BBA 76030

THE ROLE OF PHOSPHATIDYLSERINE IN THE HORMONAL CONTROL OF ADENYLATE CYCLASE OF RAT LIVER PLASMA MEMBRANES

ANTAL RETHY*, VITTORIO TOMASI,
AGOSTINO TREVISANI AND OTTAVIO BARNABEI**
Institute of General Physiology, University of Ferrara, Ferrara (Italy)

(Received February 10th, 1972)
(Revised manuscript received May 23rd, 1972)

SUMMARY

The involvement of phospholipids in the responsiveness of adenylate cyclase of rat liver plasma membranes to hormones was studied, using a mild extraction procedure and phospholipase A and C treatment for the removal of membrane phospholipids.

Mild extraction of membranes caused a reduction in the basal activity of adenylate cyclase which was also no longer sensitive to hormones and F^-. Addition of phosphatidylserine almost completely restored the epinephrine responsiveness of the enzyme, but only partially restored the glucagon and F^- sensitivity. Phosphatidylinositol, which restored the basal cyclase activity, had no effect on hormonal and F^- sensitivity. Phosphatidylethanolamine and phosphatidylcholine had no effect.

Treatment of membranes with phospholipase A and C abolished the hormonal and F^- responsiveness of adenylate cyclase; phospholipase A also decreased its basal activity. The hormonal and F^- reponsiveness were partially reactivated with phosphatidylserine, but not with phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine.

These observations are consistent with the catalytic and regulatory subunits of plasma membrane adenylate cyclase both being lipoproteins, the former requiring phosphatidylinositol and the latter phosphatidylserine for proper functioning.

INTRODUCTION

In a previous study it was shown that membrane-bound adenylate cyclase of rat liver requires phospholipids for full activity[1]. This was indicated by several lines of evidence: (a) enzyme activity is reduced after mild treatment of membranes with ether–butanol; (b) it is partially reactivated after re-addition of endogenous phospholipids or phosphatidylinositol to the extracted membranes, (c) the enzyme is inhibited by phospholipase A.

* Present address Medical University of Debrecen, Microbiological Institute, Debrecen 12, Hungary
** Present address Institute of General Physiology, University of Rome, Rome, Italy

Since adenylate cyclase activity of rat liver plasma membranes is known to be under hormonal control[2-4], we have now studied the role of phospholipids in the response to hormones. The results described here indicate that mild solvent extraction, as well as treatment with phospholipase A or C, results in the loss of the enzyme's sensitivity to epinephrine, glucagon and F^-. Re-addition of phosphatidylserine results in full restoration of the epinephrine effect, while that of glucagon or F^- is only partially restored. Part of these results have been reported elsewhere[5].

While this manuscript was in preparation, a paper from Rodbell's group[6] appeared which described a role of phospholipids in the reactivation of glucagon-sensitive adenylate cyclase in liver plasma membranes previously treated with phospholipase A or digitonin.

MATERIALS AND METHODS

Plasma membranes were isolated from rat liver as previously described[1,7]. Assays for proteins were carried out by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

Lipid extraction and determination

Mild lipid extraction, total lipid extraction, purification of lipids, separation and determination of phospholipids were performed as previously described[1]. For the preparation of rat liver phospholipid components the method of Skipski *et al.*[9] was used. Phospholipid phosphorus was assayed according to the method of Bartlett[10].

Treatment of membranes with phospholipase A and C

The incubation medium contained, in a final volume of 1 ml: 5 mM Tris–HCl, pH 7.3; 5 mM $CaCl_2$; 0.5 M NaCl; 3 mg of membrane protein and 45 μg of phospholipase A or C, unless otherwise stated. The tubes were incubated 45 min at 37 °C and the reaction was terminated by the addition of cold Na_2EDTA (1 mM final concn). After centrifugation the pellets were washed twice with cold 0.01 mM Na_2EDTA and finally suspended in distilled water. Controls were incubated without phospholipase (see also ref. 11).

The liberated fatty acids and phosphorus were assayed according to the methods of Skipski *et al.*[12] and Bartlett[10], respectively.

