The effects of Triton X-100 and chlorpromazine on the Mg^{2+} -dependent and Mg^{2+} -independent phosphatidate phosphohydrolase activities of rat lung

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Lung contains both Mg^{2+} -dependent and Mg^{2+} -independent phosphatidate phosphohydrolase activities. Addition of Triton X-100 (0.5%) or chlorpromazine (1 mM) leads to a marked increase in the total phosphatidate phosphohydrolase activity in rat lung microsomes (microsomal fractions), but a decrease in the Mg^{2+} -dependent activity. These observations suggest that the Mg^{2+} -independent activity is stimulated, whereas the Mg^{2+} -dependent activity is inhibited. However, the possibility exists that Triton X-100 could stimulate the Mg^{2+} -dependent enzymic activity in an Mg^{2+} -independent manner. In addition, the positively charged amphiphilic drug could be replacing the enzyme's requirement for Mg^{2+} . These two possibilities were examined by using subcellular fractions in which the Mg^{2+} -dependent phosphatidate phosphohydrolase had been abolished by heat treatment at 55 °C for 15 min. Heat treatment does not affect the microsomal Mg^{2+} -independent phosphohydrolase to any great extent. Since the 6–8-fold stimulations due to Triton X-100 and chlorpromazine are retained after heat treatment of this fraction, the Mg^{2+} -independent activity must be involved. Addition of Triton X-100 and chlorpromazine to cytosol virtually abolishes the Mg^{2+} -dependent phosphatidate phosphohydrolase activity and decreases the Mg^{2+} -independent activity by half. Heat treatment also abolishes the Mg^{2+} -dependent activity and decreases the Mg^{2+} -independent activity by over half. The Mg^{2+} -independent phosphatidate phosphohydrolase activity remaining after heat treatment was not affected by Triton X-100 or chlorpromazine. These studies demonstrate that Triton X-100 and chlorpromazine specifically stimulate the heat-stable Mg^{2+} -independent phosphatidate phosphohydrolase activity in rat lung microsomes. In contrast, the heat-labile Mg^{2+} -independent phosphatidate phosphohydrolase activities in cytosol are inhibited by these reagents. Triton X-100 and chlorpromazine inhibit the Mg^{2+} -dependent phosphatidate phosphohydrolase activities in both rat lung microsomes and cytosol. These results are consistent with the view that a single Mg^{2+} -dependent phosphatidate phosphohydrolase present in both microsomes and cytosol is specifically involved in glycerolipid metabolism.

INTRODUCTION

Phosphatidate phosphohydrolase (EC 3.1.3.4) catalyses the conversion of phosphatidic acid (PA) into 1,2-*sn*-diacylglycerol (DG). This reaction lies at the initial branch-point in glycerolipid metabolism. Modulation of phosphatidate phosphohydrolase activity could influence the biosynthesis of all membrane phospholipids, with the possible exception of sphingomyelin. In lung, this could affect the relative production of unsaturated phosphatidylcholine (PC) and dipalmitoylphosphatidylcholine (DPPC), derived from DG, versus the acidic phospholipids phosphatidylinositol (PI) and phosphatidylglycerol (PG), derived from PA. These glycerolipids constitute the major components of pulmonary surfactant.

Pulmonary microsomal and cytosolic fractions contain

both Mg^{2+} -dependent and Mg^{2+} -independent phosphatidate phosphohydrolase activities. As has been suggested for other tissues, including liver and adipose tissue (Jamdar & Fallon, 1973; Sturton & Brindley, 1977; Cheng & Saggerson, 1978), the Mg^{2+} -dependent activities in lung appear to be responsible for most of the activity observed with membrane-bound PA enzymically generated on microsomal membranes (Casola & Possmayer, 1981*a,b*; Walton & Possmayer, 1985). This relationship arises from the presence of Mg^{2+} in the incubation media during the biosynthetic reaction, resulting in the formation of the magnesium salt of PA. Investigations in our laboratory have presented evidence suggesting that the Mg^{2+} -dependent activities in rat lung microsomal and cytosolic fractions are specifically involved in glycerolipid metabolism. These studies demonstrated that selectively removing the Mg^{2+} -dependent phospho-

