Inhibition of Tyrosylprotein Sulfotransferase by Sphingosine and Its Reversal by Acidic Phospholipids†

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ABSTRACT: Although tyrosylprotein sulfation has been implicated in the processing of several secretory proteins, nothing is known about the regulation of the enzyme responsible for this event. When poly(Glu_6, Ala_3, Tyr_1) (EAY; M_r 47 000) was employed as sulfate acceptor, the tyrosylprotein sulfotransferase (TPST) from Golgi membranes of submandibular salivary gland was used to study the effect of various lipids on the expression of its activity. The TPST activity in the Golgi membrane was 38 pmol (mg of protein)$^{-1}$ (30 min)$^{-1}$. Approximately 90% of the total activity present in Golgi membranes was extracted by NaCl and Triton X-100 treatment. The K_m values of solubilized TPST for EAY and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were 0.04 and 0.25 μM, respectively. Among the various lipids tested, sphingosine showed maximum inhibition of TPST activity followed by sphingomyelin and phosphatidylcholine (PC). Of the two sphingosine analogs tested, threosphinganine was as effective as sphingosine in TPST inhibition, while erythrosphinganine had little effect. In contrast, the acidic phospholipids phosphatidylinositol (PI) and phosphatidylserine (PS) and oleic acid showed slight stimulation. Half-maximal inhibition of TPST was obtained at 150 μM sphingosine (6 mol % when expressed as mole percent of sphingosine to total phospholipids plus Triton X-100). The inhibition was competitive with respect to EAY and uncompetitive with respect to PAPS. The inhibition caused by sphingosine could be reversed by PI, PS, and oleic acid but not by PC and sphingomyelin. Sphingosine inhibition of TPST activity was also observed in the enzyme isolated from several other tissues such as liver, lung, heart, and cerebellum. These results suggest that lipids may play an important role in the regulation of TPST activity.

Ever since the identification of tyrosine sulfation in bovine fibrinogen (Huttner & Baeuerle, 1988), protein sulfation has been recognized for its significant role in the processing of secretory proteins. This widespread posttranslational modification of proteins and peptides such as cholecystokinin, fibrinopeptide B, gastrin, phyllokinin, leucosulfakinin, and C_4 complement affects their biological activities (Mutt, 1980; Bettelheim, 1954; Gregory et al., 1964; Anastasi et al., 1966; Nachman et al., 1986; Hortin et al., 1988). The enzyme responsible for the sulfation of protein on tyrosine residues is tyrosylprotein sulfotransferase (TPST),[1] an integral membrane protein of Golgi (Lee & Huttner, 1985). Although TPST activity has been identified in several tissues (Domiano & Roth, 1989), including gastric mucosa and submandibular salivary gland (Sundaram et al., 1992), no information is available on the regulation of this enzyme.

Lipids, the structural components of biological membranes, and their metabolic products have been shown to play an important role in cell growth and function (Hannun & Bell, 1989; Nishizuka, 1986; Majerus et al., 1988), as well as in the regulation of several enzyme activities. Activation of protein kinase C by phosphatidylserine (PS) and diglyceride and of cytidylyltransferase by phosphatidylcholine (PC) and oleic acid are a few of these enzyme activities (Nishizuka, 1986; Paul et al., 1986). A recent addition to this biologically and physiologically active group of lipids is sphingolipids (Hannun & Bell, 1989). Sphingolipid synthesis starts in the endoplasmic reticulum (ER) by the condensation of serine with palmitoyl-CoA by serine palmitoyltransferase (Mandon et al., 1992). The condensation product, 3-dehydrosphinganine, is then converted to D-erythrosphinganine and then to sphingosine (Brady et al., 1969). D-Erythrosphinganine could also be used directly for the synthesis of dihydroceramide by sphingosine *N*-acyltransferase [for a review, see Kishimoto (1983)]. The sphingolipid synthesizing enzymes responsible for the formation of dihydroceramide are present in ER while the latter enzymes are present in Golgi (Futerman et al., 1990; Jeckel et al., 1990; Schwarzmann & Sandhoff, 1990). Sphingosine, sphinganine, and phytosphingosine, the intermediates in the biosynthesis and catabolism of sphingomyelin and glycosphingolipids, exhibit various pharmacological effects in cells such as inhibition of growth factor action (Hanai et al., 1987; Hall et al., 1988) and phorbol ester induced responses (Merril et al., 1986; Grove & Mastro, 1988), and in vitro they are known to regulate activities of casein kinase II (McDonald et al., 1991), phospholipase D (Kiss & Anderson, 1990), cytidylyltransferase (Parmjit & Rosemary, 1990), and protein kinase C (Hannun et al., 1986).

