. Data presentation

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

3. Results

Fig. 1 shows the effects of S1P and LPA on $[Ca^{2+}]_i$. Both S1P and LPA increased $[Ca^{2+}]_i$; a significant increase was detected from 0.1 μM for both lipids (Fig. 1A). The S1P-induced response was maximal at around 10 μM, while the Ca^{2+} response to LPA was saturated at around 0.3 μM (Fig. 1C,D). However, the magnitude of maximal response was much higher for S1P than that for LPA (Fig. 1C,D). Moreover, PTX treatment of

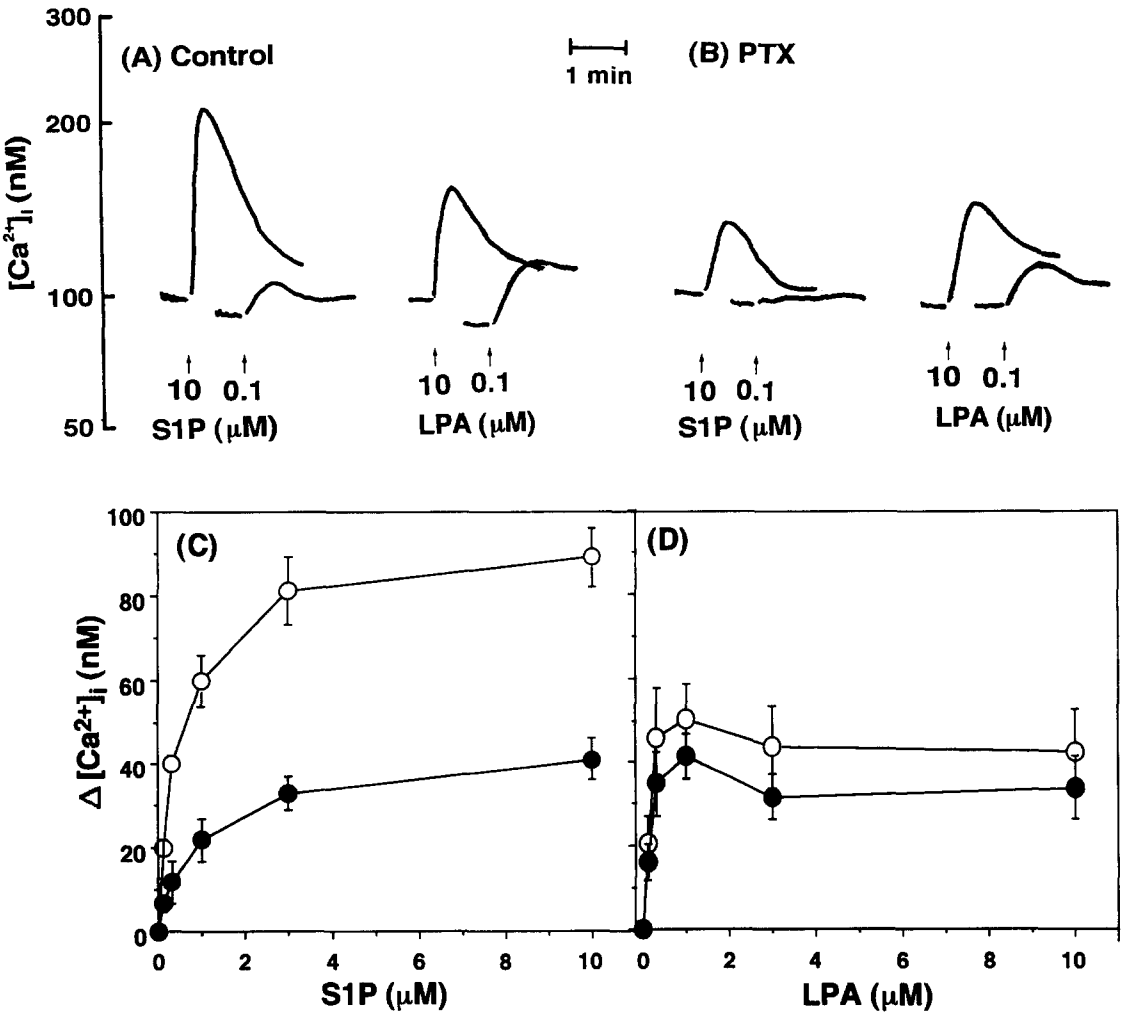

Fig. 1. Effect of S1P and LPA on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ change in control cells (non-treated with PTX) (A) or PTX-treated cells (B) was monitored. At arrows, the indicated doses of S1P or LPA were added. The results shown are representatives of four separate experiments. In (C) and (D), the cells were incubated with the indicated doses of S1P or LPA in control cells (○) or PTX-treated cells (●). The net $[Ca^{2+}]_i$ change (peak value−basal value) was plotted against the indicated doses of S1P (C) or LPA (D). Results are means ± S.E. of four separate experiments.

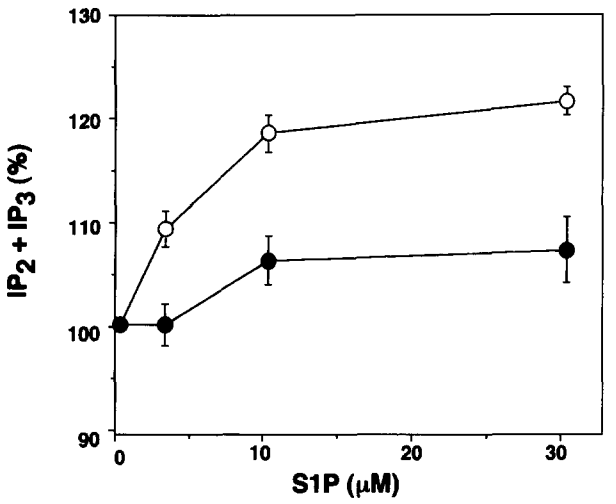

