Tissue-specific distribution and subcellular distribution of phospholipase D in rat: evidence for distinct RhoA- and ADP-ribosylation factor (ARF)-regulated isoenzymes

Joseph J. PROVOST, Jennifer FUDGE, Sallie ISRAELIT, Abdur R. SIDDIQI* and John H. EXTON†

Howard Hughes Medical Institute and Departments of Molecular Physiology and Biophysics and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

Phospholipase D (PLD) is regulated by many factors including the small G-proteins, RhoA and ADP-ribosylation factor (ARF). The present study examined the distribution of RhoA- and ARF-responsive PLD in membranes, microsomes and cytosol of rat tissues and in rat liver subcellular fractions. PLD was present in all tissue fractions examined and was stimulated by guanosine 5'-[γ-thio]triphosphate (GTP[S]), with the highest specific activities being in lung, kidney and spleen. When myristoylated recombinant ARF (mARF) was added with GTP[S], the PLD activity was stimulated further, but the addition of RhoA was without effect. However, in extracts from crude membranes both mARF and RhoA enhanced the stimulation by GTP[S], with high specific activities of PLD being observed in all tissues except muscle. The response to mARF was usually greater than to RhoA, and the responses were additive, except for liver, which showed synergism. When the PLD activity of subcellular fractions

of liver was examined, GTP[S] caused increases in all fractions except microsomes and mitochondria, which exhibited low activity. All fractions except mitochondria showed responses to RhoA and mARF, with the response to RhoA being greater in plasma membranes and that to mARF being greater in Golgi and nuclei. Western blotting showed that RhoA was located mainly in the cytosol and plasma membranes, whereas ARF was principally in the cytosol. These findings demonstrate the widespread occurrence of significant activity of both Rho- and ARF-responsive forms of PLD in membranes from all tissues except muscle, and the presence of both forms in liver subcellular fractions except mitochondria. The large variations in the relative responses of PLD to Rho and ARF observed in different tissues and fractions support the existence of different isoforms of the enzyme.

INTRODUCTION

Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PC) to choline and phosphatidic acid (PA) in response to many agonists in a wide variety of tissues [1–3]. Because PA is a biologically active lipid and is a major precursor of diacylglycerol in certain cell types [1,3], the regulation of PLD is of interest. PLD is present in crude membranes of rat liver, bovine brain and HL60 cells [4–7]. It has also been found in the Golgi apparatus of Chinese hamster ovary (CHO) cells [8], the cytosol of HL60 cells and several bovine tissues [9,10], the microsomes of brain and lung [6,11], and the nuclei of Madin–Darby canine kidney (MDCK) cells [12,13].

The mechanisms by which PLD is activated are not well defined, but the enzyme has been shown to be regulated by several effectors. Rat brain PLD is stimulated by oleate and Triton X-100 [6,11,14,15] and Ca²⁺ ions can both activate and inhibit PLD, depending on the tissue and Ca²⁺ concentration [11,15,16]. Protein kinase C (PKC) has been implicated in the activation of PLD by many agonists, but there is also evidence for PKC-independent mechanisms [1]. Regulation of PLD activity has also been found for the small G-proteins Rho and ADP-ribosylation factor (ARF). The latter is required for coatamer assembly and vesicle trafficking in Golgi [17] and has been shown to be an activator of PLD in neutrophils, HL60 cells,

liver and brain [8,9,14,18–22]. Members of the Rho family (Rho, Rac and Cdc42) are involved in structural rearrangements of the actin cytoskeleton [23–25], and in the regulation of phosphatidylinositol 3-kinase [26,27], phosphatidylinositol-4-phosphate 5-kinase [28] and certain protein kinases [29–34]. Rho and Rac proteins are involved in the regulation of PLD in rat liver, neutrophils and HL60 membranes, nuclei from MDCK cells, and brain [4,5,9,12,22,35].

The existence of different mechanisms of PLD regulation raises the possibility that there are different isoenzymes. The recent cloning of a human PLD [36] may lead to the discovery of different forms of the enzyme. Here we report the distribution of PLD and its response to RhoA and ARF in six rat tissues, and also the subcellular localization of RhoA- and ARF-responsive PLD in rat liver.

