Effects of glycosaminoglycans and glycosphingolipids on cytosolic phospholipases A₂ from bovine brain

Hsiu-Chiung YANG, Akhlaq A. FAROOQUI* and Lloyd A. HORROCKS

Department of Medical Biochemistry, The Ohio State University, Columbus, OH 43210, U.S.A.

Two forms of Ca²⁺-independent cytosolic phospholipase A₂ activity (110 kDa and 39 kDa) were found in bovine brain. They were separated by Sephadex G-75 column chromatography. The 110 kDa phospholipase A₂ was much more active with phosphatidylethanolamine and was not affected by glycosaminoglycans, whereas the 39 kDa phospholipase A₂ was much more active with ethanolamine plasmalogen and was markedly inhibited by glycosaminoglycans. Heparan sulphate was the most potent inhibitor, followed by chondroitin sulphate, hyaluronic acid and

heparin. Gangliosides, especially the GM3 ganglioside, but not other glycosphingolipids, inhibited the activity of the 39 kDa phospholipase A₂ in a dose-dependent manner. The heat-inactivation profiles of the 110 kDa and 39 kDa phospholipases A₂ provide further evidence for the differences between these cytosolic enzymes. Interactions between glycosaminoglycans, gangliosides and phospholipases A₂ may be involved in the maintenance of membrane function.

INTRODUCTION

Phospholipases A₂ (EC 3.1.1.4) play an important role in the metabolism of membrane glycerophospholipids and signal transduction (Dennis et al., 1991; Farooqui et al., 1992). These enzymes catalyse the hydrolysis of phospholipids at the *sn*-2 position and liberate free fatty acids and 1-acyl-lysophospholipids. The free fatty acids serve as substrates for the generation of eicosanoids and the lysophospholipids may either be involved directly in membrane perturbation and membrane fusion, or may act as substrates for lysophospholipases, and acyl- or acetyl-transferases (Farooqui et al., 1992; Mayer and Marshall, 1993; Glaser et al., 1993; Chao and Olson, 1993). Intracellular phospholipases A₂ (Clark et al., 1990; Hazen et al., 1990; Gronich et al., 1990) have apparent molecular masses from 70 to 110 kDa, are generally localized in the cytosol and prefer arachidonate over other *sn*-2 fatty acids (Glaser et al., 1993; Mayer and Marshall, 1993). Some intracellular phospholipases require Ca²⁺ (10–1000 nM) and are called Ca²⁺-dependent phospholipases A₂; others do not require Ca²⁺ and thus are called Ca²⁺-independent phospholipases A₂. Two forms of phospholipase A₂ activity (PLA₂-H and PLA₂-L) have been detected in rat brain cytosol (Yoshihara and Watanabe, 1990). PLA₂-H was eluted as a broad peak of 200–500 kDa, and its activity was partially inhibited by the addition of 4 mM Ca²⁺. By contrast, PLA₂-L had a molecular mass of 100 kDa and absolutely required Ca²⁺ (Yoshihara and Watanabe, 1990). Both of these enzymes were active with 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine. The activity of PLA₂-L changed biphasically with increasing concentrations of Ca²⁺. That is, the first stimulation was observed at 1 μM and the second at 10 mM Ca²⁺. Earlier work from our laboratory had indicated the occurrence of two Ca²⁺-independent phospholipases A₂ in bovine brain cytosol (Hirashima et al., 1992), namely 110 kDa phospholipase A₂ that was active with 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PtdEtn) and a 39

kDa phospholipase A₂ that was active with both PtdEtn and 1-alk-1'-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmenylethanolamine, PlsEtn).

Glycosaminoglycans (GAGs) are sulphated polysaccharides composed of repeating disaccharide units that vary in degree of sulphation, type of sulphate linkages, sugars involved in the disaccharide unit and type of sugar linkages within and between the disaccharide units. GAGs are found in the plasma membrane and are known to interact with a variety of enzymes, including extracellular pig pancreatic phospholipase A₂ (Farooqui and Horrocks, 1984; Diccianni et al., 1991a, 1991b). Glycosphingolipids (GSLs) are another group of membrane lipids containing carbohydrate and sphingosine that are known to inhibit pig pancreatic phospholipase A₂ activity (Bianco et al., 1990). At present no information is available on the effects of GAGs and GSLs on intracellular phospholipases A₂. This paper describes the effects of GAGs and GSLs on two cytosolic Ca²⁺-independent phospholipases A₂ from bovine brain.

