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Identification of glycosylphosphatidylinositol-specific phospholipases C in mouse brain membranes

Françis FOUCHIER,*‡ Théo BALTZ† and Genevieve ROUGON*

*Institut de Chimie Biologique, CNRS URA 202, Université d'Aix-Marseille 1, 3 place Victor Hugo, 13331 Marseille Cedex 3, and †Laboratoire d'Immunologie et de Biologie Parasitaire, Université Bordeaux 2, 33076 Bordeaux Cedex, France

Using the membrane form of variant surface glycoprotein from *Trypanosoma equiperdum* labelled with [^{3}H]myristate as a substrate, we identified two glycosylphosphatidylinositol phospholipase C enzymic activities in mouse brain. These activities were associated with particulate membrane fractions. They were characterized by their pH activity maxima and sensitivity to activators and ion chelators. One of the activities was maximal at acidic pH, stimulated by butanol, sensitive to cation chelator and insensitive to manganese. The activity of the other was maximal at neutral pH, stimulated by the detergent deoxycholate and independent of the presence of cation chelator or calcium. On membrane subfractionation, the acidic butanol-stimulated activity was found mainly associated with the lysosomal compartment, whereas the neutral deoxycholate-stimulated activity sediments with the myelin and plasma membrane compartment. These activities could be differentiated from particulate phosphatidylinositol phospholipases C, whose acidic lysosomal form is sensitive to manganese and insensitive to cation chelator or butanol, whereas the deoxycholate-activated enzymes are Ca^{2+}-dependent.

INTRODUCTION

The covalent linkage of proteins to glycosylphosphatidylinositol (GPI) structures is recognized as an important means of anchoring proteins to membranes (for reviews see [1–3]). These anchors have been identified in many proteins with an extracellular orientation, including Trypanosome variant surface glycoproteins (VSG) as well as many ectoenzymes and molecules involved in cell–cell or cell–substrate adhesion [2]. However, this mode of anchorage does not seem to be restricted to the plasma membrane, as it has been shown for the GP-2 protein of pancreatic zymogen granules [4] as well as for two proteins of chromaffin cell granules [5].

Common structural features of GPI anchors are an ethanolamine residue with an amide linkage to the *C*-terminus of the protein, phosphate groups, a mannose-containing glycan and a non-acetylated glucosamine residue with a glycosidic linkage to an inositol phospholipid [1]. One characteristic of most GPI-anchored proteins is their conversion from membrane-bound amphipathic to soluble hydrophilic forms when treated with purified phosphatidylinositol-specific phospholipase C (PI-PLC) from bacteria [1–3] or anchor-specific PLC from Trypanosomes [1–3].

In studies involving cell culture, spontaneous release of soluble GPI-anchored proteins has been reported [6,7]. The mechanisms implicated in this liberation have not been elucidated. They could involve the activation of enzymic processes exhibiting phospholipase, protease or endoglycosidase specificities. So far, besides the bacterial PI-PLC enzymes, two types of enzyme with distinct specificities which are capable of hydrolysing the GPI anchors have been reported, i.e. GPI-PLC and GPI-PLD. For the GPI-PLCs, an enzyme of M_r 39000 has been purified from *Trypanosoma brucei* [8–10], and such an activity has also been observed in rat liver [11]. GPI-PLD activities have been shown predominantly in plasma [12,13]. Interestingly, the GPI-PLD enzyme is unable to release molecules which are anchored in membranes and hydrolyses only detergent-solubilized GPI–proteins [12]. Besides this activity, plasma also contains an acid lipase with unknown specificity which is able to solubilize GPI–proteins [14]. So far the roles of such enzymes are not understood.

The hypothesis that GPI-PLC or GPI-PLD plays a physiological role in liberating GPI-anchored molecules is very attractive. Indeed, besides the modulatory functions of the molecules involved, products resulting from cleavage, i.e. 1,2-diacylglycerol and phosphatidic acid, are known to be involved in signal transduction. For example, diacylglycerol, the product of GPI-PLC cleavage, is an endogenous activator of calcium- and phospholipid-dependent kinase C [15]; phosphatidic acid, the product of GPI-PLD cleavage, is also known to participate in many metabolic events [16]. Moreover, these enzymes could be possible targets for hormonal or growth factor regulation. This hypothesis is strengthened by the observation that insulin seems to increase the liberation of GPI molecules [17].

We have investigated GPI-PLC activities in mouse brains because the tissue is known to express many identified GPI-anchored proteins [7,18–20] the expression of which is developmentally regulated, and which play a role in cell adhesion or recognition. Several criteria must be met for an enzyme to be relevant in the processing of particular GPI–proteins. For example, the hydrolysing enzyme should be present in membranes where processing is thought to occur and should function at physiological pH. We report the presence of two GPI-PLC activities in brain membranes using labelled mVSG as substrate. These activities are distinguishable from the other PI-PLCs already characterized in the brain [21–24]. The acidic GPI-PLC activity is stimulated by butanol and is sensitive to divalent cation chelator. The neutral GPI-PLC is activated by deoxycholate without a shift in the pH optimum and is insensitive to a divalent cation chelator. On the contrary, the acidic

Abbreviations used: DMG, dimyristylglycerol; DMPA, dimyristylphosphatidic acid; GPI, glycosylphosphatidylinositol; mVSG, membrane form of variant surface glycoprotein; PLC, phospholipase C; PLD, phospholipase D.

