BBA 75451

HORMONE ACTION AT THE MEMBRANE LEVEL

II. THE BINDING OF EPINEPHRINE AND GLUCAGON TO THE RAT LIVER PLASMA MEMBRANE

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(Received December 15th, 1969)

SUMMARY

The binding of epinephrine and glucagon to isolated rat liver membranes shows that both hormones have a much higher affinity for the plasma membrane than for the other cell membranes (nuclear, mitochondrial, endoplasmic reticulum). The binding of glucagon to the plasma membrane is more rapid than the binding of epinephrine. Propranolol, a β-andrenergic blocking agent, inhibits the binding of epinephrine to the plasma membrane. A study of several epinephrine analogs shows that the two ring hydroxyl groups are important in the binding of epinephrine to the membrane.

p-Chloromercuribenzoate (PCMB) and 5',5'-dithio-bis-2-nitrobenzoic acid (DTNB) both inhibit epinephrine binding and glucagon binding thus implicating SH groups on the receptor proteins. Heating membranes for 1 min at 100° does not appreciably affect the hormone binding but nearly completely inactivates the adenyl cyclase. Heating for 5 min at 100° abolishes the hormone binding. These results show that the hormone receptor proteins are separate from the adenyl cyclase.

Extraction of membrane bound epinephrine with chloroform–methanol removes approx. 20–25 % of the bound hormone, a finding which indicates at least two types of membrane bound epinephrine.

The membrane bound glucagon and epinephrine were isolated by column chromatography on Sephadex G-200. The receptor proteins were eluted just ahead of the void volume and were separated from the major adenyl cyclase activity. A second peak containing adenyl cyclase activity was eluted from the column with the void volume.

INTRODUCTION

In a previous paper[1] the properties of membrane bound rat liver adenyl cyclase were presented. This enzyme is localized on the plasma membrane and is stimulated by glucagon and epinephrine in isolated membrane systems. In order to elucidate the mechanism of action of these hormones at the membrane level, studies were carried

Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5',5'-dithio-bis-2-nitrobenzoic acid.

out on the binding of glucagon and epinephrine to the different membranes of the rat liver cell and an attempt was made to correlate the hormone binding with adenyl cyclase activity. The results indicate that receptor proteins exist in the plasma membrane which bind these hormones and that this interaction precedes the activation of adenyl cyclase. However, no definitive mechanism can be made at present on the coupling of hormone binding with enzyme activation.

METHOD AND REAGENTS

The isolation of the plasma membranes and the assay for adenyl cyclase are described elsewhere[1,2]. DL-[7-^{3}H]Epinephrine and [^{3}H]ATP (uniformly labeled) were obtained from New England Nuclear Corp. [^{131}I]Glucagon was prepared by the method of BALE *et al.*[3] and purified on Sephadex G-10. L-Epinephrine and *p*-chloromercuribenzoate (PCMB) were purchased from Sigma Corp. Crystalline pork glucagon was a gift from Lilly Research Lab.

5',5'-Dithio-bis-2-nitrobenzoic acid (DTNB) was obtained from K and K Laboratories. Propranolol was purchased from Ayerst. The epinephrine analogs were obtained from Cal Biochem. These reagents were neutralized with 0.05 M Tris buffer (pH 7.5) before use.

The [^{3}H]epinephrine was diluted with 0.1 M acetic acid and aliquots of 10 μl were used for each incubation. Unlabeled L-epinephrine was dissolved just before use in a minimal volume of 0.1 M HCl and the pH was adjusted to 7.5 with 0.05 M Tris buffer. The glucagon was dissolved in 0.16 M borate buffer (pH 10.0) and diluted with Tris buffer before use.

Phospholipase A (*Naia naia*) was obtained from Ross Allen Reptile Farms. Phospholipase C was obtained from Sigma Chemical Co. Trypsin, 2 × crystalline, was obtained from Worthington Biochem. Corp.