EXPERIMENTAL

Materials

Dipalmitoyl-PC, phosphatidylethanolamine (PE), PA and phosphatidylbutanol (PBut) were purchased from Avanti Polar Lipids Corp. Guanosine 5'-[γ-thio]triphosphate (GTP[S]) was obtained from Boehringer Mannheim. LK6D silica-gel thin-layer plates were purchased from Whatman and dipalmitoyl-[2-palmitoyl-9,10-³H]PC from DuPont NEN. Reactive Green-19

Abbreviations used: PLD, phospholipase D; ARF, ADP-ribosylation factor; mARF, myristoylated recombinant human ARF3; MDCK, Madin–Darby canine kidney; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PBut, phosphatidylbutanol; GTP[S], guanosine 5'-[γ-thio]triphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; Rho GDI, Rho GDP dissociation inhibitor; DTT, dithiothreitol; PKC, protein kinase C.

*Present address: National Jewish Center for Immunology and Respiratory Medicine, Department of Pediatrics, 1400 Jackson Street, Denver, CO 80262, U.S.A.

† To whom all correspondence should be addressed.

was purchased from Sigma and OptiPrep (iodixanol) from Nycomed Pharma. Polyclonal antibodies against RhoA were from Santa Cruz Biotechnology, Inc., and human ARF3 cDNA and antibodies raised against ARF3 were kind gifts from Dr. Joel Moss (National Institutes of Health). Phosphatidylinositol 4,5-bisphosphate (PIP₂) was prepared as described by Schacht [37,38]. Polyclonal antibodies to α -mannosidase II were obtained from Dr. Kelley Moremen (University of Georgia).

Small G-protein preparation

Bovine brain RhoA was purified to near homogeneity by the method of Williamson et al. [39] with the following changes: RhoA was detected by Western blotting and its ability to stimulate PLD. The eluate from hydroxyapatite was made 500 mM NaCl, 5 mM MgCl₂ and 0.1 % sodium cholate and loaded on to a phenyl-Sepharose column. The RhoA protein was eluted from the column with 10 mM Tris/HCl, pH 8.0, 4 % sodium cholate, 0.1 mM EDTA, 0.1 mM PMSF and dithiothreitol (DTT). Fractions containing RhoA were then concentrated using an Amicon Centriprep and applied to an Ultrogel column as described in [39]. In order to remove contaminating proteins, the sample was then dialysed against 10 mM Tris/HCl, pH 8.9, 0.1 mM EDTA, 1 mM MgCl₂, 0.1 mM DTT and 0.1 mM PMSF before loading on to a Reactive Green-19 column. RhoA was eluted with a 0 to 0.5 M NaCl gradient followed by a 2 h pulse of 2 M NaCl. Silver-stained SDS/PAGE of the final preparation showed two minor proteins in addition to RhoA. The final preparation was found, by Western blotting, to be free of ARF, RhoB, RAC1, Rho GDP dissociation inhibitor (Rho-GDI) and CDC42. Recombinant human ARF3 (mARF) was co-expressed with myristoyltransferase in *Escherichia coli* and prepared as described by Weiss et al. [40].

Preparation of crude cell fractions and membrane extracts

Tissues were removed from a rat and all but the muscle and lung tissue were homogenized in a buffer (10 mM Hepes, pH 7.5, 250 mM sucrose, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF and 0.1 μ M leupeptin) with five passes using a Potter–Elvehjem homogenizer. The lungs were first frozen in liquid N₂ and blended to a powder before using the Potter–Elvehjem homogenizer, and skeletal muscle was homogenized with a Polytron homogenizer. Each homogenate was filtered through four layers of cheesecloth, centrifuged at 500 *g* for 5 min and the pellet discarded. The supernatant was centrifuged at 10000 *g* for 30 min, and the pellet was resuspended in homogenization buffer to approx. 10 mg of protein/ml and designated crude membranes. The supernatant solution was further centrifuged at 100000 *g* for 60 min. The pellet was resuspended as before and designated microsomes, whereas the supernatant was labelled cytosol.