Abbreviations used: DG, diacylglycerol; DPPC, dipalmitoylphosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

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hydrolase from rat lung microsomes by salt washing resulted in a marked decrease in the ability to incorporate [^{14}C]glycerophosphate through PA into PC (Walton & Possmayer, 1984). The Mg^{2+} -independent phosphohydrolase activity was not affected by this treatment. Addition of either the salt-washings from the microsomes or partially purified cytosolic Mg^{2+} -dependent phosphatidate phosphohydrolase restored the ability of the microsomes to degrade membrane-bound PA and to produce radioactive PC from glycerophosphate.

A number of exogenous compounds can affect phosphatidate phosphohydrolase activity *in vitro*. Extrapolation of the stimulatory or inhibitory effects of these substances to the situation *in vivo* could be misleading, owing to three separate factors. (1) Assays which do not distinguish between the Mg^{2+} -dependent and Mg^{2+} -independent phosphohydrolases could suggest an effect on glycerolipid metabolism under conditions where only the Mg^{2+} -independent activities are affected. (2) Some substances, such as detergents, could alter the substrate or the enzyme in such a manner that the Mg^{2+} -dependent enzyme could function to degrade PA in the absence of Mg^{2+} . (3) Some compounds, for example amphiphilic drugs, could affect these enzyme reactions *in vitro* in a manner other than that by which they influence these reactions physiologically within the cell.

Studies in a number of tissues have demonstrated that Triton X-100 can inhibit (McCaman *et al.*, 1965; Casola & Possmayer, 1981*b*; Jamdar *et al.*, 1984; Walton & Possmayer, 1985), or stimulate (Casola & Possmayer, 1981*a,b*; Pasquare de Garcia & Giusto, 1986), or have no effect (Sedgewick & Hübscher, 1965; Jamdar *et al.*, 1984) on, the activity of phosphatidate phosphohydrolase. As the enzyme activities were measured under a variety of conditions both with and without Mg^{2+} , the true effect of this detergent is difficult to assess. Studies from our laboratory have reported that Triton X-100 stimulates the phosphatidate phosphohydrolase activity in microsomes and cytosol observed with membrane-bound PA (Casola & Possmayer, 1981*b*), but inhibits the Mg^{2+} -dependent activities observed with PA/PC liposomes (Walton & Possmayer, 1985). This observation suggested either that the activity assayed with membrane-bound PA differed from the Mg^{2+} -dependent activity, or else that, in the presence of Triton, Mg^{2+} was not required for this activity. The present investigations sought to resolve these possibilities.

Amphiphilic cationic drugs such as chlorpromazine, propranolol and methacholine can re-direct the intracellular flux of glycerolipids from the production of triacylglycerols and PC towards the acidic phospholipids PI and PG (Brindley, 1978; Brindley & Sturton, 1982), apparently by inhibiting phosphatidate phosphohydrolase. However, addition of chlorpromazine to subcellular fractions from liver can result in stimulation *in vitro* of phosphatidate phosphohydrolase activity (Bowley *et al.*, 1977; Sturton & Brindley, 1980). Since the stimulations observed with chlorpromazine could be masked by the addition of Mg^{2+} , but were not reversed by EDTA, it was concluded that the requirement for Mg^{2+} was not absolute and could be replaced by the positively charged amphiphilic drugs.

The present studies sought to establish whether the effects of Triton X-100 and chlorpromazine were on the Mg^{2+} -dependent or -independent phosphohydrolase activities of rat lung microsomal and cytosolic fractions, in

order to remove the ambiguity created by the results summarized above.

EXPERIMENTAL

Materials

The method for the preparation of PC and PA and [^{32}P]PA have been described previously (Walton & Possmayer, 1984, 1985). [^{32}P]ATP was purchased from New England Nuclear, Toronto, Ontario, Canada. Biochemicals, including chlorpromazine hydrochloride, were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Reagent-grade chemicals were obtained from Fisher Scientific.

