Biochimica et Biophysica Acta, 551 (1979) 368–381

BBA 78285

EFFECTS OF PHOSPHOLIPASE A_2 AND FILIPIN ON THE ACTIVATION OF ADENYLATE CYCLASE

PRAMOD M. LAD, M. SUE PRESTON, ANN F. WELTON, THOR B. NIELSEN and MARTIN RODBELL

Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, National Institutes of Health, Bethesda, MD 20014 (U.S.A.)

(Received June 30th, 1978)

Key words: Phospholipase A_2; Filipin; Adenylate cyclase; Hormone activation; GTP regulation

Summary

Rat liver plasma membranes were incubated with phospholipase A_2 (purified from snake venom) or with filipin, a polyene antibiotic, followed by analysis of the binding of glucagon to receptors, effects of GTP on the glucagon-receptor complex, and the activity and responses of adenylate cyclase to glucagon + GTP, GTP, Gpp(NH)p, and F^-. Phospholipase A_2 treatment resulted in concomitant losses of glucagon binding and of activation of cyclase by glucagon + GTP. Greater than 85% of maximal hydrolysis of membrane phospholipids was required before significant effects of phospholipase A_2 on receptor binding and activity response to glucagon were observed. The stimulatory effects of Gpp(NH)p or F^- remained essentially unaffected even at maximal hydrolysis of phospholipids, whereas the stimulatory effect of GTP was reduced. Detailed analysis of receptor binding indicates that phospholipase A_2 treatment affected the affinity but not the number of glucagon receptors. The receptors remain sensitive to the effects of GTP on hormone binding.

Filipin also caused marked reduction in activation by glucagon + GTP. However, in contrast to phospholipase A_2 treatment, the binding of glucagon to receptors was unaffected. The effect of GTP on the binding process was also not affected. The most sensitive parameter of activity altered by filipin was stimulation by GTP or Gpp(NH)p; basal and fluoride-stimulated activities were least affected. It is concluded from these findings that phospholipase A_2 and filipin, as was previously shown with phospholipase C, are valuable tools for differentially affecting the components involved in hormone, guanyl nucleotide, and fluoride action on hepatic adenylate cyclase.

Introduction

In recent years a number of efforts have been made to resolve the various components responsible for regulation of adenylate cyclase systems in membranes. Several approaches have been used. These include resolution of components following solubilization with detergents [1—4]; transfer of components between membranes by cell fusion [5,6]; the use of genetic variants as a source of cyclase systems having defined functional deletions [7—9]; and genetic analysis of somatic cell hybrids [10]. Emerging from such efforts is the realization that hormone-sensitive adenylate cyclase systems probably contain several distinct components rather than only receptor and catalytic components.

From our earlier work, it has been proposed [11,12] that the interdependent actions of glucagon and GTP [13] on the glucagon-sensitive cyclase system can be explained by the participation of at least four components: the glucagon receptor, a guanyl nucleotide regulatory process (designated N_1) that governs the structure of the receptor required for activation by hormone, another guanyl nucleotide regulatory process (designated N_2) that regulates enzyme activity independently of N_1 and receptor, and the catalytic unit. It was suggested that N_1 and N_2 may be crucial 'coupling' processes that link receptor and enzyme in the membrane.

An observation which helped in distinguishing between various regulatory processes of the adenylate cyclase system was the finding that phospholipase C (from *Bacillus cereus*) abolished glucagon activation of hepatic adenylate cyclase without altering the stimulatory effects of either Gpp(NH)p (through N_2) or F^- [14]. In that same study it was shown that phospholipase C treatment abolished the effects of GTP (through N_1) on the hormone-receptor complex. These studies gave an early indication that N_1 and N_2 may be distinct regulatory processes of the cyclase system.

Other lipid-perturbing agents such as phospholipase A_2 and filipin, a polyene antibiotic that reacts with cholesterol-rich regions in membranes [15], have been used to explore the role of lipids in the process of hormonal activation of adenylate cyclase [16—21]. In this study we report that a purified preparation of phospholipase A_2 causes concomitant alteration of glucagon receptors and glucagon action on hepatic adenylate cyclase without either loss of GTP action through N_1 on these hormone receptors or losses in the activation of the enzyme by Gpp(NH)p (at N_2) of F^-. Thus, the major effect of this agent is on the receptor unit. By contrast, it is shown that filipin does not alter the glucagon receptor or N_1 but causes, as its major effect, loss or modification of the N_2 process.

Materials and Methods

Materials. [α-^{32}P]ATP and Gpp(NH)p were obtained from International Chemical and Nuclear Corp.; Tris base and dithiothreitol were purchased from Schwarz-Mann. ATP and GTP were obtained from Sigma Chemicals. Oxoid filters were obtained from Med-Ox Chemicals (Ontario). Purified phospholipase A_2 from snake venom (*Naja naja*) [27] was a gift from Professor D.J. Hanahan (University of Texas, San Antonio). The enzyme used in these studies has been

purified to homogeneity. It shows a single band on SDS gels and is free from protease activity. The purification of the enzyme as well as its kinetics have been published [27]. Filipin was generously supplied by Dr. Jan Wolff. Defatted serum albumin was purchased from Miles Laboratory. Phospholipids used as standards were obtained from Applied Sciences and from Supelco.

Isolation of hepatic plasma membranes. Partially purified plasma membranes were prepared from rat liver by a modification [22] of the method of Neville [23]. Membranes were stored in liquid nitrogen.