Fig. 2. Effect of S1P on inositol polyphosphate production. The cells labeled with [3H]inositol were incubated for 1 min with the indicated doses of S1P in control cells (non-treated with PTX) (○) or PTX-treated cells (●). Production of IP_2 plus IP_3 was measured. Results are expressed as percentages of the basal values obtained without S1P. Normalized basal values (cpm) (see section 2) were 788 ± 89 and 865 ± 56 for control cells and PTX-treated cells, respectively. Data are means ± S.E. of three separate experiments.

the cells inhibited about 60% the S1P-induced Ca^{2+} response at any dose employed, whereas the LPA-induced response was not appreciably affected by the toxin treatment. These results suggest that at least the PTX-sensitive part of the $[Ca^{2+}]_i$ increase is independent of the LPA receptor-mediated signaling pathway.

One of the mechanisms for increasing $[Ca^{2+}]_i$ is inositol 1,4,5-trisphosphate-dependent mobilization of Ca^{2+} from the internal store [13]. In Fig. 2, we measured the production of inositol polyphosphate including IP_2 and IP_3, which may reflect phospholipase C activity. The inositol polyphosphate production was significantly increased by S1P. Consistent with the Ca^{2+} response (Fig. 1), PTX treatment clearly inhibited its production (Fig. 2), suggesting that PTX-sensitive G-proteins are involved in the S1P-induced activation of phospholipase C. Parallelism of the inhibition by PTX of phospholipase C activation and Ca^{2+} mobilization also suggests that S1P-induced enzyme activation is responsible for the lipid-induced Ca^{2+} mobilization.