MATERIALS AND METHODS

Materials

Ethanolamine glycerophospholipid from bovine brain was purchased from Serdary Co. (London, Ontario, Canada). 1-Pyrenesulphonyl chloride was a product of Molecular Probes (Eugene, OR, U.S.A.). *Rhizopus arrhizus* lipase was obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). T.l.c. plates were from Analtech (Newark, DE, U.S.A.). Gangliosides, ceramide, cerebroside, sulphatide, Sephadex G-75, hyaluronic acid, chondroitin sulphate, heparan sulphate, heparin and phenylmethanesulphonyl fluoride (PMSF) were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). All other chemicals used in the study were of analytical grade.

Abbreviations used: PtdEtn, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PlsEtn, plasmenylethanolamine; GAGs, glycosaminoglycans; GSLs, glycosphingolipids; PMSF, phenylmethanesulphonyl fluoride; PlsEtn(Pyr), 1-pyrenesulphonyl-labelled PlsEtn; PtdEtn(Pyr), 1-pyrenesulphonyl-labelled PtdEtn; GroPEtn(Pyr), 1-pyrenesulphonyl-labelled *sn*-glycero-3-phosphoethanolamine.

* To whom correspondence should be addressed.

Synthesis of pyrenesulphonyl-labelled ethanolamine glycerophospholipids

Pyrenesulphonyl-labelled ethanolamine glycerophospholipids were synthesized as described previously (Hirashima et al., 1990).

Assay of phospholipase A₂ activity

Routinely, 100 μ l of cytosol, dialysate, or column chromatographic eluate was incubated with 74 nmol of 1-pyrenesulphonyl-labelled PlsEtn [PlsEtn(Pyr)], 5 mg of fatty acid-free BSA, 0.1% Triton X-100 and 100 mM 3-(*N*-morpholino)propanesulphonic acid buffer (pH 7.4) in a final volume of 1 ml. The assay with PtdEtn(Pyr) was performed under similar conditions. The reaction mixture was incubated for 2 h at 37 °C. The control for this assay did not include the enzyme preparation. The reaction was terminated with 4 vol. of chloroform/methanol (2:1, v/v).

The phospholipase A₂ assay with PlsEtn(Pyr) was performed according to the following reaction scheme:

1. 1-alkenyl-2-acylGroPEtn(Pyr) $\xrightarrow{\text{phospholipase A}_2}$ 1-alkenyl-GroPEtn(Pyr) + fatty acid
2. 1-alkenyl-GroPEtn(Pyr) $\xrightarrow{\text{HCl treatment}}$ GroPEtn(Pyr) + fatty aldehyde

where GroPEtn(Pyr) represents pyrenesulphonyl-labelled *sn*-glycero-3-phosphoethanolamine.

The enzymic activity was determined by the measurement of GroPEtn(Pyr) fluorescence as described earlier (Hirashima et al., 1990, 1992). For the assays using PtdEtn(Pyr) and PlsEtn(Pyr), the lower organic phases of the reaction and control mixtures were applied to t.l.c. plates (10 cm $\times$ 10 cm) and developed with a solvent system composed of chloroform/methanol/ NH_4OH (65:25:4, by vol.). Subsequently, the fluorescence of GroPEtn(Pyr) in both reaction mixture and control were measured, and the difference in values was used to calculate the enzymic activity (Hirashima et al. 1990, 1992).

Protein content was determined by a dye-binding method using BSA as the standard (Bradford, 1976).

Preparation of bovine brain cytosol fractions

Fresh whole adult bovine brain was obtained from a local slaughterhouse, trimmed of arachnoid, blood and large vessels, cut into small pieces (3 cm $\times$ 3 cm $\times$ 3 cm), and kept in a freezer at -70 °C. The blocks of brain were thawed at room temperature and homogenized in 0.32 M sucrose/25 μ M EGTA/100 μ M PMSF/50 mM Tris/HCl buffer (pH 7.4), using a Teflon-glass homogenizer (approx. 100 g of brain/300 ml of buffer). The cytosol was prepared by centrifugation at 100000 *g* for 60 min.