‡ To whom correspondence should be addressed.

lysosomal PI-PLC is insensitive to divalent cation chelator and butanol, and the neutral PI-PLC presents a shift in pH activity with deoxycholate and is sensitive to calcium.

MATERIALS AND METHODS

Labelling of *Trypanosoma equiperdum* Bo Tat-1 with [^{3}H]myristate

The parasites were labelled with [^{3}H]myristate according to Ferguson & Cross [25]. Briefly, dry [9,10-^{3}H]myristic acid (40–60 Ci/mmol) was mixed with equimolar fatty-acid-free BSA. Cells were suspended in medium supplemented with 1 mg of fatty-acid-free BSA/ml and incubated for 15 min at 37 °C. The [^{3}H]myristate–BSA complex was then added and incubated for 90 min. After labelling, the suspension was placed on ice and the cells were washed by centrifugation.

Isolation of [^{3}H]myristate-labelled mVSG

The washed *Trypanosoma* pellets which contained mVSG were extracted by the butanol procedure according to Hereld *et al.* [9]. The cells were lysed osmotically with 5 mM-sodium *p*-chloromercuribenzenesulphonate, which inhibited VSG lipase, and boiled for 5 min, after which the butanol extraction was carried out. To completely remove any lipid component non-covalently attached to the protein, the purified mVSG was treated as follows: Acetone (7 vol.) was added to the protein preparation and mixed. The mixture stood overnight at −20 °C; the precipitate was then collected by centrifugation at 10000 *g* for 30 min and washed twice with hexane/propan-2-ol (3:2 v/v). The dried protein residue obtained with resuspended in 1 % SDS and used for the subsequent analysis. The specific radioactivity was estimated to be 4800 c.p.m./μg.

Preparation of [^{3}H]myristate-labelled phosphatidic acid, PI and diacylglycerol

In order to retain comparable specific radioactivity, all molecules were prepared from [^{3}H]myristate-labelled mVSG. The labelled dimyristylphosphatidic acid (DMPA) was obtained by treatment with rat serum of mVSG (4000 c.p.m.) in 40 mM-Tris/HCl (pH 7.4)/10 mM-NaCl/2.5 mM-$CaCl_2$ in a total volume of 100 μl [12]. After 2 h of incubation, the mixtures were extracted with hexane/propan-2-ol/HCl (3:2:0.05, by vol.) as described below. The labelled dimyristyl-PI was prepared by nitrous acid deamination. mVSG (200000 c.p.m.) was incubated for 6 h at 20 °C in 0.5 M-sodium acetate (pH 4) containing fresh 0.5 M-$NaNO_2$ [26] in a total volume of 150 μl. The dimyrystoylated PI was extracted with 1.2 ml of chloroform/methanol (2:1, v/v) and two washes with chloroform. The chloroform-rich phases were combined and a sample was subjected to t.l.c. using the solvent system chloroform/methanol/acetone/acetic acid/water (6:2:8:2:1, by vol.). The position of the radioactive lipid was located by scraping and counting as described below. At least 95 % of the radioactivity co-migrated with the PI standard. The dimyristoylated glycerol (DMG) resulted from the cleavage of mVSG by the PI-PLC of *Bacillus thuringiensis*. mVSG (4000 c.p.m.) was digested for 2 h at 37 °C with 0.5 units of PI-PLC in 50 mM-Tris/HCl (pH 7.4) containing 0.1 % (w/v) sodium deoxycholate. After incubation, DMG was extracted with hexane/propan-2-ol/HCl (3:2:0.05, by vol.) as described below.

Assay of GPI-PLC and PI-PLC activities

Mouse brain membranes were incubated at 37 °C with about 4000 c.p.m. of [^{3}H]myristate-labelled mVSG or [^{3}H]myristate-labelled PI in 40 mM-buffer (Tris/acetate from pH 4 to 6, Tris/maleate from pH 6.5 to 7.0, Tris/HCl from pH 7.5 to 8.5). Butanol, deoxycholate, EGTA, $CaCl_2$ and $MnCl_2$ were added as indicated in the legends to the Figures. The mixture contained 0.01 % SDS which was introduced with the mVSG or PI preparation. The total reaction volume was 100 μl. After incubation, the mixture was extracted with 500 μl of ice-cold hexane/propan-2-ol/HCl (3:2:0.05, by vol.) by vortex-mixing for 1 min, the phases were separated by centrifugation (2000 *g*/5 min) and the upper (organic) phase was evaporated in a Speed-Vac concentrator. The extracts were resuspended in 20 μl of hexane/propan-2-ol (3:2, v/v).

Thin-layer chromatography

The extracts were spotted on a silica gel 60 plate. Standards, including ^{3}H-labelled PI, DMPA, DMG and myristate, were spotted on adjacent lanes. Development was performed with solvent A [heptane/isopropyl ether/acetic acid (20:40:3, by vol.)], solvent B [chloroform/methanol/0.25 % aqueous KCl (11:9:2, by vol.)] or solvent C [light petroleum (b.p. 35–60 °C)/diethyl ether/acetic acid (35:15:1, by vol.)]. The positions of radioactive components were determined by scraping the plates in strips of 0.5 cm. The scraped gels were mixed with 5 ml of scintillation cocktail and counted for radioactivity.

