Properties of phosphatidate phosphohydrolase in rat adipose tissue

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Previously we have identified the presence of two different phosphatidate phosphohydrolase (PPH) activities in rat adipose tissue, based on Mg^{2+} -dependency. In the present investigation, we have further characterized these isoenzymes, using both aqueous dispersed and membrane-bound phosphatidate as substrates and differentiated these activities on the basis of both Mg^{2+} -dependency and *N*-ethylmaleimide (NEM)-sensitivity. These two distinguishing criteria gave identical estimates of PPH activities present in the different subcellular fractions. The microsomal and cytosol fractions contained mainly the Mg^{2+} -dependent (NEM-sensitive) form, which was inhibited by various thiol reagents, was inactivated by heating at 55 °C for 20 min, and was decreased significantly within 2 h after intraperitoneal administration of cystamine (200 mg/kg). Such treatments had

no effects on the Mg^{2+} -independent (NEM-insensitive) form of PPH, which was mainly located in the plasma membranes, mitochondrial and microsomal fractions. Addition of Lipid A and guanosine 5'-[γ -thio]triphosphate to the assay mixture had no effect on the PPH activities. The Mg^{2+} -independent PPH form, which was thermostable in the intact subcellular fractions, became thermolabile when these fractions were disrupted in the presence of Triton X-100. The present studies demonstrate that: (1) the thermostability is not a satisfactory index to differentiate these isoenzymes; (2) the thiol/disulphide exchange may be involved in the regulation of Mg^{2+} -dependent PPH activity; and (3) the PPH isoenzymes do not seem to be under G-protein control in adipose tissue, as reported previously in the mesangial cell line.

INTRODUCTION

Phosphatidate phosphohydrolase (PPH; EC 3.1.3.4) catalyses the release of orthophosphate from *sn*-1,2-diacylglycerol 3-phosphate to form 1,2-diacylglycerol. This reaction lies at the branch point in glycerolipid metabolism and plays an important role in the synthesis of both phospholipids and neutral lipids via the *sn*-glycerol 3-phosphate pathway [1]. The presence of this enzyme has been reported in several tissues, including adipose tissue [1–10]. The properties of this enzyme in rat liver, chicken liver, pig kidney, rat brain, human neutrophils, leucocytes and erythrocytes have been studied in some detail [1–10].

Previous studies from our laboratory indicated the presence of two different PPH activities in rat adipose tissue, namely Mg^{2+} -dependent and Mg^{2+} -independent [10–13]. The evidence was based on the differential responses of these two isoenzymes to various treatments, including the effects of Mg^{2+} , temperature, proteolysis, detergents and thiol reagents, such as *N*-ethylmaleimide (NEM). Jamal and co-workers [14] have also reported the presence of two PPH activities in rat liver, based on NEM-sensitivity. The NEM-insensitive enzyme was able to sustain high temperature (55 °C for 20 min), did not require Mg^{2+} for activity and was predominantly located in the plasma-membrane fraction. On the other hand, NEM-sensitive PPH was thermolabile, required Mg^{2+} for activity, was inhibited by NEM and was mainly localized in the liver cytosol and microsomal fractions. These workers postulated that NEM-insensitive PPH may be involved in signal transduction, in conjunction with phospholipase D to generate the second messenger diacylglycerol, during agonist-induced degradation of phosphatidylcholine [7–9,15], whereas NEM-sensitive PPH may be involved in triacylglycerol formation by generating diacylglycerol via the *sn*-glycerol 3-phosphate pathway as indicated previously in other studies [1,6,10,11].

The present studies were undertaken: (1) to investigate further

the properties of PPH isoenzymes in rat adipose tissue and relate these to their location within the membranes; (2) to determine whether PPH activity in adipose tissue is mediated via thiol/disulphide exchange reactions; and (3) to ascertain the role of G-proteins in the regulation of PPH activity.

MATERIALS AND METHODS

Materials

L-[U- ^{14}C]Glycerol 3-phosphate (sp. radioactivity 153.2 mCi/mmol), [γ - ^{32}P]ATP (disodium salt; sp. radioactivity 3000 Ci/mmol) and [G- 3H]AMP (trimethylammonium salt; sp. radioactivity 25.5 Ci/mmol) were purchased from DuPont NEN Research Products, Boston, MA, U.S.A. [Arg 8]Vasotocin, 4,4'-dithiopyridine, 2,2'-dithiopyridine, cystamine dihydrochloride, thimerosal, *NN'*-*p*-phenylenedimaleimide, dithiothreitol, phosphatidate (sodium salt) from egg lecithin, guanosine 5'-[γ -thio]triphosphate (GTP[S]) and Ficoll (400 kDa) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *sn*-1,2-Diacylglycerol kinase (*Escherichia coli*) was purchased from Calbiochem, San Diego, CA, U.S.A., and fatty-acid-poor BSA was obtained from Armour Pharmaceutical Co., Kankakee, IL, U.S.A. 1,2-Diacylglycerol from egg lecithin was purchased from Serdary Research Laboratories, London, Ontario, Canada. Percoll was a product of Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A. Male Sprague–Dawley rats (175–200 g) were purchased from Charles River Laboratories, Wilmington, DE, U.S.A. Rats received chow (Ralston Purina, St. Louis, MO, U.S.A.) and water *ad libitum*.