Separation of bovine brain cytosol phospholipase A₂

Approx. 300 ml of cytosolic fraction prepared from 100 g of bovine brain was brought to 40% satn. with solid $(\text{NH}_4)_2\text{SO}_4$. After centrifugation at 12500 *g* for 25 min at 4 °C the supernatant was discarded, and the precipitate was suspended in a minimal amount (approx. 1.5 ml) of buffer composed of 25 μ M EGTA/100 μ M PMSF/50 mM Tris/HCl buffer, pH 7.4. The solution was dialysed overnight against this buffer at 4 °C. The resultant dialysate was subsequently loaded on to a column of Sephadex G-75 (2.5 cm $\times$ 75 cm) previously equilibrated with 10% (v/v)

glycerol/25 μ M EGTA/100 μ M PMSF/1 M KCl/50 mM Tris/HCl buffer (pH 8.5) and eluted at a flow rate of 18 ml/h. Fractions of 3.5 ml were collected.

Effect of GAGs on activities of bovine brain phospholipases A₂

Various GAGs were preincubated with 110 kDa and 39 kDa phospholipases A₂ for 2 min at room temperature and the reaction was started by the addition of substrate.

Effect of GSLs on activities of bovine brain phospholipases A₂

Various GSLs were sonicated with substrate on ice for 20 s, and the reaction was started by the addition of enzyme.

Effect of heat on bovine brain phospholipases A₂

Fractions (0.7 ml) from Sephadex G-75 columns containing 110 kDa and 39 kDa phospholipases A₂ were kept at the desired temperature in a water bath for various lengths of time. Enzymic activity was assayed as indicated above and the reaction was started by the addition of substrate.

RESULTS

Bovine brain has significant phospholipase A₂ activity, namely 2.1 ± 0.1 pmol/mg of protein per min for PtdEtn(Pyr) and 7.0 ± 0.1 pmol/mg of protein per min for PlsEtn(Pyr). Almost all phospholipase A₂ activity was precipitated by 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ [2.9 ± 0.1 pmol/mg of protein per min for PtdEtn(Pyr) and 14.0 ± 0.1 pmol/mg of protein per min for PlsEtn(Pyr)].

Sephadex G-75 column chromatography of the 0–40% $(\text{NH}_4)_2\text{SO}_4$ fraction resulted in the separation of two phospholipases A₂. The 110 kDa phospholipase A₂ was eluted in the void volume, and the other phospholipase A₂ was eluted with an apparent molecular mass of 39 kDa in comparison with standards (Figure 1). The 110 kDa phospholipase A₂ (150 ± 1 pmol/mg of protein per min) preferentially hydrolysed PtdEtn(Pyr), whereas the 39 kDa phospholipase A₂ (468 ± 1 pmol/mg of protein per

Figure 1 Elution profile of Ca^{2+} -independent cytosolic phospholipases A₂ through a Sephadex G-75 column

Sephadex G-75 column chromatography was performed as indicated in the Materials and methods section. Fractions (3.5 ml) were collected at a flow rate of 18 ml/h. Enzymic activity: ■, PtdEtn(Pyr); ○, PlsEtn(Pyr); and ●, protein content (A_{995}).

Table 1 Effects of GAGs on activities of bovine brain phospholipase A₂

GAGs were preincubated with enzyme solution at room temperature for 1 min and the reaction was started by the addition of substrate. After 2 h the reaction was stopped by the addition of 4 vol. of chloroform/methanol (2:1, v/v). The specific activities were 77 pmol/mg of protein per min for 110 kDa phospholipase A₂ with PtdEtn(Pyr) and 494 pmol/mg of protein per min for 39 kDa phospholipase A₂ with PlsEtn(Pyr). These values were taken as 100%. Values are means $\pm$ S.E.M. ($n = 3$).