Preparation of mouse brain membranes

Mice were decapitated and the forebrains were rapidly removed, weighed and placed in ice-cold phosphate-buffered saline (10 mM-phosphate/0.5 M-NaCl, pH 7.2. All subsequent manipulations were carried out at 0–4 °C. The tissue was homogenized in a Potter–Elvejhem homogenizer with 9 vol. of 0.32 M-sucrose containing 10 μg of α_2-macroglobulin/ml and 80 μM-leupeptin as proteinase inhibitors. The homogenate was spun at 800 *g* for 10 min, and the pellet was washed twice with 0.32 M-sucrose containing proteinase inhibitors. The washes and the original supernatant were spun at 15000 *g* for 20 min. The pellet (M) was washed once with 0.32 M-sucrose containing proteinase inhibitors and suspended in water containing proteinase inhibitors. This membrane suspension was used immediately for determination of GPI-PLC or PI-PLC activities as described above and in the Figure legends. The supernatant and the washings from the M membrane fraction were pooled and centrifuged at 100000 *g* for 90 min, and the resulting microsomal pellet (P) was suspended, without washing, in water containing proteinase inhibitors. The supernatant from this P membrane fraction was designated as the cytosolic fraction.

Isopycnic centrifugation in a discontinuous sucrose gradient

The M membrane fraction (5 ml) suspended in 0.32 M-sucrose containing proteinase inhibitors was carefully introduced to the top of the gradient system (previously chilled at 0–4 °C) and spun at 63500 *g* for 120 min. The gradient was prepared by successive layering with 5 ml of each 1.4, 1.2, 1.0 and 0.8 M-sucrose with the aid of an Auto Densi-flow. After centrifugation, separation of the visible fraction was achieved with an Auto Densi-flow. Fractions were designated A (0.32–0.8 M), B (0.8–1.0 M), C (1.0–1.2 M) and D (1.2–1.4 M). The remaining pellet was the E fraction. The A fraction was composed of myelin and plasma membranes, the B fraction of nerve endings, the C fraction of nerve endings and some mitochondria, the D fraction of mitochondria and the E fraction of lysosomes and degenerating mitochondria [27].

Measurement of protein

Protein concentration was determined using the BCA protein assay reagent from Pierce.

Materials

[9,10-^{3}H]Myristic acid was purchased from Amersham. Silica-gel 60 plates (20 cm × 20 cm) were from Merck. All solvents were

reagent or h.p.l.c. grade and were purchased from Merck or Aldrich. PI-PLC from *Bacillus thuringiensis* was prepared as described [28].

RESULTS

Degradation of the GPI anchor of mVSG by enzyme activities in mouse brain

GPI-anchor-degrading activities were assayed in both particulate (M fraction) and cytosolic (soluble) fractions of the brain using mVSG, biosynthetically labelled with [^{3}H]myristate, as a substrate. Because activities were consistently found in the M fraction, we used only this preparation in the subsequent assays. We also measured anchor-degrading activities at two different pH values, i.e. neutral and acidic. The products of cleavage were identified by their t.l.c. migration patterns in three different solvent systems after hexane/propan-2-ol/HCl extraction of the lipid products.

In each situation tested, the major peak of radioactivity co-migrated with standard DMG. The nature of this radioactive product was further confirmed by asserting its identity with product liberated from mVSG by *Bacillus thuringiensis* PI-PLC treatment. Results are shown in Fig. 1 for acidic (*a*, *b*, *c*) and neutral (*e*, *d*, *f*) incubation conditions. These results indicated that the activities present in brain membranes are of a PLC type.

The presence of small quantities of DMPA revealed by two solvent systems (Figs 1*a*, 1*c*, 1*d* and 1*f*) was probably due to the action of a GPI-PLD [26]; we cannot exclude the possibility that this activity resulted from contamination of our M preparation with plasma. A minor peak of radioactivity co-migrated with myristate. The [^{3}H]myristate corresponds to the products of hydrolysis by phospholipase A or diacylglycerol lipase. We noted that with longer incubation times (> 5 h) the quantity of DMG decreased whereas that of myristate increased. Because of this observed time lag in the release of myristate, these results (not shown) argue in favour of diacylglycerol lipase degrading DMG rather than phospholipase cleaving myristate from mVSG. Thus the major activity of brain membranes able to cleave the anchor domain of the mVSG protein is of the phospholipase C type.

These GPI-PLC activities, revealed at pH 5 and 7, are time- and concentration-dependent as indicated in Fig. 2. When the GPI-PLC activity was measured in a broad range of pH, from 4.0 to 8.5, a single peak of activity was obtained with a maximum between pH 6.5 and pH 7.0 (Fig. 3*a*). Activity was not detected beyond pH 8.5.