Adenylate cyclase assay. Adenylate cyclase was assayed according to the method of Salomon et al. [24]. The assay mixture contained 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM creatine phosphate, 3.3 units of creatine phosphokinase, 1 mM dithiothreitol, 100 μM [α-^{32}P]ATP (100—500 cpm/pmol), and 20 μM cyclic AMP. The reaction volume was 100 μl. Assays were performed for 10 min at 30°C with 30—50 μg of protein. The reaction velocity was linear with time under all conditions used in this study. The activating ligands GTP and glucagon were present in the assays at concentrations well above the saturating concentrations. It is therefore highly unlikely that the abolition of kinetic effects reported here are due to the degration of these ligands, or competition from products of such a degradation.

Glucagon-binding assay. In most cases, ^{125}I-labelled glucagon binding was performed at 30°C for 5 min in a 250 μl reaction mixture containing the components described in the table and figure legends. The pH optimum for these binding studies was 7.0 in contrast to the pH for adenylate cyclase assays. As described by Lin et al. [25,26], the selection of pH 7.0, minimizes the difference in binding properties between native and iodinated species which is particularly noted at pH 7.5—8.0. However, we found that higher pH did not affect the results obtained although the net binding was lower and the effect of GTP thereby more difficult to quantitate. Binding reactions were stopped by the addition of 1 ml of cold 20 mM Tris-HCl, pH 7.0, containing 0.2% serum albumin, followed by immediate filtration under vacuum on oxoid filters (0.45 μm) presoaked for 30 min in 10% serum albumin [25]. The filters were washed twice with 1 ml of the same buffer and counted in a gamma counter. Background ('nonspecific' binding) was determined by carrying out the binding assay in the presence of $1 \cdot 10^{-6}$ M glucagon. In general, this value was nearly identical to the binding to the filter alone.

Treatment of membranes with phospholipase A_2. Prior to treatment, liver membranes were washed routinely with 20 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol (Buffer A). The washed membranes were suspended to about 10 mg/ml in 20 mM Tris-HCl, pH 8.0, containing 1 mM dithiothreitol and 10 mM $CaCl_2$ (Buffer B). Aliquots of 100 μl were distributed in plastic tubes to which the desired concentrations of phospholipase A_2 (stock, 36 units/ml) in Buffer B were added to give a final volume of 240 μl. Incubations were carried out at 24°C with frequent mixing, and were terminated after 10 min by the addition of 240 μl of medium containing 10 mM EGTA, pH 7.5. The sample was diluted 2—5-fold with Buffer A to give a membrane concentration of 0.2—1.0 mg protein/ml. The samples were assayed for adenylate cyclase, ^{125}I-labelled glucagon binding, or, as described below, for phospholipid content. On occasion, membranes were pelleted by centrifugation at 50 000 $\times g$ for 20 min

and resuspended in Buffer A prior to analysis. As a means of removing the products of phospholipase A digestion (fatty acids and lysophospholipids), in all experiments defatted serum albumin was included in Buffers A and B during treatment and in subsequent washing of the membranes. This ensures removal of the bulk phospholipase A_2 as well as the products of the reaction fatty acids and lysophospholipids from adenylate cyclase assay or binding assays. Since calcium is required for the activity of phospholipase A_2 [27], controls were carried out in the presence of EGTA. No hydrolysis of phospholipids or effects of the phospholipase were observed under these conditions, indicating that the effects of the enzyme were due to its catalytic activity. Other controls included incubation of membranes without phospholipase but otherwise under identical incubation conditions. Under all control conditions, hormone binding and cyclase activities remained the same as untreated membranes.

Treatment with filipin. Filipin was dissolved routinely in methanol. Suitable aliquots were distributed into glass tubes and evaporated under nitrogen to give a thin film along the sides of the tubes. Membrane suspensions (1 mg/ml) in Buffer A were added to the tubes which were mixed continually for 10 min at 20°C. Aliquots were then assayed for adenylate cyclase activity and glucagon binding as described above. When it was desired to remove unbound filipin, the membrane suspensions were diluted 10-fold in Buffer A, centrifuged for 20 min at 20 000 × *g* in the cold, and resuspended in this buffer.

Analysis of phospholipids. The types and quantity of membrane phospholipids hydrolyzed by phospholipase A_2 were analyzed as follows. Control and phospholipase A-treated membranes were extracted by the procedure of Bligh and Dyer [28] as modified by Dittmer and Wells [29]. The lipid residues were dissolved in chloroform and chromatographed on Q_1 type silica gel plates (heated for 15 min at 100°C prior to use) from Quanta Gram (341 Kaplan Drive, Fairfield, NJ). The plates were developed first with acetone/petroleum ether (60 : 180) which removes all diglycerides, cholesterol and fatty acids, leaving phospholipids at the origin, and then in chloroform/methanol/acetic acid/water (250 : 150 : 40 : 10). After staining with iodine, the regions corresponding to standard phospholipids were scraped, weighed, and analyzed for phosphate by the procedure of Bartlett [30] as modified by Dittmer and Wells [29].

