Identification and subcellular localization of sphinganine-phosphatases in rat liver

Patrick DE CEUSTER, Guy P. MANNAERTS and Paul P. VAN VELDHOVEN*
Katholieke Universiteit Leuven, Campus Gasthuisberg – Afdeling Farmakologie, Herestraat, B-3000 Leuven, Belgium

One of the primary products of [4,5-^{3}H]sphinganine phosphate, added to fibroblast cultures, is sphinganine [Van Veldhoven and Mannaerts (1994) Biochem. J. **299**, 597–601], implicating the physiological action of (a) hitherto unknown phosphatase(s). We have now further characterized this activity in rat liver. In homogenates, the dephosphorylation appeared to be catalysed by multiple enzymes. A low-affinity system was active at acidic pH, whereas at physiological pH values hydrolysis was carried out by a high-affinity enzyme. The latter was sensitive to Zn^{2+} and detergents and possessed a pH optimum of 7.5. Upon cell fractionation the major portion of the high-affinity activity was recovered in the nuclear and microsomal fractions. Further separation of the microsomal fraction showed an association predominantly with vesicles derived from the plasma membrane. Likewise, when plasma membranes were prepared from the nuclear fraction, the high-affinity phosphatase co-purified with the plasma membrane markers. From the differential effects of bivalent cations, chelators, water-soluble and amphiphilic phosphate esters, detergents and other compounds, it could be concluded that the plasma membrane-associated sphinganine-phosphatase activity is not due to alkaline phosphatase, dolichol-phosphatase, the *N*-ethylmaleimide-insensitive phosphatidate phosphatase or ceramide-phosphatase. The dephosphorylation observed at acidic pH in homogenates appeared also to be enriched in purified plasma membranes and might represent a side-activity of ceramide-phosphatase. We speculate that the high-affinity phosphatase, which is especially active in neuronal tissues, plays a role in the attenuation of bioactive phosphorylated sphingoid bases such as sphingenine phosphate, and propose to name it sphingosine-phosphatase.

INTRODUCTION

Lysosomal breakdown of sphingolipids results in the formation of sphingenine and related sphingoid bases such as sphinganine and 4*D*-hydroxysphinganine (in this paper commonly referred to as sphingosine) (see [1]). The subsequent catabolic steps, i.e. phosphorylation of the primary hydroxy group of the sphingoid bases, followed by a cleavage reaction generating a fatty aldehyde and phosphoethanolamine, occur outside the lysosomal compartment. The phosphorylation of sphingosine, catalysed by sphingosine kinase, was originally attributed to a cytosolic enzyme [2–5]. More recently, membrane-associated form(s) of kinase activity have also been reported [6,7] (for a review, see [8]). The cleavage of phosphorylated sphingosine, catalysed by sphingosine-phosphate lyase, occurs at the cytosolic site of the endoplasmic reticulum membrane [9] (for a review, see [10]). Besides being an intermediary catabolite, sphingenine phosphate is also endowed with bioactivity. It stimulates calcium release from intracellular stores in Swiss 3T3 cells [11,12], is mitogenic for Swiss 3T3 cells through stimulation of a phospholipase D activity that generates phosphatidate [13], inhibits invasiveness and haptotactic cell motility of B16 melanoma cells [14,15], and is generated in quiescent 3T3 cells upon exposure to platelet-derived growth factor (PDGF) and fetal calf serum [16]. Hence sphingenine phosphate might belong to a new class of lipid second messengers. This hypothesis is somewhat refuted by the apparent stability of sphingenine phosphate, when added exogenously to or generated in 3T3 and B16 cells, as reported initially by other groups [11,13,14]. In cultured fibroblasts, however, we demonstrated that the saturated analogue, sphinganine phosphate, is prone to a very fast turnover and that, besides the known cleavage reaction, another degradation pathway, namely dephosphorylation, is present [17]. Dephosphorylation could also be (partly) responsible for the transient nature of the rise in sphingenine phosphate levels, more recently observed in PDGF-stimulated Swiss 3T3 cells [16]. This rise was only discussed in terms of formation by phosphorylation and disappearance by cleavage.

Sphingenine- and sphinganine-phosphatase activities, which were not characterized further, have been described in homogenates of different rat tissues and cultured cells [10,18]. It is tempting to speculate that a phosphatase might be involved in the attenuation of bioactive sphingenine phosphate, while the lyase would take care of the catabolic pool [17]. Therefore we decided to further characterize the phosphatase(s) involved. Although sphingenine phosphate is the prevalent phosphorylated sphingoid base in Nature [19], the saturated analogue [4,5-^{3}H]sphinganine phosphate was mainly used as substrate to monitor the activity in rat liver. This tissue, which is more amenable to cell fractionation than cultured fibroblasts, was chosen as the enzyme source. Our results indicate that rat liver contains different enzymes that are capable of dephosphorylating sphinganine phosphate. A high-affinity enzyme with a neutral pH optimum was shown to be associated mainly with the plasma membrane and to be different from ceramide-phosphatase [20].

MATERIALS AND METHODS

Materials

Acetic anhydride, hexanoic anhydride and tetramisole were purchased from Janssen Chimica (Geel, Belgium). Semicarbazide and sodium deoxycholate were from Merck (Darmstadt, Germany). Thesit, CHAPS and octanoyl *N*-methylglucamide

Abbreviations used: CMC, critical micellar concentration; NEM, *N*-ethylmaleimide; PDGF, platelet-derived growth factor; sphinganine phosphate, sphinganine 1-phosphate; sphingenine phosphate, sphingenine 1-phosphate; sphingenylphosphocholine, sphingenyl-1-phosphocholine; sphingosine phosphate, sphingosine 1-phosphate.

* To whom correspondence should be addressed.

were from Boehringer (Mannheim, Germany). Sphingenine (D-sphingosine; 2D,3D form), sphinganine (D,L-dihydrosphingosine; 2D/L,3D/L isomers), sphingenylphosphocholine (sphingosylphosphocholine), phosphoethanolamine, *O*-phospho-D,L-serine, Brij-35, *N*-dodecylsarcosine.Na (Sarkosyl), L-α-lysophosphatidate (oleoyl; Na salt) and L-α-phosphatidate (from egg yolk; Na salt) were obtained from Sigma (St. Louis, MI, U.S.A.). *N*-Octyl-*NN*-dimethyl-3-ammonio-1-propanesulphonate and hexadecyl phosphate (Na salt) were from Calbiochem (San Diego, CA, U.S.A.). Propranolol/HCl (Inderal) was from ICI (Macclesfield, U.K.) and dodecyldimethylammonium oxide (30%, w/v) was from Serva (Heidelberg, Germany).

Preparation of lipids

Sphingenine phosphate (rac. 2D,3D/L form) [21], sphinganylphosphocholine (2D,3D isomer) [9,21], [4,5-^{3}H]sphinganine (2D,3D isomer) [9] and [4,5-^{3}H]sphinganine phosphate (2D,3D isomer) [9] were prepared as described before.

N-Acetyl-[4,5-^{3}H]sphinganine phosphate was prepared by acetylation of [4,5-^{3}H]sphinganine phosphate (3 μmol), dissolved in 1 ml of chloroform/methanol (1:1, v/v) containing a 4-fold molar excess of triethylamine, with a 10-fold molar excess of acetic anhydride. After 2 h at room temperature, another aliquot of anhydride (30 μmol) was added. After removal of the solvent, the residue was treated with methylamine [22] in order to hydrolyse the *O*-ester bond of any *O*-acetyl-*N*-acetyl-[4,5-^{3}H]sphinganine phosphate formed. After drying, 0.25 M acetic acid was added to the residue and the product was extracted into 1 vol. of diethyl ether. Analysis of the product by TLC on silica G 60A plates (0.25 mm thickness) revealed a single spot [R_f values of *O*-acetyl-*N*-acetyl-sphinganine phosphate and *N*-acetyl-sphinganine phosphate were respectively 0.44 and 0.30 in solvent system chloroform/methanol/water (60:35:8, by vol.)]. The radiochemical purity as determined by TLC was 97.5%.