Effect of butanol on GPI-PLC activity

It has been reported that when GPI-anchored alkaline phosphatase is purified from various tissues using a butanol extraction procedure under acidic conditions, only the soluble form is recovered [29–31]. The nature of the enzymic processes involved is unknown. However, in placenta, the data suggest [30]

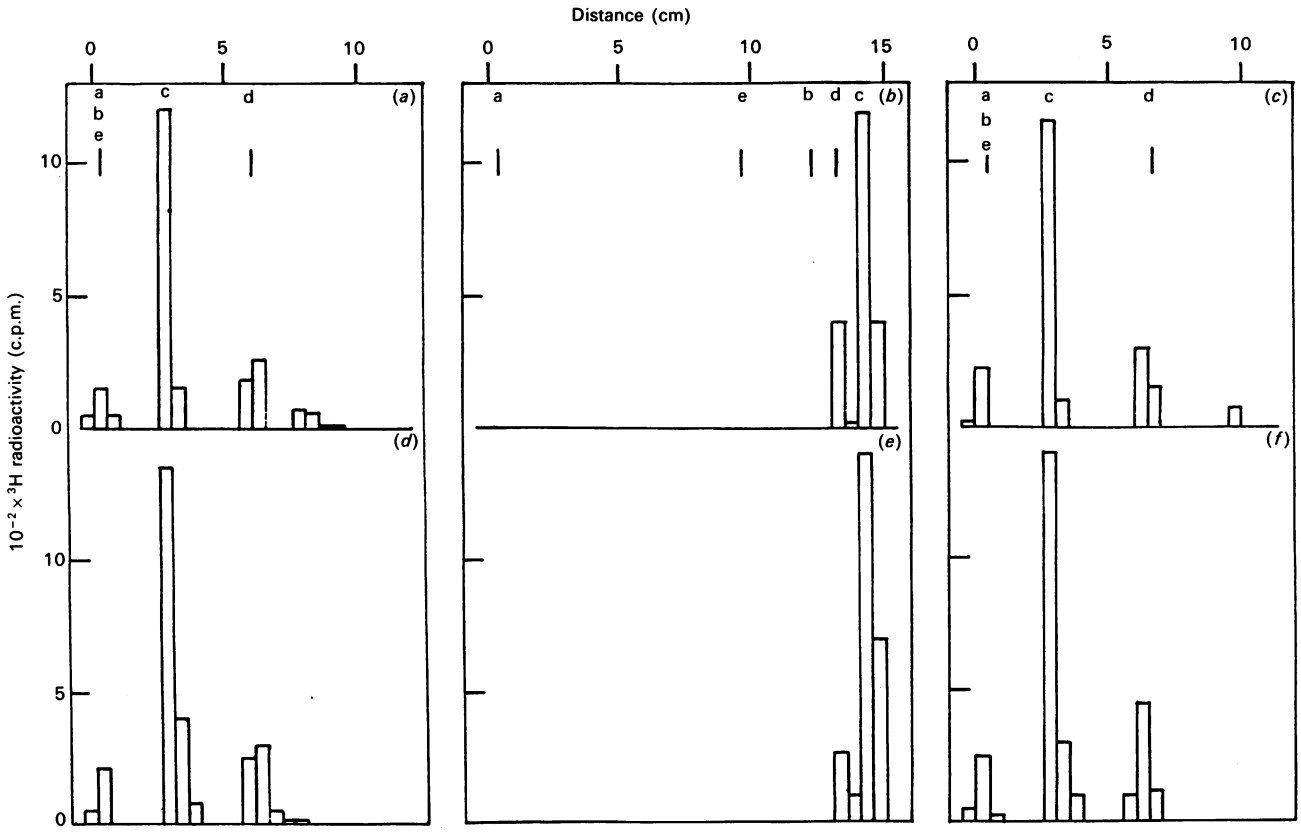

Fig. 1. Products of hydrolysis of [^{3}H]myristate-labelled mVSG by brain membranes

[^{3}H]Myristate-labelled mVSG (4000 c.p.m.) was incubated with 400 μg of mouse brain membrane (fraction M) for 4 h at 37 °C in a volume of 100 μl. Incubations were performed at pH 5 either in the presence of 2% (v/v) butanol (*a–c*) or in the presence of 0.1% (w/v) deoxycholate (*d–f*). The hydrolysates were extracted with hexane/propan-2-ol/HCl (3:2:0.05, by vol.), and products in the organic phase were examined by t.l.c. on silica-gel 60 plates. Plates were developed in various solvent systems as described in the Materials and methods section, i.e. solvent systems A (*a*, *d*), B (*b*, *e*) and C (*c*, *f*). Scrapings were eluted and ^{3}H radioactivity was determined by liquid scintillation spectrometry. The mobilities of various standards lipids in the different solvent systems are indicated: a, mVSG; b, PI; c, DMG; d, myristate; e, DMPA.

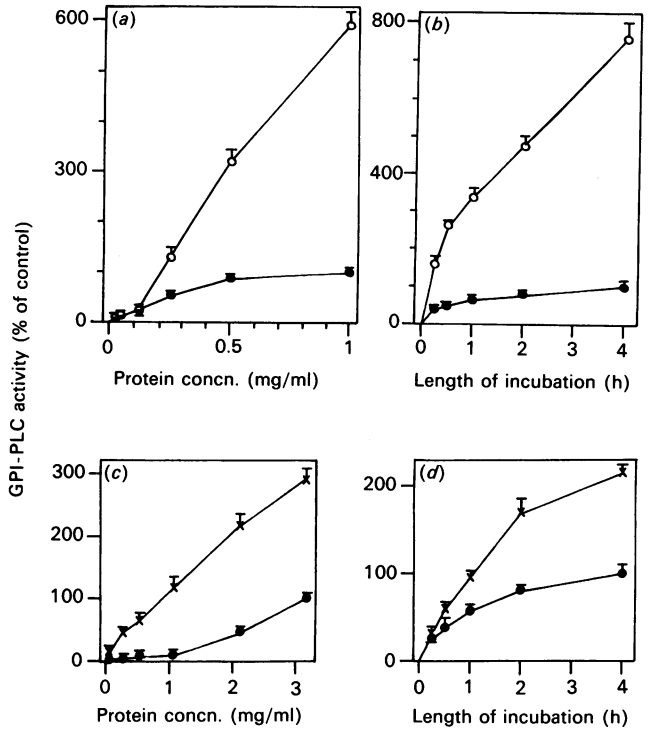

Fig. 2. Effects of protein concentration and time on the GPI-PLC activities of brain membranes