In naturally occurring substrates of TPST, the sulfated tyrosyl residues are predominantly surrounded by acidic amino acids (Hortin et al., 1986), and the frequency distribution of basic amino acids in these sites is extremely low (Niehrs et al., 1990). Recently, Lin et al. (1992) have shown increased tyrosine sulfation of substrates containing acidic amino acids by TPST. These studies suggest that acidic environments are

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[1] Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; [^{35}S]PAPS, 3′-phosphoadenosine 5′-phospho[^{35}S]-sulfate; EAY, poly(Glu_6, Ala_3, Tyr_1); MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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the important determinants for tyrosine sulfation. Since lipids, the constituents of biological membranes, are acidic and basic in nature, we have examined the effect of various lipids on TPST activity. The results demonstrate, for the first time, that the activity of TPST may be regulated by lipids.

EXPERIMENTAL PROCEDURES

Materials. Male Sprague-Dawley rats, 8 weeks old with a mean weight of 175 g, were obtained from Charles River Laboratories, Wilmington, MA. [^{35}S]PAPS (1.5 Ci/mmol) was from New England Nuclear, Boston, MA. EAY (M_r 47 000), sphingosine, psychosine, erythrosphinganine, threosphinganine, stearylamine, octylamine, sphingomyelin, oleic acid, PC, PS, phosphatidylinositol (PI), and lysoPC were obtained from Sigma.

Subcellular Fractionation. Freshly dissected rat submandibular glands were gently washed with cold 0.15 M NaCl, fat was removed, and the glands were weighed. The glands were cut into small pieces, suspended in 5 volumes of STKM buffer [50 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM Mg-$(CH_3COO)_2$, 5 mM 2-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride] containing 0.25 M sucrose, and homogenized first in a polytron PT-20 (Brinkmann Instruments Inc.) for 10 s at half-maximal speed. The resulting homogenate was again homogenized in a motor-driven Teflon homogenizer using six strokes at 300 rpm, passed through a nylon screen, and centrifuged at 5000g for 20 min. The 5000g supernatant was layered on a discontinuous gradient of 0.5–1.2 M sucrose in STKM buffer (Balch et al., 1984; Kasinathan et al., 1990). The gradients were centrifuged for 2.5 h at 25 000 rpm (90000g) in the SW 40 rotor. The light brown material at the 0.5 M/1.2 M sucrose interface (Golgi-rich) was collected, diluted with half-concentrated STKM buffer, and centrifuged at 100000g for 1 h. The pellet was suspended in 10 mM Tris-HCl, pH 6.8, containing 20% glycerol and stored at −70 °C prior to use. Protein concentration was estimated by the BCA (no. 23225) protein assay reagent (Pierce Chemical Co.).

To study the effect of sphingosine on TPST activity of different tissues, the appropriate tissues were homogenized and subjected to subcellular fractionation as for salivary gland. The Golgi membranes isolated at the 0.5/1.2 M sucrose interface were used as the enzyme source after partial purification.

Tyrosylprotein Sulfotransferase Isolation. The partial purification of tyrosylprotein sulfotransferase was carried out as described by Domiano et al. (1989). The submandibular Golgi membranes were suspended in 10 mM HEPES buffer, pH 7.2, containing 25% glycerol, 1 mM NaCl, and 1 mM DTT. The suspension was mixed on ice for 30 min and centrifuged for 1 h at 134000g. The resulting pellet was dissolved in 10 mM HEPES, pH 7.2, containing 25% glycerol, 0.2 M NaCl, 1 mM DTT, and 1% Triton X-100, mixed for 30 min on ice, and centrifuged as before. The supernatant was used for the tyrosylprotein sulfotransferase assay. Enzyme isolations from other tissues were performed as for salivary gland.

Preparation of Phospholipid Suspension. Sphingosine and other lipids were dried under N_2 to remove the organic solvents. After allowing the lipids to swell under vacuum for 18 h, phospholipid suspensions (1 or 1.3 mM) were prepared by sonication for 20 min in 10 mM MES buffer, pH 6.2, for submandibular TPST and in 10 mM MES buffer, pH 6.8, for TPST from other tissues. The lipid suspensions were used immediately (within 1 h) after preparation.