Determination of enzyme activities

Adenylate cyclase was assayed according to the method of Marinetti *et al.*[2], with some modifications. The incubation medium contained, in a final volume of 0.5 ml: 50 mM Tris–HCl, pH 8.0; 4 mM $MgCl_2$, 0.6 mM [8-^{14}C]ATP (0.5 μCi); 50 μg of albumin and 80–100 μg of membrane protein. After incubation for 15 min at 37 °C, 10 μl of a recovery mixture, containing 20 mM ATP, cyclic AMP, adenine, inosine and hypoxanthine, was added to each tube. The reaction was terminated by immersing the tubes in a boiling water bath for 2 min. In the case of blanks, the membranes were first boiled for 2 min. Cyclic AMP was isolated from 0.25 ml of the reaction mixture by two-dimensional ascending paper chromatography on rolls of Whatman 3 MM (22 cm × 22 cm), using 26 cm × 18 cm chromatographic tanks. Emmelot and Bos[14] have reported that an additional run is required for sharp separation of cyclic AMP from adenine and hypoxanthine. However, the running times used by these

authors were lower than ours (4.5 and 7 h, respectively, for the first and second direction, instead of 6–7 and 12–14 h) at about 22 °C. In our conditions, cyclic AMP is completely separated from all other metabolites of ATP and, therefore, when we tested the third eluent suggested in ref. 14, no change of activity occurred. However, the third eluent is necessary when the chromatography times in ref 14 are used.

The reaction was essentially linear with time for 15 min and proportional to the amount of membrane protein up to 100 μg. In some experiments, an ATP-regenerating system (10 mM phosphoenolpyruvate and 15 units of pyruvate kinase) was included in the assay medium. However, the results were essentially unmodified and are included in the tables*. Some batches of labeled ATP gave metabolite(s) which on chromatography partially overlapped with the cyclic AMP spot However, this difficulty was overcome by passing the ATP through a column (0.8 cm × 20 cm) of Dowex 50W-X8 (100–200 mesh, H^+ form) 4 ml of sample were adsorbed to the resin and eluted with water, the first 5 ml of eluate being collected. Mg^{2+}-ATPase and 5'-nucleotidase were assayed according to the method of Emmelot and Bos[15].

Preparation of phospholipid suspension

The method of Lester and Smith[16] was used. Phospholipids were sonicated for 15 min at 4 °C in an MSE ultrasonic disintegrator, in a medium containing 0.01 M Tris–HCl, pH 8.0, 2 mM $CaCl_2$ and 8.55 % sucrose. Phospholipid suspensions were added to the tubes containing membranes immediately after sonication, and they were incubated for 1 h at 4 °C before enzymatic or binding assays. Usually 3–4 mg of phospholipids could easily be suspended in 1 ml of medium.

Materials

[8-^{14}C]ATP ammonium salt (spec. act., 52–55 Ci/mole) was obtained from the Radiochemical Centre, Amersham, England. Crystalline glucagon (insulin free) was a gift of Dr R. Chance, E. Lilly Co., Indianapolis, Ind., U.S.A. Phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine were obtained from Koch–Light Laboratories, England, or were prepared from rat liver in our laboratory. The phospholipids were purified by thin-layer chromatography before use[9]. Other lipid components were obtained as previously described[1]. Phospholipase A (pancreatic, spec. act., 1000 μmoles/min per mg protein) was a gift of Prof L.L.M. van Deenen and Dr G. H. de Haas, Department of Biochemistry, Utrecht, The Netherlands. Phospholipase C from *Clostridium welchii* (type I), the nucleotides used as enzyme substrates, L-epinephrine·bitartrate and bovine serum albumin were products of Sigma Chemical Co., U.S.A. Phosphoenolpyruvate and pyruvate kinase were obtained from Boehringer, Mannheim, Germany.

RESULTS

Effect of phosphatidylserine on the restoration of hormonal and F^- sensitivity of mild-extracted plasma membrane

In a previous paper[1] it was shown that adenylate cyclase activity of isolated liver plasma membranes is reduced after mild extraction with solvents. The data

* This is not surprising, since the ATP-regenerating system has a poor effect when the concentration of ATP is "physiological" (*i.e.* around 0.5 mM) as in our adenylate cyclase assay medium (see also Rodbell[13])

TABLE I

EFFECT OF PHOSPHOLIPIDS ON THE RESTORATION OF HORMONAL AND F^- SENSITIVITY OF ADENYLATE CYCLASE OF EXTRACTED MEMBRANES

Enzyme activity is expressed as nmoles cyclic AMP/mg protein per h Means ± S E are given Figures in parentheses represent the number of experiments