Results

Effects of phospholipase A_2

The effects of increasing doses of phospholipase A_2 on activation of adenylate cyclase by several ligands are illustrated in Fig. 1. Activation by glucagon + GTP or by GTP alone was decreased substantially. The concentration of phospholipase A required for 50% of maximal inhibition ($K_{0.5}$) of glucagon + GTP action was 0.8—1.1 units/mg membrane protein. Basal activity was unaffected in the range of phospholipase A_2 concentrations tested. Activities stimulated by Gpp(NH)p and fluoride were increased (20—50%) with 0.1—0.5 units of phospholipase/mg membrane protein and then declined. Increases in these activities could be due to an increased access of the activators or of substrate to the relevant sites in vesicles present in the plasma membrane prepa-

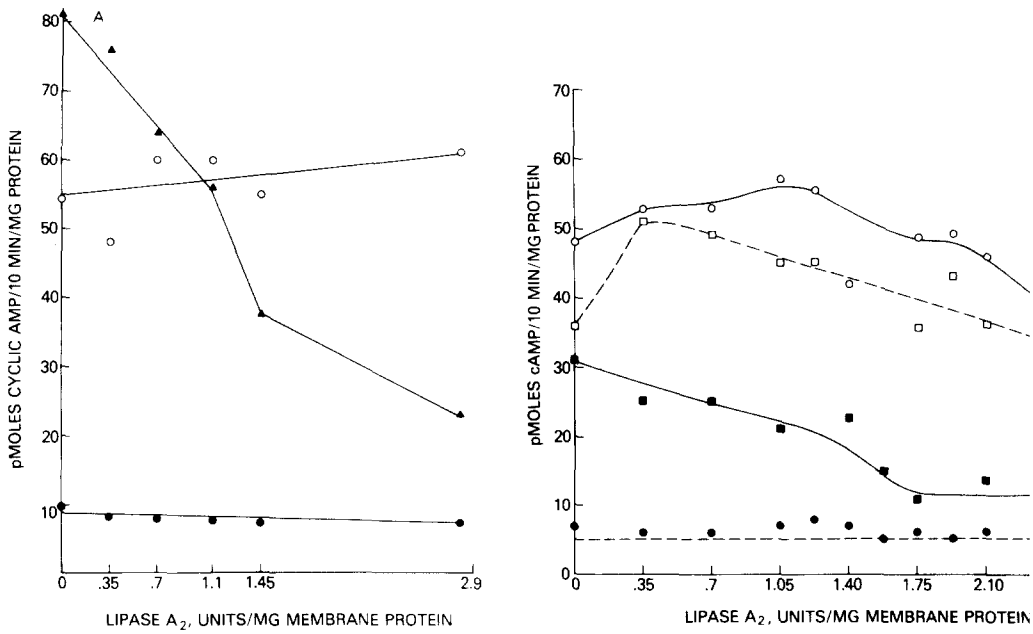

Fig. 1. The effects of phospholipase A_2 treatment on adenylate cyclase activities. Adenylate cyclase activities were measured in the presence of (A) 10 μM Gpp(NH)p (○), 1 μM glucagon + 10 μM GTP (▲), or without any additions (●); and (B) in a separate experiment in the presence of 10 μM GTP (■), 10 μM Gpp(NH)p (○), 10 mM fluoride (□) or without any additions (●). The errors in measurement were ±3—5% for activities measured with activators (Gpp(NH)p, fluoride, GTP, hormone + GTP) and ±8—10% for basal activity.

rations [31]. However, at the highest phospholipase concentration tested, stimulation by Gpp(NH)p or fluoride was only slightly below the original value whereas at these same concentrations stimulation by glucagon + GTP or GTP alone was nearly abolished. The abolition of the GTP effect under conditions were Gpp(NH)p activation is unaltered could be due to an activation of a GTPase. However, we have shown earlier that the liver plasma membrane contains a number of GTP hydrolyzing [1] activities, none of which seem related to adenylate cyclase. It was therefore not possible to establish whether a specific GTPase is indeed activated by phospholipase A_2 treatment.

In order to test whether the loss of hormone activation is due to a loss of hormone binding to receptors, both binding and cyclase activities were measured along a detailed titration curve with the phospholipase (Fig. 2).

In eight experiments of this sort the binding of ^{125}I-labelled glucagon was lost synchronously with hormone activation. Both hormone binding and hormone-stimulated adenylate cyclase activities were measured on samples which were washed with or without defatted bovine serum albumin. Essentially the same result was obtained in all cases. Also the binding was determined at two concentrations of glucagon ($1 \cdot 10^{-10}$ and $5 \cdot 10^{-10}$ M) and again the same synchronous loss of binding and activation was observed.

Two additional features of glucagon binding were studied. The first concerns the GTP regulation of the hormone receptor [11,12]. In order to test whether phospholipase A_2 treatment altered this regulation the binding of hormone to

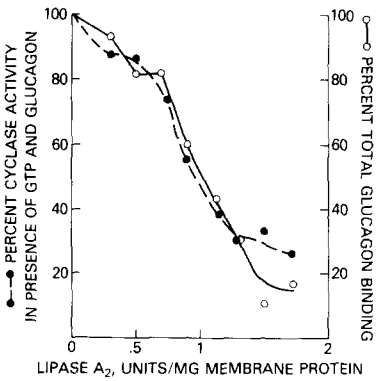

Fig. 2. The effects of phospholipase A_2 on activation of adenylate cyclase by glucagon + GTP and on the binding of ^{125}I-labelled glucagon. Details for cyclase activity by measurements were the same as in the legend to Fig. 1. The concentration of ^{125}I-labelled glucagon was $5 \cdot 10^{-10}$ M, (150 000 cpm/pmol). The error in measurement of ^{125}I-labelled glucagon binding was 4—5% at this glucagon concentration.

the receptor was measured in the presence as well as the absence of GTP. In four experiments of this sort it was found that while the binding of glucagon at concentrations below the $K_{0.5}$ were substantially reduced (70 ± 15%), the GTP effect on hormone binding was unaltered. A typical experiment is shown in Table I. This in contrast to phospholipase C treatment which also weakens the binding affintiy of the receptors but abolishes the GTP effect on binding [14].