Methods

The microsomal and cytosolic fractions were prepared from the perfused lungs of Sprague-Dawley rats as previously described (Walton & Possmayer, 1984). The cytosol used for these experiments was carefully removed from the supernatant of the 100 000 *g* centrifugation to ensure that membrane fragments were not included. The preparations of egg PC, egg PA and [^{32}P]PA are also outlined in that paper. Phosphatidate phosphohydrolase activity was estimated through the release of [^{32}P]P_i from PA/PC (1:1) liposomes in the absence and presence of 3.25 mM- MgCl_2 . The Mg^{2+} -dependent activity was calculated by difference. Microsomal fractions were assayed with 0.5 mM-PA liposomes, whereas the cytosolic fraction was assayed with 0.2 mM-PA (Walton & Possmayer, 1985). In each case an equimolar concentration of PC was added to form the liposomes.

Heat inactivation of the microsomes and cytosols was performed in a water bath at 55 °C for 15 min, after which the tubes were placed on ice until assayed. The remaining assays are detailed by Walton & Possmayer (1984).

RESULTS AND DISCUSSION

Effect of thermal inactivation

The specific activities of the total Mg^{2+} -independent and Mg^{2+} -dependent phosphatidate phosphohydrolase activities of rat lung microsomal and cytosolic fractions are listed in Table 1. Under the conditions utilized for these studies (see under 'Methods'), the Mg^{2+} -dependent activity accounts for approximately half the total activity in the microsomal fraction, but 80 % of the cytosolic activity. The activities observed in the present studies are somewhat lower than previously reported for this tissue

Table 1. Phosphatidate phosphohydrolase activities in rat lung microsomal and cytosolic fractions

Results are means $\pm$ S.D. ($n = 6$).

Fraction	Phosphatidate phosphohydrolase activity (nmol/min per mg of protein)		
	Total	Mg^{2+} -independent	Mg^{2+} -dependent
Microsomal	3.01 $\pm$ 0.58	1.59 $\pm$ 0.32	1.41 $\pm$ 0.28
Cytosolic	2.96 $\pm$ 0.56	0.64 $\pm$ 0.23	2.33 $\pm$ 0.41

Fig. 1. Thermal-inactivation profiles of the Mg²⁺-dependent (□, ○) and Mg²⁺-independent (■, ●) phosphatidate phosphohydrolase activation in rat lung microsomal (□, ■) and cytosolic (○, ●) fractions

The subcellular fractions were tested at 55 °C, then chilled on ice before assay. Assays were conducted in triplicate. Similar results were obtained in several separate assays.

(Walton & Possmayer, 1985). The reason for this difference is not known, but appears to be related to differences in the substrate rather than the enzymes.

As reported previously (Walton & Possmayer, 1985), incubation at 55 °C results in a rapid decrease in the Mg²⁺-dependent activity of rat lung microsomal and cytosolic fractions, resulting in the virtual abolition of the Mg²⁺-dependent components by 10 min (Fig. 1). The Mg²⁺-independent phosphohydrolase activities are considerably more resistant. However, this resistance varied with the subcellular source of the preparation. The cytosolic activity fell to approximately half the control value within 5 min at 55 °C, and remained at this value. The microsomal activity fell to approx. 75 % of control after 5 min, and then slowly returned to the control activity with prolonged incubation. The basis of this recovery is unknown, but may reflect an increased accessibility of the heat-stable phosphohydrolase to the PA-containing liposomes as thermally denatured proteins are removed from the microsomal surface. An increase in the Mg²⁺-independent activity was also observed with salt-washed microsomes (Walton & Possmayer, 1984), and a reversible increase in the phosphohydrolase activity assayed with membrane-bound PA is observed after treatment of microsomes with increasing amounts of trypsin and chymotrypsin (Casola & Possmayer, 1981*a*). The similarity of the inactivation curves for the Mg²⁺-dependent phosphohydrolase in the microsomes and cytosol are consistent with, but do not prove, the presence of a single Mg²⁺-dependent enzyme in these lung fractions, as previously suggested (Casola & Possmayer, 1981*b*; Walton & Possmayer, 1985). On the other hand, the stability profiles of the Mg²⁺-independent activities in the microsomes and cytosol suggest the presence of two or more phosphohydrolase activities in the cytosol capable of hydrolysing PA in the absence of Mg²⁺. Whether the Mg²⁺-independent activities are specific for

phosphatidic acid cannot be determined by the present approach. Caras & Shapiro (1975) have reported the presence of specific and non-specific phosphatidate phosphohydrolase activities in rat liver.