Preparation of subcellular fractions

We have followed the procedure of Belsham et al. [16] to isolate plasma membrane and the other subcellular fractions from adipose tissue, using an iso-osmotic self-forming Percoll gradient.

Abbreviations used: PPH, phosphatidate phosphohydrolase; NEM, *N*-ethylmaleimide; GTP[S], guanosine 5'-[γ -thio]triphosphate; MGAT, monoacylglycerol acyltransferase.

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In these experiments epididymal tissues from 6–9 animals were homogenized with a Polytron for 30 s in medium A, containing 0.25 M sucrose, 10 mM Tris/HCl and 2 mM EGTA, pH 7.4. The homogenate was centrifuged at 1000 *g* for 1 min, and the infranant was removed and re-centrifuged at 30000 *g* for 30 min. The supernatant fraction served as a source of microsomal and cytosol fractions, which were isolated by centrifugation at 105000 *g* for 1 h. The pellets containing mitochondria and plasma membrane (30000 *g* pellet) were suspended in a minimal volume of medium A, and this suspension was dispersed in 8 ml of a self-forming Percoll gradient. Percoll, dissolved in 2 M sucrose/80 mM Tris/HCl (pH 7.4)/8 mM EGTA was mixed with medium A and the 30000 *g* pellet suspension in the proportions 7:1:32 (by vol.). The Percoll mixture was centrifuged at 10000 *g* for 15 min. After centrifugation, the two visible bands containing plasma membrane (top) and mitochondrial fractions (bottom) were collected. The plasma-membrane fraction was removed with a Pasteur pipette and washed by diluting 5-fold in NaCl-based medium [0.15 M NaCl/10 mM Tris/HCl (pH 7.4)/1 mM EGTA], followed by centrifugation at 10000 *g* for 5 min. The pellet containing plasma membrane was finally resuspended in 2–3 ml of medium A with a Teflon/glass homogenizer. The mitochondrial and microsomal pellets were washed and resuspended in medium A. The purity of the resultant subcellular fractions was examined by assaying marker enzymes, as described previously [17]. Succinate dehydrogenase was used as a marker for mitochondria [18], and cytochrome *c* reductase (rotenone-insensitive) was taken as a marker for microsomes [19]. Lactate dehydrogenase [20] and 5'-nucleotidase [21] served as markers for cytosol and plasma-membrane fractions, respectively. This examination of purity of subcellular fractions indicated that the mitochondrial, plasma-membrane and cytosol fractions were substantially pure, whereas the microsomal fraction was often found contaminated by the plasma-membrane fraction, as indicated by the presence of 5'-nucleotidase activity. Contamination of the microsomal fraction by plasma membrane varied from 15 to 20% in 5 different experiments performed.

In addition, in some experiments, we have isolated different subcellular fractions from adipose tissue by Ficoll-density-gradient centrifugation, as described by McKeel and Jarrett [22].

Preparation of substrates

Phosphatidate (sodium salt) was dispersed by sonication (Heat Systems Instruments, model W-255, equipped with micro-tip probe) in deionized water. The phosphatidate concentration was adjusted to 5–6 $\mu\text{mol/ml}$. Usually, fresh substrate was used; however, when storage was necessary, it was stored at 4 °C under N_2 . Under these conditions, the substrate was stable at least for 1 week, as evidenced by the minimal liberation of P_i (blank values very low). In some experiments, we have also used ^{32}P -labelled phosphatidate and ^{14}C -labelled membrane-bound phosphatidate to measure PPH activity. The ^{32}P -labelled phosphatidate was synthesized essentially as described by Preiss et al. [23], in the presence of 1,2-diacylglycerol, ^{32}P ATP and the 1,2-diacylglycerol kinase preparation from *E. coli*. The ^{32}P -phosphatidate formed was extracted with chloroform/methanol, as described by Bligh and Dyer [24]. The lipid extract was subjected to t.l.c. on silica gel G-60 plates (Whatman, Maidstone, Kent, U.K.) with authentic phosphatidate as standard to determine the purity of nascent radioactive phosphatidate. Phosphatidate was separated on t.l.c. plates with chloroform/methanol/3 M NH_4OH (65:25:8, by vol.) or chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as solvent

systems [25,26]. The resulting phosphatidate preparation was found to be substantially pure, with more than 95% of ^{32}P radioactivity associated with the phosphatidate spot. The radioactive phosphatidate was diluted with unlabelled phosphatidate to adjust the specific radioactivity to 300–400 c.p.m./nmol to measure PPH activity.

