Evidence for the presence of multiple forms of Sph kinase in human platelets

Yoshiko BANNO^{*1}, Makiko KATO[†], Akira HARA[†] and Yoshinori NOZAWA^{*}

^{*}Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500-8705, Japan, and [†]Laboratory of Biochemistry, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502-0003, Japan

The intracellular distribution of sphingosine (Sph) kinase activity was examined in human platelets. A large proportion (72 %) of the total activity was found to be associated with the membrane fraction, and the membrane-associated fraction had higher specific activity compared with the cytosolic enzyme. Most of the membrane-associated activity could be extracted with 1 M NaCl. The cytosolic activity was unstable upon heat treatment, with 80 % of the activity being lost during incubation at 45 °C for 1 h,

whereas the NaCl-extractable fraction was stable under the same conditions. When subjected to Mono Q column chromatography, the cytosolic fraction produced two activity peaks and the NaCl-extractable fraction gave a single peak. These three Sph kinase activities showed different responses to stimulation by β -octyl-glucoside and inhibition by *N,N*-dimethylsphingosine and *L-threo*-dihydrosphingosine, suggesting the presence of multiple enzyme forms in human platelets.

INTRODUCTION

Sphingosine (Sph) and other metabolic products of sphingomyelin hydrolysis have recently been found to affect a wide variety of biological activities [1,2]. Sphingolipids may be involved in cell signalling via agonist-induced activation of the sphingomyelin cycle, which results in the hydrolysis of sphingomyelin to generate ceramide, Sph and sphingosine-1-phosphate (Sph-1-P) [3–5]. Earlier studies have implicated Sph as a negative modulator of the phosphoinositide-mediated signal transduction by inhibition of protein kinase C activity [3]. Moreover, Sph is considered to be involved in a multitude of responses in cells [1]. Ghosh et al. [6] have proposed that Sph is converted intracellularly into Sph-1-P via Sph kinase, which mediates calcium release from the inositol 1,4,5-trisphosphate (IP3)-insensitive intracellular calcium pool. Antigen receptor activation in rat mast cells has been shown to induce activation of Sph kinase, leading to production of Sph-1-P, an alternative second messenger for IP3-independent calcium mobilization [7]. Furthermore, Sph-1-P is considered to play a role in cellular proliferation as a weak mitogen in Swiss 3T3 cells [8,9] and also stimulates the production of phosphatidic acid through the activation of phospholipase D [10,11]. A recent study has demonstrated that Sph-1-P, which is stored in platelets, specifically activates the heterotrimeric G-protein-coupled orphan receptor Edg-1 [12,13]. Platelets are known to have a very high Sph kinase activity, but lack Sph-1-P lyase activity, thereby resulting in an abundant accumulation of Sph-1-P [14,15]. Recent studies have demonstrated that agonist stimulation of platelets induces Sph-1-P release into the medium, which, like lysophosphatidic acid, is capable of activating platelets as an autocrine stimulator [16,17].

Sph kinase activity is present in various tissues and cells [18,19], but complete purification of the enzyme from any source has yet to be achieved. Early studies showed that Sph kinase is a soluble cytoplasmic enzyme in platelets [20,21] and is per-

ipherally associated with membranes in rat brain [22]. In the protozoan *Tetrahymena pyriformis*, the enzyme activity is tightly associated with microsomal membranes [23]. In rat brain [20] and human platelets [24], the enzymes were inhibited by *L-threo*-dihydrosphingosine and *N,N*-dimethylsphingosine. Acidic phospholipids, particularly phosphatidylserine (PS), are known to stimulate a cytosolic Sph kinase activity in Swiss 3T3-fibroblasts [25]. The bovine brain enzyme has been partially purified and appeared to exist in multiple forms [26]. However, little is known about the structures and regulatory mechanisms of the enzymes.

The present study demonstrates that most of the enzyme activity is associated with the membrane fraction in human platelets and also suggests that, based on differences in properties between the cytosolic enzyme and the high salt-extractable membrane-associated enzyme, multiple forms of Sph kinase are present in human platelets.