N-Hexanoyl-[4,5-^{3}H]sphinganine phosphate was prepared as described above for *N*-acetyl-[4,5-^{3}H]sphinganine phosphate, using hexanoic anhydride as acylating reagent. After drying the acylation mixture, the residue was treated with 0.5 M NaOH in methanol/water (9:1, v/v) to hydrolyse the *O*-ester bond of any *O*-hexanoyl-*N*-hexanoyl-[4,5-^{3}H]sphinganine phosphate formed. After acidifying with HCl, the truncated ceramide phosphate was extracted into diethyl ether and purified by preparative TLC using chloroform/methanol/formic acid (65:25:10, by vol.) as solvent system. The R_f values for *N*-hexanoyl-sphinganine phosphate and *O*-hexanoyl-*N*-hexanoyl-sphinganine phosphate in this system were 0.48 and 0.73 respectively. The final radiochemical purity of *N*-hexanoyl-[4,5-^{3}H]sphinganine phosphate was 95%.

N-Acetyl- and *N*-hexanoyl-sphingenine phosphates were prepared in the same way as described above for the tritiated saturated analogues, except that sphingenine phosphate was used as starting product.

Subcellular fractionation

Male Wistar rats (body weight approx. 200 g), maintained on a standard chow diet, were killed by decapitation. Their livers (or other tissues) were removed and homogenized in 0.25 M sucrose/0.1% (v/v) ethanol. Fractionation of liver homogenates was done as described before [9]. The microsomal fraction was further separated by means of sucrose gradient centrifugation. Optimum resolution was obtained by layering an aliquot (2 ml) of a microsomal fraction derived from 2 g of rat liver on top of a 30–65% (w/v) linear sucrose gradient containing 5 mM Mops, pH 7.2 (25 ml), with an underlying cushion of 75% (w/v) sucrose (5 ml). The gradient was spun at 4 °C in a Beckman 55.2Ti fixed angle rotor for 2 h at 100000 *g*, using the slow acceleration mode. After centrifugation, gradient fractions were collected starting from the bottom.

The method of plasma membrane purification will be published elsewhere (details available on application to the authors). Briefly, a nuclear fraction was prepared from a 10% (w/v) liver homogenate, obtained using a hand-held Dounce homogenizer, and mixed with an isotonic Percoll solution (final density 1.077). After centrifugation at 2500 *g* for 15 min, the floating material was diluted in 1 mM $NaHCO_3$/0.5 mM $MgCl_2$ to 10 ml per g of liver. After removal of large aggregates by centrifugation at low speed, the supernatant was subjected to 100000 *g* for 60 min. The pelleted vesicles were dissolved in 50% (w/w) sucrose, overlaid subsequently with 41 and 33% (w/w) sucrose, and centrifuged in a swing-out rotor at 100000 *g* for 150 min. The material floating to the 33/41% sucrose interphase consisted of highly purified plasma membrane vesicles (see Table 1).

Marker enzymes and protein were measured as described [9]. 5′-Nucleotidase [23], cholesterol [9] and galactosyltransferase [9] were measured as described before except that, for the latter enzyme, assay volumes were reduced twice and glycerol was omitted from the reaction mixtures. Assays of alkaline phosphatase and alkaline phosphodiesterase were based on the formation of *p*-nitrophenol [24].

Sphinganine-phosphatase assay

The phosphatase activity was generally measured by following the formation of labelled sphinganine from [4,5-^{3}H]sphinganine phosphate. Assays were performed in glass tubes as follows. An aliquot of [4,5-^{3}H]sphinganine phosphate (2 nmol; 25 mCi/mmol), dissolved in methanol, was placed at the bottom of the tube and dried under nitrogen. To dissolve the lipid, 75 μl of reaction mixture (containing buffer, semicarbazide, EDTA or any other addition) was added, and the tubes were placed in a bath sonicator for 2 min to ensure complete dissolution of the substrate (confirmed by counting aliquots of this mixture). Reactions were started by adding 25 μl of sample appropriately diluted in homogenization medium so that less than 15% of the substrate was hydrolysed during the subsequent incubation. Standard final concentrations were 100 mM Hepes, pH 7.5, 10 mM semicarbazide, 10 mM EDTA and 20 μM [4,5-^{3}H]sphinganine phosphate. After 20 min of incubation at 37 °C, reactions were stopped by adding 0.75 ml of chloroform/methanol (1:2, v/v). After mixing, phase separation was obtained by adding 0.25 ml of chloroform and 0.35 ml of 0.5 M NaOH. The organic layer was re-extracted with 0.5 ml of 0.4 M NaOH/methanol (9:10, v/v). In mock extractions, spiked with [4,5-^{3}H]sphinganine or [4,5-^{3}H]sphinganine phosphate, it was shown that sphinganine phosphate was completely extracted into the upper phase while sphinganine remained quantitatively in the lower phase. An aliquot (250 μl) of the organic phase was analysed by TLC in chloroform/methanol/25% (w/v) ammonia (60:30:5, by vol.). The region corresponding to sphinganine (R_f 0.74) was scraped into 0.5 ml of 1% (w/v) SDS and counted after adding 4 ml of Instagel II. Obtained counts were always corrected by using appropriate blanks or zero-time samples.

In the absence of detergents and with 20–40 μM substrate, the highest activities in homogenates were obtained in Hepes buffer, pH 7.5, containing EDTA and semicarbazide. Under these conditions more than 90% of the label present in the lower phase appeared to be associated with sphinganine and the production of sphinganine increased linearly with time (up to 40 min) and with protein (up to 4 mg/ml of assay volume). In order to

prevent breakdown of the substrate by sphingosine-phosphate lyase, the addition of semicarbazide, an inhibitor of the lyase [9], was necessary. Omission of this compound resulted in the formation of labelled palmitaldehyde, which is also recovered in the lower organic phase. The formation of sphinganine was not influenced by semicarbazide, up to 16 mM, while the generation of palmitaldehyde became negligible above 5 mM inhibitor. The stimulatory effect of EDTA on the phosphatase activity reached a plateau at around 3 mM. Replacement of the Hepes buffer, pH 7.5, by Tris, phosphate or Mes buffers resulted in 20–30% lower phosphatase activities.

Ceramide-phosphatase assay

Ceramide-phosphatase was measured by following the formation of labelled *N*-hexanoyl-sphinganine from *N*-hexanoyl-[4,5-^{3}H]sphinganine phosphate. Substrate (2 nmol; 25 mCi/mmol), dissolved in chloroform/methanol (1:1, v/v), was placed at the bottom of glass tubes and dried. After adding 75 μl of reaction mixture, tubes were placed in a bath sonicator for 2 min, and the reactions were then started by adding 25 μl of sample, appropriately diluted in homogenization medium. Unless otherwise mentioned, final concentrations were 100 mM ammonium acetate buffer, pH 5.0, 0.3% (w/v) defatted albumin and 20 μM *N*-hexanoyl-[4,5-^{3}H]sphinganine phosphate. In order to solubilize the substrate efficiently, the addition of albumin to the reaction mixture prior to sonication was necessary. After 15 min at 37 °C, reactions were stopped with 0.75 ml of chloroform/methanol (1:2, v/v). Phases were separated by adding 0.25 ml of chloroform and 0.35 ml of 0.5 M acetic acid. An aliquot (250 μl) of the lower phase was spotted on to TLC plates, which were developed in chloroform/methanol/25% (w/v) ammonia (60:30:5, by vol.). The region corresponding to an R_f of 0.85–1.0, containing *N*-hexanoyl-[4,5-^{3}H]sphinganine (R_f = 0.95), was scraped into scintillation vials and radioactivity was measured as described above.