Membranes (100–200 μg protein) were incubated with the labeled hormones with and without test agent (0.5–1.0 μC of [^{3}H]epinephrine or 10000 counts/min of [^{131}I]glucagon) in 0.5 ml of 50 mM Tris buffer (pH 7.5) for 10 min at 37°. The reaction was stopped by addition of 2.0 ml of 5 % trichloroacetic acid in the case of epinephrine and with 2.0 ml of 0.1 M acetic acid in the case of glucagon. The membranes were washed by centrifugation in the cold with 3-ml portions of 5 % trichloroacetic acid or 0.1 M acetic acid (depending on the hormone) until the supernatant contained only background counts (usually 4 washes were required). The membrane pellets were treated with 0.3 ml of Nuclear Chicago solubilizer reagent and transferred to counting vials by washing with exactly 15 ml of Bray's Scintillation cocktail. The epinephrine binding is expressed as pmoles/mg protein per h. The glucagon binding is expressed as pmoles/mg protein per 10 min.

These different times are used since the glucagon binding was rapid and complete within 5–10 min whereas the epinephrine binding was slower and continued over a period of 30 min to 1 h. The calculations for epinephrine were made on the assumption that only the L-isomer of the DL-epinephrine was bound to the membrane.

RESULTS

The high selectivity of binding of the labeled epinephrine to the plasma membrane of rat liver was reported earlier[1]. On a mg of protein basis, epinephrine binding

was 5-fold higher for the plasma membrane than the microsomal membrane and 10–20-fold higher than the nuclear and mitochondrial membrane.

The relation between epinephrine binding and plasma membrane concentration is given in Fig. 1. The total binding increases in a linear fashion with increasing membrane protein concentration, but the binding per mg protein per h is highest between 60–80 μg of protein and decreases exponentially after this level The fall off in the relative specific binding may be due to aggregation of membrane fragments at higher levels.

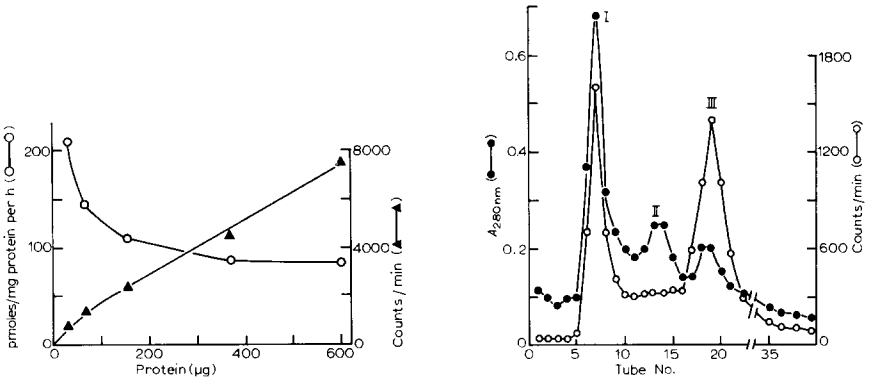


Fig. 1. The binding of epinephrine to the plasma membrane as a function of membrane concentration. Membranes in 50 mM Tris buffer (pH 7.5) were incubated with 0.62 μC of DL-[^{3}H]epinephrine for 10 min at 37°. The membranes were washed three times with cold 5% trichloroacetic acid and the amount of bound epinephrine was analyzed, as described in the text.

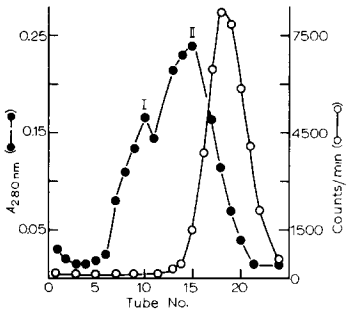
Fig. 2. The fractionation of [^{3}H]epinephrine treated membranes on Sephadex G-200. 2.0 mg of membrane protein in 50 mM Tris buffer (pH 7.5) were incubated with 6.2 μC of [^{3}H]epinephrine at 37° for 10 min. The membranes were centrifuged and washed twice with 3 ml of Tris buffer. The washed membranes were sonicated at 0° for 90 sec in 25 ml of Tris buffer containing 0.15% deoxycholate. Glycerol was added to the sonicated membranes to give a final glycerol concentration of 20%. 2 ml of this preparation were applied to a 35 cm × 1.7 cm column of Sephadex G-200 previously equilibrated with Tris–deoxycholate buffer. Fractions of 1.5 ml volume were collected. The absorbance and radioactivity of each fraction was analyzed.

