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THE EFFECT OF PHOSPHOLIPASE ON THE BINDING OF ASIALOGLYCOPROTEINS BY RAT LIVER PLASMA MEMBRANES

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SUMMARY

The ability of hepatic plasma membranes to bind desialylated glycoproteins has been shown to be markedly diminished by prior treatment of the membranes with phospholipase A or phospholipase C. In the latter case, the decreased binding capacity was correlated with the loss of membrane-bound phosphate over a wide range of enzyme concentration. However, upon solubilization of the membrane associated binding protein, the sensitivity to phospholipase-induced inhibition of binding was eliminated.

Additional evidence is presented to support the concept that the observed inhibition is a consequence of non-specific changes in the membrane phospholipids and that phospholipid, per se, does not participate directly in the mechanism of binding.

INTRODUCTION

A previous report has established a generalized role for the terminal sialic acid residues of circulating glycoproteins. Injection of desialylated glycoproteins into test animals resulted in their prompt uptake by the liver under conditions where the intact, fully sialylated proteins survived normally in the circulation [1]. With the subsequent identification of hepatic plasma membranes as the major locus of binding, it was noted that the membrane binding capacity was significantly reduced by brief exposure to phospholipase C [2]. Since phospholipases have been widely used as probes of membrane structure and function [3, 4] and have proved to be useful reagents in distinguishing between hormone and fluoride stimulation of adenylate cyclase [5, 6], the possibility was considered that membrane phospholipids might participate in the binding reaction as an integral part of the membrane–glycoprotein complex.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

* Part of this work is taken from a Ph.D. Thesis to be presented to the Department of Biology, The Johns Hopkins University.

Consequently, the present study was undertaken in an attempt to define more precisely the role of membrane phospholipids in the overall binding phenomenon.

MATERIALS AND METHODS

Phospholipase A (*Viperi rossella*), phospholipase D (cabbage), trypsin and soybean trypsin inhibitor were purchased from Sigma; phospholipase C (*Bacillus cereus*) from Calbiochem; phospholipase C and neuraminidase (both *Clostridium perfringens*) from Worthington. Alkaline phosphatase (*Escherichia coli*) was a gift from Dr Marie Lipsett of this Institute. Phosphatidylethanolamine (*E. coli*), phosphatidylserine (bovine) and lysophosphatidylcholine were obtained from Calbiochem; bovine sphingomyelin and phosphatidylcholine were from Applied Sciences. Human orosomucoid was generously provided by the American Red Cross Research Center, Bethesda, Md.

Phospholipids were extracted in chloroform–methanol (2 : 1, v/v) by the method of Folch et al. [7] and chromatographed on silica gel Q5 precoated thin-layer plates from Quantum Industries, Fairfield, N. J. Solvent systems used were: (1) chloroform–methanol–28 % ammonia (65 : 35 : 5, by vol.) and (2) chloroform–acetone–methanol–acetic acid–water (5 : 2 : 1 : 1 : 0.5, by vol.) [8]. Phospholipids were located on thin-layer plates with iodine vapor, ninhydrin or phosphate spray reagents [9].

Phosphatidylcholine was isolated by preparative thin-layer chromatography on 500 μm thick plates of Camag DO silica gel in Solvent 1 of the chloroform–methanol extract of fresh rat liver. The appropriate band was eluted from the plates with chloroform–methanol (2 : 1, v/v) and shown to be at least 90 % pure by thin-layer chromatography. All phospholipids were dried under N_2 and sonicated in 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES) buffer, pH 7.5, in an N_2 atmosphere. Sonication was carried out in closed polypropylene test tubes for three periods of 5 min each at 0 °C in a 10-kcycle Raytheon sonic oscillator at a setting of 1.0–1.5 A. The phospholipids were stored at 4 °C for periods less than 1 week and were sonicated again on the day of use. Phosphorus content was determined by the method of Ames and Dubin [10].

The commercial preparation of neuraminidase was purified by affinity chromatography [11] and assayed with orosomucoid as substrate using the Warren procedure for the estimation of free sialic acid [12]. Protease activity [13] was determined with the synthetic substrate, Azocoll (Calbiochem). All of the enzymes used were free from protease activity at levels of 0.2 μg, or less, of trypsin equivalents.