Effect of Triton X-100 on pulmonary phosphatidate phosphohydrolase

Fig. 2(*a*) shows that the total phosphatidate phosphohydrolase activity in microsomes is stimulated approx. 6-fold by 0.5 % Triton X-100. The Mg²⁺-dependent component was decreased to zero. In cytosol, 0.5 % Triton inhibits the Mg²⁺-dependent activity by close to 90 %, and the Mg²⁺-independent phosphohydrolase activity is decreased by 50 % (Fig. 2*c*). This results in an overall 80 % decline in the total phosphatidate phosphohydrolase activity. These observations suggest that 0.5 % Triton X-100 inhibits the Mg²⁺-dependent activity in both the microsomes and cytosol. Since Mg²⁺ ions were not required, it appears that the Mg²⁺-independent activity in the microsomes was stimulated by the detergent. However, previous studies (Casola & Possmayer, 1981*a,b*) demonstrated that the micro-

Fig. 2. Effects of Triton X-100 on the phosphatidate phosphohydrolase activation of control and heat-inactivated lung microsomes and cytosol

Control (*a, c*) or heat-inactivated (*b, d*) microsomes (*a, b*) and cytosols (*c, d*) were assayed in the presence and absence of Mg²⁺. The total (mean + S.D.) and Mg²⁺-independent (□; mean - S.D.) activities were determined, and the Mg²⁺-dependent activity (▨) was calculated by difference. The assays were conducted in triplicate.

somal activity observed with membrane-bound PA is enhanced by 0.5% Triton. Since membrane-bound PA is an excellent substrate for the Mg^{2+} -dependent phosphohydrolase, these observations raised the possibility that Triton X-100 could alter the physical form of PA in such a manner to make it susceptible to degradation by the Mg^{2+} -dependent enzyme even in the absence of Mg^{2+} .

The nature of the enzymic activity affected by Triton was tested by taking advantage of the differences in the thermal-inactivation profiles of the Mg^{2+} -dependent and Mg^{2+} -independent phosphatidate phosphohydrolase (Fig. 1), which reveal that the Mg^{2+} -dependent activities are essentially abolished by heating for 15 min at 55 °C, whereas the Mg^{2+} -independent activity is only slightly affected in the microsomes and decreased by 60% in the cytosol. As shown in Fig. 2(b), heat-inactivated microsomes retained the marked stimulation by Triton. This result is indicative of a specific stimulation of the Mg^{2+} -independent phosphohydrolase in the microsomes and is consistent with the view that the Mg^{2+} -dependent phosphohydrolase in both the microsomal and cytosolic fractions is inhibited by the detergent. Heat treatment abolished the Mg^{2+} -dependent phosphohydrolase activity in the cytosol and also decreased the Mg^{2+} -independent activity (Fig. 2d). In contrast with the results obtained with the microsomal fraction, the heat-stable phosphohydrolase in the cytosol was not affected by Triton. This observation suggests that the heat-stable phosphohydrolase activity present in the cytosol differs from the Mg^{2+} -independent activity associated with the microsomes. These studies have been repeated with similar results.