GAGs	Concn. (μ g/ml)	Relative specific activity (%)	
		110 kDa phospholipase A ₂	39 kDa phospholipase A ₂
Heparin	100	87 $\pm$ 10	76 $\pm$ 5
	200	103 $\pm$ 27	75 $\pm$ 9
Chondroitin sulphate	100	103 $\pm$ 13	90 $\pm$ 9
	200	130 $\pm$ 21	70 $\pm$ 8
Hyaluronate	100	94 $\pm$ 17	61 $\pm$ 24
	200	120 $\pm$ 18	46 $\pm$ 22
Heparan sulphate	10	122 $\pm$ 59	62 $\pm$ 14
	50	154 $\pm$ 99	32 $\pm$ 21

min) preferentially hydrolysed PlsEtn(Pyr). It is not known whether the hydrolysis of PlsEtn(Pyr) by the 110 kDa phospholipase A₂ is due to contamination with 39 kDa phospholipase A₂ (Figure 1) or due to the specificity of 110 kDa phospholipase A₂. A similar argument can be made for the PtdEtn(Pyr)-hydrolysing activity of the 39 kDa phospholipase A₂.

GAGs had no effect on 110 kDa phospholipase A₂, whereas the 39 kDa phospholipase A₂ was markedly inhibited by various GAGs (Table 1). Heparan sulphate was the most potent GAG to inhibit 39 kDa phospholipase A₂ activity. At concentrations of 10 and 50 μ g/ml it produced 40% and 70% inhibition of phospholipase A₂ activity respectively. Heparin, chondroitin sulphate and hyaluronic acid at 10 and 50 μ g/ml had no effect on enzymic activity. However, when the concentration of these GAGs was increased, inhibition of phospholipase A₂ activity was observed. At 100 and 200 μ g/ml, heparin produced 25% and

Table 2 Effects of GSLs on activities of bovine brain phospholipase A₂

GSLs were sonicated with phospholipid substrates in water and the reaction was started by the addition of enzyme. After 2 h the reaction was stopped by the addition of 4 vol. of chloroform/methanol (2:1, v/v). The specific activities of controls were 45 pmol/mg of protein per min for 110 kDa phospholipase A₂ with PtdEtn(Pyr) and 470 pmol/mg of protein per min for 39 kDa phospholipase A₂ with PlsEtn(Pyr). These values were taken as 100%. Values are means $\pm$ S.E.M. ($n = 3$).

GSLs	Concn. (μ M)	Relative specific activity (%)	
		110 kDa phospholipase A ₂	39 kDa phospholipase A ₂
GM1	37	124 $\pm$ 13	117 $\pm$ 8
	74	138 $\pm$ 14	117 $\pm$ 8
GM1	148	165 $\pm$ 11	75 $\pm$ 4
	37	68 $\pm$ 1	79 $\pm$ 0.2
GM3	74	71 $\pm$ 3	44 $\pm$ 3
	148	61 $\pm$ 6	17 $\pm$ 2
Ceramide	74	83 $\pm$ 5	92 $\pm$ 7
	74	110 $\pm$ 7	121 $\pm$ 3
Sulphatide	74	81 $\pm$ 2	107 $\pm$ 11

25% inhibition, chondroitin sulphate 11% and 31% inhibition and hyaluronic acid 39% and 54% inhibition respectively. DNA (350 μ g) produced 25% stimulation of 39 kDa phospholipase A₂ activity. The stronger inhibition of 39 kDa phospholipase A₂ by hyaluronic acid (a non-sulphated GAG) indicated that degree of sulphation may not be responsible for the inhibition. The inhibition of GAGs was reversed by the addition of protamine sulphate or histone IIA to the reaction mixture (Figure 2). It is interesting to note that both phospholipases A₂ were retained on the heparin-agarose column and were eluted with 0.5 M NaCl (results not shown).

The bovine brain 110 kDa phospholipase A₂ was not affected significantly by the addition GM1 ganglioside. At 148 μ M this ganglioside produced 25% inhibition of the 39 kDa phospholipase A₂. Both enzymes were markedly inhibited by the GM3 ganglioside. At 148 μ M it caused 40% and 83% inhibition of 110 kDa and 39 kDa phospholipase A₂ respectively. Other

Figure 2 Reversal of GAG inhibition by protamine sulphate or histone IIA

GAGs and protamine sulphate (●) or histone IIA (○) were added to the reaction mixture and preincubated for 2 min with 39 kDa phospholipase A₂ at room temperature. The reaction was started by the addition of substrate. Details of the assay are included in the Materials and methods section. (a) Reversal of heparan sulphate inhibition. (b) Reversal of chondroitin sulphate inhibition. (c) Reversal of hyaluronic acid inhibition.