	Enzyme activity				
	Control	*Extracted*	*Extracted + phosphatidylinositol**	*Extracted + phosphatidylserine***	*Extracted + phosphatidylethanolamine***
None	6 49 ± 0 3 (4)	2 48 ± 0 23 (4)	5 49 ± 0 17 (4)	3 70 ± 0 52 (4)	2 2 (1)
Epinephrine ($1 \cdot 10^{-5}$ M)	10 99 ± 0 7 (3)	2 68 ± 0 39 (3)	4 80 ± 0 3 (3)	8 97 ± 0 19 (3)	2 2 (1)
Glucagon ($2\ 10^{-6}$ M)	15 83 ± 2 0 (3)	2 56 ± 0 27 (3)	4 74 ± 0.24 (2)	5 97 ± 0 12 (3)	2 1 (1)
NaF ($1 \cdot 10^{-2}$ M)	16 5 ± 1 78 (3)	2 9 ± 0 29 (3)	5 23 ± 0 52 (3)	6 38 ± 0 99 (3)	2 1 (1)

* Concentration of phosphatidylinositol: 8 μg P per mg membrane protein
** Concentrations of phosphatidylserine and phosphatidylethanolamine· 12 μg P per mg membrane protein.

presented in Table I indicate that this treatment abolishes stimulation of the enzyme by hormones and F^-.

The stimulatory effect of F^- became evident only when the concentration of Mg^{2+} was sufficiently high (around 4 mM in the present experimental conditions). In fact, it has been found[3,17,18] that at lower Mg^{2+} concentrations, F^- either has no effect or even inhibits adenylate cyclase.

Phosphatidylinositol largely restores the basal adenylate cyclase activity, but is ineffective toward the hormone and F^- sensitivity. On the other hand, when phosphatidylserine was added at a concentration of 12 μg P per mg membrane protein, it had a poor effect on the basal activity but almost restored the responsiveness to epinephrine, and partially that to glucagon and F^- (Table I). In the same conditions, phosphatidylethanolamine which constitutes the major component (on a weight basis) removed by the mild extraction procedure[1], affected neither basal activity nor hormonal or F^- stimulation.

It is worth noting that phospholipids from two different sources (bovine brain and rat liver, see Material and Methods) were used for these experiments, and because they showed the same effect, the two sets of data are combined. The fatty acid composition of phospholipids therefore probably does not influence the reactivation of adenylate cyclase.

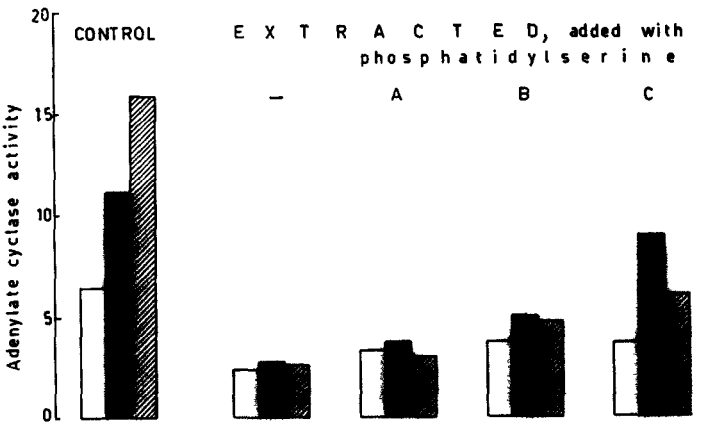
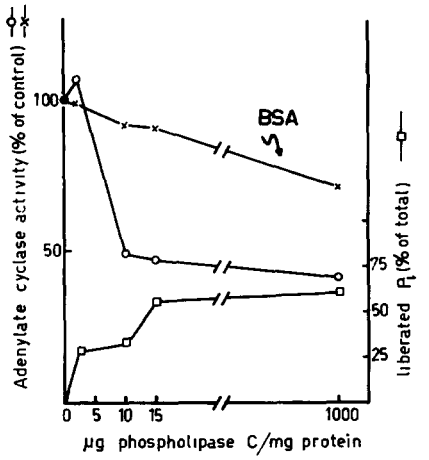


Fig. 1 Partial restoration of hormonal responsiveness of plasma membrane adenylate cyclase by phosphatidylserine Membranes were incubated as described in Materials and Methods; 3 μg P (A), 6 μg P (B) and 12 μg P (C) of phosphatidylserine were added, respectively Activity was determined in the absence (▭) and the presence of either $1 \cdot 10^{-5}$ M epinephrine (■) or $2 \cdot 10^{-6}$ M glucagon (▨). Basal, epinephrine- and glucagon-stimulated activities of control membranes in the presence of phosphatidylserine (12 μg P) were 7 0, 11 6 and 18 3 nmoles/mg protein per h, respectively

Fig. 1 shows the effect of three concentrations of phosphatidylserine. Epinephrine and glucagon sensitivity reappeared when phosphatidylserine was added to the extracted membranes at 6 μg P per mg membrane protein.