These results indicate that the Mg^{2+} -independent phosphatidate phosphohydrolase activity associated with the microsomal fraction, which appears to be a heat-stable integral protein (Bell & Coleman, 1980; Ballas & Bell, 1980; Casola & Possmayer, 1981a,b; Walton & Possmayer, 1984), is specifically stimulated by Triton. The previous observation that the pulmonary phosphohydrolase activities observed with membrane-bound PA were also stimulated by 0.5% Triton (Casola & Possmayer, 1981a,b) appears to conflict with the suggestion that these activities are due to the Mg^{2+} -dependent phosphohydrolase activity. However, it should be noted that, in the earlier studies, heat treatment abolished only 65% of the microsomal activity observed with the membrane-bound substrate. Stimulation of the remaining heat-stable activity, presumably corresponding to the Mg^{2+} -independent component, could account for the increase in activity with Triton.

Effect of chlorpromazine on phosphatidate phosphohydrolase

Although cationic amphiphilic drugs such as chlorpromazine can act as competitive inhibitors of phosphatidate phosphohydrolase (Bowley *et al.*, 1977; Sturton & Brindley, 1980), a stimulation is observed in the absence of Mg^{2+} (Bowley *et al.*, 1977). This has led to the suggestion that the positively charged chlorpromazine can replace Mg^{2+} under certain circumstances, and therefore this enzyme should be referred to as Mg^{2+} -stimulated rather than Mg^{2+} -dependent (Brindley & Sturton, 1982).

Fig. 3(a) reveals that exposure of rat lung microsomes to 1.0 mM-chlorpromazine stimulates total phosphatidate

Fig. 3. Effects of chlorpromazine on the phosphatidate phosphohydrolase activation of control and heat-inactivated lung microsomes and cytosols

Control (a, c) or heat-inactivated (b, d) microsomes (a, b) and cytosols (c, d) were assayed in the presence and absence of Mg^{2+} . The total (mean + s.d.) and Mg^{2+} -independent ($\square$; mean - s.d.) activities were determined, and the Mg^{2+} -dependent activity (hatched) was calculated by difference. The assays were conducted in triplicate.

phosphohydrolase activity. The Mg^{2+} -dependent activity was decreased, suggesting that chlorpromazine could be replacing the requirement for Mg^{2+} . After heat treatment to inhibit the Mg^{2+} -dependent activity, the microsomal fraction retained the stimulation observed with chlorpromazine (Fig. 3b). This stimulation must be associated with the Mg^{2+} -independent activity. Hence it can be concluded that the decrease in the Mg^{2+} -dependent activity shown in Fig. 3(a) reflects the inhibition of this enzymic activity rather than the replacement of Mg^{2+} by chlorpromazine.

The presence of chlorpromazine resulted in a marked decrease in the total phosphatidate phosphohydrolase in the cytosolic fraction (Fig. 3c). The Mg^{2+} -dependent activity was almost completely inhibited by the cationic drug, and the Mg^{2+} -independent activity was decreased by half. Heat treatment abolished the Mg^{2+} -dependent phosphohydrolase activity in the cytosol and lowered the Mg^{2+} -independent activity by 60% (Fig. 3d). The remaining heat-stable Mg^{2+} -independent activity was not affected by chlorpromazine. These experiments have been repeated several times with the same results.

These observations provide strong evidence indicating that chlorpromazine cannot replace Mg²⁺ with the Mg²⁺-dependent phosphohydrolase activities of rat microsomes or cytosol. Rather, this cationic amphiphilic drug inhibits the Mg²⁺-dependent activity, particularly with the cytosolic fraction. The inhibition of the Mg²⁺-dependent activity by chlorpromazine is consistent with the ability of chlorpromazine to divert the incorporation of radioactive precursors from neutral lipids and PC into the acidic phospholipids with cells and tissue slices (Pappu & Hauser, 1983; Weiss *et al.*, 1982; see Brindley & Sturton, 1982; Brindley, 1984, for review). The contrast between the inhibitory effects of chlorpromazine on phosphatidate phosphohydrolase *in situ* and the stimulatory effects on the Mg²⁺-independent activity as assayed *in vitro* serve to support the contention that the Mg²⁺-dependent phosphohydrolase activity is specifically involved in glycerolipid metabolism (Casola & Possmayer, 1981a; Walton & Possmayer, 1984, 1986).