The preparation and assay of rat liver plasma membranes was carried out as described previously [14]. In the standardized binding assay, 50 μg of membrane protein was incubated, in a final volume of 0.5 ml, in a solution containing 0.2 M NaCl, 0.01 M $CaCl_2$, 0.1 % bovine serum albumin, 0.02 M HEPES, pH 7.5, and a saturating amount of ^{125}I-labelled asialo-orosomucoid (approx. 1 μg). The suspension was incubated for 1 h at 37 °C in a shaking water bath and the labelled membranes were recovered by filtration on Whatman GF/C glass filters. After extensive washing, the filters were counted in a Packard auto-gamma-spectrometer. Blanks were maintained in an ice bath and the ^{125}I-labelled asialo-orosomucoid added just prior to filtration.

The membrane binding protein was solubilized by homogenizing the membrane

pellet in 1 % Triton X-100 containing 0.4 M KCl and 0.01 M Tris–HCl, pH 7.8. For each milligram of membrane protein, 1 ml of the Triton solution was added and the extraction continued for at least 30 min at 4 °C on a gyrating shaker. The binding capacity of the resulting suspension was assayed by $(NH_4)_2SO_4$ precipitation as follows: Aliquots of the solubilized membranes were incubated in a final volume of 1 ml containing 0.1 % Triton X-100, 0.6 % bovine serum albumin, 0.04 M $CaCl_2$, 1M NaCl, 0.05 M Tris, pH 7.8, and 0.6–1.0 μg of ^{125}I-labelled asialo-orosomucoid (spec. act. 0.5–0.8 Ci/g). After 30 min at 25 °C, the reaction was stopped by the addition of an equal volume of cold, saturated $(NH_4)_2SO_4$. The precipitate was kept in an ice bath for 10 min, filtered on Whatman GF/C glass filters, washed with cold 40 % $(NH_4)_2SO_4$ and counted as above. Blanks were stored in ice and the ^{125}I-labelled asialo-orosomucoid added immediately after precipitation with $(NH_4)_2SO_4$.

Units of activity for each of the enzymes are expressed as micromoles of product formed per minute.

TABLE I

EFFECT OF PHOSPHOLIPASES ON THE BINDING OF ^{125}I-LABELLED ASIALO-OROSOMUCOID

The binding capacity is expressed as ng ^{125}I-labelled asialo-orosomucoid per 50 μg membrane protein. The added enzyme is expressed as munits per 50 μg membrane protein.

Treatment	Enzyme added	Binding capacity	Control (%)
Phospholipase C*			
C. perfringens	0	16.1	100
	60	14.5	90
	320	10.4	65
B. cereus	0	12.1	100
	80	10.1	83
	400	9.3	77
Phospholipase A**	0	7.6	100
	60	5.3	70
	300	4.4	58
Phospholipase D***	0	14.7	100
	12	10.6	72
Alkaline phosphatase†	0	18.8	100
	51 000	19.7	105

* Phospholipase C was added to membranes suspended in the standard assay medium, in the absence of ^{125}I-labelled asialo-orosomucoid, and incubated for 15 min at room temperature with shaking. The membranes were then recovered by centrifugation, rehomogenized and aliquots assayed for binding as described in Materials and Methods. Similar results were obtained when the membranes were assayed without removal of the enzyme by centrifugation.

** Conditions were identical to those described above except for the addition of phospholipase A.

*** For optimal activity, the pH of the incubation mixture was lowered to 5.6 during the initial 15-min incubation with phospholipase D. The pH was then readjusted to 7.3 and the membranes assayed directly without prior centrifugation.

† In the presence of alkaline phosphatase the pH was raised to 8.0 and maintained at that pH throughout the assay.

RESULTS

Initial observations, suggestive of the possible participation of membrane phospholipids in the glycoprotein binding reaction, are presented in Table I. It is apparent that brief exposure of rat liver plasma membranes to any of several phospholipase preparations resulted in a marked diminution in the ability of these membranes to bind ^{125}I-labelled asialo-orosomucoid. In contrast, alkaline phosphatase was without discernible effect even at significantly higher concentrations of enzyme.