This possibility was further examined in Figs. 3 and 4. S1P-induced Ca^{2+} mobilization was hardly affected by an addition of excess EGTA over extracellular Ca^{2+} (Fig. 3A,B), suggesting that the source of Ca^{2+} was internal. More direct evidence showing the involvement of phospholipase C in the Ca^{2+} mobilization is that U73122, a potent phospholipase C inhibitor [14], markedly suppressed S1P-induced Ca^{2+} response (Fig. 3A,B), which was associated with the inhibition of the lipid-induced inositol polyphosphate production (Fig. 3C). Failure of U73343, an inactive analogue of U73122 ([14] and Fig. 3C), to inhibit the Ca^{2+} response to S1P (Fig. 3A,B) suggests that the

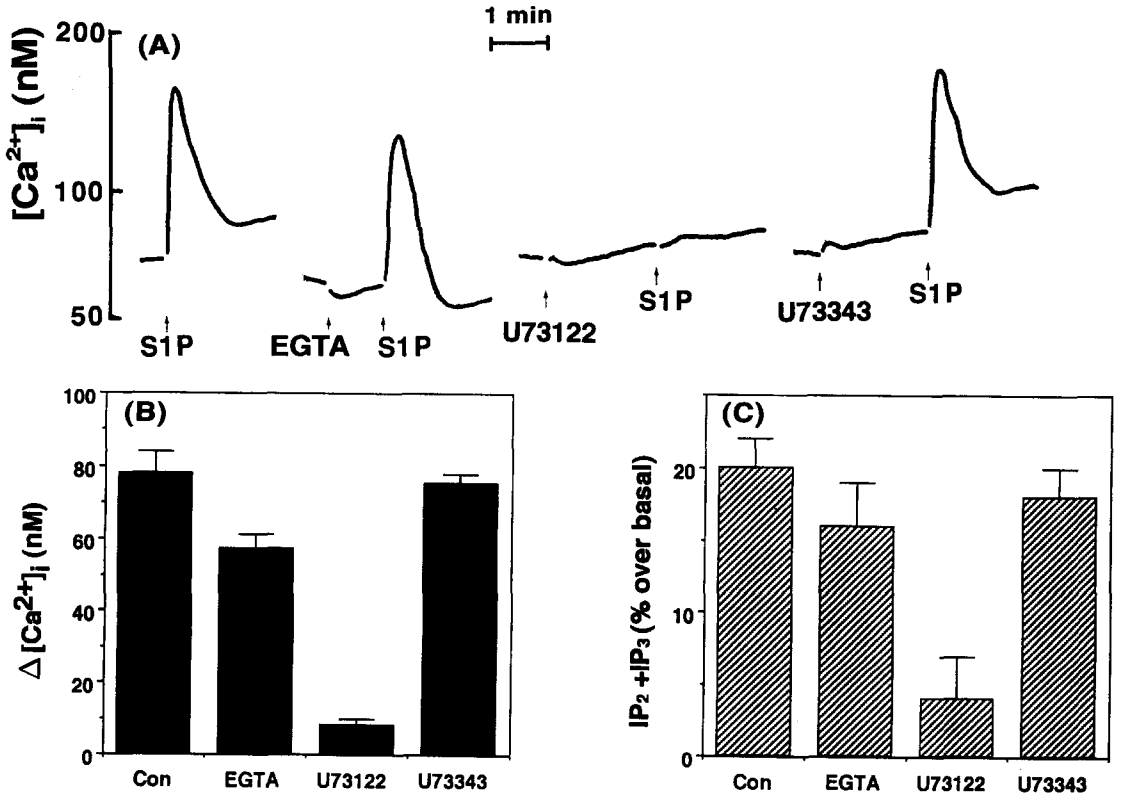

Fig. 3. Effect of extracellular Ca^{2+}, U73122 or U73343 on S1P-induced increase in $[Ca^{2+}]_i$. Representative traces of $[Ca^{2+}]_i$ change from three separate experiments by S1P (10 μM) in the absence or presence of 2.5 mM EGTA, 2.5 μM U73122 or 2.5 μM U73343 are shown in (A). In (B), the net change in $[Ca^{2+}]_i$ by S1P under the conditions shown in (A) was expressed as means ± S.E. In (C), the cells labeled with [^{3}H]inositol were incubated for 1 min with 10 μM S1P under the same conditions as those for (A). Results are expressed as percentages over basal value without S1P. The basal value taken as 100% were 850 ± 52 for control cells, 867 ± 26 for EGTA-treated cells, 799 ± 24 for U73122-treated cells and 977 ± 20 for U73343-treated cells.