In conclusion, the observations reported in the present paper are consistent with the view that the Mg²⁺-dependent and -independent phosphatidate phosphohydrolase activities reside in different proteins, and that it is the Mg²⁺-dependent phosphatidate phosphohydrolase activities that are involved in glycerolipid metabolism. Whether these working hypotheses will withstand future examination is unknown. Nevertheless, it is clear that previous work which did not take into account the presence of separate Mg²⁺-dependent and -independent phosphatidate phosphohydrolases must be re-interpreted with respect to surfactant lipid synthesis (Schultz *et al.*, 1974; Ravinuthala *et al.*, 1978; Mavis *et al.*, 1978; Brehier *et al.*, 1977; Johnston *et al.*, 1978; Ballard *et al.*, 1980; Benson, 1980; Crecelius & Longmore, 1983; Filler & Rhoades, 1982; Freese & Hallman, 1983). In particular, those studies which have employed phosphatidate phosphohydrolase solubilized with Triton X-100 as the substrate to study lung fractions (Johnston *et al.*, 1978) or type II cells (Douglas *et al.*, 1983) may be monitoring a lamellar-body-associated phosphatase (Casola *et al.*, 1982) rather than the capacity for enhanced PC synthesis. In the same vein, it should be acknowledged that, since the work described in the present investigations was conducted with fractions from whole lung rather than isolated type II cells, the possibility exists that the results may not be relevant to surfactant production.

One of the difficulties associated with studying this enzymic reaction relates to the amphipathic, and therefore insoluble, nature of the substrate, which requires artificial conditions that do not necessarily mimic the conditions *in vivo*. Kinetic analyses conducted in our laboratory are consistent with the idea, but do not prove, that the Mg²⁺-dependent phosphatidate phosphohydrolase hydrolyses primarily, if not only, the magnesium salt of PA. Whether this represents a universal property for this enzyme in all tissues is not clear. Studies by Martin *et al.* (1987) have revealed that, after dialysis of rat liver microsomal and cytosolic fractions against EDTA to remove bivalent cations, the Mg²⁺-dependent stimulation rose over 100-fold. These authors noted that, although chlorpromazine stimulated the hepatic phosphatidate phosphohydrolase activity assayed with PA alone, little effect was noted with PA/PC liposomes.

The studies in the present report demonstrate that both Triton X-100 and chlorpromazine inhibit the Mg²⁺-dependent activities in rat lung microsomes and cytosol.

The Mg²⁺-independent activity in the microsomes, but not the cytosol, is stimulated by these agents. The similarity in the effects of Triton X-100 and chlorpromazine is interesting and could indicate a similar mechanism. The inhibitory effects of chlorpromazine and Triton X-100 on the Mg²⁺-dependent phosphatidate phosphohydrolase activities in pulmonary microsomal and cytosolic fractions are consistent with our working hypothesis that these activities reside in a single enzyme, which is highly dependent on Mg²⁺. Treatment of tissue slices or isolated cells with Triton X-100 (Toth *et al.*, 1987) or amphiphilic cationic drugs (Pappu & Hauser, 1983; Weiss *et al.*, 1982; Brindley, 1984) results in alterations in glycerolipid metabolism which can be explained through an inhibition (Mg²⁺-dependent activity) rather than a stimulation (Mg²⁺-independent activity) of phosphatidate phosphohydrolase. It has become apparent that the Mg²⁺-dependent activity in lung can be controlled through translocation of the cytosolic enzyme to the endoplasmic reticulum, where its substrate PA is produced (Walton & Possmayer, 1986). The studies in the present paper are in accord with previous studies indicating that amphiphilic cationic drugs can affect the physiological activity of the Mg²⁺-dependent phosphatidate phosphohydrolase through inhibiting its translocation from cytosol, as has been observed in studies with hepatocytes (Brindley, 1984; Hopewell *et al.* 1985; Martin *et al.*, 1987). Further investigations are required to confirm whether these drugs affect pulmonary phosphatidate phosphohydrolase via this mechanism and to determine its effect on the biosynthesis of phospholipids for pulmonary surfactant.

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