Effect of phospholipase A and C on phospholipid composition and adenylate cyclase activity of rat liver plasma membrane

There is considerable evidence that phospholipases can specifically hydrolyze phospholipid substrates attached to biological membranes[19–21].



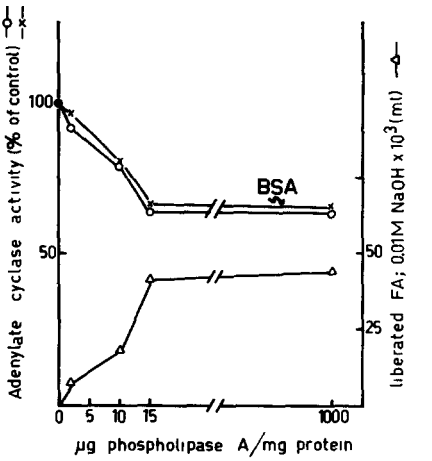


Fig 2 Adenylate cyclase activity of phospholipase A-treated membranes For experimental conditions see Materials and Methods. The concentration of bovine serum albumin (BSA) in the incubation medium was 0 25 mg FA, fatty acids

Fig. 3 Adenylate cyclase activity of phospholipase C-treated membranes. For experimental conditions see Materials and Methods BSA, bovine serum albumin.

It was shown previously that both phospholipase A and C produced a decrease of adenylate cyclase activity of plasma membranes[1]. Fig. 2 describes the effect of different concentrations of phospholipase A. When the concentration of phospholipase A was increased from 2 μg to 15 μg per mg membrane, both the inhibition of adenylate cyclase and the release of fatty acids were increased; no further modification was observed at concentrations as high as 1000 μg per mg membrane protein. Since addition of 0.25 mg bovine serum albumin to the assay medium of adenylate cyclase did not reverse the inhibition, it seems likely that the hydrolysis products of phospholipids are not involved in the inhibition by phospholipase A. Similar results were obtained using ten times as much bovine serum albumin, which is presumably enough to tie up the fatty acids and lysophospholipids (not shown).

Fig. 3 shows the effect of different concentrations of phospholipase C. In the presence of 15 μg of phospholipase C per mg membrane protein, adenylate cyclase was inhibited; however, this inhibitory effect was largely prevented by bovine serum

TABLE II

AMOUNT OF PHOSPHOLIPIDS OF PLASMA MEMBRANES AFTER PHOSPHOLIPASE A AND C TREATMENT

The concentration of phospholipases was 15 μg per mg membrane protein.

	mg phospholipids/100 mg membrane protein *	Δ (%)
Control	22 3 ± 0.4 (3)	—
Phospholipase A treated	10 5 ± 0 3 (3)	−53
Phospholipase C treated	9 8 ± 0.25 (3)	−56

* Means ± S E. Figures in parentheses represent number of experiments.

albumin, suggesting that diglycerides (liberated by the enzyme) are involved in the reduction of adenylate cyclase activity.

Both phospholipases slightly decreased Mg^{2+}-ATPase and 5'-nucleotidase activities (not shown), which is in accordance with the data of Emmelot and Bos[11]

To compare the action of phospholipases and that of mild solvent extraction, it seemed opportune to study the changes of membrane phospholipid produced by these enzymes. As shown in Table II, after the membrane had been treated with phospholipase A, about 53 % of the total phospholipids were hydrolyzed, whereas 56 % of phospholipids was hydrolyzed by phospholipase C

The phospholipid composition of control and phospholipase-treated membranes is reported in Table III. The data for the control membrane preparation differ only slightly from those reported in the previous paper[1], the concentration of lysophosphatidylcholine is lower and those of diphosphatidylglycerol *plus* phosphatidic acid are higher. This is probably due to the fact that, to minimize artifacts and decomposition of phospholipids, we carried out two-dimensional thin-layer chromatography immediately after the preparation of membranes. On the other hand, the concentration of the major phospholipids was very similar to that recorded previously[1].