Table I: Isolation of Tyrosylprotein Sulfotransferase Activity from Golgi Membranes of Rat Submandibular Salivary Gland[a]

fraction	tyrosylprotein sulfotransferase act. [pmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]
homogenate	1.9 ± 0.1
Golgi	37.9 ± 3.2
Triton X-100 supernatant	46.5 ± 5.6

[a] The sulfate acceptor EAY of the tyrosylprotein sulfotransferase assay was present at a concentration of 3 μM for the homogenate and Golgi membrane and at 1 μM for the Triton X-100 supernatant. A concentration of EAY above 1 μM inhibited the TPST activity of the Triton X-100 supernatant. The other condition for tyrosylprotein sulfotransferase assay was as described under Experimental Procedures. Values are the mean ● SE of three determinations.

Assay of Tyrosylprotein Sulfotransferase. The tyrosylprotein sulfotransferase assay mixture contained the following components: acceptor, unsulfated EAY at concentrations indicated in the text, 2 μM [^{35}S]PAPS (400 000 cpm), 0.1% Triton X-100, 50 mM NaF, 20 mM $MnCl_2$, 2 mM 5′-AMP, 20 mM MES buffer at pH 6.2 for salivary TPST and at pH 6.8 for TPST from other tissues, and 10–40 μg of enzyme protein, in a final volume of 50 μL. Sphingosine and other lipids were added at the concentrations indicated in the text. Assays were initiated by the addition of the enzyme protein and run for 30 min at 37°C. The reactions were stopped by spotting a 40-μL aliquot of the reaction mixture on 2.4 × 2.4 cm Whatman 3MM filter paper (Domiano & Roth, 1989). The papers were washed 3 times for 15 min in 10% trichloroacetic acid/10 mM Na_2SO_4 and rinsed 5 min in 95% ethanol. The dried papers were placed in vials containing scintillation solution and counted in a scintillation counter, TriCarb-1500.

RESULTS

In our earlier report, we presented evidence for a highly active TPST in rat submandibular salivary gland (Sundaram et al., 1992) which had a pH optimum different from that of TPST from other tissues (Domiano & Roth, 1989). In this study, we have solubilized the enzyme from submandibular gland Golgi membranes and investigated the effect of various lipids on its activity. Table I shows the isolation of TPST activity from Golgi membranes. The specific activity present in the Golgi membrane was 20 times higher than that of the homogenate. Upon NaCl treatment and solubilization with Triton X-100, over 90% of the membrane-bound enzyme activity was released into the soluble fraction with 24-fold higher activity compared to homogenate. Solubilization of the enzyme protein from Golgi membranes prior to NaCl treatment released only 40% of the membrane-bound activity.

Initially, experiments were carried out to determine the substrate concentrations required for the partially purified TPST. The effect of EAY and PAPS on TPST activity is shown in Figure 1. The sulfation of EAY increased with increasing concentration of EAY and PAPS, reaching saturation at 0.5 and 1 μM concentrations, respectively. The K_m value of the enzyme for EAY was 0.04 μM and for PAPS 0.25 μM.

Sphingosine inhibited TPST activity in a concentration-dependent manner (Figure 2). Half-maximal inhibition was observed at approximately 150 μM. Sphingomyelin, the parental lipid of sphingosine, was only 10% inhibitory at 200 μM, the concentration at which sphingosine showed 75% inhibition. At lower EAY concentration (0.1 μM), the sphingosine concentration required for half-maximal inhibition

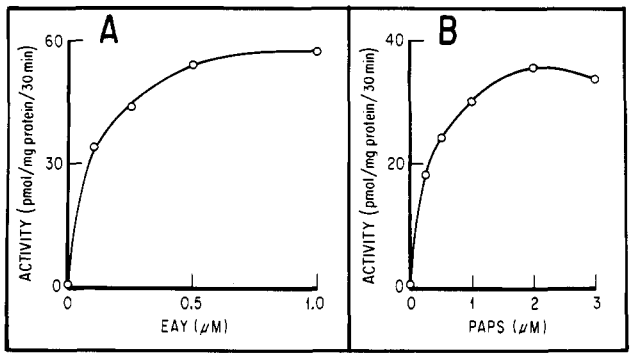

FIGURE 1: Effect of EAY and [^{35}S]PAPs concentrations on the activity of solubilized submandibular tyrosylprotein sulfotransferase. (A) The activity was measured with varying EAY concentration. (B) The enzyme was assayed at different PAPS concentrations. Reactions were carried out for 30 min at 37 °C and processed as described under Experimental Procedures.