Membrane-bound substrate was prepared by using adipose-tissue microsomes, as described previously [17]. Adipose-tissue microsomes were incubated in the presence of ^{14}C glycerol 3-phosphate, palmitate, ATP, CoA, Mg^{2+} and NaF for 60 min at 37 °C in a shaking water bath. In a total volume of 18 ml, the reaction mixture contained 24 mM Tris/HCl buffer, pH 7.5, 50 mM KCl, 1.6 mM *sn*-glycerol 3-phosphate, 0.2 μCi of *sn*- ^{14}C glycerol 3-phosphate, 0.7 mM dithiothreitol, 1.05 mM ammonium palmitate, 3 mM ATP, 3.6 mM MgCl_2 , 0.01 mM CoA, 6.25 mg of fatty-acid-poor albumin, 100 mM NaF and 8.5 mg of adipose-tissue microsomal protein. After 60 min the reactions were terminated by addition of sufficient ice-cold medium B (0.25 M sucrose/1 mM dithiothreitol/1 mM EDTA/1 mM Tris, pH 7.5). The microsomal pellets, containing bound lipids, were isolated by centrifugation at 105000 *g* for 60 min. The pellets were washed with medium B and extracted with butanol saturated with water as described by Daae and Bremer [27]. The butanol layer was extracted twice by water saturated with butanol and was dried under nitrogen and dissolved in 1 ml of chloroform/methanol (2:1, v/v). A portion (100 μl) of this mixture was counted for radioactivity in the presence of Cytosint (ICN Biochemicals, Irvine, CA, U.S.A.) to determine the total ^{14}C radioactivity present in the lipid fraction. The other portion was subjected to t.l.c. as noted above [25,26], to determine the radioactivity associated with the different lipids formed from ^{14}C glycerophosphate. Under these conditions, more than 96% of the total radioactivity was recovered in the phosphatidate, with 2.5% in lysophosphatidate and the remaining in the di- and tri-acylglycerol fractions.

PPH assay

PPH was assayed in the presence of aqueous-dispersed substrate as previously described [10], and the release of P_i was taken as a measure of PPH activity. In a final volume of 1 ml, the reaction mixture contained 60 mM Tris/maleate buffer, pH 7.5, 0.5 mM phosphatidate, or ^{32}P -labelled phosphatidate, 0, 2.5 or 5 mM MgCl_2 and 0.4 mg of BSA. The reaction was started by addition of 50–100 μl (50–120 μg of protein) of enzyme preparation. After incubation at 37 °C for 20 min, the reaction was terminated by addition of 1 ml of 10% (w/v) trichloroacetic acid, and the protein-free filtrate was collected by centrifugation. A portion of this filtrate (1 ml) was used to measure P_i content as described by Ames and Dubin [28]. The difference in the phosphate content between the experimental reaction and the reaction stopped at zero time was taken as a measure of PPH activity.

When radioactive phosphatidate was used as substrate, 1 ml of protein-free filtrate was washed with 2 $\times$ 4 ml of water-saturated diethyl ether. The aqueous layer, containing ^{32}P P_i , was mixed with 10 ml of Cytosint and counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter. There was a good agreement between these two methods of phosphate determination (correlation coefficient, $r = 0.98$). The Mg^{2+} -dependent PPH activity was calculated from total activity measured in the presence of Mg^{2+} minus the activity measured in the absence of Mg^{2+} . In some experiments, the PPH activities were differentiated on the basis of NEM-sensitivity. Protein was determined as described by Lowry et al. [30], with crystalline BSA as standard.

PPH activity with the membrane-bound substrate was de-

terminated on the basis of conversion of ^{14}C -labelled phosphatidate into [^{14}C]diacylglycerol, as described previously [10]. In a total volume of 0.55 ml, the reaction mixture contained 60 mM Tris/maleate, pH 7.5, and 100–200 μl of membrane-bound substrate (about 0.18–0.2 mg of protein, containing 46–48 nmol of radioactive phosphatidate) and also contained GTP[S], Lipid A or tetrahydrolipstatin (lipase inhibitor), when the effects of these agents were tested. The contents were incubated for 0–90 s at 37 °C in a shaking water bath. The reactions were terminated by addition of 2 ml of chloroform/methanol and the lipids were extracted as described previously [24]. The lipids were separated and identified by t.l.c. [29]. The areas containing diacylglycerol were scraped, mixed with 10 ml of CytoScint and counted for radioactivity in a Packard 480 scintillation counter to monitor the conversion of [^{14}C]phosphatidate into [^{14}C]diacylglycerol. Under all conditions initial enzyme-reaction rates were measured, and appropriate substrates and enzyme blanks were included to estimate the true enzyme activity. The agents GTP[S] and Lipid A were dissolved in 0.05 M Tris/HCl buffer, pH 7.5, and tetrahydrolipstatin was dissolved in dimethyl sulfoxide and added (10 μl) to give a final concentration of approx. 200 μM . This concentration of tetrahydrolipstatin has been shown previously to preserve the PPH product, diacylglycerol, against lipases [31]. Tetrahydrolipstatin was kindly provided by Dr. M. K. Meier, Hoffman La Roche, Switzerland.