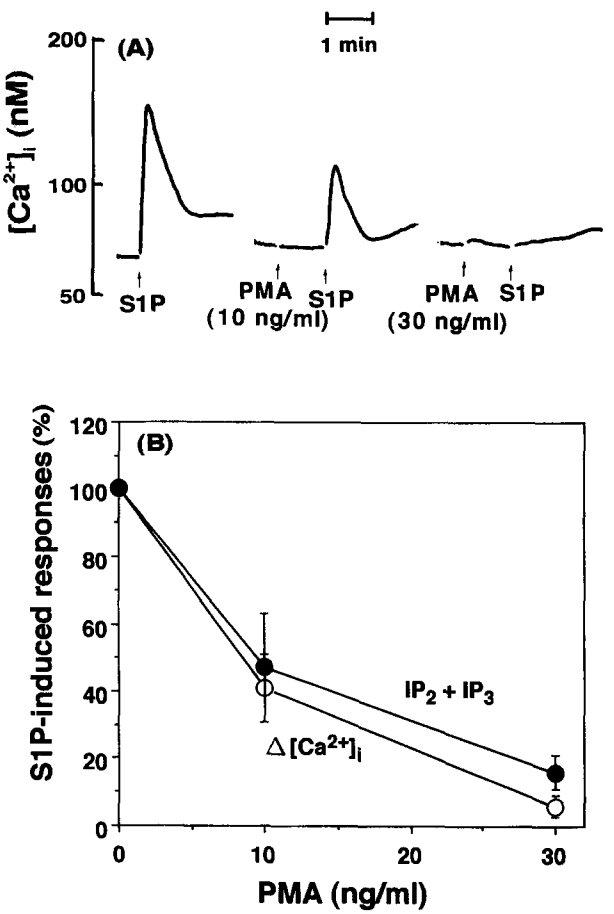

Fig. 4. Effect of PMA pretreatment on S1P-induced $[Ca^{2+}]_i$ increase and inositol polyphosphate production. Representative traces of $[Ca^{2+}]_i$ change from three separate experiments by S1P (10 μM) in the absence or presence of 10 ng/ml or 30 ng/ml PMA are shown in (A). In (B), the net $[Ca^{2+}]_i$ changes (○) (data are from (A)) or increase in IP_2+IP_3 production (●) by 10 μM S1P were expressed as percentage of control cells without PMA pretreatment. For IP_2+IP_3 production, the cells labeled with [^{3}H]inositol were incubated with or without 10 μM S1P in the absence or presence of the indicated doses of PMA. PMA was added 1 min before S1P addition. PMA treatment did not significantly change the basal value without S1P. Data are means ± S.E. of three separate experiments.

inhibitor effect is specific. Finally, we examined the effect of short term treatment of PMA on S1P-induced responses (Fig. 4). In HL60 cells, short term treatment of PMA has been reported to inhibit the receptor-mediated phospholipase C activation [15]. PMA dose-dependently inhibited S1P-induced inositol polyphosphate production (Fig. 4B). Under the conditions, the lipid-induced Ca^{2+} mobilization was also inhibited, in parallel with the inositol polyphosphate production, by PMA treatment. These results are well consistent with the idea that S1P-induced Ca^{2+} mobilization is dependent on the phospholipase C activity.