TABLE III

PHOSPHOLIPID COMPOSITION OF CONTROL AND PHOSPHOLIPASE A- AND C-TREATED RAT LIVER PLASMA MEMBRANES

	*mg phospholipids/100 mg membrane protein**			*% of control*	
	Control	*Phospholipase A***	*Phospholipase C***	*Phospholipase A*	*Phospholipase C*
Phosphatidylcholine	7 96 ± 0 17	2 26 ± 0 16	0 87 ± 0 05	28	11
Sphingomyelin	3 44 ± 0 14	2 82 ± 0 03	0 50 ± 0 06	82	15
Lysophosphatidylcholine	0 92 ± 0 03	5 27 ± 0 1	0 34 ± 0 07	573	37
Phosphatidylethanolamine	3 75 ± 0 05	1 08 ± 0 05	1 62 ± 0 03	29	43
Phosphatidylserine	2 36 ± 0 2	0 86 ± 0 07	0 69 ± 0 05	36	29
Phosphatidylinositol	1 46 ± 0 12	0 86 ± 0 02	1 03 ± 0 06	59	70
Lysophosphatidylserine	—	0 73 ± 0 14	+	—	—
Lysophosphatidylethanolamine	—	2 17 ± 0 09	+	—	—
Phosphatidylglycerol	0 85 ± 0 02	0 56 ± 0 01	1 06 ± 0 07	66	125
Cardiolipin + phosphatidic acid	0 67 ± 0 02	0 54 ± 0 05	1 2 ± 0 04	80	179
Unidentified I	0 29 ± 0 03	0 15 ± 0 03	0 9 ± 0 03	52	172
Unidentified II	+	—	0 9 ± 0 03	—	—
Origin	0 8 ± 0 06	1 4 ± 0 08	1 1 ± 0 04	—	—

* Average of three separate experiments ± S E. In each experiment two different plasma membrane preparations were used.

** For concentration see Table II.

The comparison of the phospholipid composition of intact and phospholipase A-treated membranes indicates that phospholipase A extensively hydrolysed phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. On the other hand, phospholipase C extensively hydrolysed phosphatidylcholine, sphingomyelin and phosphatidylserine but to a lesser extent phosphatidylethanolamine and phosphatidylinositol. Other minor components were not affected by either phospholipase.

TABLE IV

EFFECT OF PHOSPHOLIPIDS ON THE RESTORATION OF HORMONAL AND F^- SENSITIVITY OF ADENYLATE CYCLASE OF PHOSPHOLIPASE A-TREATED MEMBRANES

Basal, epinephrine- and glucagon-stimulated activities of control membranes in the presence of phosphatidylserine were 6 5, 11 6 and 19 6, respectively (average of two experiments) No appreciable modification of these data was observed when phosphatidylinositol was tested Control membranes were preincubated for 45 min at 37 °C (see Materials and Methods). The amounts of phosphatidylserine and phosphatidylinositol used were the same as in Table I Means ± S E are given Figures in parentheses represent number of experiments

	Enzyme activity			
	Control	*Phospholipase A*	*Phospholipase A + phosphatidylserine*	*Phospholipase A + phosphatidylinositol*
None	6 62 ± 0 16 (6)	4 33 ± 0 07 (4)	4 96 ± 0 02 (3)	5 78 ± 0 14 (3)
Epinephrine ($1 \cdot 10^{-5}$ M)	10 75 ± 0 29 (3)	4 59 ± 0 04 (3)	7 37 ± 0 16 (3)	5 31 ± 0 14 (2)
Glucagon ($2 \cdot 10^{-6}$ M)	20 75 ± 0 36 (3)	4 77 ± 0 12 (3)	8 11 ± 0 16 (3)	5 52 ± 0 17 (2)
NaF ($1 \cdot 10^{-2}$ M)	19 9 ± 0 32 (3)	4 85 ± 0 17 (3)	7 28 ± 0 02 (3)	5 96 ± 0 22 (2)

Restoration of adenylate cyclase of membrane treated with phospholipase A

Treatment of membranes with phospholipase A decreased the basal activity of adenylate cyclase and destroyed its hormonal as well as its F^- sensitivity (Table IV). The addition of phosphatidylinositol (as expected from experiments on mild-extracted membranes) partially restored basal enzyme activity, but not the hormonal and F^- sensitivity. Addition of phosphatidylserine, however, partially restored the epinephrine, glucagon and F^- responsiveness of adenylate cyclase.