To prepare extracts from the crude membrane fractions, equal volumes of membranes (4–8 mg of protein) and 1.2 M NaCl in 10 mM Tris/HCl, pH 7.5, 0.1 mM DTT, 1 mM EDTA and 0.5 mM PMSF were mixed at 4 °C for 30 min, centrifuged at 14000 *g* for 30 min and the supernatant (15–30 μ g of protein) assayed for PLD as described below.

Preparation of rat liver subcellular fractions

Plasma membranes, mitochondria, microsomes and Golgi apparatus were prepared using differential centrifugation through various concentrations of sucrose as described by Siddiqui and Exton [41]. Rat liver nuclei were prepared using iodixanol density centrifugation. Briefly, fresh rat liver was homogenized in a

buffer containing 10 mM Tricine/NaOH, 250 mM sucrose, 1 mM DTT, 1 mM EDTA, 0.5 mM PMSF, 0.1 μ M leupeptin, 25 mM KCl and 5 mM MgCl₂. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1000 *g* for 10 min to yield a pellet that was resuspended and recentrifuged. The pellet was then made to 25 % iodixanol, underlaid with equal volumes of 30 % and 35 % iodixanol in homogenization buffer, and centrifuged for 10000 *g* for 20 min. The nuclei were found at the 30–35 % interface, resuspended and washed in homogenization buffer. Subcellular liver fractions were characterized by their specific markers. Lactate dehydrogenase and cytochrome *c* oxidase activities were assayed as described by Storrie and Madden [42]. Glucose 6-phosphatase was assayed according to the method of Nordlie and Arion [43]. 5'-Nucleotidase activity was measured according to the method of Siddiqui and Exton [41]. Fractions enriched with Golgi were determined by Western blotting using antibodies to the Golgi-specific enzyme α -mannosidase II [44]. Nuclear fractions were analysed by subjecting 5 μ g of each fraction to sonication and then to DNA agarose electrophoresis. The nuclear fractions were determined by the presence of DNA laddering after staining with ethidium bromide.

PLD assay

PLD activity was determined by the formation of PBut in the presence of 1 % butanol using phospholipid vesicles (PE/PIP₂/PC, 16:1.4:1, by vol.) as described by Brown et al. [19]. Assays proceeded for 30 min before termination by addition of chloroform/methanol/HCl (50:98:2, by vol.). The lipids were extracted and analysed by the formation of PBut as described by Siddiqui et al. [9].

Immunoblots

Tissue samples were subjected to SDS/14 %-PAGE (Novex Experimental Corp.) and transferred to poly(vinylidene difluoride) (PVDF) paper (Novex Experimental Corp.). The paper was blocked for 1 h (with 5 % non-fat dry milk for anti-RhoA blots and 1 % BSA and goat antiserum for anti-ARF), rinsed, and incubated overnight with either 1:500 anti-RhoA or 1:1000 anti-ARF sera. After rinsing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (Vector Laboratories) and developed by enhanced chemiluminescence (Amersham Corp.).

RESULTS

Tissue distribution of PLD

The unique transphosphatidylation activity of PLD was used to determine the distribution of this enzyme in rat tissues and its responses to exogenously added RhoA or mARF (Figures 1A–1F). Additionally, Western blotting was also employed to examine the presence of RhoA and ARF in the fractions (Figure 2). Crude membranes from all tissues examined showed basal PLD activity, but lung, spleen and kidney showed the highest specific activity (Figures 1A–1F). There was some variation in the distribution of basal PLD activity between the crude membrane, microsome and cytosol fractions in each tissue, but this was 2-fold or less, except in the case of muscle where activity in the crude membranes was very low relative to the other fractions (Figures 1A–1F).

In all tissues and all fractions, addition of GTP[S] produced a large stimulation of PLD activity. The specific activity of guanine nucleotide-sensitive PLD in crude membranes was highest in

Figure 1 Effects of GTP[S], RhoA and mARF on PLD activity in the crude cell fractions of (A) rat liver, (B) brain, (C) kidney, (D) lung, (E) spleen and (F) muscle

The cell fractions of rat liver (113–151 μ g of protein) (A), rat brain (90–173 μ g of protein) (B), rat kidney (88–124 μ g of protein) (C), rat lung (71–142 μ g of protein) (D), rat spleen (102–132 μ g of protein) (E) and rat muscle (67–90 μ g of protein) (F) were prepared by differential centrifugation and assayed for PLD activity in the presence or absence of 30 μ M GTP[S], 0.37 nM RhoA, or 1 μ M mARF as described in the Experimental section. Data are representative of three experiments performed in duplicate.