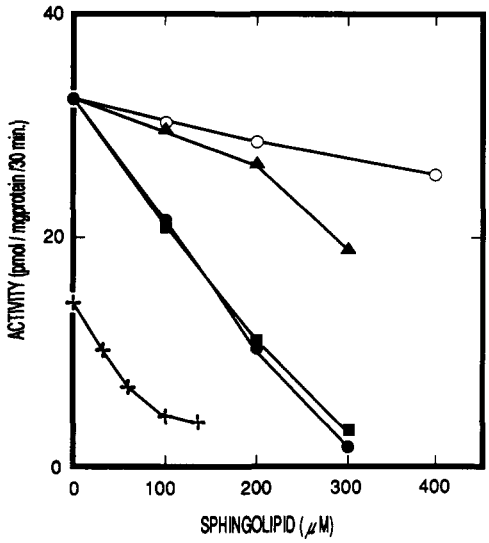

FIGURE 2: Inhibition of tyrosylprotein sulfotransferase activity by sphingolipids. The reactions were carried out as described under Experimental Procedures at 0.25 μM EAY and increasing concentrations of sphingosine (●), erythrosphinganine (▲), threosphinganine (■), and sphingomyelin (○). The effect of sphingosine at 0.1 μM EAY is also shown (+).

was decreased to 30 μM. Next, to test the specificity of sphingosine inhibition, the effect of sphingosine analogs such as threo- and erythrosphinganine on TPST activity was examined. Threosphinganine showed similar inhibition, while erythrosphinganine was less effective compared to sphingosine. These analogs showed a similar effect on protein kinase C (Igarashi et al. 1989) and phospholipase D (Kiss & Anderson, 1990). The effect of various lipids and sphingosine-related compounds on TPST is shown in Table II. At 200 μM, sphingosine and the sphingosine-related lipid psychosine were the most potent inhibitors of TPST activity. Psychosine inhibited the enzyme slightly more than sphingosine. Sphingomyelin and PC also inhibited TPST, however, to a lesser extent compared to sphingosine, while oleic acid, PS, PI, and lysoPC slightly stimulated the enzyme. Further, to determine if the inhibition is attributable solely to the presence of free amino groups, the effect of stearylamine and octylamine on TPST activity was ascertained. Stearylamine inhibited TPST, whereas octylamine had less effect on the enzyme activity. Amino acids such as glutamate, aspartate, glutamine, asparagine, and serine at 100 or 300 μM produced no change in TPST activity (data not given). These results suggest that the inhibitory potency is a function of the hydrocarbon chain

Table II: Effect of Lipids and Sphingosine-Related Compounds on Tyrosylprotein Sulfotransferase Activity in Rat Submandibular Salivary Glands[a]

lipid (200 μM)	tyrosylprotein sulfotransferase act. (% control)
none	100
phosphatidylcholine	81 ± 5
phosphatidylserine	117 ± 10
phosphatidylinositol	123 ± 12
sphingomyelin	80 ± 8
sphingosine	25 ± 5
psychosine	8 ± 6
stearylamine	44[b]
octylamine	78[b]
oleic acid	119 ± 5
lysophosphatidylcholine	120 ± 3

[a] The EAY concentration was 0.25 μM. Phospholipids were added as suspensions to incubation medium. Tyrosylprotein sulfotransferase activity was measured as described under Experimental Procedures. One hundred percent activity is 35.7 pmol (mg of protein)$^{-1}$ (30 min)$^{-1}$. Values are mean ± SE of three determinations. [b] Mean of duplicate determinations.

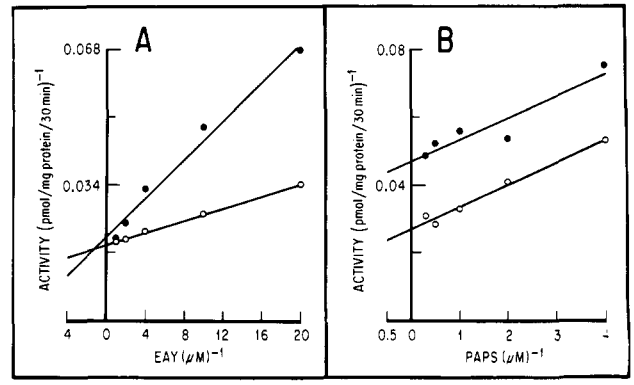

FIGURE 3: Effect of sphingosine on tyrosylprotein sulfotransferase activity at varying substrate concentration. Increasing concentrations of EAY (A) and [^{35}S]PAPS (B) were added to the assay medium with 150 μM sphingosine (●) or without sphingosine (○). The tyrosylprotein sulfotransferase activity was measured as described under Experimental Procedures.

length. The structural requirements for TPST inhibition appear to include a free amino group and a long alkyl chain.