Fig. 4 shows the effect of different concentrations of phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine on the epinephrine-stimulated

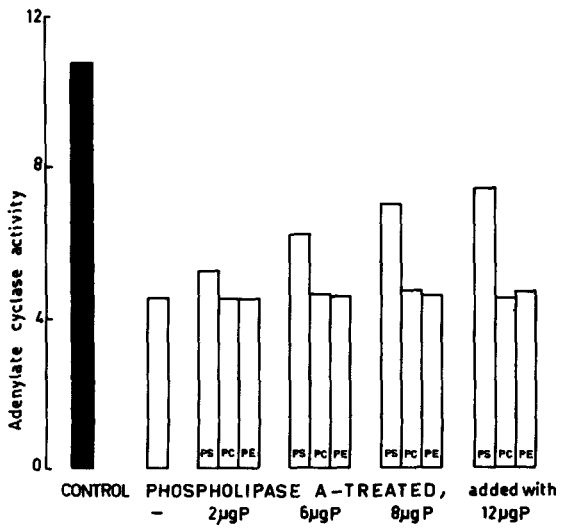


Fig. 4 Effect of phospholipids on the restoration of epinephrine-sensitive adenylate cyclase of phospholipase A-treated membranes Membranes were incubated as described in Materials and Methods, in the presence of $1 \cdot 10^{-5}$ M epinephrine.

adenylate cyclase of phospholipase A-treated membranes. Phosphatidylcholine and phosphatidylethanolamine, which were more extensively hydrolyzed, failed to modify the activity. In contrast, phosphatidylserine enhanced the activity linearly when its concentration was raised from 2 to 12 μg P per mg membrane protein. Higher concentrations did not appreciably modify the activity further.

The specificity of phosphatidylinositol and phosphatidylserine cannot be due simply to their dispersion in micellar form in aqueous buffers, since phosphatidylcholine is suspended at least as well as phosphatidylserine but is completely ineffective.

Birnbaumer *et al.*[22] reported that treatment of rat liver plasma membranes with a purified preparation of phospholipase A (from snake venom) caused a selective loss of the glucagon response and enhanced the stimulatory effect of F^-. In our hands, purified phospholipase A (from pancreas) caused a loss of the responsiveness of adenylate cyclase to both glucagon and F^-. The reason for this discrepancy may be the amount and the source of the phospholipase A used, and possibly also to the different concentration of ions (Ca^{2+} and Na^+) in the incubation mixture. Another important difference is that we treated membranes with the enzyme for a longer period of time and at a higher temperature. Therefore, marked differences probably exist in the type and amount of phospholipids hydrolyzed in our conditions and those of Birnbaumer *et al.*[22].

Restoration of adenylate cyclase activity of membranes treated with phospholipase C

As shown above, treatment of membranes with phospholipase C, in the presence of bovine serum albumin, did not modify the basal activity of adenylate cyclase; the enzyme was, however, made unresponsive to hormones and F^- (Table V). Again, a small but significant reactivation of the sensitivity towards epinephrine, glucagon and F^- was achieved by adding phosphatidylserine.

TABLE V

EFFECT OF PHOSPHATIDYLSERINE ON THE RESTORATION OF HORMONAL AND F^- SENSITIVITY OF ADENYLATE CYCLASE OF PHOSPHOLIPASE C-TREATED MEMBRANES

Means ± S E are given Figures in parentheses represent number of experiments Control values are the same as those in Table IV

	Enzyme activity		
	Control	*Phospholipase C*	*Phospholipase C + phosphatidylserine**
None	6 62 ± 0 16 (6)	6 24 ± 0 03 (4)	5 88 ± 0 1 (3)
Epinephrine ($1 \cdot 10^{-5}$ M)	10 75 ± 0 29 (3)	6 36 ± 0 01 (3)	7 68 ± 0 13 (3)
Glucagon ($2 \cdot 10^{-6}$ M)	20 75 ± 0 36 (3)	6 41 ± 0 1 (3)	8 84 ± 0.1 (3)
NaF ($1 \cdot 10^{-2}$ M)	19 90 ± 0 32 (3)	6 21 ± 0 1 (3)	7 64 ± 0 1 (3)