Figure 2 Distribution of RhoA and ARF in crude cell fractions of various rat tissues

(A) Crude cell fractions (15 μ g of protein for liver, kidney, lung and brain and 50 μ g of protein of spleen and muscle) were subjected to SDS/14%-PAGE and immunoblotted for the presence of RhoA as described in the Experimental section. (B) Samples (50 μ g of protein) of the cell fractions for each tissue were prepared and blotted for ARF as described in the Experimental section.

lung, kidney and spleen, but the other tissues also showed large (5- to 12-fold) responses to GTP[S] (Figures 1A–1F). When RhoA was added to the crude membranes from any tissue in the presence of GTP[S], it caused no significant increase in PLD

activity compared with GTP[S] alone. In contrast, exogenous mARF caused a further increase in the formation of PBut in the crude membranes in all tissues except muscle (Figures 1A–1F).

The microsomal fractions of all the tissues showed a variable (2- to 8-fold) increase in PLD activity with GTP[S]. However, as was found for the crude membranes, the further addition of RhoA caused little or no change, whereas significant increases were observed with the addition of mARF (Figures 1A–1F). In all tissues except muscle, the PLD activity of the cytosol increased markedly with addition of GTP[S]. However, in no case did RhoA cause a further stimulation. (In the case of crude kidney cytosol, an inhibitory effect of added RhoA was consistently observed. However, when the PLD activity was partially purified using chromatography on DEAE-Sephacel and hydroxyapatite, the inhibition was no longer observed.) In contrast, the addition of mARF caused a 1.5- to 2.5-fold increase in the cytosolic activity of liver, brain, spleen and muscle (Figures 1A–1F).

Because the addition of GTP[S] alone to the subcellular fractions produced significant increases in PLD activity, it seemed likely that members of the Rho and ARF families were present in the fractions. In support of this idea, Western blotting revealed the presence of RhoA in crude membranes, microsomes and cytosol in all tissues, except muscle, where it was found almost exclusively in the microsomal fraction (Figure 2). ARF was found predominantly in the cytosol in most tissues. It was also detected in the microsomes, but was not detected in crude membranes from lung and muscle (Figure 2).

PLD activity in membrane extracts

Because of the low activity of PLD in crude membranes and the lack of a response to RhoA, the membranes were extracted with

Figure 3 Tissue distribution of PLD in salt extracts of crude membranes

Crude membranes of each tissue were extracted as described in the Experimental section and the supernatants (15–30 μ g of protein) were assayed for PLD activity in the presence or absence of 30 μ M GTP[S], 0.37 nM RhoA and/or 1 μ M mARF as described in the Experimental section. Data are representative of two experiments performed in duplicate.

Figure 4 Subcellular distribution of PLD activity in rat liver

Rat liver subcellular fractionation was performed by density gradient centrifugation and the fractions (51–76 μ g of protein) were assayed for PLD activity as described in the Experimental section in the presence or absence of 30 μ M GTP[S], 0.37 nM RhoA, or 1 μ M mARF. Data are representative of two experiments performed in duplicate.

0.6 M NaCl, centrifuged and the resulting supernatant analysed for PLD activity (Figure 3). In the case of liver, brain and muscle, this treatment caused a very large increase in PLD specific activity [cf. Figures 1(A)–1(F) with Figure 3]. Addition of GTP[S] to these extracts produced large increases in PLD activity, except in the case of muscle (Figure 3). The response of PLD to GTP[S] in the extracts was significantly enhanced by addition of RhoA except for muscle and spleen (Figure 3), in contrast to what was observed with crude membranes. Addition of mARF produced increases which were greater than those with RhoA, except in brain and liver. When RhoA and mARF were added together, the increases were additive within experimental error, except in the case of liver, where a synergistic interaction was observed (Figure 3).