Membranes were incubated with [^{3}H]epinephrine, solubilized with deoxycholate, sonicated for 90 sec and fractionated on Sephadex G-200. This procedure gave rise to 3 major peaks (see Fig. 2). Protein bound epinephrine was associated with the fast moving Peak I. Another peak for epinephrine was eluted with the slowest moving Peak III. This may represent either unbound epinephrine or epinephrine bound to different proteins. Free epinephrine in absence of protein was found to be eluted in the same fraction as the slower moving Peak III.

The specificity of binding of epinephrine to the plasma membrane was shown by control studies where [^{3}H]epinephrine was incubated with albumin, γ-globulin and hemoglobin under the same conditions as incubation of membranes and then subjected to deoxycholate treatment and column fractionation on Sephadex G-200. The elution profile given in Fig. 3 shows that the epinephrine was eluted after the protein peaks. Hence, very little or no epinephrine binds to albumin, hemoglobin and γ-globulin.

The effect of pH on the binding of epinephrine to the membranes is given in Fig. 4. An unusually high pH optimum was observed at pH 10.5. This may in part

be due to "solubilization" of the membrane at high pH with subsequent exposure of more binding sites. The physiological significance of this high pH optimum is not clear.



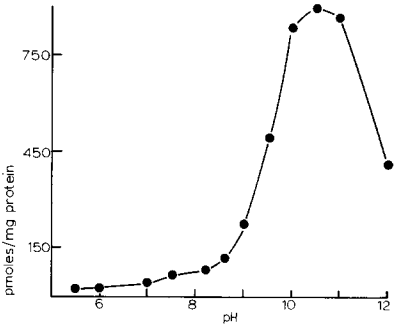


Fig. 3. Column fractionation of γ-globulin, hemoglobin and albumin incubated with [^{3}H]epinephrine. A mixture of γ-globulin and albumin (2.0 mg each in 1 ml of Tris buffer) was incubated with 6.2 μC of [^{3}H]epinephrine at 37° for 10 min. Deoxycholate and glycerol was added to give a concentration of 0.15% and 20%, respectively. The material was fractionated on Sephadex G-200 as explained in Fig. 2. Peak I, γ-globulin; Peak II, hemoglobin + albumin.

Fig. 4. Effect of pH on the binding of [^{3}H]epinephrine to the plasma membrane. The binding of epinephrine was carried out as explained in the text. For pH 5.5–8.2, 30 mM phosphate buffer was used. For pH 8.6–12, 25 mM glycine–NaOH buffer was used.

TABLE I

EFFECT OF ATP AND Mg^{2+} ON EPINEPHRINE BINDING TO THE PLASMA MEMBRANE

See text for experimental details.

System	*pmoles epinephrine per mg protein per h*
Control	43.2
ATP (1 mM)	34.1
ATP (2 mM)	36.2
Mg^{2+} (2 mM)	42.5
Mg^{2+} (8 mM)	31.7
ATP (1 mM) *plus* Mg^{2+} (2 mM)	39.5

TABLE II

EFFECT OF GLUCAGON AND PROPRANOLOL ON EPINEPHRINE BINDING TO THE PLASMA MEMBRANE

See text for experimental details.

System	*pmoles epinephrine per mg protein per h*
Control	70
Propranolol (100 μg)	51
Propranolol (200 μg)	42
Glucagon (10 μg)	77
Glucagon (30 μg)	96

The effects of ATP and Mg^{2+} on the binding of epinephrine are given in Table I. 1–2 mM ATP and 8 mM Mg^{2+} inhibited the hormone binding.

The effects of glucagon, and propranolol on the epinephrine binding are given in Table II. Propranolol gave the expected inhibition. Propranolol, a β-adrenergic blocking agent was tested because of its known ability to suppress the stimulatory effect of epinephrine on adenyl cyclase[4–6]. The finding that it also inhibits the binding of epinephrine to the membrane supports the hypothesis that hormone binding has a relationship to activation of adenyl cyclase. Glucagon at higher levels may stimulate epinephrine binding.

The effects of SH agents, albumin and heating on epinephrine binding are shown in Table III. PCMB and DTNB inhibited the binding of epinephrine thus implicating SH groups in the receptor protein. PEARLMAN *et al.*[7] reported that SH reagents block the binding of testosterone to a protein present in serum. MARSH AND GEORGE[8] found that SH groups are essential for the action of hormones such as thyroid stimulating hormone on fat cells.