EXPERIMENTAL

Materials

Sph, Sph-1-P, *L-threo*-dihydrosphingosine, *N,N*-dimethylsphingosine, PS (bovine) and phosphatidic acid (egg) were purchased from Matreya, Inc. (Pleasant Gap, PA, U.S.A.). Caldiolipin was from Sigma (St. Louis, MO, U.S.A.). *D-erythro*-[³H]Sph (specific radioactivity, 16.7 Ci/mmol) was from Du Pont-New England Nuclear (Boston, MA, U.S.A.). The Mono Q (HR5/5) column was obtained from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). *trans*-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64, a cysteine proteinase inhibitor) was from Peptide Institute, Inc. (Osaka, Japan).

Preparation of the cytosol, membrane and NaCl-extractable fractions from human platelets

Out-dated human platelet concentrates obtained from The Red Cross were centrifuged for 5 min at 200 *g* to sediment erythro-

Abbreviations used: E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; IP3, inositol 1,4,5-trisphosphate; PS, phosphatidylserine; Sph, sphingosine; Sph-1-P, sphingosine-1-phosphate.

¹ To whom correspondence should be addressed (e-mail banno@cc.gifu-u.ac.jp).

cytes, and the supernatants were then centrifuged for 15 min at 2000 *g*. The pelleted platelets were washed three times with Hepes/Tyrode buffer (129 mM NaCl/8.9 mM NaHCO₃/0.8 mM KH₂PO₄/0.8 mM MgCl₂/10 mM Hepes, pH 7.4/2 mM EGTA). The washed platelets were resuspended in buffer A (20 mM Tris/HCl, pH 7.4/1 mM EGTA/20 µg/ml E-64/1 mM PMSF/1 mM dithiothreitol), sonicated and centrifuged at 1 × 10⁵ *g* for 1 h at 4 °C. The cytosolic and membrane fractions were stored separately at -80 °C.

The membrane fraction was resuspended in buffer A containing 1 M NaCl, stirred for 1 h at 4 °C and then centrifuged for 1 h at 1 × 10⁵ *g* to obtain the NaCl-extractable fraction. This fraction was dialysed against buffer A for 16 h at 4 °C, then centrifuged at 1 × 10⁵ *g* for 1 h.

Assay for Sph kinase activity

The Sph kinase activity was assayed as described by Olivera et al. [25], with minor modification. Briefly, Sph and [³H]Sph substrate were delivered from ethanol solution and dried under a stream of nitrogen. The substrate (0.25 mM; specific radioactivity 1600 c.p.m./nmol) was resuspended by bath sonication in 0.4 % (w/v) BSA solution. The reaction mixture (160 µl) contained 20 mM Tris/HCl, pH 7.4, 2.5 mM MgCl₂, 0.25 mM EDTA, 5 % (w/v) glycerol, 1.2 mM dithiothreitol, 1 mM ATP and enzyme protein. The reaction was started by addition of 40 µl of [³H]Sph-BSA complex substrate, allowed to proceed for 30 min at 30 °C, then terminated by addition of 20 µl of 1 M HCl followed by 0.8 ml of chloroform/methanol/HCl (100:200:1). After vigorous vortexing, 240 µl of chloroform and 240 µl of 1 M KCl were added and the suspension was separated by centrifugation at 2000 *g* for 10 min. The lipids in the organic phase were separated, concentrated and applied to a silica gel 60 HPTLC plate. The plate was developed in butan-1-ol/acetic acid/water (3:1:1), then sprayed with ninhydrin reagent to reveal lipids. The silica gel areas corresponding to authentic standard Sph-1-P were scraped off and their radioactivity was counted in a liquid-scintillation counter (Beckman LS 6500).

Separation of the Sph kinases by Mono Q column chromatography

The cytosolic and NaCl-extractable fractions were dialysed against buffer A for 16 h and loaded onto a Mono Q HPLC column (HR5/5) equilibrated with buffer A. The columns were eluted at a flow rate of 1 ml/min with an increasing NaCl gradient of 0–0.05 M for 2 min, 0.05–0.5 M for 30 min, 0.5–1.0 M for 5 min and 1.0 M for 5 min. Each fraction was assayed for Sph kinase activity as described above.