To determine whether or not the salt treatment of the membranes extracted RhoA or ARF, Western blotting of the extracts was performed. The results showed that RhoA was high in the extracts from brain and spleen and detectable in that from kidney, but absent in the other tissue extracts (results not shown). ARF immunoreactivity was high in the brain extract, detectable in the kidney and spleen extracts, but absent from the other extracts (results not shown). These results suggest that the very large stimulation of PLD activity induced by GTP[S] alone in the brain, spleen and kidney extracts was due to the presence of both RhoA and ARF.

Subcellular localization of PLD in rat liver

Liver was chosen as the tissue for examination of the subcellular distribution of PLD because methods for subfractionation of this tissue are well developed [41] and because PLD activity in liver is under agonist regulation [1]. Fractions enriched in plasma membranes, Golgi, nuclei, microsomes, mitochondria and cytosol were prepared and assayed for PLD activity (Figure 4). Analysis of the subcellular liver fractions showed a 6- to 15-fold enrichment of enzyme activities in the appropriate fractions (Table 1). Significant glucose-6-phosphatase activities were observed in the plasma membrane and nuclear fractions, but the highest specific activity was associated with the microsomal fraction. When the fractions were immunoblotted against α -mannosidase II, a Golgi-specific enzyme [44], only the Golgi fraction displayed immunoreactivity (results not shown). Additionally, only the nuclei exhibited DNA laddering after DNA agarose gel electrophoresis (results not shown).

Table 1 Marker enzyme analysis of rat liver subcellular fractions

Rat liver subcellular fractions were prepared and analysed for marker enzymes as described in the Experimental section. Liver fractions (10–20 μ g) were characterized by the enzymic activities (μ mol/min per mg of protein) of 5'-nucleotidase (plasma membrane), lactate dehydrogenase (cytosol), glucose 6-phosphatase (microsomes) and cytochrome *c* oxidase (mitochondria). Values are means $\pm$ S.D. of assays performed in triplicate. Abbreviation: ND, not detected.

Cell fraction	Specific activity (pmol/min per mg of protein)			
	5'-Nucleotidase	Lactate dehydrogenase	Glucose-6-phosphatase	Cytochrome <i>c</i> oxidase
Plasma membrane	0.229 $\pm$ 0.005	0.31 $\pm$ 0.02	0.097 $\pm$ 0.003	0.05 $\pm$ 0.06
Cytosol	0.015 $\pm$ 0.002	2.13 $\pm$ 0.27	ND	ND
Golgi	0.025 $\pm$ 0.009	0.10 $\pm$ 0.04	0.041 $\pm$ 0.001	0.04 $\pm$ 0.03
Nuclei	0.040 $\pm$ 0.017	0.17 $\pm$ 0.11	0.154 $\pm$ 0.003	0.13 $\pm$ 0.01
Microsomes	ND	0.21 $\pm$ 0.02	0.208 $\pm$ 0.003	0.11 $\pm$ 0.05
Mitochondria	0.024 $\pm$ 0.007	0.09 $\pm$ 0.02	0.032 $\pm$ 0.001	3.30 $\pm$ 0.34

Figure 5 Subcellular distribution of RhoA and ARF in rat liver

Cell fractions (50 μ g of protein) were subjected to SDS/14%-PAGE and immunoblotted for the presence of RhoA and ARF as described in the Experimental section.

The highest specific activity of GTP[S]- and G-protein-stimulated PLD was associated with the plasma membrane (Figure 4, note difference in ordinate). Significant stimulated PLD activity was found in the Golgi and nuclei, but the activity in the cytosol and microsomes was lower, and that in the mitochondria was negligible. Immunoanalysis of the subcellular fractions showed that RhoA was present in the cytosol and plasma membrane, but was barely detectable in Golgi (Figure 5). In contrast, ARF was only detected in the cytosol (Figure 5). (The nature of the faster-migrating protein in nuclei that reacted with anti-ARF antibody was not explored.)