Figure 3 Effects of temperature on bovine brain phospholipases A₂

The zero-time point is expressed as 100%. The remaining activity is expressed as a relative percentage of the zero-time activity. (a) The heating time was 10 min and variations with temperature are shown in the figure. The 110 kDa phospholipase A₂ before dialysis (2.4 mg/ml, ●); the 110 kDa phospholipase A₂ after dialysis (1.27 mg/ml, ○); the 39 kDa phospholipase A₂ before dialysis (0.214 mg/ml, ■); and the 39 kDa phospholipase A₂ after dialysis (0.218 mg/ml, ▲). (b) The temperature was kept constant (45 °C) and variations with time are shown in the figure. The enzyme activities were assayed as described in the Materials and methods section. Change in pH with temperature was not taken into account. Before dialysis, ○, 39 kDa phospholipase A₂; and ●, 110 kDa phospholipase A₂.

sphingolipids, such as ceramide, cerebroside and sulphatide, had no effect on 110 kDa and 39 kDa phospholipases A₂ (Table 2).

Dialysis of Sephadex G-75 fractions against 0.05 M Tris/HCl buffer, pH 8.5, at 4 °C for 4 h resulted in marked inactivation of bovine brain phospholipases A₂. Thus, the 110 kDa phospholipase A₂ lost 57% of its activity, whereas the 39 kDa phospholipase A₂ lost 43% of its activity.

Heat-inactivation profiles of bovine brain phospholipases A₂ are shown in Figure 3(a). The enzyme solutions from Sephadex G-75 chromatography were heated in sealed tubes at different temperatures for 10 min. The 110 kDa phospholipase A₂ lost 40% and all of its activity after exposure to 45 °C and 55 °C respectively. Dialysed preparations of 110 kDa phospholipase A₂ were more sensitive to heat inactivation than preparations containing 1 M KCl, 25 μM EGTA, 100 μM PMSF and 10% (v/v) glycerol. The dialysed preparations lost 80% of their activities in 10 min at 45 °C. By contrast, 39 kDa phospholipase A₂ was more stable to heat inactivation than 110 kDa phospholipase A₂. It lost 20% of its activity at 45 °C and retained at least 10% of its activity after exposure to 55 °C. Like the 110 kDa phospholipase A₂, the dialysed preparations of 39 kDa phospholipase A₂ were more sensitive to heat inactivation than were the non-dialysed preparations of both 110 and 39 kDa phospholipases A₂. The 39 kDa phospholipase A₂ lost 95% of its activity in 10 min at 45 °C. The heat-inactivation profile clearly indicates that the rates of inactivation of the two phospholipases A₂ are quite different, and that the 110 kDa phospholipase A₂ is more sensitive to thermal inactivation than the 39 kDa phospholipase A₂ (Figure 3b).

DISCUSSION

The unique structure and surface-charge distribution of GAGs allow them to interact strongly with lysosomal enzymes, enzymes of nucleic acid metabolism, lipoprotein lipases, diacylglycerol

lipases, tyrosine hydroxylase, protein kinases and extracellular pancreatic phospholipases (Farooqui and Horrocks, 1984; Farooqui et al., 1984; Herbert and Maffrand, 1991). Intracellular Ca²⁺-independent phospholipases A₂ of bovine brain cytosol are unique enzymes. Unlike pancreatic phospholipases A₂, they do not require Ca²⁺ for their activities and they prefer arachidonate over other *sn*-2 fatty acids. The present study reports for the first time the effect of GAGs and sphingolipids on intracellular Ca²⁺-independent phospholipases A₂. The 110 kDa phospholipase A₂, that prefers PtdEtn, was not affected, whereas the 39 kDa phospholipase A₂ enzyme that prefers PlsEtn was markedly inhibited by GAGs. The pattern of inhibition was heparan sulphate > hyaluronic acid > chondroitin sulphate > heparin. The pig pancreatic phospholipase A₂ is also inhibited by GAGs (Diccianni et al., 1991b), but the pattern of inhibition in this case was heparin > chondroitin sulphate > de-N-sulphated heparin > heparan sulphate > hyaluronic acid. Also unlike the bovine cytosolic 39 kDa phospholipase A₂, the pancreatic enzyme hydrolyses phosphatidylcholine and requires Ca²⁺ for its activity.