RESULTS AND DISCUSSION

Distribution of Sph kinase in human platelets

Despite many recent studies, the intracellular distribution of Sph kinase remains controversial. It has been suggested, but not proven, that most of the enzyme activity is located in the cytosol in human platelets [20,21]. However, the Sph kinase has been reported to be associated with membranes in other sources such as bovine brain and protozoan *Tetrahymena* cells [22,23]. Although a cytosolic platelet Sph kinase has been characterized, little information is available regarding the biochemical properties of the membrane-associated Sph kinase.

To further examine the intracellular distribution of Sph kinase in human platelets, the sonicated lysates were fractionated into membrane and cytosolic fractions by centrifugation at 1 × 10⁵ *g* for 60 min. Consistent with previous reports [18,20], Sph kinase

Figure 1 Dependence of Sph kinase activity on protein concentration in cytosolic and membrane fractions of human platelets

The Sph kinase activity was measured at indicated protein concentrations of the cytosolic (A) and membrane (B) fractions from human platelets as described in the Experimental section. Results are the means ± S.D. of three experiments.

Table 1 Distribution of Sph kinase activity in human platelets

Human platelets were sonicated and the cytosol, membrane and NaCl-extractable fractions were separated. Sph kinase activity was measured in the three fractions as described in the Experimental section. Results are means ± S.D. of three experiments.

Fractions	Distribution (%)	Total activity (nmol/h)	Specific activity (nmol/h per mg of protein)
Lysate	100	363.00 ± 18.58	12.80 ± 2.55
Cytosol	24.41 ± 1.79	91.72 ± 4.70	9.36 ± 0.65
Membrane	72.31 ± 2.51	262.71 ± 10.31	13.35 ± 1.20
NaCl extract	—	183.25 ± 6.98	22.25 ± 1.57

activities in these two fractions displayed similar requirements for magnesium and ATP, and also pH optimum (7.0–7.6). When incubated with [³H]p-erythro-sphingosine under the assay conditions described previously [23], both fractions caused a dose-dependent increase in Sph-1-P formation (Figures 1A and 1B). However, the membrane-associated Sph kinase activity was much higher than that of the cytosolic enzyme and increased linearly for up to 300 µg of protein.

The membrane-associated Sph kinase activity was extracted with NaCl in a concentration-dependent manner (0.2–1.0 M). Half of the activity was extracted by stirring in 1 M NaCl solution for 1 h at 4 °C, and more than 80 % of the activity was released by the repeated extraction. On the other hand, the buffer without 1 M NaCl had only a marginal ability to release the activity from the membrane fraction, suggesting that the Sph kinase is loosely associated with the membranes in human platelets.

A quantitative comparison of the Sph kinase activity in the cytosol, membranes and 1 M NaCl-extractable fractions revealed that approx. 72 % of the activity was located in the membranes, with its specific activity being 1.5-fold higher than that of the cytosolic fraction (Table 1). Since out-dated human platelets were employed, we re-examined the proportional distribution in the above three fractions in freshly prepared human platelets and no significant differences were observed. Thus, the activity

Figure 2 Heat-stability of Sph kinase activity in cytosol and NaCl-extract of human platelets

The cytosol (□) and NaCl-extract (●) were incubated at protein concentrations of 0.2 mg/ml in 20 mM Tris/HCl buffer, pH 7.4, at the indicated temperature for 1 h. Sph kinase activity was then measured as described in the Experimental section. Results are the mean $\pm$ S.D. of three experiments.

Figure 3 Elution profiles of Sph kinase activity in cytosol and NaCl-extract from human platelets on Mono Q column chromatography

Cytosol (□) and NaCl-extract (●) were applied to Mono Q columns and eluted with increasing NaCl gradients (broken line 0.05–0.5 M), and Sph kinase activity in each 5 μ l fraction was measured as described in the Experimental section. The results are representative of three experiments. The three activity fractions (C1, C2 and M1) were collected as indicated.

distribution in the out-dated platelets may reflect the proportional distribution in fresh platelets.