TABLE III

EFFECT OF SH REAGENTS, ALBUMIN AND HEAT TREATMENT ON EPINEPHRINE BINDING TO THE PLASMA MEMBRANE

See text for experimental conditions.

System	*pmoles epinephrine per mg protein per h*
Control	30
plus PCMB (1.10^{-4} M)	0.5
plus DTNB (1.10^{-4} M)	2
plus albumin (100 μg)	38
Heated membranes (1 min, 100°)	34
Heated membranes (5 min, 100°)	2

Heating the membrane for 5 min in boiling water inhibited the binding. However, the protein receptors are stable to boiling for 1 min. These findings are particularly informative when compared to the results in the previous paper which show that PCMB stimulated adenyl cyclase activity. It was found that PCMB also enhanced the epinephrine stimulation of adenyl cyclase. Moreover, the adenyl cyclase activity is markedly reduced by heating the membranes for 1 min in boiling water, whereas the epinephrine binding is not affected.

It was of interest to ascertain which functional groups of the epinephrine molecule were involved in its binding to the membrane. Therefore the effect of epinephrine analogs and metabolites on the binding were tested (Table IV). The results show that although phenylalanine, tyrosine and metanephrine do not inhibit epinephrine binding, norepinephrine and 3,4-dihydroxymandelic acid, DOPA and 3,4-dihydroxyphenylglycol were strongly inhibitory. This data suggests that analogs containing the two hydroxyl groups in positions 3 and 4 of the benzene ring act as competitive inhibitors of epinephrine and that these two functional groups are important for the binding of epinephrine to the membrane. FISHER AND JOST[9] recently reported the results of NMR studies on the binding of epinephrine to mouse liver cells. They conclude that the hormone does bind to the liver cell and that the ring and the side chain

TABLE IV

EFFECT OF EPINEPHRINE ANALOGS ON THE BINDING OF [^{3}H]EPINEPHRINE TO THE PLASMA MEMBRANE

Tested at $1.3 \cdot 10^{-4}$–$2.0 \cdot 10^{-4}$ M. Average of two experiments. See text for experimental details.

Analog	% *of control*
None–Control	100
Unlabeled L-epinephrine	0
L-Norepinephrine	0
L-Phenylalanine	87
L-Tyrosine	97
DL-3,4-Dihydroxymandelic acid	3
DL-3-Methoxy-4-hydroxymandelic acid	98
L-3,4-Dihydroxyphenylalanine (DOPA)	32
DL-3,4-Dihydroxyphenylglycol	47
DL-Metanephrine	129

TABLE V

EFFECT OF SOLVENT EXTRACTION ON MEMBRANE BOUND EPINEPHRINE

Average of two experiments. Membranes (2 mg protein) were incubated with 2.5 μC [^{3}H]epinephrine at 37° for 10 min at pH 7.5 and 10.5, then washed 3 times with 2 ml of 5% trichloroacetic acid. The pellets were extracted 2 times with 3 ml of chloroform–methanol (2:1, by vol.) and two times with 3 ml of chloroform–methanol–HCl (200:100:1, by vol.). The chloroform–methanol extract (after removal of aliquots for counting) was washed with 0.9% NaCl.

Fraction	*Counts/min*	
	pH 7.5	*pH 10.5*
I. Chloroform–methanol extract	2185	9 688
II. Chloroform–methanol extract I after washing with 0.9% NaCl	686	2 610
III. Chloroform–methanol–HCl extract	280	670
IV. Protein residue	5925	31 650

nitrogen atom are involved in the binding. Our data suggest that the ring hydroxyl groups are important and that the side chain is less important for binding.

In order to determine whether the epinephrine was bound to the protein or lipid components of the membrane, the membranes were allowed to bind [^{3}H]epinephrine, washed, and then extracted with chloroform–methanol (2:1, by vol.) followed by chloroform–methanol–HCl. The data given in Table V shows that the major part of the counts remains bound to the protein fraction. About one-third of the counts are removed by extraction with organic solvents but most of these counts are removed from the organic phase by water extraction. This may represent a more loosely bound epinephrine or epinephrine loosely associated with lipoprotein. Experiments with trypsin indicate that the hormone receptor is a protein. When membranes are pre-incubated with trypsin for 30 min to 1 h and then washed to remove the trypsin, these trypsin treated membranes were found to bind much less epinephrine than did control membranes.