Preparation of microsomal membranes devoid of Mg^{2+} -dependent PPH activity

It has been shown previously by Moller and Hough [32] in adipocytes and by Possmayer [33] in lung that the treatment of microsomal membranes with 0.5 M NaCl completely removes the Mg^{2+} dependent PPH from this fraction, without affecting the Mg^{2+} -independent activity. Our previous studies show that this can also be achieved by specific inactivation of Mg^{2+} -dependent PPH in adipose tissue at 55 °C for 10–20 min [11]. Therefore, these two procedures were used in some experiments to isolate microsomal membranes free from Mg^{2+} -dependent PPH. The plasma membrane ([14], and the present studies) and the mitochondrial fractions [10] did not contain Mg^{2+} -dependent PPH activity, and such treatment was not necessary when these fractions were used.

Effect of temperature on Mg^{2+} -independent PPH in intact and disrupted membranes

In some experiments, we have used disrupted membrane preparations to compare the thermolability of Mg^{2+} -independent PPH in the intact and disrupted membrane preparations. The disruption of mitochondria, microsomes (NaCl-washed or preheated at 55 °C for 20 min) and plasma-membrane fractions (1 mg of protein/ml) was carried out in the presence of 100 μl of 0.2 % Triton X-100 on ice for 30 min. This concentration of Triton X-100 was found previously to disrupt membranes, without affecting the Mg^{2+} -independent PPH activity [11].

Effect of thiol compounds on the PPH activity

Since the Mg^{2+} -dependent PPH in adipose tissue required a thiol group for its activity [1,11], the effects of various thiol compounds on the PPH activity were investigated. In addition, we have injected (intraperitoneally) cystamine into rats (200 mg/kg) to monitor its effect on adipose PPH at 2 h after cystamine

administration. This dose of cystamine has been shown previously to produce protein mixed disulphides in different organs and to decrease the liver glycogen content by nearly 90 % within 2 h, due to derangement in carbohydrate metabolism [34].

RESULTS AND DISCUSSION

Previously we have demonstrated the presence of two PPH isoenzymes in rat adipose tissue, based on Mg^{2+} -dependency [10]. Jamal and co-workers [14] have also reported the presence of two isoenzymes in rat liver, on the basis of NEM-sensitivity. To determine whether these two identifying procedures lead to identical estimates of these two isoenzymes in adipose tissue, we have used both of these criteria to measure the PPH activities in adipose-tissue subcellular fractions. Results in Table 1 show that these two distinguishing procedures give identical estimates of the PPH activities present in the different subcellular fractions in adipose tissue, and further demonstrate that Mg^{2+} -dependent PPH is equivalent to NEM-sensitive activity and that Mg^{2+} -independent activity represents the NEM-insensitive PPH (Table 1).

As noted by Jamal et al. for the liver enzyme [14], the adipose plasma-membrane fraction contained mainly the Mg^{2+} -independent PPH (NEM-insensitive) (Table 2). In the present studies, the microsomal fraction was often found to be contaminated by the plasma membrane (as much as 20 %, as evidenced by the presence of 5'-nucleotidase activity). Therefore, it is likely that a portion (20 %) of the Mg^{2+} -independent PPH associated with the microsomal fraction may represent a contamination from plasma membrane, whereas the remaining (80 %) activity may be of true microsomal origin. A major portion of the Mg^{2+} -dependent (NEM-sensitive) activity was present in the cytosol and microsomal fractions, with little or no activity in the plasma membrane and the mitochondrial fractions. Thus the microsomal fraction contained both of these isoenzymes, as noted in our previous studies [9,10]. A similar distribution of the hepatic Mg^{2+} -dependent and Mg^{2+} -independent PPH activities has been reported recently by Day and Yeaman [35].