In order to test whether the binding of glucagon was affected only at concentrations around the dissociation constant ($1 \cdot 10^{-9}$ M) or saturation ($1 \cdot 10^{-8}$ M) the binding of glucagon was carried out at various concentrations on control as well as phospholipase A_2-treated membranes (Table II). The results show again that the binding of glucagon at concentrations below $1 \cdot 10^{-9}$ M is substantially lowered. However at saturating concentrations the binding to control and phospholipase A_2-treated membranes is similar. The predominant effect of phospholipase A_2 treatment is on the affinity of the hormone receptor but not on receptor number. It should be noted that there are two limiting aspects of methodology which have precluded a finer computation of the effect

TABLE I

EFFECT OF PHOSPHOLIPASE A_2 TREATMENT ON THE REGULATION OF GLUCAGON BINDING BY GTP

The total concentrations of glucagon were $1 \cdot 10^{-10}$ M (1) and $5 \cdot 10^{-10}$ M (2). The specific activity was 400 000 cpm/pmol. Other details were as described in Table II and in Materials and Methods. The error in measurement is ±5—7%. The GTP concentration was $1 \cdot 10^{-5}$ M.

Lipase concentration (units/mg membrane protein)	Glucagon bound (cpm × 10^{-3}/mg membrane protein)			
	1		2	
	no GTP	+GTP	no GTP	+GTP
None	40	25	196	108
1.0	34	18	127	74
2.0	19	18	96	61

TABLE II

EFFECT OF PHOSPHOLIPASE A_2 ON BINDING OF GLUCAGON TO RECEPTOR

Glucagon concentration is the molar concentration of glucagon free in the solution. Bound denotes the amount of ^{125}I-labelled glucagon not displacable by the addition of $1 \cdot 10^{-5}$ M unlabelled glucagon in mol glucagon bound/g protein. Liver membranes were incubated for 10 min at 23°C with 5 units phospholipase A_2/mg protein, then washed twice by resuspending in 4% defatted bovine serum albumin and centrifuging. Control membranes were also washed with bovine serum albumin. σ values are averages of quadruplicate measurements. Standard deviations are based on the error in the total bound plus the error in the glucagon bound at $1 \cdot 10^{-5}$ M glucagon.

	Glucagon concn.	Bound	σ
Control membranes	$9.5 \cdot 10^{-10}$	$8.0 \cdot 10^{-11}$	$\pm 0.4 \cdot 10^{-11}$
	$3.8 \cdot 10^{-9}$	$2.2 \cdot 10^{-10}$	$\pm 0.2 \cdot 10^{-10}$
	$9.8 \cdot 10^{-9}$	$3.0 \cdot 10^{-10}$	$\pm 0.2 \cdot 10^{-11}$
	$3.9 \cdot 10^{-8}$	$7.6 \cdot 10^{-10}$	$\pm 0.5 \cdot 10^{-10}$
Lipase-treated membranes	$9.8 \cdot 10^{-10}$	$4.6 \cdot 10^{-11}$	$\pm 0.4 \cdot 10^{-11}$
	$3.9 \cdot 10^{-9}$	$1.3 \cdot 10^{-10}$	$\pm 0.1 \cdot 10^{-10}$
	$1.0 \cdot 10^{-8}$	$2.1 \cdot 10^{-10}$	$\pm 0.2 \cdot 10^{-10}$
	$4.0 \cdot 10^{-8}$	$9.1 \cdot 10^{-10}$	$\pm 1.6 \cdot 10^{-10}$

on hormone binding. Firstly, variability is seen in the final level of binding obtained at high lipase concentrations (2—5 units/mg membrane protein). In the experiment shown in Table I for example, a 50% change was obtained at glucagon concentration $1 \cdot 10^{-9}$ M whereas a larger change, 80%, was obtained in the results shown in Fig. 1. In five experiments the average change observed at these concentrations was 70% (±15%). The cause of this variability is not known to us. The second experimental limitation concerns the accuracy of the estimation of hormone receptor number. As can be seen from the results in Table I, at a high concentration of ^{125}I-labelled glucagon ($4.0 \cdot 10^{-8}$ M) the accuracy in measurement is poor (±20%). This is due to very high filter background at high hormone concentrations. However given these limitations it can be stated that the major effect of the phospholipase A_2 treatment is on the affinity of the glucagon receptor. If there are changes in the GTP regulation of the receptor or receptor number then these are by comparison, much smaller.

Phospholipase A_2 treatment was carried out also on plasma membranes pretreated with glucagon. Under conditions where glucagon remains bound to the receptor [20,21] occupancy of the receptor by hormone did not alter the inactivation curves shown in Fig. 1A, indicating that the binary hormone-receptor complex is as affected by phospholipase A_2 as is the unoccupied receptor. This point was tested directly by prebinding ^{125}I-labelled glucagon to the receptor, treating the membranes with phospholipase A_2, followed by centrifugation. The treated membranes showed an increased number of counts in the supernatant. Chromatography of the supernatant on Agarose (1.5 M) showed that all counts eluted with free ^{125}I-labelled glucagon (data not shown).