RESULTS

Characterization of sphinganine-phosphatase activities in rat liver homogenates

To monitor dephosphorylation, [4,5-^{3}H]sphinganine phosphate was chosen as substrate. This lipid can be easily prepared from [4,5-^{3}H]sphinganylphosphocholine, in its turn obtained from tritiated sphingomyelin, by treatment with a phospholipase D from *Streptomyces chromofuscus* [9], and it possesses the natural 2D,3D-configuration [9]. Furthermore, the position of the label allows for a straightforward separation of labelled substrate and product. The sphinganine produced remains in the lower organic phase, while the phosphorylated base is quantitatively extracted into the alkaline aqueous phase [19]. In the presence of semicarbazide, added to prevent cleavage of the substrate by sphingosine-phosphate lyase [9], dephosphorylation of sphinganine phosphate in rat liver homogenates appeared to be catalysed by several enzymes, as suggested by the pH profiles (Figure 1a). In the absence of EDTA, a rather broad pH profile ranging from pH 4 to 8.5, with weak optima around pH 4.5 and 7.5, was observed. The neutral activity appeared to be stimulated several-fold by the presence of chelator, while the acidic activity was less influenced, resulting in a clear optimum at pH 7.5. Screening of different bivalent cations showed that Zn was the most potent inhibitor of the neutral activity ($IC_{50} \approx 6$ μM in the absence of EDTA). Also, the kinetics obtained at neutral and acidic pH, although not offering conclusive evidence due to the peculiar solubility of the substrate [10], differed. At pH 7.5 in Hepes buffer, high- and low-affinity components were involved

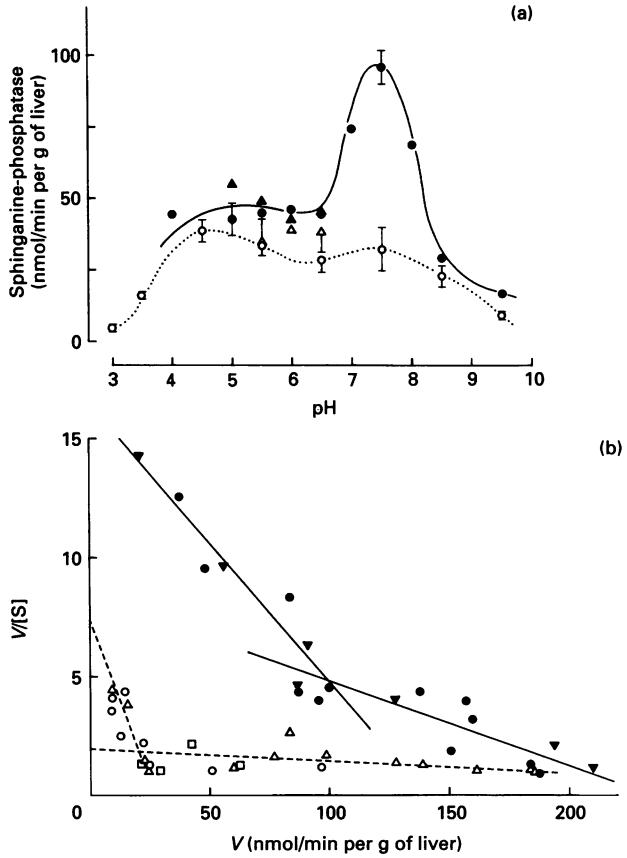

Figure 1 pH profile and substrate-dependence of sphinganine-phosphatase in rat liver homogenates

Sphinganine-phosphatase activities, measured in rat liver homogenates at different pH values with 20 μM substrate and 10 mM semicarbazide in the absence (open symbols) or presence (closed symbols) of 10 mM EDTA, are shown in (**a**). The buffers (100 mM final concentration) indicated by circles were glycine (pH 3.0, 3.5 and 4.0), ammonium acetate (pH 4.5, 5.0 and 5.5), Mes (pH 6.0 and 6.5), Hepes (pH 7.0, 7.5 and 8.0) and diethanolamine (pH 8.5 and 9.0). The triangles represent values obtained with histidine (pH 5.0 and 5.5) and imidazole (pH 6.0 and 6.5) as buffer. Data are means ± S. D. for two or more values obtained in separate experiments. In (**b**), the Eadie–Hofstee plots of the dephosphorylation velocities observed at various substrate concentrations in homogenates in 100 mM Hepes, pH 7.5, in the absence (open symbols) or presence (closed symbols) of 10 mM EDTA are shown. The different symbols refer to data obtained in separate experiments.

in the hydrolysis of sphinganine phosphate, as revealed by the Eadie–Hofstee plots (Figure 1b). The K_m and $V_{max.}$ values of the high-affinity component were 9 μM and 142 nmol/min per g of liver respectively (in the presence of EDTA; means for two experiments). The dephosphorylation reaction occurring at pH 5.0 displayed a single low-affinity component with an (extrapolated) K_m of 120 μM (results not shown).

In order to study the high-affinity phosphatase, the substrate concentration was fixed at 20 μM and reactions were performed in a Hepes-buffered assay at pH 7.5 containing only EDTA and semicarbazide. Under these standard conditions, an activity of 96 ± 7 nmol/min per g of tissue (mean ± S.D.; n = 7) was obtained in rat liver homogenates. Therefore dephosphorylation of sphinganine phosphate is more active than the cleavage reaction catalysed by sphingosine-phosphate lyase (13 nmol/min per g at 40 μM substrate) [9]. Also in fibroblasts supplemented with sphinganine phosphate, dephosphorylation is more prominent than cleavage, as was deduced from analysis of the metabolites recovered [17].

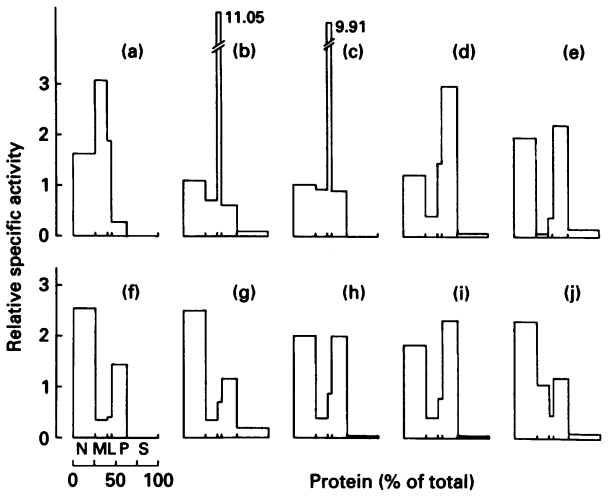

Figure 2 Subcellular distribution of sphinganine-phosphatase in rat liver

A fresh rat liver homogenate was fractionated into a nuclear (N), a heavy mitochondrial (M), a light mitochondrial (L), a microsomal (P) and a cytosolic (S) fraction. In each fraction, marker enzymes, sphinganine-phosphatase (20 μM sphinganine phosphate in Hepes buffer, pH 7.5, containing 10 mM semicarbazide and 10 mM EDTA; **l**), ceramide-phosphatase (20 μM *N*-hexanoyl-sphinganine phosphate in ammonium acetate buffer, pH 5.0, containing 0.3% albumin; **j**) and protein were measured. Marker enzymes were glutamate dehydrogenase (mitochondria; **a**), acid phosphatase (lysosomes; **b**), urate oxidase (peroxisomes; **c**), glucose-6-phosphatase (endoplasmic reticulum; **d**), galactosyltransferase (Golgi; **e**), 5′-nucleotidase (plasma membrane; **f**), alkaline phosphatase (plasma membrane; **g**) and alkaline phosphodiesterase (plasma membrane; **h**). Results of a representative experiment are shown and are expresssed as relative specific activities versus cumulative percentage of total protein. Relative specific activity is defined as the percentage of the total recovered activity present in each fraction divided by the corresponding percentage of total protein. Recoveries for sphinganine-phosphatase and ceramide-phosphatase were 96 and 108% respectively; recoveries for marker enzymes were between 97 and 119%, except for galactosyltransferase (59%) and glutamate dehydrogenase (134%).