* Concentration of phosphatidylserine. 12 μg P per mg membrane protein

DISCUSSION

Sutherland *et al.*[23] first suggested that adenylate cyclase may be bound to membrane phospholipids and may be lipoproteic in nature. This hypothesis induced a

number of workers[6,24], including ourselves[1], to study the effect on the enzyme activity of removing and then adding back phospholipids. Membrane lipids are commonly removed either with a solvent mixture[23,25-28] or by incubating with phospholipase A and C[12,29-33]. The solvent extraction method developed by Emmelot and Bos[11] for plasma membranes appears to be particularly mild, since little of the total lipids is removed and, whereas enzymes like Mg^{2+}-ATPase, (Na^+–K^+)-ATPase and adenylate cyclase are inhibited, 5′-nucleotidase is not affected[1,11]. Moreover, this procedure characteristically removes a small part of neutral lipids and only phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol phospholipids[1]. Adding back phosphatidylinositol to the extracted membranes enhanced the adenylate cyclase activity almost to control values. We believe, therefore, that phosphatidylinositol is involved in the catalytic moiety of adenylate cyclase

In view of the specificity of their action and the generally mild conditions under which the treatment can be carried out, phospholipases are useful for the selective modification of membranes[30-33]. Treatment of plasma membranes with purified pancreatic phospholipase A resulted in an extensive hydrolysis of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine; whereas phospholipase C (from *Cl. welchii*) hydrolysed phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidyl serine and, to a lesser extent, other phospholipids (see Table III). Phospholipase treatment was, in our hands, more drastic than mild extraction. This is indicated, besides the more profound alteration of phospholipid composition, by the fact that reactivation of adenylate cyclase by phosphatidylinositol is less complete in phospholipase-treated membranes. Both solvent and phospholipase destroyed the enzyme's responsiveness to hormones and F^-, which was restored specifically by re-addition of phosphatidylserine. Epinephrine sensitivity was restored by phosphatidylserine to a much greater extent than glucagon and F^- sensitivity. This may be interpreted to mean that other (phospho)lipids are required. However, it cannot be excluded that the glucagon receptor is more easily damaged by solvent and phospholipase treatment, and is hence more difficult to reactivate than the epinephrine receptor. A different situation probably exists in myocardial cells, since Levey[24,34] observed that phosphatidylserine specifically reactivates glucagon sensitivity and phosphatidylinositol norepinephrine sensitivity of hormone-unresponsive adenylate cyclase that has been detergent-solubilized.

In our opinion, the specificity exhibited by phosphatidylserine in restoring hormone and F^- sensitivity rules out the possibility that the reactivation of adenylate cyclase is due simply to a detergent-like action of the phospholipids, which would remove traces of fatty acids or diglycerides formed by phospholipases and not bound by bovine serum albumin. In their study on the reactivation of glucagon-sensitive adenylate cyclase of phospholipase A-treated membranes, Pohl *et al.*[6] found that, although phosphatidylserine is the most active phospholipid, other phospholipids can partially restore the enzyme's activity. This partial discrepancy with our data may be due to the different source, and hence specificity, of phospholipases and to the amount used or to the different method used to prepare the membranes. The fatty acid composition of phospholipids seems to be of limited importance, since purified brain phospholipids were only slightly less effective than purified rat liver phospholipids.

In preliminary experiments the effect of phospholipids on the binding of [^{3}H]-

epinephrine to mild-extracted or phospholipase-treated liver plasma membranes has been studied using a recently described method[35]. The data showed that removal of phospholipid reduced the binding of hormone, but this was restored almost completely by phosphatidylserine, while phosphatidylinositol and phosphatidylethanolamine were ineffective. These data, which parellel the results obtained assaying the adenylate cyclase activity, suggest that phosphatidylserine may have an important role in the binding of hormone to liver plasma membrane. In this connection, it may be of interest to recall that the NMR studies of Hammes and Tallman[36] are in accordance with a strong interaction between epinephrine and phosphatidylserine, but not phosphatidylethanolamine and phosphatidylcholine.

Robinson *et al.*[37] have depicted the adenylate cyclase molecule as being composed of at least two distinct subunits: a regulatory subunit and a catalytic subunit The hormones acting on this enzyme are thought to interact with the regulatory subunit. If we accept this view we can tentatively interpret our data to indicate that phosphatidylinositol is essential for the function of the catalytic subunit whereas phosphatidylserine, at least in the case of epinephrine, is essential for that of the regulatory subunit.