In the previous study [12], we have shown that dibutyryl cAMP-induced differentiation of HL60 cells into neutrophil-like cells is associated with an increase in the amount of G_{i2} and G_{i3}, and enhancement of AlF_4^-- and fMLP-induced activation of phospholipase C and Ca^{2+} mobilization. This suggests that the PTX-sensitive G-protein-mediated signaling pathway leading to activation of phospholipase C and Ca^{2+} mobilization is rather enhanced in the differentiated cells. However, sphingosylphosphorylcholine (lysosphingomyelin)-induced response was conversely attenuated [12]. In Fig. 5, we confirmed the enhancement of fMLP-induced Ca^{2+} response and the attenuation of a sphingosylphosphorylcholine-induced one by dibutyryl cAMP-induced differentiation. In this figure, we also examined S1P and LPA effects in the differentiated HL60 cells. The Ca^{2+} response to S1P, like sphingosylphosphorylcholine, was significantly attenuated in the differentiated cells, whereas the LPA-induced response was not appreciably affected under the conditions.

In the differentiated cells, PTX markedly inhibited the Ca^{2+} response to fMLP (about 95% inhibition), whereas that to S1P was still significant (about 50% inhibition) in the toxin-treated cells; the net $[Ca^{2+}]_i$ increase (nM) was 10 ± 1 for fMLP and 26 ± 3 for S1P, respectively (number of observations was 3, see Fig. 5 for the control value in the cells not treated with PTX).

4. Discussion

In the present paper, we have shown that S1P induces phospholipase C activation and Ca^{2+} mobilization in HL60 leukemia cells. Our results clearly indicate that the enzyme activation by the lipid is responsible for Ca^{2+} mobilization, as evidenced from the parallelism of the inhibition of both responses by several agents including PTX (a G-protein inhibitor), U73122 (a phospholipase C inhibitor) and PMA (an inhibitor for the receptor-mediated phospholipase C activation). In the previous study with Swiss 3T3 fibroblasts [5], the authors concluded that S1P-induced phospholipase C activation is not involved in Ca^{2+} mobilization, although exogenous S1P induced both the enzyme activation and Ca^{2+} mobilization. This conclusion was based on the observation that PMA treatment abolished S1P-induced activation of the enzyme but never affected the lipid-induced Ca^{2+} mobilization [5]. At present, we cannot clearly explain the difference in the PMA effect between the fibroblasts and HL60 cells. As mentioned in section 1, S1P, like inositol

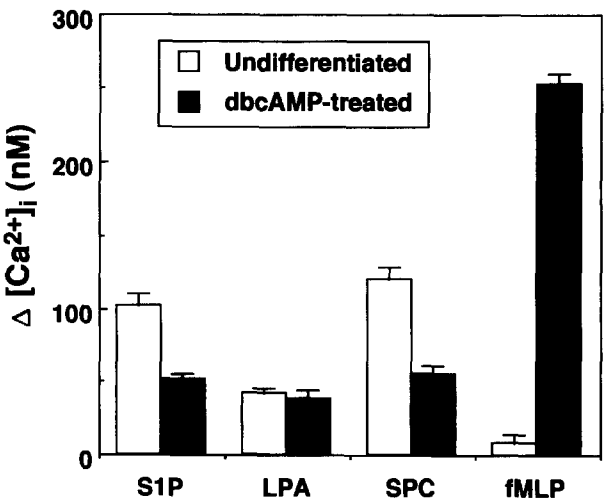

Fig. 5. Differentiation into neutrophil-like cells by dibutyryl cAMP attenuates Ca^{2+} mobilization induced by S1P or SPC, but not by LPA. The cells were incubated with S1P (10 μM), LPA (10 μM), SPC (10 μM) or fMLP (10 nM) to monitor $[Ca^{2+}]_i$ change in undifferentiated cells (white column) or dibutyryl cAMP-induced differentiated cells (black column). The net $[Ca^{2+}]_i$ changes by these agents were shown. Data are means ± S.E. of four separate experiments.