In addition to Mg^{2+} -dependency and NEM-sensitivity, thermostability has also been used previously to distinguish the properties of these two isoenzymes in several tissues, including adipose tissue [6,11,14]. The Mg^{2+} -dependent (NEM sensitive) activity present in the cytosol and microsomal (loosely bound) fractions was extremely labile (more than 95 % loss at 55 °C within 20 min; Figure 1), whereas the Mg^{2+} -independent activity in the microsomal, mitochondria and plasma-membrane fraction (tightly bound) was relatively more stable. However, our recent studies on the monoacylglycerol acyltransferase (MGAT) in neonatal-rat liver [36] suggest that the thermostability is not an intrinsic property of an enzyme, but is an acquired one, arising perhaps from the location of an enzyme within the membranes. This conclusion was based on the thermostability of MGAT in the intact and disrupted microsomes. The MGAT activity present in intact membranes was thermostable. However, the same enzyme present in disrupted membranes was thermolabile (over 95 % loss of activity within 10 min at 52 °C). To determine whether the same situation also exists for the Mg^{2+} -independent PPH in adipose tissue, the thermolability studies were conducted in the presence of intact and disrupted subcellular fractions. In these experiments, the plasma-membrane, mitochondria and microsomal fractions (free from Mg^{2+} -dependent PPH activity) were used. As noted in our previous studies [11], the Mg^{2+} -independent activity present in the intact fraction was stable at 55 °C; whereas the activity in the disrupted membrane prep-

Table 1 PPH activity in different subcellular fractions in rat adipose tissue

PPH activity was measured on the basis of both Mg^{2+} -dependency and NEM-sensitivity and was expressed as nmol of P_i released/min per mg of protein. The Mg^{2+} -dependent PPH activity (A) was calculated from total activity measured in the presence of Mg^{2+} minus the activity measured in the absence of Mg^{2+} . The activity measured in the absence of Mg^{2+} represented the Mg^{2+} -independent PPH (B). The NEM-sensitive PPH activity (C) was determined from the total activity measured with Mg^{2+} minus the activity measured in the presence of both Mg^{2+} and NEM (10 mM). The NEM-insensitive PPH (D) represented the PPH activity measured in the presence of NEM, without Mg^{2+} . Each value is the mean $\pm$ S.D. of 3–4 different experiments, using 6–9 animals per experiment, with assays conducted in duplicate. The present data show that (A) is equivalent to (C) and (B) is equivalent to (D).

Subcellular fraction	Mg^{2+} -dependent (A)	Mg^{2+} -independent (B)	NEM-sensitive (C)	NEM-insensitive (D)
Homogenate	12.14 $\pm$ 2.44	5.10 $\pm$ 1.38	13.28 $\pm$ 2.0	4.99 $\pm$ 0.66
Mitochondria	0	3.15 $\pm$ 0.78	0.51 $\pm$ 0.47	2.24 $\pm$ 2.0
Plasma membrane	0	14.57 $\pm$ 5.0	0	13.47 $\pm$ 2.0
Microsomes	30.71 $\pm$ 0.87	14.50 $\pm$ 5.45	33.5 $\pm$ 2.0	17.61 $\pm$ 3.11
Cytosol	14.71 $\pm$ 5.39	2.48 $\pm$ 1.48	13.32 $\pm$ 4.72	1.57 $\pm$ 0.3

Table 2 Intracellular distribution of marker enzymes, PPH and protein

The enzymes were assayed as described in the Materials and methods section. The results are expressed both as a percentage of total of fat-free homogenates recovered in each subcellular fraction and as a relative specific activity (SA) with respect to protein, by setting homogenate activity equal to 1. Each value is the mean $\pm$ S.D. from 2–4 experiments. Abbreviations: SDH, succinate dehydrogenase; Cyto Red, cytochrome *c* reductase; LDH, lactate dehydrogenase.

Subcellular fraction	SDH		5'-Nucleotidase		Cyto Red		LDH		Mg^{2+} -independent PPH		Mg^{2+} -dependent PPH		Protein (% of total)
	% of total	SA	% of total	SA	% of total	SA	% of total	SA	% of total	SA	% of total	SA	
Homogenate	100	1	100	1	100	1	100	1	100	1	100	1	100
Mitochondria	80 $\pm$ 6	24.3	8 $\pm$ 2	2.43	2.53	0.76	1.83	0.55	15 $\pm$ 3	4.55	0.5 $\pm$ 0.2	0.15	3.29 $\pm$ 0.5
Plasma membrane	4 $\pm$ 1	1	40 $\pm$ 6	9.69	3.79	0.91	1.2	0.74	47 $\pm$ 5	11.35	0.5 $\pm$ 0.1	0.12	4.14 $\pm$ 1.0
Microsomes	4 $\pm$ 1	0.5	20 $\pm$ 3	2.5	65 $\pm$ 8	8.12	5.34	0.66	23 $\pm$ 3	2.87	32 $\pm$ 6	4.4	8.0 $\pm$ 1.52
Cytosol	2 $\pm$ 1	0.03	15 $\pm$ 3	0.22	8 $\pm$ 2	0.11	76 $\pm$ 8	1.1	6 $\pm$ 2	0.08	70 $\pm$ 5	1.1	68 $\pm$ 13