Sphinganine phosphate hydrolysis in homogenates under standard conditions was not inhibited by high concentrations of glycerol 3-phosphate (6 mM), or by the phosphate esters of ethanolamine or serine, not even when present in a 500-fold excess over the substrate (results not shown). Since the latter phosphate esters possess some structural analogy to the substrate, the dephosphorylation of sphinganine phosphate observed at neutral pH seems to be a highly specific process. With pyrophosphate (10 mM), a moderate activation (1.5–1.8-fold) was observed in the absence of EDTA. This is most likely due to the chelating properties of pyrophosphate, since no effect was seen in the presence of EDTA (results not shown). L-Tartrate (10 mM), an inhibitor of lysosomal acid phosphatase [25], did not affect the neutral activity in homogenates, but NaF, an aspecific inhibitor of many phosphatases, was inhibitory in a concentration-dependent manner ($IC_{50} \approx 10$ mM). Further tests revealed that the enzymic activity at pH 7.5 was barely influenced by *N*-ethylmaleimide (NEM) (see below) and was insensitive to several freeze–thaw cycles. However, the dephosphorylation reaction was severely suppressed by a variety of detergents (results not shown). Deoxycholate, octanoyl *N*-methylglucamide, dodecyldimethyl-ammonium oxide, CHAPS, octyl glucoside, Triton X-100 and *N*-octyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate were inhibitory when used at concentrations equal to their reported critical micellar concentrations (CMCs) [26]. Tween-20, Thesit and *N*-dodecylsarcosine were slightly stimulatory (20–40% at their CMCs), and Brij-35, at its CMC, resulted in a 2-fold stimulation. At 10 times their CMC values, all detergents were inhibitory, with the exception of Tween-20.

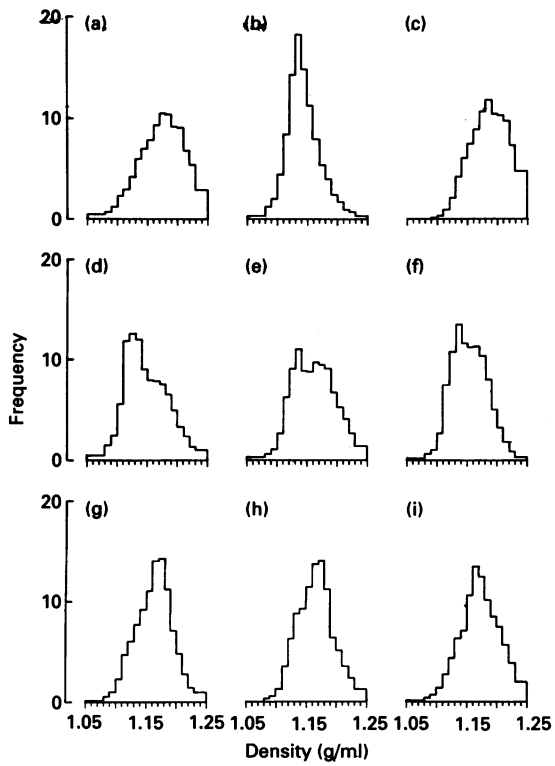

Figure 3 Subfractionation of a microsomal fraction on a sucrose gradient

A microsomal fraction, prepared by differential centrifugation, was subfractionated on a sucrose gradient as described in the Materials and methods section. The gradient fractions were analysed for protein (**a**), galactosyltransferase (**b**), glucose-6-phosphatase (**c**), alkaline phosphatase (**d**), cholesterol (**e**), 5′-nucleotidase (**f**), alkaline phosphodiesterase (**g**), sphinganine-phosphatase (20 μM sphinganine phosphate in Hepes buffer, pH 7.5, containing 10 mM EDTA and 10 mM semicarbazide; (**h**), ceramide-phosphatase (20 μM *N*-hexanoyl-sphinganine phosphate in ammonium acetate buffer, pH 5.0, in the presence of 0.3% albumin; (**i**), density by refraction and weight. Results are shown in the form of a density-distribution histogram [29], divided into 20 normalized fractions of identical density increment (starting at 1.05 and extending to 1.25). Recoveries of sphinganine-phosphatase and ceramide-phosphatase were 148 and 100% respectively; recoveries of protein and marker enzymes were between 76 and 129%.

Subcellular distribution of the high-affinity phosphatase

Analysis of subcellular fractions obtained by differential centrifugation demonstrated that the highest sphinganine-phosphatase activities, measured under standard conditions, were present in the nuclear and microsomal fractions (Figure 2). The distribution pattern of sphinganine-phosphatase fitted best with those of the known plasma membrane markers 5′-nucleotidase, alkaline phosphatase and alkaline phosphodiesterase. To exclude the presence of a sphinganine-phosphatase in other structures such as vesicles derived from the endoplasmic reticulum or the Golgi stacks, microsomal fractions were further separated by sucrose density gradient centrifugation. Comparison of the histograms in Figure 3 shows that the gradient distribution of the microsomal sphinganine-phosphatase activity most closely coincided with that of the plasma membrane marker alkaline phosphodiesterase. Surprisingly, the endoplasmic reticulum (marker, glucose-6-phosphatase) did not seem to contain sphinganine-phosphatase activity. Whether the weak shoulder of sphinganine-phosphatase activity observed at lower sucrose density ($d = 1.13$) is due to some association with Golgi vesicles, which equilibrate at this density, is not known. The plasma membrane markers other than alkaline phosphodiesterase also showed a peak at this density. It

Table 1 Co-purification of sphinganine-phosphatase with plasma membrane fragments

Plasma membrane fragments were purified from rat liver as described in the Materials and methods section, and marker enzymes, protein, sphinganine-phosphatase (in Hepes buffer, pH 7.5, containing EDTA and semicarbazide) and ceramide-phosphatase (*N*-hexanoyl-sphinganine phosphate in ammonium acetate buffer, pH 5.0, containing 0.3% albumin) activities were measured at each purification step. Results are expressed as a percentage of the activity found in the total homogenate and as relative specific activity (RSA).