Mouse brain membranes (fraction M) were incubated as detailed in the Materials and methods section at either pH 5 (*a* and *b*) or pH 7 (*c* and *d*). The activity was measured in the presence of 2% (v/v) butanol (○), or 0.1% (w/v) deoxycholate (×), or without butanol and deoxycholate (●). GPI-PLC activity was estimated by measuring [^{3}H]DMG liberated after incubation and development of the released products in solvent system A on t.l.c. plates. Each point represents the mean of triplicate assays, with S.D. indicated by error bars. Each experiment is representative of two replicate experiments. The data are expressed as percentages of the maximum activity without butanol or deoxycholate. The 100% activity was 0.14 (*a*) and 0.44 (*c*) pmol of mVSG cleaved/h and 2.48 (*b*) and 3.53 (*d*) pmol of mVSG cleaved/mg, respectively. The length of incubation was 4 h (*a* and *c*). The protein concentration was 260 μg (*b*) and 400 μg (*d*).

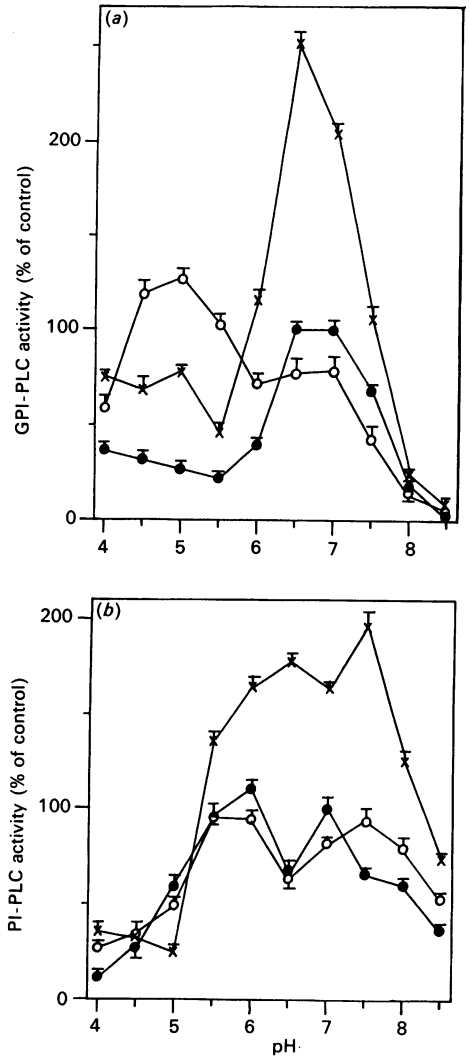

Fig. 3. pH-dependence of GPI-PLC and PI-PLC activities of brain membranes

Mouse brain membranes (fraction M; 400 μg) were incubated for 4 h at 37 °C and at different pH values under conditions detailed in the Materials and methods section, in the presence of about 4000 c.p.m. of [^{3}H]myristate-labelled mVSG (*a*) or PI (*b*). The activity was tested in the presence of either 2% (v/v) butanol (○) or 0.1% (w/v) deoxycholate (×), or without butanol or deoxycholate (●). Each point represents the mean of triplicate assays (bars are S.D.). The data are representative of two replicate experiments, and are expressed as percentages of the activity at pH 7 obtained without butanol and deoxycholate. GPI-PLC activity was estimated by measuring the [^{3}H]DMG liberated after incubation and development of the released products in solvent system A on t.l.c. plates. The 100% GPI-PLC activity was 0.90 pmol of mVSG cleaved/h per mg (*a*) and the 100% PI-PLC activity was 1.21 pmol of PI cleaved/h per mg (*b*). Whatever the pH tested, no hydrolysing activities of mVSG or PI occurred when they were incubated with or without butanol or deoxycholate in the absence of brain membranes.

that the cleavage of the anchor domain could be attributed to the action of a GPI-PLD. In order to ascertain the effect of butanol on the enzyme activities in the M fraction from mouse brain, we tested the influence of 2% (v/v) butanol in the incubation medium.

Experiments were conducted in a broad pH range (from 4.0 to 8.5). Results presented in Fig. 3(*a*) show that, under these conditions, the maximal GPI-PLC activity at neutral pH was not significantly modified by butanol. Interestingly, butanol revealed a peak of maximum GPI-PLC activity at acidic pH (Fig. 3*a*). Thus the GPI-PLC activities measured are very likely borne by two distinct molecular entities. We demonstrated that the stimulatory effect was dependent on butanol concentration (Fig. 4*a*) when assayed at acidic pH. A maximal effect was observed at a butanol concentration of 3%; higher concentrations tested (5–10%) were found to exert opposite effects. The intensity of the stimulation was variable, and in some experiments we noticed a 7-fold increase in activity (Fig. 2*b*).