Figure 1 Effect of temperature on Mg^{2+} -dependent and Mg^{2+} -independent PPH activity in the intact and disrupted subcellular fractions

PPH activity was measured in the presence of aqueous-dispersed phosphatidate as substrate, and the release of P_i was taken as a measure of PPH activity. (a) The intact microsomal fractions were preheated at various times at 55 °C before assaying Mg^{2+} -dependent and Mg^{2+} -independent PPH activities to monitor the thermolability of these two PPH isoenzymes. (b) The intact and disrupted subcellular fractions (MC, microsomes; MT, mitochondria; PM, plasma membranes) were preheated at 55 °C for various times before assaying Mg^{2+} -independent activity. The disruption of different subcellular fractions was carried out in the presence of Triton X-100 on ice for 30 min, as described in the Materials and Methods section. Activity before the preincubation at 55 °C was considered as 100% to calculate the residual activity at the end of this treatment. Note that Mg^{2+} -independent PPH activity was thermolabile in the disrupted preparations, whereas in the intact preparation it was stable. Since microsomal fractions also contained Mg^{2+} -dependent PPH, it was either removed by washing with buffer containing 0.5 M NaCl or preheated at 55 °C, before the experiment reported in (b) was performed. Each value is the mean of two experiments, conducted at different times.

Table 3 Effects of thiol reagents on adipose-tissue PPH

Adipose-tissue cytosol served as an enzyme source to measure Mg^{2+} -dependent PPH activity. Adipose-tissue microsomal fraction heated at 55 °C for 20 min and plasma-membrane fractions were used to measure Mg^{2+} -independent PPH. Each value is the mean of 2–3 experiments. Although several concentrations of thiol reagents (0.1–10 mM) were used to monitor their effects on PPH, effects of mainly two concentrations have been reported here. PPH activity (nmol of P_i released/min per mg of protein) was measured in the presence of aqueous dispersed substrate as described in the Materials and methods section. Activity in the absence of these reagents is considered as 100% to calculate % inhibition. *In these experiments, cytosol fractions were preincubated at 37 °C in the presence of 10–200 μ g of vasotocin for 10–60 min before assaying PPH activity.

Reagent	Concn. (mM)	Inhibition (%)	
		Mg^{2+} -dependent PPH	Mg^{2+} -independent PPH
NEM	1	80	5
	10	95	10
<i>NN'</i> - <i>p</i> -phenylenedimaleimide	0.4	44	7
	4.0	62	9
Thimerosal	0.5	85	12
	5.0	100	18
Cystamine dihydrochloride	1	25	8
	10	85	18
2,2'-Dithiodipyridine	0.45	20	5
	9.0	90	16
4,4'-Dithiodipyridine	0.45	22	5
	9.0	96	20
5,5'-dithiobis-(2-nitrobenzoic acid)	0.2	80	0
	2.0	100	23
[Arg ⁸]Vasotocin* (acetate salt)	0.01	0	0

aration was extremely thermolabile (95% loss within 10 min; Figure 1). Thus it seems that the thermostability of adipose-tissue Mg^{2+} -independent PPH, like liver MGAT [36], is an acquired property resulting from its anchoring within the mem-

brane, and does not seem to be a satisfactory index to differentiate the two PPH isoenzymes in adipose tissue. On the basis of sensitivity to proteolytic enzymes, we have previously demonstrated that the anchoring sites of these PPH isoenzymes indeed differ [11]. The Mg^{2+} -dependent PPH resides on the cytoplasmic surface of the microsomal membrane, whereas the Mg^{2+} -independent PPH resides on the luminal surface or is buried in the membranes [11].

Any enzyme bearing an accessible thiol group essential for its activity is capable of forming protein mixed disulphides or intramolecular disulphides [34]. This thiol/disulphide exchange is considered as a general mechanism regulating various metabolic reactions in animals [34]. Table 3 and Figure 2 show the effects of various thiol compounds on both the Mg^{2+} -dependent and Mg^{2+} -independent PPH activities. In these experiments, we have used cytosol, plasma-membrane and microsomal fractions (free of Mg^{2+} -dependent PPH) to determine the effect of these compounds on the Mg^{2+} -dependent and Mg^{2+} -independent PPH activities. These compounds were able to inhibit or inactivate Mg^{2+} -dependent PPH activity when included in the assay mixture, and preincubation of enzyme at 37 °C for 10 min, with different thiol reagents, did not further decrease the enzyme activity. In this respect, thimerosal was more effective than the other disulphides and alkylating agents. Addition of dithiothreitol to the assay mixture provided a 100% protection of Mg^{2+} -dependent PPH against NEM. However, dithiothreitol was less effective against the other thiol compounds (Figure 2). Thus the present studies demonstrate that the active thiol group present in the Mg^{2+} -dependent PPH remains accessible to various thiol compounds, and support the possibility that thiol/disulphide exchange interaction may be involved in the regulation of Mg^{2+} -dependent PPH activity. This possibility is further strengthened by the fact that the administration of cystamine to animals resulted in a significant decrease in the Mg^{2+} -dependent activity within 2 h (Table 4).