Figure 6 Concentration dependence of PLD activity on RhoA and mARF in isolated liver plasma membranes and nuclei

PLD activity was assayed in the presence of 1% butanol of 65 μ g of protein of rat liver plasma membranes (A) and 35 μ g of protein of rat liver nuclei (B) in the presence of 30 μ M GTP[S] and the indicated concentrations of RhoA or mARF as described in the Experimental section. Results are representative of two experiments performed in duplicate.

The activity of PLD was markedly increased by addition of GTP[S] to all subcellular fractions except microsomes and mitochondria (Figure 4). When RhoA was added in addition to GTP[S], there was a further increase of PLD activity in all the fractions except the cytosol and mitochondria, and the increase observed with plasma membranes was very large. Addition of mARF also increased the action of GTP[S] in all the fractions except in mitochondria. The increases were most marked in Golgi, nuclei and cytosol. Thus, the plasma membrane fraction showed the greatest response to RhoA, and the Golgi and nuclei were most responsive to ARF.

Dose response of PLD to small G-proteins

As noted above, the PLD activity of rat liver plasma membranes was more responsive to RhoA than ARF, whereas the reverse was true for the activity in nuclei (Figure 4). To explore this difference in more detail, PLD activity from both plasma membrane and nuclei was determined in the presence of 30 μ M GTP[S] and increasing concentrations of mARF and RhoA (Figure 6). Plasma membrane PLD was stimulated by both RhoA and mARF in a dose-dependent manner (Figure 6A). Although full dose-response curves could not be performed due to the lack of large amounts of the small G-proteins, it was very clear that much higher concentrations of mARF were required compared with RhoA and RhoA appeared to have a greater effect than mARF (Figure 6A). In contrast, the PLD activity of nuclei was stimulated more by mARF than by RhoA (Figure 6B). The Figure shows a very large difference in the potency of RhoA and mARF as activators of either form of PLD. However, the effective concentrations of the two G-proteins were similar to those observed in other studies [4,9,14,19,22,35,36,45].

DISCUSSION

The importance of PLD is evident in its widespread occurrence and the wide variety of agonists that control its activity [1–3]. While the existence of PLD in particulate and soluble fractions of bovine tissues has been shown [10], its response to small G-proteins in various tissues was not known. Studies of its regulation have been done mostly with either RhoA or ARF and few studies have compared the effects of both G proteins [9,22,45]. While a human PLD whose activity is stimulated by ARF has recently been cloned [36], it is unclear how many isoenzymes of PLD exist. In this study we present the tissue and subcellular distribution of RhoA- and ARF-responsive PLD activity.

The present study showed basal PLD activity in crude membranes and microsomal and cytosolic fractions of all the tissues examined. However, its specific activity was highest in lung, kidney and spleen (Figures 1A–1F). The presence of PLD in cytosol has been recognized in only a few studies [9,10]. The failure of most previous studies to detect cytosolic PLD is almost certainly due to the absence of PIP₂ in the assays, since this lipid is required for basal PLD activity as well as stimulation by small G-proteins [9,19,20,22].

The fact that GTP[S] stimulated PLD activity in all the crude tissue fractions examined indicates that they contain G-proteins, which are likely to be members of the Rho and ARF families. The presence of such proteins was shown by Western blotting, except that RhoA was not detectable in crude membranes and cytosol from muscle, and ARF was apparently absent in the membranes from muscle and lung (Figure 2). In all the tissues, except muscle, cytosol contained the highest concentration of ARF. The absence or low level of the G-proteins in muscle membranes and cytosol accounts for the negligible effects of

GTP[S] in these fractions (Figure 1F). The low level of Rho proteins in muscle extracts has been noted previously [46].

When the effects of RhoA and mARF were tested in the crude tissue fractions, a striking difference was observed, namely the effects of GTP[S] were enhanced by mARF, but not RhoA. The simplest explanation is that the crude fractions already contain sufficient Rho proteins to provide a full response. As noted above, all the tissue fractions except muscle membranes and cytosol contained RhoA (Figure 2) and it is likely that other Rho-type proteins were also present. In support of this idea, when PLD was extracted from crude membranes with NaCl, stimulation by exogenous RhoA became apparent (Figure 3). Furthermore, other studies on membranes from rat liver, neutrophils and HL60 cells [4,5,9] have shown that treatment with Rho GDI is necessary in order to demonstrate stimulation of PLD by Rho proteins. Rho GDI acts in part by extracting Rho proteins from the membranes [4,5,48].