This finding prompted further examination of the phospholipase preparations in an attempt to determine whether the loss of binding capacity could be ascribed to the presence of unrelated enzymatic contaminants. For this purpose, advantage was taken of the unusual heat stability of phospholipase C which has been reported to tolerate high temperatures [15]. Thus, exposure of membranes to phospholipase C which had been heated at 95 °C for 5 min was accompanied by full retention of enzymatic activity as manifested by a significant loss in both the membrane binding capacity and the membrane phosphate content identical to that observed with the unheated enzyme; phospholipase A behaved similarly [16] (Table II).

A second property characteristic of both enzymes, an absolute requirement for calcium [16, 17], was tested by addition of chelating agents. Upon either removal or

TABLE II

EFFECT OF HEAT AND CALCIUM ON PHOSPHOLIPASE ACTIVITY

Preliminary incubations in the presence of phospholipase were carried out as described in the footnotes to Table I. Where indicated, phospholipase A was maintained at 60 °C for 5 min and phospholipase C at 95 °C for 5 min prior to the first incubation. Removal of calcium was accomplished by washing the membranes with 10 mM EGTA followed by incubation with phospholipase A in a medium devoid of calcium. The effect of calcium chelation on the enzymatic activity of *C. perfringens* phospholipase C involved a preliminary incubation of the enzyme with 10 mM EDTA for 30 min at room temperature at which time the membranes were added and the incubation continued for an additional 15 min. The membranes were recovered by centrifugation and the binding activity determined in the standard assay system. Control membranes were subjected to the same conditions in the absence of phospholipase. The phosphate content of control and enzyme-treated membranes was assayed as described in Materials and Methods. The binding capacity is expressed as ng ^{125}I-labelled asialo-orosomucoid per 50 μg membrane protein and the added enzyme as munits per 50 μg membrane protein.

Enzyme	Enzyme added	Enzyme treatment		Binding capacity	Total phosphate (nmoles)	Control (%)	
		Heated	Calcium present			Binding	Membrane phosphate
Phospholipase A	0	—	—	5.6	n.t.	100	n.t.
	300	—	—	3.0	n.t.	54	n.t.
	300	+	—	2.6	n.t.	46	n.t.
	300	+	—	5.7	n.t.	102	n.t.
Phospholipase C	0	—	—	6.9	26.7	100	100
	320	—	—	4.3	13.5	62	51
	320	—	—	4.5	14.7	65	55
	320	—	—	6.8	26.0	98	97

n.t., not tested.

chelation of calcium both enzymes were completely inhibited as manifested by complete retention of the membrane binding capacity (Table II). This inhibition appeared to be irreversible in that subsequent addition of excess calcium failed to reactivate phospholipase C when tested with either membranes or phosphatidylcholine as substrate.

These results were interpreted as supporting the presumption that diminution of binding to membranes resulted from the intrinsic activity of the phospholipase studied. More specifically, these preparations were tested for the presence of the two degradative enzymes known to destroy membrane binding activity: neuraminidase and protease [2]. Within the limits of the assay procedures described in Materials and Methods no significant concentration of either enzyme was detectable in the preparations of phospholipase A and phospholipase C. However, a small but perceptible amount of neuraminidase, 0.15 munit, was shown to be present at the level of phospholipase D used. Consequently, further studies with this preparation were discontinued.

Effect of ligand concentrations

In order to determine whether the phospholipase-induced inhibition was influenced by the amount of ligand added, membrane binding was determined over a wide concentration range of ^{125}I-labelled asialo-orosomucoid. The upper curve of Fig. 1 represents a typical binding curve for untreated membranes; the lower curve reflects the effect of phospholipase C. Identical patterns of inhibition were obtained with phospholipase C prepared from either *C. perfringens* or *B. cereus*. It is apparent