In contrast with Mg^{2+} -dependent PPH, most of the thiol compounds were less effective in inhibiting or inactivating Mg^{2+} -independent PPH, and cystamine administration had no effect on the enzyme activity (Table 4). Further studies with the disrupted subcellular preparations gave essentially similar results (not shown). This differential sensitivity of PPH isoenzymes

Figure 2 Effect of various thiol compounds on the cytosolic Mg^{2+} -dependent PPH activity

Mg^{2+} -dependent PPH was measured (with aqueous-dispersed phosphatidate as substrate; the release of P_i is taken as a measure of PPH activity) in the presence of increasing concentrations of NEM (alkylating agent), cystamine (disulphide agent) and thimerosal (mercaptide-forming agent) with or without 10 mM dithiothreitol (DTT). Activity in the absence of these thiol reagents was considered as 100% (control) to calculate the residual activity in the presence of increasing concentrations of these reagents. DTT, when present in the incubation mixture, offered 100% protection of the PPH activity measured against NEM. However, DTT was less effective in protecting PPH against disulphide- and mercaptide-forming thiol reagents.

Table 4 Effect of cystamine administration on adipose-tissue PPH activity

Rats were injected with either cystamine (200 mg/kg, dissolved in 0.9% NaCl) or 0.9% NaCl (controls) intraperitoneally. After 2 h, animals were killed. Epididymal fat-pads were harvested to prepare cytosol and microsomal fractions. Cytosol was used to measure Mg^{2+} -dependent PPH and microsomal fractions (preheated at 55 °C for 20 min) were used to measure Mg^{2+} -independent PPH. The release of P_i from aqueous-dispersed substrate was monitored to determine PPH activities. Each value is the mean $\pm$ S.D. of the number of animals given in parentheses: * $P < 0.02$.

Group and expt. no.	Body wt. (g)	Adipose-tissue wt. (g)	PPH (nmol/min per mg of protein)	
			Mg^{2+} -dependent	Mg^{2+} -independent
Expt. 1				
Control (4)	202 $\pm$ 4.40	1.03 $\pm$ 0.23	15.38 $\pm$ 1.83	22.04 $\pm$ 2.32
Cystamine-treated (4)	206 $\pm$ 4.34	0.92 $\pm$ 0.15	10.05 $\pm$ 3.87*	20.55 $\pm$ 6.1
Expt. 2				
Control (3)	254 $\pm$ 7.04	1.20 $\pm$ 0.14	20.74 $\pm$ 3.51	17.54 (2)
Cystamine-treated (3)	265 $\pm$ 6.11	1.22 $\pm$ 0.16	13.23 $\pm$ 2.50*	18.28 $\pm$ 6.46

Figure 3 Effect of GTP[S], Lipid A, NEM and EDTA on the Mg^{2+} -dependent PPH measured in the presence of membrane-bound substrate

PPH activity was measured in the presence of microsomal membranes containing 46 nmol of ^{14}C -labelled phosphatidate and 0.19 mg of microsomal protein as described in the Materials and methods section. The membrane-bound substrate was incubated at various times in the absence (control) or presence of GTP[S] (100 μ M), Lipid A (1 μ g), NEM (10 mM) and EDTA (10 mM). After termination of the reactions, the conversion of ^{14}C phosphatidate into ^{14}C diacylglycerol was monitored by t.l.c. to estimate Mg^{2+} -dependent PPH activity. Each value is the mean of two experiments, with assays conducted in duplicate.

against the various thiol reagents suggests the possibility that the Mg^{2+} -independent PPH either possesses no thiol group essential for its activity, or that this group is located in a unique microenvironment and remains inaccessible to various thiol reagents.

Bursten and Harris [37] suggested that PPH activity in the mesangial-cell membranes is regulated by a pertussis-toxin-sensitive G-protein. The evidence was based on the increased conversion of a membrane-bound (*cis*-parinaric) phosphatidate into (*cis*-parinaric) diacylglycerol in the presence of Lipid A and GTP[S]. To determine whether the adipose-tissue PPH is under G-protein control, we have investigated the effects of Lipid A and GTP[S] on the microsomal Mg^{2+} -dependent PPH, measured in the presence of membrane-bound substrate. The rate of conversion of phosphatidate into diacylglycerol did not change

in the presence of either GTP[S] or Lipid A, but was inhibited completely in the presence of both EDTA and NEM (Figure 3). However, recent studies by Jamal and co-workers [31] suggest that adipose-tissue PPH activity, as estimated by the formation of diacylglycerol, is underestimated because of the degradation of the product diacylglycerol by lipases present in the different subcellular fractions in adipose tissue. These workers [31] overcame this problem by using tetrahydrolipstatin (200 μ M), a lipase inhibitor, while measuring the activity of PPH in adipose tissue. Therefore, in some experiments, we have measured the adipose-tissue PPH in the presence of tetrahydrolipstatin, but observed no effect of either GTP[S] or Lipid A on the PPH activity, as noted previously (Figure 3).