To further understand the mechanism involved in sphingosine inhibition of TPST, we investigated the sphingosine effect at various concentrations of EAY and PAPS (Figure 3). At fixed PAPS concentration and varying EAY concentration, sphingosine increased the K_m value for EAY from 0.04 to 0.23 μM (Figure 3A). The V_{max} remained the same. When the PAPS concentration was varied, sphingosine decreased both the K_m (from 0.24 to 0.14 μM) and the V_{max} [from 37.3 to 21.3 pmol (mg of protein)$^{-1}$ (30 min)$^{-1}$]. Thus, as evidenced from the double-reciprocal plots, the sphingosine effect on TPST activity appears to be competitive with respect to EAY and uncompetitive with respect to PAPS.

To test the role of other phospholipids in relieving sphingosine inhibition, the experiment shown in Figure 4 was carried out. Tyrosylprotein sulfotransferase activity was assayed at 0.25 μM EAY with increasing concentrations of sphingosine. At 200 μM sphingosine, exhibiting an inhibition of approximately 75% enzyme activity, the sphingosine concentration was fixed, and the concentrations of other lipids were increased. Increasing the concentration of PI from 0 to 200 μM gradually abolished the sphingosine inhibition of TPST activity with complete removal of inhibition at 200 μM PI. Phosphatidylserine showed a similar effect. Oleic acid also reversed the

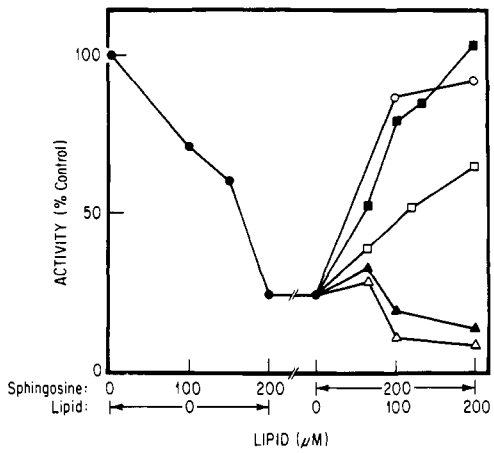

FIGURE 4: Reversal of the inhibition of tyrosylprotein sulfotransferase by acidic phospholipids and oleic acid. Tyrosylprotein sulfotransferase activity was measured as described under Experimental Procedures at 0.25 μM EAY and increasing concentrations of sphingosine. At 200 μM sphingosine, approximately 75% inhibition was attained; the PI (■), PS (○), oleic acid (□), PC (△), and sphingomyelin (▲) concentrations were increased as indicated.

Table III: Effect of Sphingosine on Tyrosylprotein Sulfotransferase Activity of Various Rat Tissues[a]

tissue	tyrosylprotein sulfotransferase act. (% control)
submandibular salivary gland	28
liver	76
lungs	29
heart	10
cerebellum	3

[a] Tyrosylprotein sulfotransferase activity was isolated from the Golgi membranes obtained from various tissues of rat as described under Experimental Procedures. Enzyme activity was measured in the presence of 0.25 μM EAY and 2 μM [^{35}S]PAPS and in the presence and absence of 200 μM sphingosine. The pH optimum was 6.2 for salivary enzymes and 6.8 for the enzymes from other tissues. The protein concentration in a 50-μL assay varied from 20 to 40 μg. The 100% activities in salivary gland, liver, lungs, heart, and cerebellum were 37.6, 21.2, 2.7, 1.7, and 32.1 pmol (mg of protein)$^{-1}$ (30 min)$^{-1}$, respectively.

inhibition but to a lesser extent. These results suggest that sphingosine inhibition of TPST activity was prevented by PI, PS, and oleic acid and that the action of these lipids was competitive with sphingosine. However, PC and sphingomyelin not only failed to remove the inhibition but rather increased the inhibition from approximately 80% to 95% at 200 μM lipid concentration.

To determine the sphingosine effect on the TPST activity in other tissues, the enzyme was solubilized from Golgi membranes of liver, lung, heart, and cerebellum, and its activity was measured in the presence and absence of sphingosine (Table III). Sphingosine at 200 μM exhibited maximum inhibition on the cerebellum TPST activity followed by heart, lung, and liver. However, it must be noted that the sphingosine effect observed on TPST in other tissues is only approximate and the true inhibition, after optimization of the enzyme activity in appropriate tissues, may differ. Further, the variations observed with TPST inhibition by sphingosine in different tissues are most probably due to the differences in the solubilized membranes rather than the differences in the enzymes of various organs.