1,4,5-trisphosphate, has the ability to interact rather directly with the internal Ca^{2+} store resulting in Ca^{2+} mobilization [4,5]. To interact with the internal store, S1P first must enter the cells. Therefore, the differences in the efficiency of the cell machinery that leads S1P to penetrate inside the cells and to mobilize Ca^{2+} may partly account for the discrepancy of the results. In any event, in HL60 cells, at least a short term of Ca^{2+} mobilization during 2 min after S1P addition is almost completely dependent on phospholipase C activation.

The phospholipase C activation and the subsequent Ca^{2+} mobilization was partly PTX-sensitive, suggesting the involvement of PTX-sensitive G-proteins in the S1P signaling. As shown in Fig. 5, dibutyryl cAMP-induced differentiation clearly attenuated the S1P-induced Ca^{2+} response. Under the differentiated conditions, G-protein-induced phospholipase C activation and Ca^{2+} mobilization were rather enhanced reflecting an increase in the amount of G_i-proteins [12]. This excludes the possibility that S1P directly activates G_i-proteins and suggests that S1P action is attenuated at a step before G-proteins in the lipid signaling pathway. Hence we propose the existence of receptors for S1P.

Although we did not directly prove the complete ADP-ribosylation of G_i/G_o proteins by PTX in this study, the toxin treatment almost completely abolished fMLP-induced Ca^{2+} response in dibutyryl cAMP-induced differentiated cells, whereas the S1P response was partly inhibited by the toxin. This suggests that the putative receptors for S1P couple to both PTX-sensitive and insensitive G-proteins. The putative receptors may not be the same as those for LPA, because, in contrast to S1P-induced Ca^{2+} response, LPA-induced action was insensitive to PTX (Fig. 1) and dibutyryl cAMP (Fig. 5). However, we cannot exclude the possibility that the PTX-insensitive part of the Ca^{2+} response to S1P is mediated through the LPA receptor. We have recently proposed the presence of a novel G-protein-coupled receptor for a group of lysosphingolipids including sphingosylphosphorylcholine and galactosylsphingosine (psychosine) in HL60 cells [12]. It is also unclear, however, whether S1P shares with these lysosphingolipids the same receptor and signaling pathway. This problem is our current subject of investigation.

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References

[1] Hannun, Y.A. and Bell, R.M. (1989) Science 243, 500–507.
[2] Hannun, Y.A. (1994) J. Biol. Chem. 269, 3125–3128.
[3] Kolesnick, R. and Golde, D.W. (1994) Cell 77, 325–328.
[4] Ghosh, T.K., Bian, J. and Gill, D.L. (1994) J. Biol. Chem. 269, 22628–22635.
[5] Mattie, M., Brooker, G. and Spiegel, S. (1994) J. Biol. Chem. 269, 3181–3188.
[6] Olivera, A. and Spiegel, S. (1993) Nature 365, 557–560.
[7] Goodemote, K.A., Mattie, M.E., Berger, A. and Spiegel, S. (1995) J. Biol. Chem. 270, 10272–10277.
[8] Durieux, M.E., Carlisle, S.J., Salafranca, M.N. and Lynch, K.R. (1993) Am. J. Physiol. 264, C1360–C1364.
[9] Moolenaar, W.H. (1995) J. Biol. Chem. 270, 12949–12952.
[10] Van Veldhoven, P.P., Fogelsong, R.J. and Bell, R.M. (1989) J. Lipids Res. 30, 611–616.
[11] Zhang, H., Desai, N.N., Olivera, A., Seki, T., Brooker, G. and Spiegel, S. (1991) J. Cell. Biol. 114, 155–167.
[12] Okajima, F. and Kondo, Y. (1995) J. Biol. Chem. 270, 26332–26340.
[13] Berridge, M.J. (1993) Nature 361, 315–325.
[14] Bleasdale, J.E., Thankur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J. and Bunting, S. (1990) J. Pharm. Exp. Ther. 225, 756–768.
[15] Kikuchi, A., Ikeda, K., Kozasa, O. and Takai, Y. (1987) J. Biol. Chem. 262, 6766–6770.