	5′-Nucleotidase		Alkaline phosphatase		Alkaline phosphodiesterase		Sphinganine-phosphatase		Ceramide-phosphatase	
Fraction	(%)	RSA	(%)	RSA	(%)	RSA	(%)	RSA	(%)	RSA
Nuclear fraction	37.9	2.0	43.2	2.3	47.9	2.6	30.1	1.6	36.4	2.0
Percoll flotation	29.0	4.2	25.8	3.7	26.7	3.9	22.0	3.2	22.4	3.2
Hypotonic pellet	17.0	19.3	19.1	21.7	21.5	24.4	19.4	22.0	13.1	14.8
Sucrose flotation	7.1	34.3	8.8	42.8	7.4	35.9	6.8	33.2	4.6	22.3

is generally accepted that alkaline phosphatase and phosphodiesterase are plasma membrane markers, but controversial data are found in the literature concerning their presence or absence in the Golgi stacks [27–31]. According to Smith and Peters [31] the low-density peak might be associated with a distinct zone of the plasma membrane. Manipulation of the sucrose density and the bivalent cation concentration of the gradients did not provide more conclusive answers, but a predominant association of the sphinganine-phosphatase with the plasma membrane was always clear. A similar conclusion could be drawn after separating the microsomal fractions by means of Percoll gradient centrifugation under isotonic conditions (results not shown). In another approach to localizing sphinganine-phosphatase, plasma membrane fragments were purified from the nuclear fraction as starting material. As shown in Table 1, the co-purification of sphinganine-phosphatase and the plasma membrane markers was evident.

Sphinganine-phosphatase, a novel phosphatase?

Because of the multitude of hepatic phosphatases that hydrolyse water-soluble as well as amphiphilic phosphate esters, the almost exclusive hydrolysis of sphinganine phosphate at pH 7.5 (in presence or absence of EDTA) by a plasma membrane-associated enzyme, as shown above, was rather unexpected. Equally surprising was the observation that the sphinganine-phosphatase activity present in plasma membranes enriched approx. 35–40-fold also showed a bimodal pH profile that was influenced by EDTA, like that found in homogenates (Figure 4a). Further kinetic characterization of the activities in plasma membranes revealed a high K_m at pH 5.0 (150 μM). At pH 7.5, as in homogenates, two systems with different affinities seemed to be active. The K_m and $V_{max.}$ values of the high-affinity system were 10 μM and 19.3 nmol/min per mg of protein respectively (in the presence of EDTA; means for two experiments) (Figure 4b).

At a sphinganine phosphate concentration of 20 μM, high concentrations (5–10 mM) of soluble phosphate esters such as phosphoethanolamine, phosphoserine and glycerol 3-phosphate had no effect on the neutral phosphatase activity, measured in either microsomal fractions (results not shown) or purified plasma membranes (Table 2). Hexadecyl phosphate, which can be regarded as a substrate analogue lacking the 2-amino and 3-hydroxy groups, was also without effect. Sphingenylphosphocholine was inhibitory ($IC_{50} \approx 750$ μM). Since hexadecylphosphocholine produced a similar degree of inhibition as sphingenylphosphocholine (results not shown), the effect of the latter might be caused by its detergent action and not by its structural analogy with the substrate. *N*-Acetyl-sphingenine phosphate was a potent inhibitor ($IC_{50} = 6$ μM in the presence of 0.3% albumin). The inhibition was of a mixed non-competitive type ($K_i = 0.9$–3 μM; results not shown). Product inhibition by sphinganine ($IC_{50} \approx 70$ μM in the presence of 0.3% albumin) was small. *N*-Acetyl-sphinganine was only slightly inhibitory (Table 2). As in homogenates, the phosphatase was not sensitive to tartrate and was inhibited by NaF ($IC_{50} \approx 8$ mM).

At this stage, we wished to rule out the possibility that the sphinganine-phosphatase activity was catalysed by alkaline phosphatase. Despite the widespread use of this enzyme, its physiological substrate remains unknown, but pyridoxal phosphate might be a good candidate [32,33]. Alkaline phosphatase activity is dependent on the presence of Zn^{2+} ions, is stimulated by Mg^{2+} ions, and is inhibited by chelators such as EDTA [34]. The opposite situation was seen with sphinganine-phosphatase (Table 2). The activity in purified plasma membranes was stimulated by the addition of chelators and potently inhibited by low concentrations of Zn^{2+} ions ($IC_{50} \approx 5$ μM in the absence of albumin). In contrast to Mn^{2+}, Mg^{2+} had no effect at low concentration. Above 0.5 mM, Mg^{2+} became slightly inhibitory, possibly due to substrate complexation. Ca^{2+} at a low concentration (10 μM) had no effect. Whereas the alkaline phosphatase activity was destroyed by heating at 55 °C, sphinganine-phosphatase remained active (85% of initial activity recovered after 30 min of heating). Finally, tetramisole, a specific inhibitor of alkaline phosphatase [35], had no effect on sphinganine-phosphatase (Table 2). Together, these data rule out a significant contribution of alkaline phosphatase to the hydrolysis of sphinganine phosphate by plasma membranes, at least when measured at a substrate concentration of 20 μM. Even at higher substrate concentrations or at a more alkaline pH the contribution must be low, since the addition of EDTA, a potent inhibitor of the alkaline phosphatase, resulted in higher activities (see Figures 4a and 4b).

Other plasma-membrane-associated phosphatase activities have been reported in the literature [36–39]. Except for dolichol-phosphatase, they have been poorly characterized, so that it is difficult to discuss their (non)identity with sphinganine-phosphatase. Compared with sphinganine-phosphatase, dolichol-phosphatase has a lower pH optimum (pH 6.5), is not inhibited by high concentrations of Triton X-100, is only moderately stimulated by EDTA (10–20%), is inhibited by phosphatidate and is affected by high ionic strength [39]. The other reported plasma membrane-bound phosphatases include an NEM-insensitive phosphatidate phosphohydrolase and ceramide-phosphatase. Brindley and co-workers [40] showed the presence in rat liver plasma membranes of an NEM-insensitive phosphatidate phosphohydrolase with different properties from the endoplasmic reticulum-associated enzyme and a cytosolic activity. Whereas the microsomal enzyme is thought to be involved in glycerolipid synthesis, the plasma membrane enzyme might be involved in signal transduction, being responsible for the degradation of phosphatidate formed via hydrolysis of phosphatidylcholine

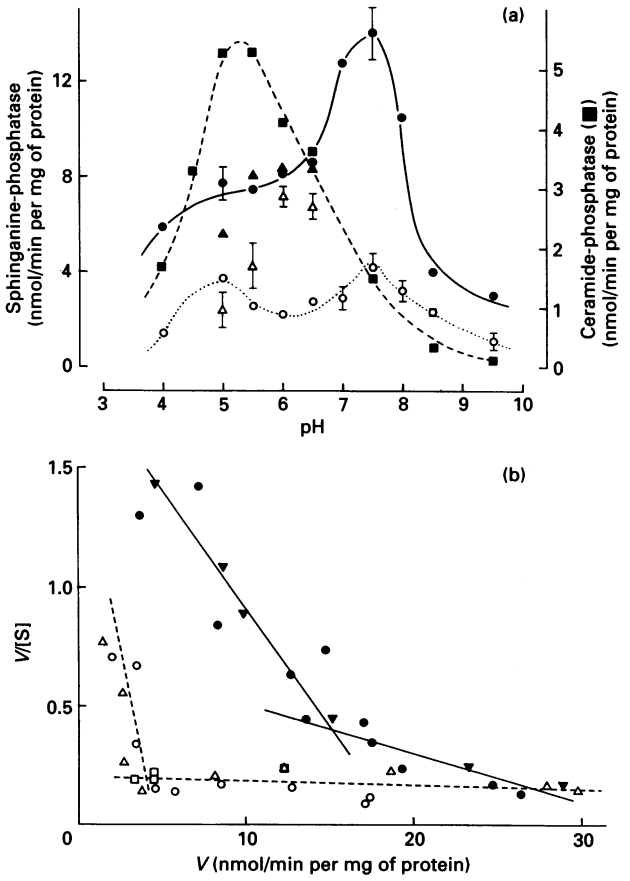

Figure 4 pH profile and substrate-dependence of sphinganine-phosphatase in purified plasma membranes