Biochemical properties of Sph kinase activities in cytosol and NaCl-extract of human platelets

Sph kinase activity is reported to be highly unstable and to decrease during purification [18]. The heat-stability of platelet cytosolic and NaCl-extractable Sph kinases was examined by incubating them for 1 h at between 4 °C and 45 °C. As shown in Figure 2, the activities of the two fractions displayed different responses to heat treatment. The cytosolic Sph kinase was unstable on heat treatment, 80 % of the activity was lost during incubation at 45 °C for 1 h, whereas the NaCl-extractable enzyme was highly stable, showing no significant changes in activity after 1 h at 45 °C.

Figure 4 Concentration-dependent inhibition of Sph kinase activity in C1 and M1 fractions of human platelets by *N,N*-dimethylsphingosine and *L*-threo-dihydrosphingosine

C1 (□) and M1 (●) fractions were incubated with 50 μ M [3 H]Sph-BSA and various concentrations of *N,N*-dimethylsphingosine (A) and *L*-threo-dihydrosphingosine (B), as described in the Experimental section. Results are expressed as percentage inhibition of the control without the inhibitors. Results are means $\pm$ S.D. of three experiments.

For further characterization, the cytosolic and NaCl-extractable Sph kinases were chromatographed on Mono Q columns. The cytosolic fraction yielded two major activity peaks (C1, fractions 3–6; and C2, fractions 7–10) in the fractions eluted with approx. 0.15 M and 0.25 M NaCl respectively (Figure 3). On the other hand, the NaCl-extractable fraction gave a single broad activity peak (M1, fractions 5–9) eluted with between 0.15 and 0.25 M NaCl. The fractions constituting these three peaks were pooled for further characterization. Cytosolic Sph kinase activity in Swiss 3T3 cells is reported to be stimulated by acidic phospholipids, particularly PS, and to a lesser extent by phosphatidic acid, phosphatidylinositol and caldiolipin [25]. However, none of the three platelet Sph kinase activities was affected by these phospholipids without Triton X-100 (results not shown). This discrepancy may be due to differences in isoforms.

N,N-Dimethylsphingosine and *L*-threo-dihydrosphingosine are known to be potent inhibitors of Sph kinase [22,24]. Their effects on the above three Sph kinase activities from human platelets were examined. As shown in Figure 4(A), dimethylsphingosine markedly inhibited the enzyme activity in the M1 fraction, IC_{50} being about 5 μ M. It also inhibited the activity in the C1 fraction, but to a lesser extent, with 50 % inhibition at about 80 μ M. *L*-threo-Dihydrosphingosine similarly inhibited the Sph kinase activity in the M1 fraction: more than 70 % of the activity was inhibited at 10 μ M (Figure 4B). The activity of the cytosolic fraction (C1) was less sensitive to the inhibitor; the two inhibitors had similar effects on the activities of two cytosolic fractions (C1 and C2).

Detergents such as Triton X-100 and β -octyl glucoside have been shown to exert stimulatory effects on rat brain Sph kinase activity [22]. The effects of β -octyl glucoside were examined on the three Sph kinase fractions from human platelets. The activity

Figure 5 Effect of β -octyl glucoside on Sph kinase activity in the three fractions (C1, C2 and M1) of human platelets

β -Octyl glucoside was added in sonicated [3 H]Sph-BSA at the indicated concentrations and the Sph kinase activity was measured in the C1 (□), C2 (△) and M1 (●) fractions as described in the Experimental section. Results are the means $\pm$ S.D. of three experiments.

in the C1 fraction was most greatly stimulated by β -octyl-glucoside, showing approx. 2.5-fold enhancement at 3 mM (Figure 5). The M1 fraction was much less stimulated, and the activity in the C2 fraction was slightly inhibited at low concentration of the agent.

The results presented here suggest that multiple forms of Sph kinase activity are present and also that the major part of Sph kinase is associated with the membrane fraction in human platelets. A recent study has demonstrated that a cytosolic Sph kinase activity of Swiss 3T3 cells is stimulated by PS and other acidic phospholipids [25]. However, the platelet membrane-associated Sph kinase was less sensitive to these acidic phospholipids without Triton X-100. These results imply that differences in Sph kinase isoenzymes may reflect differences in cell types. However, the presence of isoenzymes of Sph kinase in human platelets can only be proved by purifying each enzyme to

homogeneity. Further purification of Sph kinases from human platelets is now in progress in our laboratory.

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