Since the products of phospholipase A_2 digestion (lysolipids and fatty acids) are detergents it is possible that, rather than depletion of lipids being the causal factor, accumulation of products may be responsible for receptor loss. Serum albumin binds both products [32] and was included (at 4%) during incubation with phospholipase A_2 in some experiments. No change in effects of phospho-

TABLE III

EFFECT OF REPEATED WASHED WITH DEFATTED BOVINE SERUM ALBUMIN ON CONTROL AND LIPASE A_2 TREATED MEMBRANES

Liver plasma membranes were treated with phospholipase A_2 as described in Materials and Methods. The control as well as treated membranes were suspended in Buffer A with 3% defatted bovine serum albumin. The samples were centrifuged at 20 000 × *g* for 20 min and the pellets resuspended in the same buffer. The 'number of washed' refers to the number times this operation was carried out; the activities are expressed as pmol/10 min per mg.

Sample	Number of washes in 3% defatted bovine serum albumin			
	1	2	3	4
I. Control — bovine serum albumin				
Basal	9	11	16	26
+ 10^{-5} M Gpp(NH)p	68	76	66	72
+ 10^{-5} M GTP + 10^{-7} M glucagon	118	123	88	83
II. Control bovine serum albumin				
Basal	8	14	16	12
+ 10^{-5} M Gpp(NH)p	67	73	64	67
+ 10^{-5} M GTP + 10^{-7} M glucagon	112	120	95	94
III. Lipase treated + bovine serum albumin				
Basal	11	18	14	12
+ 10^{-5} M Gpp(NH)p	75	76	46	52
+ 10^{-5} M GTP + 10^{-7} M glucagon	30	34	27	27

lipase A_2 was observed; nor did extensive washings of the treated membranes with serum albumin reverse the loss of hormone action (Table III). Finally, it was observed that albumin did not modify the binding curve of ^{125}I-labelled glucagon or the change in receptor affinity due to phospholipase A_2 treatment (Table I, Fig. 2).

The amount of lysophospholipid generated and the resulting phospholipid composition of the membranes treated with phospholipase A are shown in Table IV. As judged by the quantity of lysophospholipids formed, a maximum of about 50% of the membrane phospholipids were hydrolyzed over the range of phospholipase A_2 used in these studies. At concentrations of the enzyme which produces less than 20% loss of hormone binding (or action), near maximal formation (85%) of lysophospholipids had occurred. The loss of hormone binding and action (Table IV, Fig. 2) occurred in a range of phospholipase A_2 concentrations in which there was only a small further increase in hydrolysis of membrane lipids. These findings suggest that the effects of phospholipase A_2 on receptors are not due simply to accumulation of endogenous products of phospholipid digestion. Indeed, the relatively selective effects seen with phospholipase A_2 on the receptor were not seen when exogenous lysophospholipid was added. As shown in Fig. 3, all activities (hormone + GTP, Gpp(NH)p, fluoride, basal) decreased in parallel with added lysolecithin. The concentration of lysolecithin required in two experiments to give 50% inhibition of these activities was 100 ng/mg (±8 ng/mg) membrane protein wheras only 55 ng/mg of lysophospholipid generated by the action of phospholipase A_2 was sufficient to give complete loss of the actions of glucagon + GTP without change in responses to Gpp(NH)p or F^-.

TABLE IV

COMPARISON OF THE EFFECTS OF LIPASE A_2 ON PHOSPHOLIPID HYDROLYSIS AND GLUCAGON-STIMULATED ACTIVITY

Membranes were treated with phospholipase A_2 and analyzed for glucagon-stimulated adenylate cyclase activity and phospholipid composition as described in Materials and Methods. Abbreviations and relative mobilities (R_F) are as follows: PE, phosphatidylethanolamine (0.95); PS, phosphatidylserine (0.82); PI, phosphatidylinositol (0.76); SPM, sphingomyelin (0.50); LYSO, lysophospholipid (0.36). The data is representative of three experiments. The accuracy in determining the amount of phospholipid was ±10% in most samples. Only in samples where the amount of phospholipid is low (PS + PI) was the error substantial (±20%).

Lipase units/mg membrane protein	% Loss of stimulated activity	nmol lipid/mg membrane protein				
		PE	PS + PI	PC	SPM	LYSO
0	0	37	14	65	16	1
0.2	0	26	14	46	16	40
0.4	30	12	7	48	16	43
1.0	70	11	12	50	16	49
3.0	80	10	11	38	16	51