The sphinganine-phosphatase activities measured in plasma membranes, purified from rat liver nuclear fractions, at different pH values with 20 μM substrate and 10 mM semicarbazide in the absence (○, △) and presence (●, ▲) of 10 mM EDTA are shown in (**a**). Buffers and symbols are as indicated in the legend to Figure 1(**a**). Data represent means ± S.D. for two or more values obtained in separate experiments. For comparison, the pH profile of ceramide-phosphatase in purified plasma membranes with 20 μM *N*-hexanoyl-sphinganine phosphate as substrate in the presence of 0.3% albumin is also shown (■). Buffers used were glycine (pH 4.0), ammonium acetate (pH 4.5 and 5.0), Mes (pH 5.5, 6.0 and 6.5), Hepes (pH 7.5) and diethanolamine (pH 8.5 and 9.5). In (**b**), the Eadie–Hofstee plots of the sphinganine-phosphatase activities versus substrate concentration observed in purified plasma membranes in 100 mM Hepes, pH 7.5, in the absence (open symbols) or presence (closed symbols) of 10 mM EDTA are shown. The different symbols refer to data obtained in separate experiments.

by phospholipase D (see [41]). Like phosphatidate phosphohydrolase, sphinganine-phosphatase was affected by lysophosphatidate and was not influenced by NEM, but, in contrast to phosphatidate phosphohydrolase [20,40], it was not activated by heat treatment, was inhibited by truncated ceramide phosphates and by Triton X-100 [$IC_{50} \approx 0.03$ and 0.1% (w/v) in the absence and presence of 0.3% albumin respectively], and was only slightly affected by aliphatic cations (propranolol, sphingenine) or high concentrations of $MgCl_2$ (Table 2). Furthermore, excess phosphatidate did not affect the hydrolysis of sphinganine phosphate; the inhibition by lysophosphatidate might be due to a detergent action or to some structural analogy with the substrate.

During the course of our experiments, a phosphatase acting on ceramide phosphate and enriched in plasma membrane fractions from rat liver was described [20]. Ceramide phosphate is formed during the turnover of sphingomyelin in the plasma membrane [42,43]. Most probably it is one of the attenuation products of bioactive ceramide (for reviews see [44–46]). Therefore we also measured ceramide-phosphatase activity using a truncated ceramide phosphate analogue synthesized by acylation of [4,5-^{3}H]sphinganine phosphate with hexanoic anhydride. Addition of albumin to the reaction mixture was necessary in order to

Table 2 Effect of different additions on sphinganine-phosphatase and ceramide-phosphatase activities in purified rat liver plasma membranes

The effects of the different additions on sphinganine-phosphatase (SaPase; 20 μM sphinganine phosphate, 10 mM semicarbazide) and ceramide-phosphatase (CerPase; 20 μM *N*-hexanoyl-sphinganine phosphate) activities were determined in plasma membranes purified from a rat liver nuclear fraction. Assays were performed as described in the Materials and methods section, except that both assays contained 0.1 M Hepes buffer, pH 7.5, and 0.3% albumin was also added to the sphinganine-phosphatase reaction mixture in order to minimize differences due to substrate solubility and to allow the addition of amphipathic compounds. Results are expressed as percentages of control (no addition) activities. Except for the heat treatment, data were obtained with two different plasma membrane preparations in two (both values given) or more (means ± S.D.) separate experiments. Similar results were obtained when the effects of the additions were studied on the sphinganine- and ceramide-phosphatase activities in microsomal fractions. n.d., not determined.

	Activity (%)	
Addition/treatment	SPase	CerPase
10 mM NaF	55.6 ± 6.7	5, 6
10 mM Tartrate	93.6 ± 2.7	n.d.
6 mM Glycerol 3-phosphate	103 ± 2	103, 115
10 mM Pyrophosphate	150 ± 13	n.d.
10 mM Phosphoserine	103 ± 2	105, 123
10 mM Phosphoethanolamine	101 ± 4	106, 130
100 μM Hexadecyl phosphate	99.1 ± 11.7	106.6 ± 12.7
100 μM Lysophosphatidate	66, 61	17, 24
250 μM Phosphatidate	96, 98	72, 76
30 μM *N*-Acetyl-sphingenine phosphate	6, 8	28, 29
30 μM *N*-Hexanoyl-sphinganine phosphate	4, 7	n.d.
30 μM *N*-Acetyl-sphingenine	71.4 ± 8.8	113, 114
500 μM Sphingenylphosphocholine	69.8 ± 9.6	57, 57
30 μM Sphinganine phosphate	n.d.	59, 66
50 μM Sphinganine	63.5 ± 9.2	115 ± 5
4 mM Propranolol	89.0 ± 10.5[a]	71.5 ± 19.2[b]
5 mM NEM	98.0 ± 8.9[a]	87.0 ± 1.6[b]
10 μM $ZnCl_2$	2.5 ± 2.3[c]	90.0 ± 12.0
10 μM $CuCl_2$	103, 108[d]	90, 96
10 μM $MnCl_2$	45.4, 40.9	62, 80
10 μM $FeCl_2$	89.9, 93.0	100, 105
10 μM $CoCl_2$	98.6, 112	96, 119
10 μM $CaCl_2$	97.6 ± 4.3	112 ± 8
10 mM $MgCl_2$	94.8 ± 15.3[c]	113 ± 11
1 mM EDTA	126 ± 10	103 ± 4
10 mM EDTA	366 ± 19[c]	114, 123
Minus albumin	70, 77	–[e]
0.2 mM Tetramisole	114, 116[c]	110, 135
100 mM NaCl	93, 97	176, 183
0.2% (w/v) Triton X-100	28.1 ± 7.1	95.6 ± 23.3
20 min at 55 °C	90[a,c,f]	92[b]

[a] Assayed in the absence of albumin.

[b] Data reported by Boudker and Futerman [20].

[c] The influence of these additions or treatments was also tested on alkaline phosphatase present in the plasma membranes using *p*-nitrophenyl phosphate as substrate in glycine/NaOH buffer, pH 10.5. The results, expressed as a percentage of the control activity, were 111% ($ZnCl_2$), 123% ($MgCl_2$), 0% (EDTA), 16% (tetramisole) and 0% (heat treatment).

[d] In the absence of albumin, this value decreased to 31%.

[e] Substrate solubilization not adequate.

[f] Data derived from a time course experiment in which purified plasma membranes were kept at 55 °C for 5, 10, 20 and 30 min and subsequently assayed for sphinganine-phosphatase activity. The activity decreased slowly, approaching 85% of the initial value after heating for 30 min.

Table 3 Tissue distributions of sphinganine-phosphatase and ceramide-phosphatase

Sphinganine-phosphatase and ceramide-phosphatase activities were measured in homogenates of the tissues listed, as described in the Materials and methods section, with 20 μM sphinganine phosphate (in Hepes buffer, pH 7.5, containing EDTA and semicarbazide) and 20 μM *N*-hexanoyl-sphinganine phosphate (in ammonium acetate buffer, pH 5.0, containing albumin) respectively as substrates. Results are given as nmol/min per g of tissue and as specific activity (S.A.; nmol/min per mg of protein).