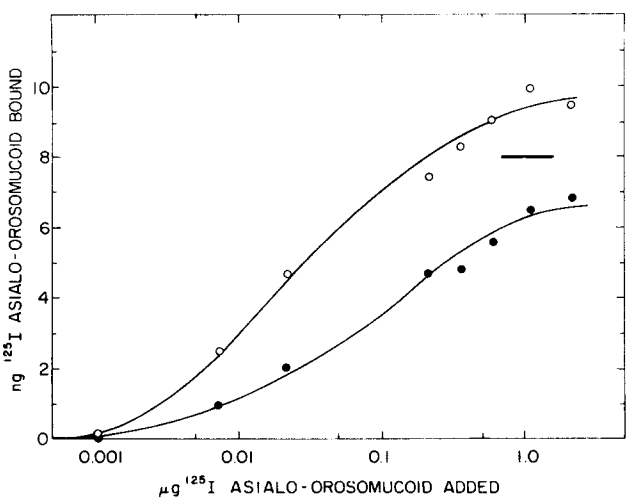

Fig. 1. Effect of ligand concentration on binding to control (○–○) and (●–●) phospholipase C-treated membranes. Membranes were incubated with 320 munits of phospholipase C. (*C. perfringens*) or 400 munits of phospholipase C (*B. cereus*) per 50 μg of membrane protein as described in Table I. Aliquots of the rehomogenized membranes were then assayed for binding with increasing amounts of ^{125}I-labelled asialo-orosomucoid. Since the response to each of the phospholipases was identical, within experimental variation, the values obtained at each level of ligand were combined and each point represents the average of 1–5 determinations. The horizontal bar indicates the range of ligand normally added when the assay is conducted under saturating conditions.

from these data that the decreased binding of asialo-orosomucoid by the enzyme-treated membranes does not depend on ligand concentration, in contrast to analogous studies wherein the phospholipase C-mediated inhibition of ^{125}I-labelled glucagon binding was abolished at high levels of ligand [6].

Correlation of phospholipid release with inhibition of binding

The effect of phospholipase C on binding was initially presumed to result from perturbation or cleavage of essential phospholipid constituents from the binding complex of the membranes. Consequently, an examination of these components was undertaken. Upon increasing the amount of phospholipase C over several orders of magnitude, the exponential decrease in membrane binding capacity was paralleled closely by the loss of membrane-bound phosphate (Fig. 2). Most significantly, in the presence of EDTA, the membranes retained their full complement of phosphate together with an undiminished binding capacity.

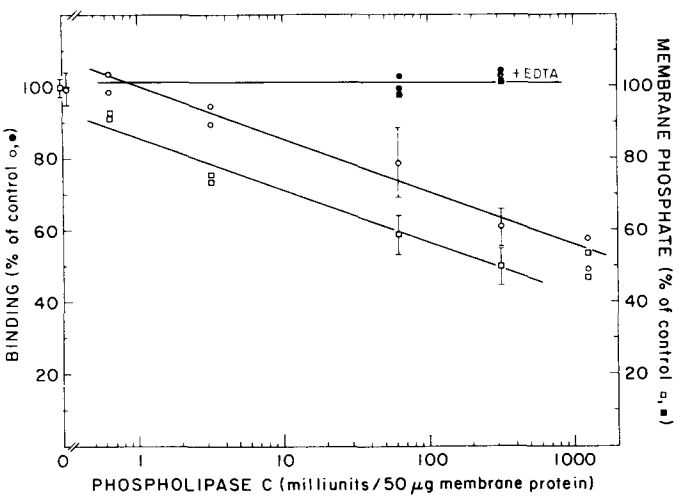

Fig. 2. Effect of increasing amounts of phospholipase C on binding and on membrane phosphate content. Membranes were incubated in the presence and absence of phospholipase C (*C. perfringens*) and the binding assay carried out as described in Table I. Calcium chelation and binding were carried out as described in Table II. The values with perpendicular bars represent the average of at least three duplicate determinations ± one standard deviation.

The organic phosphate released from the membranes by the action of phospholipase C was recovered quantitatively in the ambient fluid and was determined as inorganic phosphate following the addition of alkaline phosphatase. This point is illustrated in Fig. 3 wherein the sum of membrane-bound and -soluble phosphate remained constant over the entire concentration range of phospholipase added. Here, as in Fig. 1, identical results were obtained with phospholipase C from either *B. cereus* or *C. perfringens*. Furthermore, when the several sequential combinations of the two phospholipases were tested, no additional decrease in either binding or membrane phosphate was observed.