Modulating effects of detergents on GPI-PLC activities

To characterize further the GPI-PLC activities present in brain membranes, we tested the modulating effects of detergents. It was already known that PI-PLC activities are modulated by deoxycholate [32,33]. In our assay system, deoxycholate at a concentration of 0.1% (w/v) stimulated GPI-PLC activity as shown in Fig. 3(*a*). This effect was pH-dependent, and the maximum stimulation was clearly obtained at neutral pH. The effect was also dependent on the detergent concentration, which had to reach a value of around 0.5–1 mg/ml before its action could be detectable. At these concentrations the activity was enhanced by 2–3-fold, as shown in Figs. 2(*c*) and 2(*d*). However,

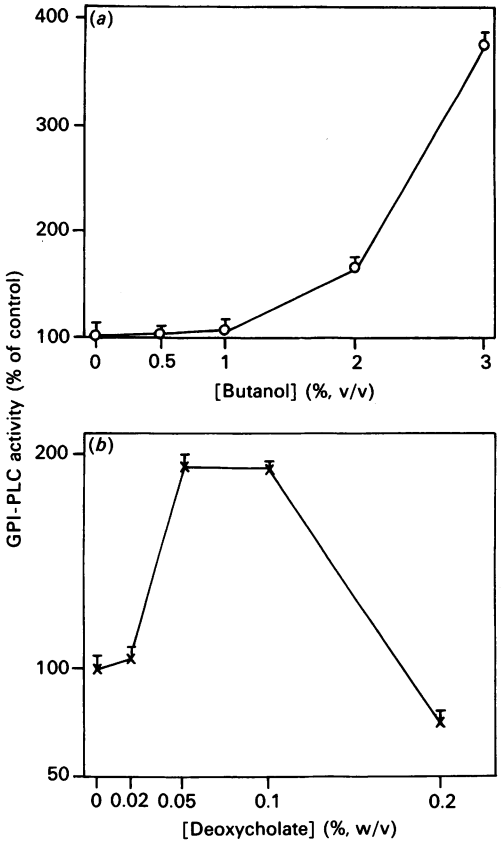

Fig. 4. Effects of increasing concentrations of butanol or deoxycholate on the GPI-PLC activities of brain membranes

Mouse brain membranes (fraction M; 360 μg) were incubated as detailed in the Materials and methods section at pH 5 (*a*) in the presence of different concentrations of butanol (○) or at pH 7 (*b*) with different concentrations of deoxycholate (×) for 4 h at 37 °C. Each point represents the mean of triplicate assays, with S.D. values indicated by bars. Each experiment is representative of two replicate experiments. The data are expressed as percentages of the activity without butanol or deoxycholate. GPI-PLC activity was estimated by measuring the [^{3}H]DMG liberated after incubation and development of the released products in solvent system A on t.l.c. plates. The 100% activity at pH 5 was 0.35 pmol of mVSG cleaved/h per mg (*a*) and at pH 7 it was 1.02 pmol of mVSG cleaved/h per mg (*b*).

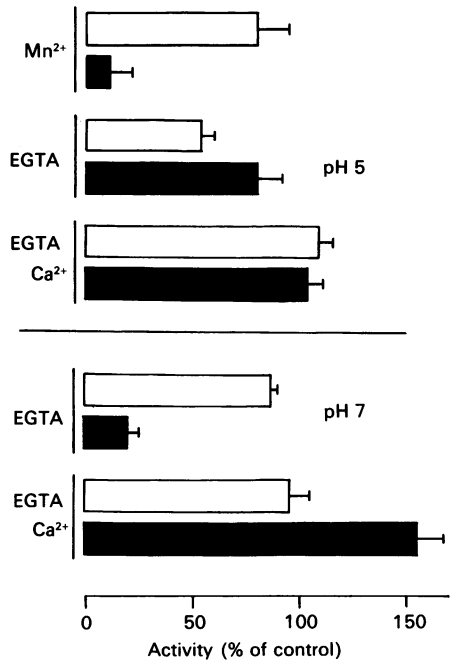

Fig. 5. Modulation by manganese, EGTA and Ca^{2+} of the GPI-PLC and PI-PLC activities of brain membranes

Mouse brain membranes (fraction M; 240 μg) were incubated for 4 h at 37 °C at pH 5 or 7 with about 4000 c.p.m. of [^{3}H]myristate-labelled mVSG (□) or [^{3}H]PI (■) as detailed in the Materials and methods section with or without 3 mM-$MnCl_2$, 2 mM-EGTA or 5 mM-$CaCl_2$. Each diagram shows the mean of triplicate assays with S.D. The data are representative of three replicate experiments and are expressed as percentages of the activity of pH 5 or 7 without $MnCl_2$, EGTA or $CaCl_2$. GPI-PLC activity was estimated by measuring the [^{3}H]DMG liberated after incubation and development of the released products in solvent system A on t.l.c. plates. The 100% GPI-PLC activities were 0.78 pmol of mVSG cleaved/h per mg at pH 5 and 2.22 pmol of mVSG cleaved/h per mg at pH 7. The 100% PI-PLC activities were 1.57 pmol of PI cleaved/h per mg at pH 5 and 4.2 pmol of PI cleaved/h per mg at pH 7.

when the concentration was raised above 1 mg/ml, the effect was lost and inhibition occurred (Fig. 4*b*).