	Sphinganine-phosphatase		Ceramide-phospatase	
Tissue	(nmol/min per g)	S.A.	(nmol/min per g)	S.A.
Skeletal muscle	7.7	0.12	1.9	0.03
Heart	25.1	0.21	12.8	0.11
Testis	44.0	0.65	18.1	0.27
Lung	70.4	0.61	19.6	0.17
Liver	95.5	0.53	55.6	0.31
Intestinal mucosa	97.8	1.48	34.7	0.53
Kidney	99.2	0.69	42.4	0.29
Spleen	135	1.04	45.0	0.35
Cerebrum	298	3.12	76.6	0.80
Cerebellum	437	4.28	103	1.01

solubilize the substrate. Under the conditions described in the Materials and methods section, formation of *N*-hexanoyl-sphinganine in homogenates was linear with time for up to 60 min of incubation and with protein concentration up to 2.5 mg/ml of assay volume. An activity of 59.8 ± 3.6 nmol/min per g of liver (mean $\pm$ S.D.; $n = 5$) was obtained. Although originally described as a plasma membrane-bound phosphatase [20], we observed multiple subcellular localizations (see Figure 2). Compared with the action of sphinganine-phosphatase, dephosphorylation of *N*-hexanoyl-sphinganine phosphate was clearly more pronounced in the heavy mitochondrial and lysosomal fractions. The activity present in the microsomal fractions was associated mainly with plasma membrane vesicles, as revealed by sucrose gradient centrifugation (Figure 3). In purified plasma membranes, the enrichment of ceramide-phosphatase was approx. one-third lower than that of sphinganine-phosphatase (Table 2). The pH profile obtained in this preparation displayed an optimum at pH 5–5.5 (Figure 4a). Kinetic analysis at pH 5.0 revealed a K_m of 67 μM and a $V_{max.}$ of 32 nmol/min per mg of plasma membrane protein (results not shown). A comparable K_m value of 55 μM was found by Boudker and Futerman [20].

Table 2 shows the differences between sphinganine-phosphatase and ceramide-phosphatase observed with purified plasma membranes. In order to investigate the possible interference of ceramide-phosphatase in the hydrolysis of sphinganine phosphate under our standard conditions, both activities were measured at pH 7.5 in the presence of albumin. The strict dependence of ceramide-phosphatase on albumin cannot be used to draw conclusions about the (non)identity of the two phosphatases, since it is related to substrate solubilization. Ceramide-phosphatase was more sensitive to NaF ($IC_{50} \approx 4$ mM) and less sensitive to Zn^{2+} cations than was sphinganine-phosphatase. The addition of 0.2 % (w/v) Triton X-100 inhibited sphinganine-phosphatase, while it had no effect on ceramide-phosphatase. Higher concentrations of Triton X-100 inhibited ceramide-phosphatase ($IC_{50} \approx 1$ %), in agreement with the data of Boudker and Futerman [20]. Furthermore, ceramide-phosphatase preferred a higher ionic strength, was inhibited (non-competitively) by phosphatidate, and showed a clearly more acidic pH optimum (see Figure 4a) [20] (Table 2).

In separate experiments, ceramide-phosphatase activity was also measured using *N*-acetyl-[4,5-^{3}H]sphinganine phosphate as substrate. The subcellular distribution, pH profile and influence of the additions were almost identical to those seen with *N*-hexanoyl-sphinganine phosphate as substrate (results not shown), indicating that both truncated analogues are metabolized by the same enzyme.

Tissue distributions of sphinganine-phosphatase and ceramide-phosphatase

The sphinganine-phosphatase activities found in different rat tissues are shown in Table 3. Consistent with earlier preliminary data [10], remarkably high activities were present in nervous tissue. Ceramide-phosphatase activities (although a part of the measured activity might be due to a lysosomal activity; see above), using either *N*-acetyl- or *N*-hexanoyl-sphinganine phosphate as substrate, were also higher in nervous tissue than in liver, but not to the same degree as for sphinganine-phosphatase.

DISCUSSION

The formation of sphinganine upon addition of sphinganine phosphate to cultured fibroblasts suggested the occurrence of a physiologically important dephosphorylation step [17]. In the present study we have identified a high-affinity sphinganine-phosphatase that was associated with the plasma membrane. All available evidence indicates that it is distinct from other plasma-membrane-bound phosphatases reported to hydrolyse other lipophilic phosphate esters such as dolichol phosphate [39], phosphatidate [40] and ceramide phosphate [20]. The high-affinity phosphatase was studied with sphinganine phosphate as substrate. However, purified plasma membranes were also able to dephosphorylate sphingenine phosphate at rates equalling those observed with sphinganine phosphate, and both the saturated and unsaturated phosphate esters competed for dephosphorylation (P. De Ceuster and P. P. Van Veldhoven, unpublished work), indicating that the identified sphinganine-phosphatase also hydrolyses the unsaturated compound, which is prevalent *in vivo* [19]. Therefore we propose to name the enzyme sphingosine-phosphatase.

The plasma-membrane-associated low-affinity phosphatases, which consisted of a neutral and an acidic activity, were not investigated in detail and their nature remains speculative. Because of the peculiar solubility of the substrate [10], the high- and low-affinity components observed at neutral pH could perhaps reflect the differential recognition of the same substrate, present in two different physical states (monomer versus micelle; soluble versus aggregated), by a single enzyme. With regard to the acidic low-affinity component, the data suggest that it may be a side-activity of ceramide-phosphatase, as indicated by the following lines of evidence: (1) in the presence of detergents such as dodecyldimethylammonium oxide, which suppresses sphinganine-phosphatase but does not influence ceramide-phosphatase activity (results not shown), the pH profile for the hydrolysis of sphinganine phosphate shifted to an optimum at pH 5.0–5.5; and (2) in the presence of 10 mM NaF, which almost completely suppresses ceramide-phosphatase (Table 2), the sphinganine-phosphatase activity at acidic pH disappeared whereas the optimum at pH 7.5 was maintained (P. De Ceuster and P. P. Van Veldhoven, unpublished work). A definitive answer as to the identity of the low-affinity activities awaits the purification of the phosphatases involved.

Whatever the nature of these low-affinity activities, it is unlikely that they play a role of any significance in the degradation of phosphorylated sphingoid bases, since only small amounts of sphingenine phosphate, estimated at 0.2–0.3 nmol/g wet weight for rat liver, are present in biological systems [19]. Assuming a homogeneous cytosolic distribution, sphingenine phosphate concentrations in hepatocytes are therefore approx. 200–600-fold lower than the K_m values of the low-affinity activities, which range between 50 and 150 μM. The K_m of the high-affinity neutral phosphatase, as well as that of sphingosine-phosphate lyase (9 μM) [9], is approx. 30-fold higher than the estimated substrate concentrations.

In addition to plasma membrane phosphatase activities, we also observed a sphinganine-phosphatase in highly purified lysosomes. The lysosomal phosphatase was not affected by bivalent cations or chelators, was insensitive to tartrate, and was severely suppressed by NaF. The phosphatase is apparently non-specific since, in addition to phosphoserine and phosphoethanolamine, compounds structurally unrelated to sphinganine phosphate such as glycerol phosphate and pyrophosphate were also inhibitory. As expected, the pH optimum was acidic and the activity was stimulated by dodecyldimethylammonium oxide, suggesting latency (P. De Ceuster and P. P. Van Veldhoven, results not shown). The lysosomal contribution to the overall phosphatase activity in whole liver homogenates, when measured at pH 7.5 in the absence of detergents, was estimated to represent, at most, 5%.