DISCUSSION

Sphingosine and lysosphingolipids, the metabolic products of sphingolipids, regulate the activities of several enzymes, including activation of casein kinase II (McDonald et al., 1991), epidermal growth factor receptor kinase (Faucher et al.,1988), and phospholipase D (Kiss & Anderson, 1990) and inhibition of protein kinase C (Hannun et al., 1986), cytidylyltransferase (Parmjit & Rosemary, 1990), phosphatidate phosphohydrolase (Mullmann et al., 1991), and calmodulin-dependent protein kinase (Jefferson & Schulman, 1988). It also inhibits the binding of factor VII to tissue factor (Conkling et al., 1989) and thyrotropin releasing hormone to its receptor (Winicov et al., 1990). The concentrations at which sphingosine affects thses biological functions vary from 5 mol % for protein kinase C (Hannun et al., 1986) to 25 mol % for cytidylyltransferase (Parmjit & Rosemary, 1990). In the study presented herein, we report the inhibition of TPST, a Golgi enzyme, in rat submandibular salivary glands and also in other tissues such as liver, cerebellum, heart, and lung by sphingosine. In submandibular salivary gland, 6 mol % sphingosine was required for the half-maximal inhibition of TPST, which is in the range of concentrations required for the inhibition of protein kinase C in vitro and in platelets (Hannun et al., 1986). Under the assay condition of TPST which contained 1400 μM Triton X-100, the bulk concentration of phospholipid from Golgi was 924 μM. Therefore, 150 μM sphingosine is equivalent to 6 mol %. At lower EAY concentration (0.1 μM), the IC_{50} of sphingosine for TPST was further reduced to 1.2 mol %, suggesting that the activity of the enzyme would depend on the concentrations of tyrosylprotein and sphingosine, and also of the acidic phospholipids, PI and PS, since they exert a positive effect on TPST activity. Thus, long-chain bases and acidic lipids may play a role in the regulation of TPST-catalyzed posttranslational sulfation of secretory proteins as they regulate other cellular processes (Hannun et al., 1986; Hannun & Bell, 1989).

In PC 12 cells, 2-chloroadenosine, an adenosine receptor agonist, decreases the TPST activity in the Golgi apparatus (Lin et al., 1991). The mechanism by which 2-chloroadenosine regulates TPST activity is not known. Since sphingomyelin, PS, and PI are the lipid components of Golgi membrane (Hof & Meer, 1990; Keenan & Morre, 1970; Vance & Vance, 1988), which houses TPST, it is possible that sphingosine and acidic phospholipids may participate in regulating the TPST activity. Extracellular stimulation of sphingomyelinase by agonist to form ceramide and then to sphingosine by ceramidase has been suggested earlier (Hannun & Bell, 1989; Meril & Stevens, 1989). Sphingosine thus formed in response to agonists may modulate TPST activity in cells. Currently, no information is available on the subcellular location of these long-chain bases (Hannun & Bell, 1989). Determination of the subcellular localization of sphingosine and its analogs and then their intracellular levels along with acidic lipids in response to 2-chloroadenosine may provide some information.

Our data show that sphingosine inhibition was reversible by such lipids as PI and PS and also by oleic acid. The participation of PI in TPST regulation is quite interesting since it may provide a link between PI metabolism and tyrosine sulfation. A similar phenomenon was observed with protein kinase C (Hannun et al., 1986) and cytidylyltransferase (Parmjit & Rosemary, 1990). Furthermore, the above findings are in accord with the finding by Lin et al. (1992). In their study, substitution of basic residues in place of acidic amino acids adjacent to tyrosyl residues in protein substrates decreased the affinity of TPST. Conversely, the acidic residues in this region promoted tyrosine sulfation by increasing the affinity of TPST for protein substrate, suggesting that tyrosine sulfation may be regulated by acidic and basic environments.

Thus, the presence of either acidic lipids or acidic amino acids, provided they are adjacent to tyrosyl residues in protein substrates, would stimulate the enzyme, while the positive charges of sphingosine or basic amino acids would inhibit it. Alternatively, the presence of an acidic lipid environment may promote the interaction of TPST with protein substrates containing a relatively fewer number of acidic residues near the tyrosine.

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