Experiments were carried out in an attempt to separate sonicated membranes on Sephadex G-200 and to relate the receptor protein to adenyl cyclase activity.

Membranes were sonicated for 90 sec at 0° with 0.2 % deoxycholate and separated on Sephadex G-200. The fractions were assayed for protein and adenyl cyclase activity (Fig. 5). Fraction 7 was previously shown to contain the bound epinephrine. The adenyl cyclase activity was eluted in two fractions, namely Tube 5 and Tubes 10–11. The major part of the adenyl cyclase activity occurred in Tubes 10–11. Hence the receptor protein for epinephrine is different from the adenyl cyclase. ROSEN AND ROSEN[15] have reported the column separation of two adenyl cyclases from frog erythrocyte only one of which was hormone sensitive.

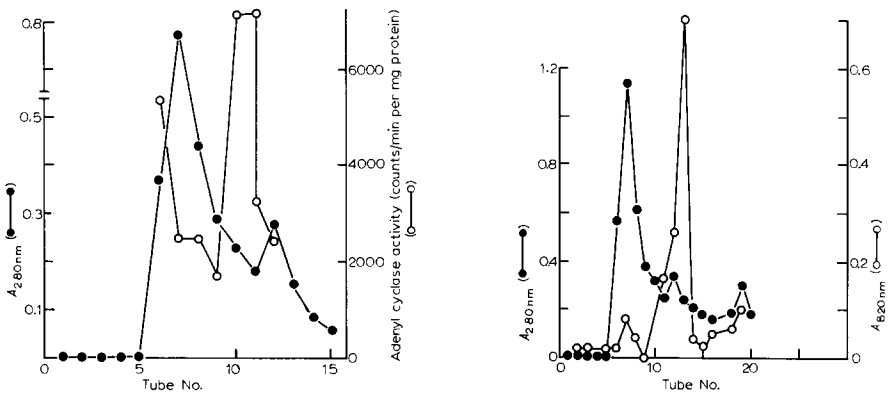


Fig. 5. Column fractionation of sonicated membranes and assay of adenyl cyclase activity. 2 mg of membrane protein were sonicated in 2.5 ml of Tris–deoxycholate buffer and fractionated on Sephadex G-200 as explained in Fig. 2. As indicated in the figure analyses were carried out for adenyl cyclase activity and protein. Adenyl cyclase was assayed as described previously[1]. 0.2-ml aliquots of the column fractions were incubated with 2 mM ATP and 4 mM Mg^{2+} and 5 μC of [^{3}H]ATP (uniformly labeled). The volume was diluted to 0.5 ml with 20 mM Tris buffer (pH 7.5).

Fig. 6. Fractionation of sonicated plasma membranes on Sephadex G-200; lipid and protein analysis. 4.0 mg of membrane protein were dissolved in 2.5 ml of Tris–deoxycholate buffer and sonicated for 90 sec at 0°. The material was fractionated on Sephadex G-200 as explained in Fig. 6. Each fraction was analyzed for lipid phosphorus and protein. ●—●, protein; ○—○, lipid phosphorus.

Membranes were sonicated in the presence of deoxycholate and chromatographed on Sephadex G-200. The fractions were analyzed for protein and total phosphorus. Most of the phosphorus is due to membrane phospholipid. It can be seen in Fig. 6 that the usual three protein peaks are obtained, but the main part of the phosphorus emerges in Peak 13, just after the protein Peak 12. Phosphorus is also associated with protein Peaks 7 and 17. The major phosphorus Peak 13 is believed to represent membrane phospholipids which are released by sonication and complexed with deoxycholate.

The next phase of the research was directed toward elucidating the binding of glucagon to the membranes of rat liver. A high degree of selectivity of binding to the plasma membrane was observed. These data are shown in Table VI. It is significant that both glucagon and epinephrine bind more avidly to the plasma membrane than to other cellular membranes.