In our studies, the microsomal PPH activity, as measured by diacylglycerol production, was 38 ± 2.5 in the absence and 36.88 ± 3 nmol/min per mg of protein in the presence of tetrahydrolipstatin (3 determinations). Thus this lipase inhibitor had very little effect on the PPH activity. The reason for the discrepancy between these studies and those reported by Jamal et al. [31] is not at present clear, but could be related to the differences in the assay procedures, enzyme source and the preparation of substrates. The PPH activity measured on the basis of P_i release was 32 ± 2 nmol/min per mg of protein (3 determinations), which was equivalent to the activity based on diacylglycerol production measured with membrane-bound substrate.

Figures 4(a) and 4(b) show the effect of GTP[S] and Lipid A on the PPH activity measured on the basis of P_i release, which is considered as a valid measure for the PPH activity in adipose tissue in many laboratories, including our own [9–13]. These agents (GTP[S] and Lipid A) also failed to show any effect on the adipose-tissue PPH activity measured under these conditions. Thus the present studies do not support the possibility that the regulation of adipose-tissue PPH is under G-protein control. A similar conclusion has been drawn recently by Siddiqui and Exton [38] for the liver PPH, using different experimental conditions from those reported here. It is now known that the conversion of membrane-bound phosphatidate into 1,2-diacylglycerol can be mediated via both PPH and phospholipase C in rat liver membranes [5], and since there is an evidence to suggest that the regulation of phospholipase C is under G-protein control [39,40], it is more likely that the increased conversion of phosphatidate into 1,2-diacylglycerol noted previously by Bursten and Harris [37] in the presence of GTP[S] and Lipid A may be related to an activation of phospholipase C rather than of PPH.

Previous studies from our laboratory [10–13] and the present

Figure 4 Effect of Lipid A and GTP[S] on Mg²⁺-dependent and Mg²⁺-independent PPH

The Mg²⁺-dependent and Mg²⁺-independent PPH activities were measured in the presence of increasing concentrations of GTP[S] and Lipid A; ³²P-labelled phosphatidate was used as substrate to avoid the interference of non-specific release of P_i from GTP[S]. Neither Lipid A nor GTP[S] influenced the PPH activity. In these experiments, microsomal fraction was used to monitor the release of [³²P]P_i, which was taken as a measure of PPH.

examination of the properties clearly demonstrate the presence of two PPH isoenzymes in adipose tissue. The effects of various thiol compounds on the adipose-tissue PPH activities suggest the possibility that the thiol/disulphide interaction may be important in the regulation of Mg²⁺-dependent PPH activity in adipose tissue. Regarding the involvement of the Mg²⁺-dependent PPH in triacylglycerol formation, we agree with the proposal by Jamal et al. [14]. Previously, we have also observed a positive correlation between the changes in Mg²⁺-dependent PPH activity and triacylglycerol formation in different experimental conditions, including obesity, aging and various nutritional states [41–43]. As noted for the liver enzyme [14], the adipose-tissue plasma membrane did contain a major portion of Mg²⁺-independent PPH activity. However, a substantial activity of the Mg²⁺-independent PPH was also present in the microsomal and mitochondrial fractions in adipose tissue. Since the Mg²⁺-independent PPH is an integral part of the membranes, and it is always present in an active state, it is likely that Mg²⁺-independent PPH may mainly be involved in the metabolism of membrane phospholipids, including in the process of signal transduction, as proposed previously by Jamal et al. [14] for the liver PPH. Thus these PPH isoenzymes may serve distinct functions in adipocyte metabolism, as demonstrated previously in liver and in transfected cell lines [14,44].

Although the physical separation of Mg²⁺-dependent and Mg²⁺-independent forms of hepatic PPH has been achieved recently by Day and Yeaman [35], attempts to purify mammalian PPH to homogeneity so far remained unsuccessful [13,45]. Therefore, definitive proof of the existence of two PPH isoenzymes is still lacking. Yeast PPH has been purified to homogeneity [46], and the scheme of purification of the yeast enzyme may be useful in isolating homogeneous preparations of PPH isoenzymes to investigate their differential properties and their

roles in triacylglycerol and phospholipid metabolism in adipose and other tissues.

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