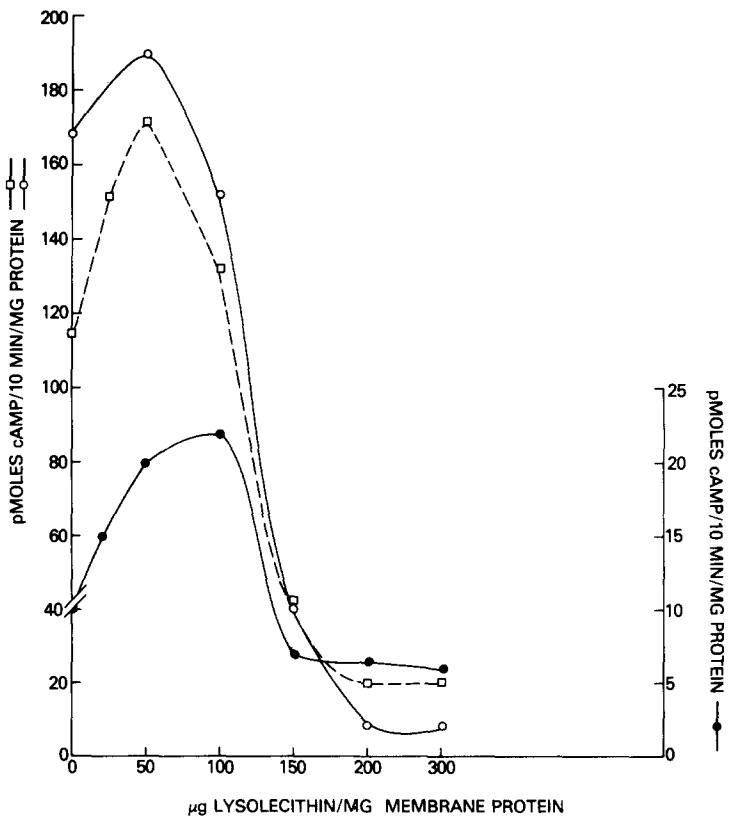

Fig. 3. The effect of lysolecithin of adenylate cyclase activities. The cyclase activities were measured in the presence of Gpp(NH)p (□), glucagon + GTP (○) or without any additions (●). The concentrations of these ligands were the same as in the legend to Fig. 1. Incubations were carried out under conditions identical to those for Fig. 1.

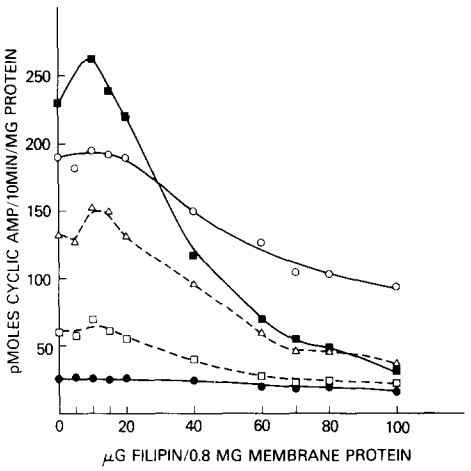

Fig. 4. The effect of filipin on various adenylate cyclase activities. Activity measurements were carried out as described in Materials and Methods, in the presence of GTP (□), Gpp(NH)p (△), glucagon + GTP (■) fluoride (○) and with no additions (●). Treatment with filipin was carried out as described in Materials and Methods, and the concentrations of activity of ligands are the same as in the legend to Fig. 1.

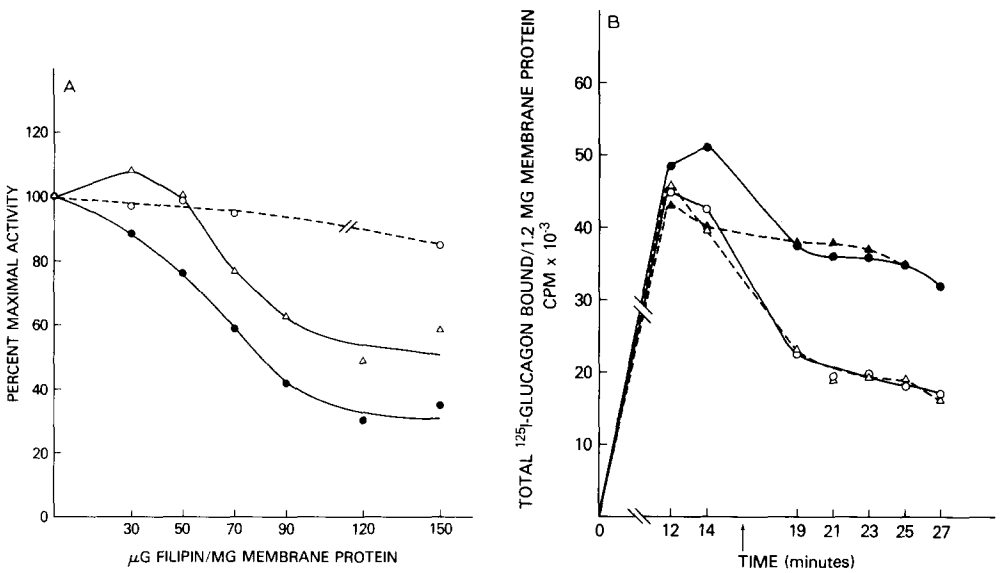

Fig. 5. (A) The effect of filipin on glucagon binding and on adenylate cyclase activities. Filipin treatment and activity measurements were carried out as described in Materials and Methods. ○, ^{125}I-labelled glucagon binding, displaceable by cold glucagon ($1 \cdot 10^{-6}$ M), at a hormone concentration of $5 \cdot 10^{-10}$ M. Adenylate cyclase activities were measured in the presence of 10 μM Gpp(NH)p (△), 1 μM glucagon + GTP (●). (B) Control (○; ●) and filipin-treated membranes (△; ▲) (100 mg filipin/mg membrane protein) were incubated in 10-ml aliquots. At 10 min and 12 min 1 ml aliquots were withdrawn and the amount of ^{125}I-labelled glucagon bound was determined by filtration as described under Materials and Methods. After 14 min, 1 μM glucagon (●; ▲) or 1 μM glucagon + 10 μM GTP (○; △) were added (arrow). The displacement of ^{125}I-labelled glucagon from plasma membranes was assessed by filtering 1 ml aliquots at 19, 21, 23, 25, and 27 min. The difference between the top curves (●; ▲) and the bottom curves (○; △) at 27 min denotes the amount of hormone displaced by GTP.