Qualitative identification of the membrane phospholipids destroyed by the

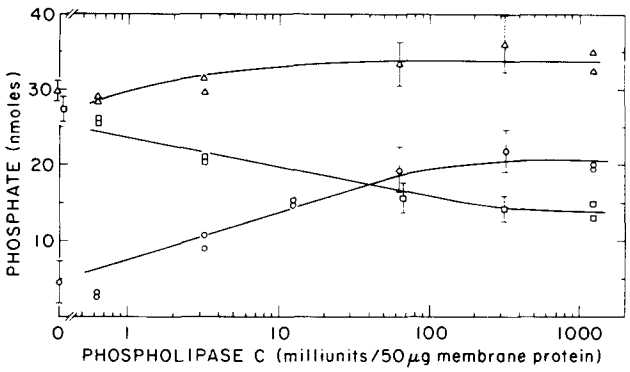

Fig. 3. Distribution and recovery of membrane-bound phosphate released by phospholipase C. The data provided in this figure were obtained from the appropriate fractions isolated as described in Fig. 2. Aliquots of the enzyme-treated membranes were assayed for organic phosphate; aliquots of the supernatant fluid, after centrifugation, were incubated for 2 h at 37 °C with 100 units of alkaline phosphatase at pH 8.0 and the released inorganic phosphate measured as described in Materials and Methods. Total phosphate refers to the sum of these values. The experimental points with perpendicular bars represent the average of at least three duplicate determinations ± one standard deviation. □, membrane phosphate; ○, supernatant fluid phosphate; △, total phosphate.

action of *C. perfringens* phospholipase C was obtained by thin-layer chromatography of the chloroform–methanol extracts of control and enzyme-treated membranes. The strongly positive-stained areas on the control plates corresponding to phosphatidylcholine and phosphatidylethanolamine were either missing entirely or only

TABLE III

ATTEMPTED RECONSTITUTION OF PHOSPHOLIPASE C-TREATED MEMBRANE

Pooled membranes were treated with 60 munits of phospholipase C (*C. perfringens*) for each 50 μg of membrane protein as described in Table I. Aliquots of the rehomogenized membranes containing 50 μg of membrane protein were incubated separately or together in 0.02 M HEPES buffer, pH 7.5, with bovine sphingomyelin (780 nmoles), rat liver phosphatidylcholine (125 nmoles) and or *E. coli* phosphatidylethanolamine (38 nmoles) for a total of 60 min at room temperature in a final volume of 200 μl. At the end of this period, the volume was adjusted to 0.5 ml by addition of the standard assay medium and binding was determined after an additional hour at 37 °C. The binding capacity is expressed as ng ^{125}I-labelled asialo-orosomucoid per 50 μg membrane protein.

Expt No.	Phospholipase C present	Additions			Binding capacity	Control (%)
		Sphingomyelin	Phosphatidyl-choline	Phosphatidyl-ethanolamine		
1	–	–	–	–	18.3	100
2	+	–	–	–	12.5	68
3	+	+	–	–	13.1	71
4	+	–	+	–	2.9	16
5	–	–	–	+	11.5	63
6	+	–	–	+	3.3	18
7	+	+	+	–	11.4	62
8	+	+	–	+	12.8	70
9	+	+	+	+	10.9	60

faintly visible on the experimental plates. Alterations in the content of the minor membrane constituents such as sphingomyelin, phosphatidylserine and phosphatidylinositol were less pronounced and no conclusions could be drawn on the basis of their staining intensity.

Effect of phospholipid additions

In view of the close correlation between the decrease in membrane binding capacity and the loss of membrane-bound phosphate following exposure to phospholipase C, reconstruction experiments were attempted whereby various phospholipids were added back to the enzyme-treated membranes. In no instance was it possible to demonstrate successful restoration of binding activity. Indeed, an augmented inhibition of binding was noted when unfractionated chloroform–methanol extracts of plasma membranes were added to the incubation mixture. One of the inhibitory components in the extract was tentatively identified as phosphatidylcholine on the basis of the data in Table III. It is noteworthy that whereas neither phosphatidylethanolamine nor sphingomyelin were stimulatory, the latter compound appeared to reverse the inhibitory effect of phosphatidylcholine.

TABLE IV

EFFECT OF PHOSPHOLIPASE C ON NATIVE VERSUS ASIALO-OROSOMUCOID-CHARGED MEMBRANES

The binding capacity is expressed as ng ^{125}I-labelled asialo-orosomucoid per 50 μg membrane protein and the added enzyme as munits per 50 μg membrane protein.