TABLE VI

BINDING OF [^{131}I]GLUCAGON TO SUB-CELLULAR FRACTIONS OF RAT LIVER

0.2 ml of each fraction (100–200 μg protein) in 0.05 M Tris buffer (pH 7.5) was incubated at 37° for 10 min with 10000 counts/min of [^{131}I]glucagon. To each sample were added 2.0 ml of ice cold 0.1 M acetic acid. Appropriate blanks were used in which the acetic acid was added to the samples immediately after adding the labeled glucagon. The samples were centrifuged at 3000 rev./min and the pellets washed three times with 2 ml portions of cold 0.1 M acetic acid. The washed pellets were dissolved in Nuclear Chicago solubilizer reagent and counted in Brays cocktail.

Expt.	*Cell fraction*	*pmoles/mg protein*	*Relative %*
1	Plasma membranes	6.1	100
	Homogenate	0.1	1.7
	Nuclei	0.17	2.9
	Mitochondria	0.05	1.0
	Microsomes	1.0	17.8
2	Plasma membranes	9.4	100
	Homogenate	0.25	2.7
	Nuclei	0.32	3.5
	Mitochondria	0.03	0.4
	Microsomes	2.44	25.9

The kinetics of glucagon binding to the isolated plasma is shown in Fig. 7. There is a rapid uptake of glucagon within a period of 2 min. The binding is increased by Mg^{2+} *plus* ATP. Glucagon binding is more rapid than epinephrine binding. It is noteworthy however, that more epinephrine is bound per mg of plasma membrane protein than is glucagon even though the binding of epinephrine occurs at a slower rate.

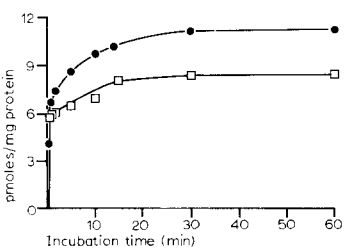


Fig. 7. The effect of ATP and Mg^{2+} on the binding of [^{131}I]glucagon to the plasma membrane. The control contained 200 μg membrane protein, 20 mM Tris buffer (pH 7.5), and 10000 counts/min of [^{131}I]glucagon. The other sample contained 4 mM Mg^{2+} and 1 mM ATP. □—□, control; ●—●, *plus* Mg^{2+} and ATP.

The relationship between amount of membrane protein and glucagon binding is shown in Table VII. The binding increases up to 100 μg of protein and falls off at higher protein concentration. This effect was also seen with epinephrine. Unlabeled glucagon but not unlabeled iodide brought about a large decrease in binding. This demonstrates that the binding is due to glucagon and not to $^{131}I^-$ which might be a contaminant in the radioactive glucagon sample since radioactive iodide is used in generating ^{131}ICl for the labeling of glucagon.

TABLE VII

BINDING OF GLUCAGON TO THE PLASMA MEMBRANE

See text for experimental details.

Sample	*pmoles/mg protein*
50 μg membrane protein	8.7
100 μg membrane protein	10.7
200 μg membrane protein	9.1
200 μg membrane protein + 10 μg unlabeled glucagon	0.4
200 μg membrane protein + 150 μg unlabeled I^-	7.6

TABLE VIII

EFFECT OF PCMB, DTNB AND HEAT TREATMENT ON THE BINDING OF GLUCAGON TO THE PLASMA MEMBRANE

See text for experimental details.

System	*pmoles/mg protein*	*% of control*
Control	18.6	100
PCMB ($5 \cdot 10^{-4}$ M)	9.1	49
DTNB ($5 \cdot 10^{-4}$ M)	7.5	40
Heating, 1 min, 100°	14.4	78

TABLE IX

EFFECT OF Ca^{2+} ON THE BINDING OF GLUCAGON TO THE MEMBRANE

Range of 2 different experiments. See text for experimental details.

Ca^{2+} concn. (M)	*pmoles/mg protein*
0	11.9–14.2
10^{-5}	11.4–12.0
10^{-4}	10.2–10.7

The effects of PCMB, DTNB and heating on the binding of glucagon to the plasma membrane are shown in Table VIII. PCMB, DTNB and heating the membrane for 1 min at 100° inhibited glucagon binding. Similar results were seen with epinephrine binding (Table III).