Effects of filipin

Filipin caused marked reduction in the ability of glucagon + GTP to stimulate adenylate cyclase activity (Fig. 4). However, the effects of the polyene were less selective in distinguishing between the various activity parameters than were seen with phospholipase A_2 or with phospholipase C [10,13]. The ratio of filipin concentrations required to produce 50% inhibition of cyclase activities were 1.0 glucagon + GTP; 1.5 Gpp(NH)p; 3.0 fluoride. The amount of filipin (per mg membrane protein) required in six experiments for 50% inhibition of these activities varied from 30 to 50 μg for glucagon + GTP to greater than 100—150 μg for fluoride-stimulated activity.

In three experiments of the sort shown in Fig. 5, filipin did not alter the binding of ^{125}I-labelled glucagon to its receptor over the entire range of filipin concentrations tested (Fig. 5A) at either low ($1 \cdot 10^{-10}$ M) or high ($1 \cdot 10^{-9}$ M) concentrations of labelled hormone. Furthermore, whereas the stimulatory effects of guanyl nucleotides on adenylate cyclase activity were reduced markedly, the effect of GTP on the binding of glucagon to the receptors remained unaltered (Fig. 5B). From this, we conclude that filipin selectively modifies the N_2 regulatory process, which may be the basis of the loss of glucagon + GTP action on adenylate cyclase activity.

Discussion

The purpose of this study was to examine the use of phospholipase A_2 and filipin as probes for adenylate cyclase structure and function in the plasma membrane. Three different types of information may be derived from the use of these probes: (1) information about the linkage of various components involved in adenylate cyclase activities; (2) inferences about the organization of the various components, and (3) the production of selectively altered membrane systems which may be used for further biochemistry with the adenylate cyclase system.

Phospholipase A_2 treatment causes a decrease in the affinity of the glucagon receptors without altering receptor number. It also abolishes activation of the catalytic unit by GTP. In the same range of phospholipase A_2 concentrations, activation by hormone is also abolished. The question which has to be addressed in this: how do the alteration of the receptor and/or the loss of GTP stimulation contribute to the synchronous loss of activation by hormone? A few alternatives may be considered. We have suggested earlier [11,12] that the low affinity form of the receptor induced by GTP may be involved in the coupling process. It is clear however that the low affinity forms of the receptor generated by phospholipase A_2 (or C) may be quite inadequate for the coupling process, i.e. a unique low affinity form generated by the action of GTP may be required for activation by hormone. We had also suggested that the nucleotide regulation of the receptor (the N_1 process) as well as the nucleotide activation of the catalytic unit (the N_2 process) may both be required for hormonal coupling. Thus, a loss of GTP activation of the catalytic unit could in itself cause a loss of activation by hormone. In summary the loss of hormone action after phospholipase A_2 treatment, may be brought about by multiple modifications of the cyclase system.

Two other intriguing features should be noted. Of the various modifications which have been attempted, none has provided a high affinity form ($K_0 = 1 \cdot 10^{-9}$ M) of the receptor which is no longer susceptible to regulation by GTP. This implies that the receptor is very closely linked to its nucleotide regulation. Also, no structural or functional assay exists for the N_1 component, independent of hormone binding to the receptor. Both aspects are important in defining the exact state of the receptor that is required for hormone action. Efforts to overcome these difficulties are in progress. Secondly, this study shows that GTP activation can be abolished under conditions where Gpp(NH)p activation remains intact. We have shown earlier that GTP is a competitive inhibitory of Gpp(NH)p activation. These nucleotides act at the same activation site [1]. However it is possible that GTP has requirements for activation different from that for Gpp(NH)p. A pertinent possibility, which further experiments will test, is whether both N_1 (the receptor dependent) as well as N_2 (the catalytic unit related) components are required for activation by GTP, while N_2 alone is sufficient for activation by Gpp(NH)p.

The basis of the selective effects of phospholipase A_2 on the receptor component is unknown. It was shown in earlier studies [16] that addition of phospholipids to phospholipase A_2-treated hepatic membranes resulted in partial restoration of hormone binding and action. Several possibilities were considered to explain restoration, including removal of the detergent products by the added lipid micelles. Aware of this possibility, we attempted to deplete the membranes of lysophospholipids and fatty acids by extensive washings with defatted serum albumin which avidly binds these products. Restoration of binding was not observed. However, the possibility has not been excluded that the products of digestion bind with considerably higher affinity to the receptor than to albumin. It was also suggested in the earlier studies that phospholipids are integral to the structure and function of the glucagon receptor [16]. The present study supports this possibility. However, it was surprising to find that substantial hydrolysis of membrane phospholipids occurred without significant loss of glucagon binding (or action); loss of receptor binding occurred over a narrow range of further phospholipid hydrolysis. Similar findings have been reported for the effects of phospholipase C on glucagon action [11,14]. Thus, if the glucagon receptor is a lipoprotein, lipids essential for functional response seem not to be as sensitive to hydrolysis as is the bulk (or unbound) phospholipid in the membrane. A possible explanation for this apparent insensitivity to phospholipase digestion is that the essential lipids adjacent to the receptor have restricted asccessibility to the phospholipases until the bulk lipids are extensively hydrolyzed. In any case, the use of phospholipases for selective alterations of the components of the cyclase system has decided advantages over lysophospholipids or other detergents which are relatively non-selective in their effects and cause large losses of enzyme activity over a narrow range of detergent concentrations [1].