At present it is not known how GAGs interact with intracellular Ca²⁺-independent phospholipase A₂. However, on the basis of their interactions with other proteins (such as apolipoprotein and antithrombin), there are probably two types of interactions between GAGs and proteins: a positive, cooperative binding and a specific binding (Zhou et al., 1992; Bourin and Lindahl, 1993; Margolis and Margolis, 1993; Timpl, 1993). In positive co-operative binding, the polysaccharide binds to a protein at different sites, and the affinity shown at each individual interacting site contributes to the overall affinity of the polysaccharide for its protein ligand. In specific binding, a specific GAG is required and other GAGs cannot substitute (Zhou et al., 1992). Finally, GAGs are known to bind phospholipids (Drake et al., 1992) and may interfere with the interactions between pyrene-labelled phospholipids and bovine brain phospholipases A₂. In the case of pancreatic phospholipase A₂, it has been suggested

that GAGs bind directly to the N-terminus of this enzyme and block the interfacial recognition site. This results in inhibition of its enzymic activity (Diccianni et al., 1991b). Reversal of GAGs inhibition by protamine sulphate or histone IIA suggests that at least some interactions of GAGs with Ca²⁺-independent phospholipases A₂ are electrostatic. The retention of 110 kDa and 39 kDa phospholipases A₂ on a heparin-agarose column and their elution with NaCl indicates that heparin-agarose chromatography can be used for the purification of bovine brain cytosolic phospholipases A₂ (Yang et al., 1993). In fact, heparin-agarose chromatography has also been used recently in the purification of rabbit platelet and human monocytic cytosolic phospholipases A₂ (Kim et al., 1991; Rehfeldt et al., 1993).

Bovine brain cytosolic phospholipases A₂ are very sensitive to thermal denaturation. The rate of inactivation of 110 kDa phospholipase A₂ was very different from that of 39 kDa phospholipase A₂. The heat-inactivation profiles of bovine brain cytosolic phospholipase A₂ are also different from those of pig pancreatic phospholipases A₂, human placental phospholipases A₂ and guinea-pig brush-border phospholipases A₂ (Diagne et al., 1987; Buhl et al., 1991).

Like pig pancreatic phospholipases A₂ (Bianco et al., 1990, 1992), intracellular Ca²⁺-independent phospholipases A₂ are also inhibited by gangliosides. The effect was rather specific because sulphatide had no effect and GM3 was much more effective than GM1. The inhibition of phospholipases A₂ by gangliosides is of considerable interest as both molecules are involved in the regulation of membrane-mediated processes. Phospholipases A₂ maintain cellular function by providing a variety of second messengers such as arachidonate, eicosanoids, and platelet-activating factor (Dennis et al., 1991). Gangliosides are apparently involved in the modulation of protein kinases (Schengrund, 1990), diacylglycerol and monoacylglycerol lipases (Freysz et al., 1991) and phospholipases (Bianco et al., 1990, 1992). Both gangliosides and phospholipases affect receptor function and ion-channel activity by regulating various membrane properties such as molecular surface organization, membrane molecular thermodynamics, and membrane fluidity and permeability.

GAGs and gangliosides are normal components of plasma membrane. GAGs are embedded in membrane through their protein core, whereas gangliosides are inserted in the outer leaflet of the membrane bilayer through their hydrophobic tail (Schengrund, 1990; Margolis and Margolis, 1993; Timpl, 1993). Recent studies have indicated that phospholipases A₂ are translocated from cytosol to plasma membrane in the presence of Ca²⁺ ions (Channon and Leslie, 1990; Krause et al., 1991; Rehfeldt et al., 1993). It seems possible that the interactions of GAGs and gangliosides reported in this paper may be involved in the binding and translocation of phospholipases A₂ to the plasma membrane.

In summary, based on the effects of GAGs, GSLs and heat-inactivation profiles, it can be concluded that 110 kDa and

39 kDa phospholipases A₂ are different enzymes found in bovine brain cytosol. The interactions between GAGs or GSLs and phospholipase A₂ may be involved in maintaining membrane integrity and receptor function.

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