The effect of Ca^{2+} on glucagon binding is shown in Table IX. Ca^{2+} over the concentration range $1 \cdot 10^{-3}$–$1 \cdot 10^{-5}$ M had a slight inhibiting effect on the binding of glucagon.

The effects of proteolytic and lipolytic enzymes on glucagon binding are given in Table X. All these enzymes which include phospholipase A, phospholipase C, and trypsin inhibited glucagon binding. Trypsin gave the highest inhibition.

Membranes were incubated with excess [^{131}I]glucagon for 10 min and then

TABLE X

EFFECT OF HYDROLYTIC ENZYMES ON GLUCAGON BINDING TO THE PLASMA MEMBRANE

The membrane samples (200 μg protein in 0.5 ml of 0.05 M Tris buffer, pH 7.4) were incubated with 500 μg of enzyme at 37° for 45 min, centrifuged and washed with 2 ml of 0.05 M Tris buffer. The membrane pellets were resuspended in 0.5 ml Tris buffer, the labeled glucagon (10000 counts/min) added, and the membranes incubated for 10 min at 37°. The bound glucagon was determined as explained in the text.

System	*pmoles/mg protein*	*% of control*
Control	3.2	100
Phospholipase A	1.2	38
Phospholipase C	2.2	67
Trypsin	0.85	26

fractionated on Sephadex G-200 after solubilizing the membranes with 0.15 % deoxycholate and glycerol. The fractions were analyzed for protein content and radioactivity. The results are shown in Fig. 8. The peak at Tube 6 represents the membrane bound glucagon. The peak at Tube 17 represents in large part the unbound (or free) glucagon but may also represent glucagon bound to a smaller membrane fragment. Control studies showed that free [^{131}I]glucagon was eluted as a single band with the peak at Tube 17.

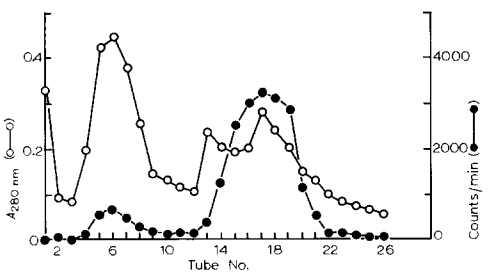


Fig. 8. Column fractionation of [^{131}I]glucagon-labeled plasma membranes. The membrane (4.2 mg protein) in 4.2 and 0.2 M Tris buffer (pH 7.5) was incubated with excess [^{131}I]glucagon for 10 min at 37°. The material was spun at 3000 rev./min and the supernatant fluid removed. The membrane pellet was suspended in 2 ml of 0.05 M Tris buffer (pH 7.5) containing 0.15 % deoxycholate and sonicated for 90 sec. 0.7 ml was removed, mixed with 1 ml glycerol and placed on a 2 cm × 20 cm Sephadex G-200 column. Elution was carried out with 0.05 M Tris buffer (pH 7.5) containing 0.15 % deoxycholate. Fractions of 1.5 ml were collected. 0.5 ml of each fraction was used for measuring radioactivity. The remaining fraction material was used for absorbance measurements and protein analysis.

It is of interest that both epinephrine and glucagon are bound to the membrane fragment which is eluted early (Peak 6–7) and thus represents a large macromolecular component. The counts in Peak 6 representing bound glucagon represents a minimal number since control studies showed that 0.15 % deoxycholate inhibited glucagon binding to the membrane by about 30 %.

DISCUSSION

The binding of glucagon and epinephrine was studied in the hope of correlating hormone binding with adenyl cyclase activation. One aim was to determine whether the hormones were interacting directly with adenyl cyclase or whether binding occurred at a receptor protein and this binding in some way led to the activation of adenyl cyclase.

Recently several studies have appeared in the literature[10–13] concerning the binding of hormones to components of their target organs. However, very little has been done on the binding of hormones to isolated plasma membranes. In 1963 POTTER AND AXELROD[14] reported the subcellular localization of catecholamines in the vas deferens, submaxillary gland, pineal gland and heart of the rat. They reported that most of the injected hormone was located or bound to "storage vesicles" which sedimented with the microsomal fraction. The uptake of [^{3}H]norepinephrine was temperature dependent and did not require ATP and Mg^{2+} (at least in most of the experiments with heart).