The effect of filipin nicely extends our previous studies [1,11,12] in which we have shown, using a variety of methods, that the regulation of the receptor by nucleotide is distinct from the activation of adenylate cyclase by nucleotides. Filipin, which affects activation by nucleotides while leaving receptor and its nucleotide regulation unaltered, offers additional support for this view. The

two regulatory processes of enzyme activation and receptor regulation are distinct regardless of the type of nucleotide used (GTP or Gpp(NH)p). It is very suggestive indeed that phospholipases (A_2 and C) both impair receptor and its regulation by nucleotides while filipin selectively affects the nucleotide activation of the catalytic unit. Such a distinction could arise from the location of these complexes in structurally distinct domains. Inferential evidence for the existence of the receptor and catalytic unit in different domains has also been provided by other workers from very different types of experiments [5,33].

A last aspect of this work concerns the usefulness of the membrane preparations derived from these modifications. It is hoped that these membrane preparations can be used in the sort of complementation studies reported by Gilman and his colleagues [8,9]. Here, chemically modified components would be used instead of the genetic variants used by these workers in their studies. For example the phospholipase A_2-modified membranes could serve as acceptors for those components required to restore hormone activation or activation by GTP. Filipin-modified membranes could provide a bioassay for the activating, nucleotide component. Efforts along these lines are being pursued in this laboratory.

Acknowledgements

We wish to thank Professor D.J. Hanahan and Dr. Jan Wolff for providing us with some of the materials used in this study. We are grateful to Professor Michael Wells for helpful suggestions on phospholipid analysis. We would like to express our thanks to Dr. M.C. Lin and Dr. C. Londos for many helpful discussions. We would also like to acknowledge the fine technical assistance of Mr. James Oden for the preparation of liver plasma membranes.

References

1 Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S. and Rodbell, M. (1977) Biochim. Biophys. Acta 552, 625—639
2 Pfeuffer, T. (1977) J. Biol. Chem. 252, 7224—7234
3 Limbird, L.E. and Lefkowitz, R.J. (1977) J. Biol. Chem. 252, 799—802
4 Dufau, M.L., Baukal, A.J., Ryan, D. and Catt, K.J. (1977) Mol. Cell. Endocrinol. 6, 253—269
5 Orly, J. and Schramm, M. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4410—4414
6 Schwarzmeier, J.D. and Gilman, A.G. (1977) J. Cyclic Nucl. Res. 3, 227—238
7 Haga, T., Ross, E.M., Anderson, H.J. and Gilman, A.G. (1977) Proc. Natl. Acad. Sci. U.S. 74, 2016—2020
8 Ross, E.M. and Gilman, A.G. (1977) J. Biol. Chem. 252, 6966—6969
9 Ross, E.M. and Gilman, A.G. (1977) Proc. Natl. Acad. Sci. U.S. 74, 3715—3719
10 Brunton, L.L., Maguire, M.E., Anderson, H.J. and Gilman, A.G. (1977) J. Biol. Chem. 252, 1293—1302
11 Lad, P.M., Welton, A.F. and Rodbell, M. (1977) J. Biol. Chem. 252, 5942—5946
12 Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S. and Rodbell, M. (1977) J. Biol. Chem. 252, 5947—5950
13 Rodbell, M., Lin, M.C. and Salomon, Y. (1974) J. Biol. Chem. 249, 59—65
14 Rubalcava, B. and Rodbell, M. (1973) J. Biol. Chem. 248, 3831
15 Sessa, G. and Weissmann, G. (1968) J. Lipid Res. 9, 310—318
16 Pohl, S.L., Krans, H.M.J., Kozyreff, V., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 4447
17 Wolff, J. and Jones, A.B. (1971) J. Biol. Chem. 248, 3939—3947
18 Puchwein, G., Pfeuffer, T. and Helmreich, E.J.M. (1974) J. Biol. Chem. 249, 3232—3240
19 Rethy, A., Tomasi, V. and Trevisani, A. (1971) Arch. Biochem. Biophys. 147, 36—40

20 Rethy, A., Tomasi, V., Revisani, A. and Barnabei, O. (1972) Biochim. Biophys. Acta 290, 58—69
21 Limbird, L.E. and Lefkowitz, R.J. (1976) Mol. Pharmacol. 12, 559—567
22 Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849—1856
23 Neville, D.R. (1968) Biochim. Biophys. Acta 154, 540—552
24 Salomon, Y., Londos, C. and Rodbell, M. (1977) Anal. Biochem. 58, 541—548
25 Lin, M.C., Nicosia, S. and Rodbell, M. (1976) Biochemistry 15, 4537—4540
26 Lin, M.C., Nicosia, S., Lad, P.M. and Rodbell, M. (1977) J. Biol. Chem. 252, 2790—2792
27 Martin, J.K., Luthra, M.G., Wells, M.A., Watts, R.P. and Hanahan, D.J. (1975) Biochemistry 14, 5400—5408
28 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Phys. 37, 911—917
29 Dittmer, J.C. and Wells, M.A. (1969) Methods Enzymol. 14, 482—530
30 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
31 Wallach, D.F.H. (1967) in The Specificity of Cell Surfaces (Davis, B.D. and Warren, L., eds.), pp. 129—163, Prentice-Hall
32 Azhar, S., Hajra, M. and Menon, K.M.J. (1976) J. Biol. Chem. 251, 7405—7412
33 Sahyown, N., Hollenberg, M.D., Bennett, V. and Cuatrecasas, P. (1977) Proc. Natl. Acad. Sci. U.S. 74, 2860—2864