ACKNOWLEDGMENT

This work was aided by a grant from the Consiglio Nazionale delle Ricerche to O.B. (Contract 70.0091904).

REFERENCES

1 A Rethy, V Tomasi and A Trevisani, *Arch Biochem Biophys*, 147 (1971) 36
2 G V Marinetti, T K Ray and V Tomasi, *Biochem. Biophys Res Commun*, 36 (1969) 185.
3 T. K Ray, V Tomasi and G V Marinetti, *Biochim Biophys Acta*, 211 (1970) 20
4 S L Pohl, L Birnbaumer and M Rodbell, *Science*, 164 (1969) 566
5 V Tomasi, A Rethy, A Trevisani and O Barnabei, *Int Conf Biol Membranes, Gargnano, June 1971*, North-Holland, Amsterdam, in the press
6 S L Pohl, H. M J Krans, V Kozyreff, L Birnbaumer and M Rodbell, *J Biol Chem*, 246 (1971) 4447
7 T K Ray, *Biochim Biophys Acta*, 196 (1970) 1
8 O H Lowry, N J Rosebrough, A L Farr and R J Randall, *J Biol Chem*, 193 (1951) 265
9 V. P Skipski, R. F Peterson and M Barclay, *Biochem J*, 90 (1964) 374
10 G R Bartlett, *J Biol Chem*, 234 (1959) 466
11 P Emmelot and C J Bos, *Biochim. Biophys Acta*, 150 (1968) 341
12 V. P. Skipski, J. J Good, M Barclay and R B Reggio, *Biochim Biophys Acta*, 152 (1968) 10.
13 M. Rodbell, *Acta Endocrinol*, Suppl 153 (1971) 337
14 P Emmelot and C J Bos, *Biochim Biophys Acta*, 249 (1971) 285
15 P Emmelot and C. J Bos, *Biochim Biophys Acta*, 120 (1966) 369
16 R. L Lester and A L Smith, *Biochim Biophys Acta*, 47 (1961) 475
17 L Birnbaumer, S L Pohl and M Rodbell, *J Biol Chem*, 244 (1969) 3468
18 G. I Drummond, D L Severson and L. Duncan, *J Biol Chem*, 246 (1971) 4166.
19 S Fleischer and B. Fleischer, *Methods Enzymol*, 10 (1967) 406
20 S Ebashi, *Arch Biochem Biophys*, 76 (1958) 410
21 J. B Finean and A Martonosi, *Biochim Biophys Acta*, 98 (1965) 547
22 L Birnbaumer, S L Pohl and M Rodbell, *J Biol Chem*, 246 (1971) 1857
23 E W Sutherland, T W Rall and T Menon, *J Biol Chem*, 237 (1962) 1220
24 G S Levey, *Biochem Biophys Res. Commun*, 43 (1971) 108
25 A K Morton, *Methods Enzymol*, 1 (1955) 25
26 A H Maddy, *Biochim Biophys Acta*, 117 (1966) 193
27 R F A Zwaal and L L M van Deenen, *Biochim Biophys Acta*, 150 (1968) 323.
28 R F A. Zwaal and L L M van Deenen, *Biochim Biophys Acta*, 163 (1968) 44

29 V Tomasi, S Koretz, T. K Ray, J Dunnick and G V Marinetti, *Biochim. Biophys Acta*, 211 (1970) 31
30 R Coleman, J B Finean, S Knutton and A R. Limbrick, *Biochim Biophys Acta*, 219 (1970) 81
31 A Martonosi, J Donley and R A Halpin, *J. Biol Chem*, 243 (1968) 61
32 K Taniguchi and Y. Tonomura, *J Biochem Tokyo*, 69 (1971) 543.
33 P Cerletti, M A Giovenco, M G Giordano, S Giovenco and R Strom, *Biochim Biophys. Acta*, 146 (1967) 380
34 G S Levey, *J Biol Chem*, 246 (1971) 7405.
35 P Freychet, J Roth and D. M Neville, Jr, *Proc. Natl Acad Sci U S*, 68 (1971) 1883.
36 G. G Hammes and D E Tallman, *Biochim Biophys Acta*, 233 (1971) 17
37 G A Robinson, R. W. Butcher and E W Sutherland, *Ann. N Y. Acad. Sci*, 139 (1967) 703.