Expt.	Phospholipase C added	Charged membrane			Binding capacity	Total membrane phosphate	Control (%)	
		^{125}I-labelled asialo-orosomucoid added					Binding	Membrane phosphate
		Before enzyme*	After enzyme**	After dissociation				
A-1	0	+	–	–	18.0	21.6	100	100
A-2	300	+	–	–	18.1	10.0	100	46
A-3	300	–	+	–	14.2	11.5	79	53
B-1	0	+	–	–	12.4	n.t.	100	n.t.
B-2	300	+	–	–	13.2	n.t.	106	n.t.
B-11***	–	+	–	+	11.1	n.t.	100	n.t.
B-12***	–	+	–	+	6.7	n.t.	60	n.t.

* At the end of the standard assay incubation period, phospholipase C (*C. perfringens*) was added and the incubation continued, with shaking, for an additional 15 min at room temperature prior to filtration and counting.

** Carried out as described in Table I.

*** The charged membranes obtained in Expts B-1 and B-2 were centrifuged separately, homogenized and dissociated by adjusting the pH to 6.0 in the absence of calcium. After a 10-min incubation at 37 C°, the membranes were again collected by centrifugation, homogenized and resuspended at pH 7.3. The corresponding membranes, B-11 and B-12, respectively, were assayed for total binding capacity as described in the text except that binding was complete after 20 min at 37 °C. Note that phospholipase C was not present during the second binding reaction.

n.t., not tested.

Action of phospholipase C on the membrane–ligand complex

In order to determine whether the presence of ligand would serve to protect membranes from the destructive effect of phospholipase C, the order of addition was changed and the enzyme was added after binding of ^{125}I-labelled asialo-orosomucoid had taken place. In contrast to the inhibition of binding noted with uncharged membranes, the complex appeared to be stable in that dissociation was not observed (Table IVA). However, despite full retention of the bound ligand, the phospholipid content of these membranes remained accessible to the enzyme and the loss of total phosphate was indistinguishable from that seen with native membranes.

In view of this unexpected finding, advantage was taken of an earlier observation [2] that ligand can be quantitatively dissociated and rebound to membranes by altering the calcium concentration and the pH of the incubation mixture. As shown in Table IVB, when membranes which had been charged with ^{125}I-labelled asialo-orosomucoid and exposed to phospholipase C were dissociated and then rebound in the absence of phospholipase C, there was a significant loss in binding ability which was entirely comparable to that seen with the enzyme-treated native membranes.

TABLE V

EFFECT OF TRITON X-100 ON RECOVERY OF BINDING ACTIVITY

Enzymes were added at the indicated levels for each 50 μg of membrane protein present. Assay conditions for the intact and Triton-solubilized membranes were carried out as described in Materials and Methods and preliminary incubations with phospholipase as given in Table I. The Triton-extracted membranes were incubated with the phospholipases for 15 min at room temperature after the addition of $CaCl_2$ (10 mM). Aliquots were then assayed for binding activity. Neuraminidase was added to membranes suspended in 0.01 M $CaCl_2$, 0.1 M sodium acetate, pH 6.5, 0.1 % bovine serum albumin. After shaking for 15 min at room temperature, the membranes were centrifuged, rehomogenized and assayed as usual. Neuraminidase was added to the Triton suspension of intact membranes in a final concentration of 0.05 M sodium acetate, pH 6.5, 0.25 % Triton X-100, 0.1 M KCl, 0.002 M Tris and incubated for 15 min at room temperature. The pH was subsequently readjusted to 7.8 with 1 M Tris buffer and the usual binding assay was carried out.

Enzyme added	Enzyme added		Binding capacity		Control (%)	
	To intact membranes	To Triton-extracted membrane	In absence of Triton	In presence of Triton	In absence of Triton	In presence of Triton
None			16.0	—	100	—
		—	—	15.8	—	99
Phospholipase C (320m units)	—	—	9.1	—	57	—
	—	—	—	16.0	—	100
	—	—	—	17.1	—	107
Phospholipase A (300 munits)	—	—	7.8	—	49	—
	—	—	—	14.3	—	90
	—	—	—	14.3	—	90
Neuraminidase (15 munits)	—	—	3.7	—	23	—
	—	—	—	5.9	—	37
	—	—	—	5.4	—	34