The rate of binding of glucagon to the membrane was faster than the rate of binding of epinephrine but more epinephrine was bound per mg of membrane protein than was glucagon. These results correlate with the observation that glucagon stimulates adenyl cyclase more rapidly and to a greater extent than does epinephrine and does so at a lower concentration than epinephrine. These findings agree with *in vivo* studies by others[4], which show that glucagon at low concentration is a more potent stimulator of adenyl cyclase than is epinephrine.

The evidence for specific receptor proteins for glucagon and epinephrine comes from column fractionation of the detergent treated membranes which were first allowed to bind hormone. The data with epinephrine clearly showed that the epinephrine was bound to a fast moving protein fraction which was separated from the fraction containing the bulk of the adenyl cyclase activity.

ROSEN AND ROSEN[15] reported that frog erythrocyte adenyl cyclase emerges from Sephadex as two peaks. Only the fast moving peak, which they believe is membrane bound, was responsive to hormone stimulation.

Further evidence that the receptor protein and the enzyme are separate entities comes from the effects of PCMB, heating, and Ca^{2+} on the hormone binding and the enzyme activity. PCMB at $1 \cdot 10^{-4}$ M decreased epinephrine binding (and glucagon binding) but increased adenyl cyclase activity[2]. Heat treatment for 1 min at 100° increased epinephrine binding but markedly inhibited adenyl cyclase activity. Ca^{2+} at a level of $1 \cdot 10^{-3}$ M decreased epinephrine binding, but increased the basal level of adenyl cyclase and also increased the epinephrine stimulation of adenyl cyclase.

One might expect that if binding of hormone to the membrane is essential for the hormone stimulation of adenyl cyclase, then agents which diminish binding of the hormone should diminish the stimulation of the enzyme by the hormone. However 3 mM Ca^{2+} and $1 \cdot 10^{-4}$ M PCMB decreased the binding of epinephrine but enhanced the epinephrine stimulation of adenyl cyclase. PCMB also increased the basal level of adenyl cyclase in absence of added epinephrine. Apparently PCMB and Ca^{2+} may have multiple effects on the membrane. It is possible that only a relatively small portion of the hormone binding is related to adenyl cyclase activation and the major binding may be relatively non-specific and concerned with its entry into the cell for

its further metabolism (degradation). Hence Ca^{2+} and PCMB may influence more the latter type of binding.

The effect of 1 min heating the membrane is of interest since this treatment leads to an increase in epinephrine binding but nearly completely abolished the adenyl cyclase activity. However, heating for 5 min abolished epinephrine binding. These results indicate that the receptor protein is more stable than the enzyme.

The effect of pH on the epinephrine binding and enzyme activity demonstrated that maximal binding of hormone occurred at pH 10.5 whereas maximal enzyme activity occurred at pH 8.0. These effects are difficult to correlate since the very high pH 10.5 is probably not physiologically significant and the membrane structure is undoubtedly drastically altered at this high pH.

The effects of Ca^{2+} and PCMB on glucagon binding to the membrane and on adenyl cyclase show that 3 mM Ca^{2+} decreased slightly the binding of glucagon but increased the glucagon stimulation of adenyl cyclase. Ca^{2+} at 3 mM also increased the basal level of adenyl cyclase. These effects are similar to those seen with epinephrine in that Ca^{2+} and PCMB decrease hormone binding but increase adenyl cyclase activity. In the previous paper it was shown that $1 \cdot 10^{-4}$–$1 \cdot 10^{-5}$ M Ca^{2+} inhibited the glucagon stimulation of adenyl cyclase but enhanced the epinephrine stimulation of adenyl cyclase. It was also shown that glucagon inhibited the binding of Ca^{2+} to the membrane whereas epinephrine stimulated Ca^{2+} binding to the membrane. Moreover, Ca^{2+} has little or no effect on the binding of either glucagon or epinephrine to the membrane. These results suggest that Ca^{2+} and glucagon act antagonistically to each other whereas Ca^{2+} and epinephrine act cooperatively, but the concentration of Ca^{2+} is critical and the interplay is complex.

ACKNOWLEDGMENTS

This work was supported in part by a grant HE 02063 from the National Heart Institute. One of the authors (V.T.) is a Post-Doctoral Fellow of the Institute of General Physiology, University of Ferrara, Italy.

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