The present findings reveal interesting differences in the relative responses of various tissues to RhoA and ARF. Since the crude fractions did not respond to RhoA (Figures 1A–1F), the comparison was made with membrane extracts (Figure 3). All the tissue extracts exhibited both RhoA- and ARF-responsive PLD activity, with the response to ARF being greater than that to RhoA, except in the case of brain and liver. Although these differences could represent different tissue distributions of ARF- and Rho-responsive PLD isoenzymes, they could also be due to differences in the extraction of these isoenzymes from the membranes or of proteins that modulate the effects of ARF and Rho [35,42,49,50]. The additive responses observed in most cases when RhoA and mARF were added together are suggestive of different isoenzymes. However, this conclusion is only tentative since it was not established that the concentrations of the G-proteins were maximally effective. In one case, liver, a synergistic response was observed. This could suggest the presence of a PLD isoenzyme that responds to both Rho and ARF, but other interactions, e.g. with other factors that regulate PLD, are possible.

Subcellular fractions from liver also showed differences in the responses of PLD to RhoA and ARF (Figure 4). Enzyme marker and DNA analyses indicated a high degree of purity of the fractions (Table 1). The highest GTP[S]-stimulated PLD activity was in the plasma membrane fraction, but high activities were also observed in Golgi, nuclei and cytosol. However, based on the 5'-nucleotidase data of Table 1, the PLD activity in these fractions could only partly be accounted for by plasma membrane contamination. Furthermore, the large difference in the relative effects of RhoA and mARF in these fractions compared with plasma membranes argues strongly against contamination. Thus the results support the concept that RhoA- and ARF-responsive PLD isoenzymes differ in their intracellular distribution. In contrast to the present results, Malcolm et al. [4] did not observe stimulation of rat liver plasma membrane PLD activity by ARF. However, they used an assay involving endogenous PC and studied membranes pretreated with Rho GDI.

There have been many reports of ARF stimulation of PLD activity in membranes and membrane extracts [8,9,14,18–22] and a report of ARF stimulation of cytosolic PLD [9]. As shown in Figures 1(A)–1(F), ARF stimulates PLD in both these fractions in all tissues examined except muscle. As found by Ktiskakis et al. [8], Golgi also contain a PLD activity that is very responsive to ARF and this is also true for nuclei (Figure 4). In accord with the present study, Balboa and Insel [12] observed that nuclei from MDCK cells showed an increase in PLD activity with GTP[S] which they attributed to the presence of Rho proteins [12].

Several groups have reported that a cytosolic factor enhances the action of ARF on PLD from brain, neutrophils and HL60 cells [42,46,49], and Kwak et al. [35] have obtained similar findings with respect to RhoA on the PLD activity of neutrophils. Such factors could be playing a role in modulating the effects of RhoA and ARF observed in the present study. In the absence of purified preparations of these factors, their possible role could not be assessed.

The present findings indicate that 0.1–3 nM RhoA is stimulatory on PLD in plasma membranes and nuclei, but that 0.5–5 μ M mARF is required for an effect (Figure 6). This difference in the effective concentrations of the two G-proteins has been observed in many studies with other PLD preparations [4,9,14,22,35,36,45,49] and does not seem to reflect differences in the acylation (isoprenylation or myristoylation) state of the proteins [12,14,19,20,22].

Certain issues of interest arise from the present results. The first is the widespread tissue distribution of both ARF- and RhoA-responsive PLD isoenzymes and the finding that both types of isoenzyme are present in extracts of membranes from these tissues, with the exception of skeletal muscle. The second is that significant PLD activity is detectable in all subcellular fractions of liver except mitochondria, with plasma membranes exhibiting the highest specific activity and the largest response to Rho. Other sites of enrichment are Golgi and nuclei, where there is the greatest response to ARF. These results strongly support the existence of different isoforms of PLD in mammalian tissues.

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