When the neutral detergent Nonidet P-40 was used at neutral pH, concentrations above 0.1% (w/v) inhibited the GPI-PLC activity (results not shown). This was also true for the cationic detergent cetrimide (alkyltrimethylammonium bromide) which completely abolished the neutral GPI-PLC activity at 0.3 mg/ml (results not shown).

Discrimination between GPI-PLC and PI-PLC in mouse brain membranes

In brain, two major types of PI-PLC activities have been described. One is measurable in a broad range of pH from 5.0 to 7.5 [21–23]; the other, of lysosomal origin, exhibits an acidic pH optimum [24]. Recently, two immunologically distinct PI-PLCs from brain have been purified [21]; although they exhibit similar substrate specificities, they respond differently to activators such as calcium and divalent metal ions. In our experiments we also demonstrated the presence of two GPI-PLC activities which can be discriminated on the basis of their pH maxima and their responses to stimulation by butanol and deoxycholate. A critical point was to ascertain, unambiguously, that the measured GPI activities were not due to the action of PI-PLC on mVSG. In the first set of experiments we incubated the M fraction with either [^{3}H]myristate-labelled mVSG or [^{3}H]myristate-labelled PI (under the same conditions). When PI was used as a substrate, two pH optima were revealed at 6 and 7 (Fig. 3*b*). Moreover, butanol did not significantly modify these activities (Fig. 3*b*), but led to a shift in pH optimum from 7.0 to 7.5. When deoxycholate was added, the PI-PLC activities were significantly stimulated above pH 5.0 and shifts in pH optima were noted from 6.0 to 6.5 and 7.0 to 7.5 respectively (Fig. 3*b*). These results are in agreement with the literature [32]. On the contrary, GPI-PLC activities exhibited no shift in pH optima with either butanol or deoxycholate (Fig. 3*a*) and the acidic activity was stimulated by butanol (Fig. 3*a*).

It is also known that the non-lysosomal PI-PLCs are Ca^{2+}-requiring enzymes [21–23,32]. We measured, at neutral pH, the GPI- and PI-PLC activities in the presence of 2 mM-EGTA; the PI enzymes were strongly inhibited whereas GPI-PLC activities were not (Fig. 5). The addition of 5 mM-Ca^{2+} to EGTA stimulated the PI-PLC, but not the GPI-PLC enzymes. At acidic pH, 2 mM-EGTA weakly inhibited the PI-PLC activities (Fig. 5), which is in agreement with the fact that, at this pH, the contribution of the lysosomal PI-PLC is dominant. This conclusion was con-

firmed by the action of 3 mM-$MnCl_2$, which strongly inhibited the lysosomal PI-PLC [24] (Fig. 5). On the contrary, the GPI-PLC activities at pH 5 were inhibited by 50% by 2 mM-EGTA; this effect was nullified by 5 mM-Ca^{2+} (Fig. 5). Manganese had no measurable effect on GPI-PLC activities (Fig. 5).

PI-PLC enzymes may be particulate or cytosolic [32]; as already mentioned, 98% of the GPI-PLC activities we detected in mouse brain was in the pellet fraction after a 100000 *g*/90 min centrifugation of the tissue homogenates (results not shown). Cetrimide detergent at 0.3 mg/ml inhibited about 90% of the GPI-PLC activity (results not shown), whereas the particulate PLC is revealed with this cationic detergent [23]. All of these results argue in favour of the existence of two particulate GPI-PLC activities which are distinct from the PI-PLC enzymes.

Subcellular distribution of GPI-PLC activities in mouse brain membranes

To obtain information on the subcellular location of the GPI-PLC particulates, we layered the M fraction from brain membranes on a discontinuous sucrose gradient according to Koenig *et al.* [27]. This separation procedure resulted in five different subfractions. We tested the butanol- and deoxycholate-stimulated activities in these five subfractions from M and in the microsomal P fraction. The two activities exhibited a maximum relative specific activity in fractions A and E. Interestingly, the acidic butanol-stimulated activity was preferentialy recovered in fraction E, which is composed essentially of lysosomes [27]. The deoxycholate-activated GPI-PLC was preferentially associated with the fraction A, which contains myelin and plasma membranes [27] (Fig. 6). Thus these activities appeared to be confined to particular subcellular domains.