Recovery of binding activity

As a consequence of the demonstration that phospholipase failed to dissociate membrane-bound ^{125}I-labelled asialo-orosomucoid, despite extensive loss of membrane phospholipid, the presumed participation of these components in the binding complex became questionable. Moreover, since it was shown recently [18] that the glycoprotein binding activity could be effectively solubilized from a particulate fraction of rabbit liver by means of the non-ionic detergent, Triton X-100, a direct experimental approach to this problem became available. To this end, plasma membranes were suspended in 1 % Triton, ^{125}I-labelled asialo-orosomucoid added and, after a suitable incubation time to permit maximal binding, the reaction was stopped by the addition of an equal volume of cold, saturated $(NH_4)_2SO_4$. Under these conditions, the bound ligand was precipitated; excess ^{125}I-labelled asialo-orosomucoid remained in solution and was removed by filtration [19]. When assayed by this technique, all of the binding activity of the original membranes was recovered on the filter as bound ^{125}I-labelled asialo-orosomucoid and was counted in the usual way.

Table V summarizes the results obtained when the binding capacity of membranes, exposed to either phospholipase A or phospholipase C, was assayed in the presence or absence of Triton X-100. It is apparent that all of the binding activity of the membranes remained intact as shown by its complete recovery in soluble form in the presence of detergent. Further evidence for the resistance of the native binding activity to phospholipolytic attack is provided by the demonstration that the addition of these phospholipases to the detergent-solubilized membranes was totally without effect. In contrast, treatment of the membranes with neuraminidase resulted in extensive loss of binding capacity which was not recoverable in the Triton extract.

DISCUSSION

The data provided in this paper confirm and extend an earlier observation on the inhibitory effect of phospholipase on the binding of asialoglycoproteins by hepatic plasma membranes. At the outset, it was considered essential to demonstrate that the inhibitory effect resulted from the intrinsic activity of the phospholipases studied rather than from non-specific enzymatic contamination. The data in Table II provide reasonable support for this requirement whereby, in both of the phospholipases tested, the enzymatic activity and the membrane binding capacity were equally resistant to high temperatures and equally sensitive to the removal of calcium. Furthermore, in the case of phospholipase C, a close correlation was shown to exist between the decrease in membrane binding and the loss of membrane-bound phospholipids over a wide range of enzyme concentration (Fig. 2).

Despite this presumptive evidence of a role for phospholipid involvement in the mechanism of glycoprotein binding, all attempts at reconstruction were unsuccessful (Table III). More important was the observation that exposure of the membrane–glycoprotein complex to phospholipase C resulted in no disruption of the bound protein although the membrane phospholipids continued to remain accessible to the enzyme. However, after dissociation of the bound ligand the resulting membranes were shown to have suffered a significant loss of their original binding capacity (Table IV).

Further investigation of this problem became possible with the development of techniques capable of solubilizing and quantitating the soluble hepatic binding protein [18, 19]. As shown in Table V, complete recovery of the original membrane binding activity was observed in Triton X-100 extracts of membranes "damaged" by prior treatment with either phospholipase A or phospholipase C. Similarly, no inhibitory effect was found when either of these phospholipases were added to soluble extracts of native membranes. In contrast, neuraminidase, an enzyme known to destroy binding, caused a definite inhibitory effect in the presence as well as in the absence of Triton.

These findings are consistent with the hypothesis that phospholipids do not constitute an integral part of the membrane–glycoprotein binding complex and that the phospholipase-induced inhibition of binding is a secondary phenomenon arising from non-specific disruption of the lipid constituents of the membrane surface. However, at least two alternative explanations must be considered.

Initially, it is possible that bound ligand may act to stabilize the receptor and that the membrane phospholipids play no role in the stabilization of the complex but are, nevertheless, essential for the binding reaction. Secondly, it is conceivable that Triton, per se, may act as a lipid and provide an adequate substitute for essential membrane phospholipids. Both of these considerations, however, are rendered highly unlikely by the recent demonstration that the purified, and highly active, binding protein has been isolated in water-soluble form which is free from detectable amounts of Triton X-100 (less than 0.01 %) [19]. Moreover, the purified binding protein, which is devoid of phospholipids has subsequently been shown to be unaffected by exposure to amounts of phospholipase C which are capable of producing a marked inhibition of binding by intact membranes (Hudgin, R. L. and Ashwell, G., unpublished).

In view of the totality of evidence available, it appears reasonable to infer that phospholipids do not constitute an integral part of the membrane–glycoprotein binding complex. Of possibly greater general significance, however, is the realization that assignment of a structure–function relationship cannot be based solely on the results obtained by enzyme-induced alterations of intact membranes.

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