In view of the proposed second messenger role for sphingenine phosphate, the presence of a plasma-membrane-bound sphingosine-phosphatase suggests a role for this enzyme in the attenuation of bioactive sphingenine phosphate. The subcellular site where sphingenine phosphate is generated remains unknown. Earlier reports mentioned that sphingosine kinase activity is cytosolic, but, more recently, membrane-bound kinase forms have also been described (see [8]). A specific association of sphingosine kinase with the plasma membrane remains to be demonstrated. Sphingenine phosphate can either be cleaved by sphingosine-phosphate lyase at the cytosolic aspect of the endoplasmic reticulum [9] or dephosphorylated at the plasma membrane, as demonstrated in the present paper. It is tempting to suppose that the lyase is involved in the overall catabolism of sphingolipids, whereas sphingosine-phosphatase may fulfil a more specific role in the degradation of signalling sphingenine phosphate. Interestingly, the tissue distributions of the lyase and the phosphatase are remarkably dissimilar. The lyase is most active in liver and intestinal mucosa but shows low activity in neuronal tissues [10], whereas the phosphatase is very active in neuronal tissues and less so in liver. Sphingenine phosphorylation and sphingenine phosphate dephosphorylation may therefore be of significant importance in the nervous system, which is very rich in sphingolipids including sphingenine phosphate [19]. Sphingenine (de)phosphorylation seems also to be linked to ceramide (de)phosphorylation, as indicated by the subcellular localization and tissue distribution of the phosphatases hydrolysing sphinganine and ceramide phosphates. Moreover, ceramide phosphate, or at least its truncated analogues, are potent inhibitors of sphingosine-phosphatase, suggesting that this enzyme may be regulated by the plasma membrane ceramide phosphate levels.

This work was supported in part by grants from the Belgian Fonds voor Kollektief Fundamenteel Onderzoek and from the Geconcerteerde Onderzoeksakties van de Vlaamse Gemeenschap. P.P.V.V. was supported by a grant 'Krediet aan Navorsers' from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek.

REFERENCES

1 Sweeley, C. C. (1991) New Compr. Biochem. **20**, 205–240
2 Keenan, R. W. and Haegelin, B. (1969) Biochem. Biophys. Res. Commun. **37**, 888–894
3 Hirschberg, C. B., Kisic, A. and Schroepfer, G. J., Jr. (1970) J. Biol. Chem. **245**, 3084–3090
4 Stoffel, W., Hellenbroich, B. and Heimann, G. (1973) Hoppe-Seyler's Z. Physiol. Chem. **354**, 1311–1316
5 Louie, D. D., Kisic, A. and Schroepfer, G. J., Jr. (1973) J. Biol. Chem. **251**, 4557–4564
6 Ghosh, T. K., Bian, J. and Gill, D. L. (1990) Science **248**, 1653–1656
7 Buehrer, B. M. and Bell, R. M. (1992) J. Biol. Chem. **267**, 3154–3159
8 Buehrer, B. and Bell, R. M. (1993) Adv. Lipid Res. **26**, 59–67
9 Van Veldhoven, P. P. and Mannaerts, G. P. (1991) J. Biol. Chem. **266**, 12502–12507
10 Van Veldhoven, P. P. and Mannaerts, G. P. (1993) Adv. Lipid Res. **26**, 69–98
11 Zhang, H., Desai, N. N., Olivera, A., Seki, T., Brooker, G. and Spiegel, S. (1991) J. Cell Biol. **114**, 155–167
12 Mattie, M., Brooker, G. and Spiegel, S. (1994) J. Biol. Chem. **269**, 3181–3188
13 Desai, N. N., Zhang, H., Olivera, A., Mattie, M. E. and Spiegel, S. (1992) J. Biol. Chem. **267**, 23122–23128
14 Sadahira, Y., Ruan, F., Hakomori, S.-I. and Igarashi, Y. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9686–9690
15 Sadahira, Y., Zheng, M., Ruan, F., Hakomori, S.-I. and Igarashi, Y. (1994) FEBS Lett. **340**, 99–103
16 Olivera, A. and Spiegel, S. (1993) Nature (London) **365**, 557–560
17 Van Veldhoven, P. P. and Mannaerts, G. P. (1994) Biochem. J. **299**, 597–601
18 Stoffel, W., LeKim, D. and Sticht, G. (1969) Hoppe-Seyler's Z. Physiol. Chem. **350**, 1233–1241
19 Van Veldhoven, P. P., De Ceuster, P., Rozenberg, R., Mannaerts, G. P. and de Hoffmann, E. (1994) FEBS Lett. **350**, 91–95
20 Boudker, O. and Futerman, A. H. (1993) J. Biol. Chem. **268**, 22150–22155
21 Van Veldhoven, P. P., Foglesong, R. J. and Bell, R. M. (1989) J. Lipid Res. **30**, 611–616
22 Van Veldhoven, P. P., Matthews, T. J., Bolognesi, D. P. and Bell, R. M. (1992) Biochem. Biophys. Res. Commun. **187**, 209–216
23 Van Veldhoven, P. P., Brees, C. and Mannaerts, G. P. (1991) Biochim. Biophys. Acta **1073**, 203–208
24 Beaufay, H., Amar-Costesec, A., Feytmans, E., Thinès-Sempoux, D., Wibo, M., Robbi, M. and Berthet, J. (1974) J. Cell Biol. **61**, 188–200
25 Brightwell, R. and Tappel, A. L. (1968) Arch. Biochem. Biophys. **124**, 333–343
26 Neugebauer, J. M. (1990) Methods Enzymol. **182**, 239–253
27 Bergeron, J. J. M., Ehrenreich, J. H., Siekevitz, P. and Palade, G. E. (1973) J. Cell Biol. **59**, 73–88
28 Farquhar, M. G., Bergeron, J. J. M. and Palade, G. E. (1974) J. Cell Biol. **60**, 8–25
29 Beaufay, H., Amar-Costesec, A., Thinès-Sempoux, D., Wibo, M., Robbi, M. and Berthet, J. (1974) J. Cell Biol. **61**, 213–231
30 Amar-Costesec, A., Wibo, M., Thinès-Sempoux, D., Beaufay, H. and Berthet, J. (1974) J. Cell Biol. **62**, 717–745
31 Smith, G. D. and Peters, T. J. (1980) Eur. J. Biochem. **104**, 305–311
32 Whyte, M. P. (1989) in Bone and Mineral Research (Peck, W. A., ed.), pp. 175–218, Elsevier, Amsterdam
33 Fedde, K. N. and Whyte, M. P. (1990) Am. J. Hum. Genet. **47**, 767–775
34 Bretaudier, J.-P. and Spillman, T. (1984) Methods Enzym. Anal. 3rd Ed. **4**, 75–86
35 Van Belle, H. (1972) Biochim. Biophys. Acta **289**, 158–168
36 Strout, H. V., Vicario, P. P., Saperstein, R. and Slater, E. E. (1988) Biochem. Biophys. Res. Commun. **151**, 633–640
37 Yokota, S., Himeno, M. and Kato, K. (1989) Cell Struct. Funct. **14**, 163–171
38 Sarrouilhe, D., Lalegerie, P. and Baudry, M. (1993) Cell. Mol. Biol. **39**, 13–19
39 Rip, J. W., Rupar, A., Chaudhary, N. and Carroll, K. K. (1981) J. Biol. Chem. **256**, 1929–1934
40 Jamal, Z., Martin, A., Gomez-Muñoz, A. and Brindley, D. N. (1991) J. Biol. Chem. **266**, 2988–2996
41 Exton, J. H. (1990) J. Biol. Chem. **265**, 1–4
42 Dressler, K. A. and Kolesnick, R. N. (1990) J. Biol. Chem. **265**, 14917–14921
43 Kolesnick, R. N. and Hemer, M. R. (1990) J. Biol. Chem. **265**, 18803–18808
44 Hannun, Y. A. and Linardic, C. M. (1993) Biochim. Biophys. Acta **1154**, 223–236
45 Mathias, S. and Kolesnick, R. (1993) Adv. Lipid Res. **25**, 65–89
46 Hannun, Y. A. (1994) J. Biol. Chem. **269**, 3125–3128

Received 8 February 1995/4 May 1995; accepted 17 May 1995