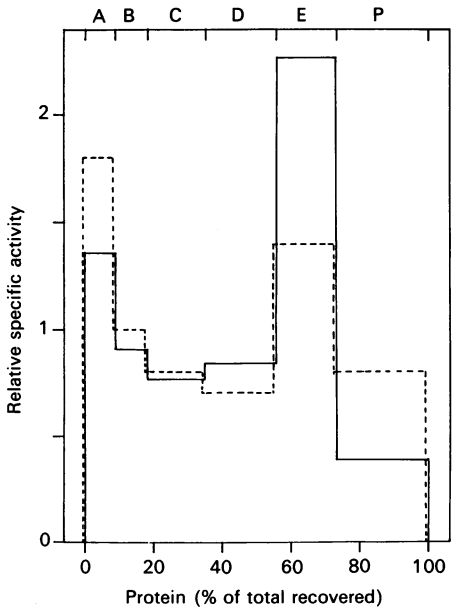

Fig. 6. Subcellular localization of GPI-PLC activities of brain membranes

The GPI-PLC activities of the different subfractions of mouse brain membranes were measured using 4000 c.p.m. of [^{3}H]myristate-labelled mVSG at pH 5 with 2% (v/v) butanol (——) or at pH 7 with 0.1% (w/v) deoxycholate (----) as detailed in the Materials and methods section. The subfractions of brain membranes (A,B,C,D,E,P) were obtained as described. GPI-PLC activity was estimated by measuring the [^{3}H]DMG liberated after incubation and development of the released products in solvent system A on t.l.c. plates. The data are representative of two replicate experiments. The results are expressed as relative specific activity: (% of total activity in the fraction)/(% of total protein in the fraction). Specific activity of the acidic butanol-activated enzyme in fraction E was 2.7 pmol of mVSG cleaved/h per mg. Specific activity of the deoxycholate-activated enzyme in fraction A was 5.1 pmol of mVSG cleaved/h per mg.

DISCUSSION

The observation that GPI-anchored molecules could be spontaneously released in soluble form (see [7]), together with the fact that purification of alkaline phosphatase using butanol extraction at acidic pH led to the recovery of the soluble form [29–31], led us to search for GPI-PLC activities in mammalian tissues. We used the brain because it is a structure known to express many GPI-anchored glycoproteins [7,18–20], of which several are identified as adhesion molecules whose expression is regulated during development.

The major activities detected using mVSG as a substrate were exclusively of the C type. They were also associated with particulate fractions of membranes, a location compatible with the membrane expression of the potential substrates. Our results are identical with those obtained in the liver [11]. It is clear from the low specific activities we measured either that the concentrations of the molecules bearing activities are low or that the substrate we used is different from physiological substrates. The activities of the brain membranes are comparable with those reported for liver [11] with the same substrate. However, in the brain they differ in many criteria, particularly the activation by detergents.

We have shown the presence of two GPI-PLC activities which can be differentiated by their relative activation properties in the presence of butanol and deoxycholate, as well as by their pH maxima. We could speculate that either deoxycholate or butanol facilitates the accessibility of the substrate to the membrane enzymes or stimulates the activity of the enzymes themselves. The acidic activity stimulated by butanol appeared different from the GPI-PLD activity described in placenta [30] which shares some common properties. The acidic GPI-PLD was not inhibited by 2.0 mM-trifluoperazine; by contrast, we measured an inhibition of 87.1 ± 4.5% (results not shown) when assays were conducted in the presence of this drug. However, we cannot formally exclude the possibility that diacylglycerol measured under these acidic conditions could result from the combined action of a GPI-PLD and a phosphatidic acid phosphatase. This is unlikely, as phosphatidic acid phosphatases are not active on substrates bearing fatty acid chains longer than C_{14} [35], and our substrate bears myristate.

Another property differentiating the two measured activities was their sensitivity to cation chelator. The neutral activity was insensitive to EGTA, whereas the acidic enzyme was sensitive. The acidic butanol-stimulated activity, which converts GPI–alkaline phosphatase from a hydrophobic to a hydrophilic form, is Ca^{2+}-dependent [33,36,37]; this could indeed correspond to the acidic butanol-stimulated activity we have demonstrated in the brain.

Activities able to release alkaline phosphatase and acetylcholine esterase GPI-anchored molecules into soluble forms have been observed in rat heart and kidney [34] as well as in sheep platelets [38]. These activities were also triggered by deoxycholate. They could correspond to the activity we have observed in mouse brain. However, these previous authors favoured the action of soluble enzymes similar to PI-PLC [34,38] capable of solubilizing the membrane GPI-alkaline phosphatase and acetylcholinesterase. The possibility could not be excluded that a cytosolic activator of membrane GPI-PLC exists in cells.

Our experiments confirmed that GPI-PLC and PI-PLC enzymes are distinguishable on the basis of their responses towards

cation chelator, detergents and manganese for the acidic activity, and thus they are very likely to be different molecular entities. Our attempts to adapt the liver purification technique [11] to brain tissue failed, confirming further the differences in molecules bearing the activities in these two tissues.

The existence of two different GPI-PLC activities which are unequally distributed in cell compartments suggests that both the function and the regulation of these enzymes should differ. The demonstration of an acidic GPI-PLC supports the hypothesis of Ferguson & Williams [2], who proposed an internalization of GPI-anchored proteins prior to their release as the soluble form. These activities could also play a role in catabolism and hypothetical recycling of GPI-anchored molecules.

The purification of molecules bearing the GPI-PLC activities is the next goal of future studies. So far there is no clear demonstration that such enzymes play a physiological role in the release of GPI-anchored molecules. The only available evidence is the exhibition of a cross-reactive determinant by the cleaved molecules; however, this is not the case for all molecules examined [7]. This observation suggested that enzymes with other specificities could also be involved. Thus the availability of antibodies directed against GPI-PLC, or the design of specific inhibitors, should be instrumental in the understanding of their role and regulation. Moreover, should these enzymes have a physiological role in liberating GPI-anchored molecules, they could constitute an important link coupling cell external events and cell activation.

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