Biochimica et Biophysica Acta, 436 (1976) 489–494

BBA 77350

THE GLUCAGON RECEPTOR OF RAT LIVER PLASMA MEMBRANE CAN COUPLE TO ADENYLATE CYCLASE WITHOUT ACTIVATING IT

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(Received October 20th, 1975)

SUMMARY

1. Activation of adenylate cyclase in rat liver plasma membranes by fluoride or GMP-P(NH)P yielded linear Arrhenius plots. Activation by glucagon alone, or in combination with either fluoride or GMP-P(NH)P resulted in biphasic Arrhenius plots with a well-defined break at 28.5±1 °C.

2. The competitive glucagon antagonist, des-His-glucagon did not activate the adenylate cyclase but produced biphasic Arrhenius plots in combination with fluoride or GMP-P(NH)P. The break temperatures and activation energies were very similar to those observed with glucagon alone, or in combination with either fluoride or GMP-P(NH)P.

3. It is concluded that although des-His-glucagon is a potent antagonist of glucagon, it nevertheless causes a structural coupling between the receptor and the catalytic unit.

INTRODUCTION

The adenylate cyclase activity associated with rat liver plasma membranes may be stimulated by a number of ligands, including fluoride, 5'-guanylyl-imidodiphosphate (GMP-P(NH)P) and glucagon (see e.g. refs. 1 and 2). Fluoride and GMP-P(NH)P are believed to act directly on the catalytic unit exposed on the inside surface of the membrane, whereas the action of glucagon is mediated by a receptor with a specific binding site on the outer surface of the membrane [1–7]. The interaction between the receptor and catalytic unit which leads to activation has been termed coupling. However, the structural basis of this trans-membrane interaction is obscure, although phospholipids have been strongly implicated (see e.g. ref. 6 for a review). We have used the competitive glucagon antagonist, des-1-histidine glucagon [8, 9], to show that there must be a structural coupling between the catalytic unit and the receptor on binding des-His-glucagon which is distinguishable from the coupling caused by glucagon.

MATERIALS AND METHODS

Membrane preparation

Rat liver plasma membranes were prepared from male Sprague-Dawley rats

weighing 250–300 gm by the method of Pilkis et al. [10]. The fraction collecting at the 48.2–42.5 % sucrose interface was removed and washed 4 times with 1 mM $KHCO_3$, pH 7.2 prior to storage in liquid nitrogen. Similar results were obtained with both fresh membrane fractions and those stored in liquid nitrogen at a protein concentration of 8–10 mg/ml in 1 mM $KHCO_3$ pH 7.2.

Assay of adenylate cyclase

Membranes were added to a cocktail at pH 7.4 containing final concentrations of 25 mM triethanolamine/HCl, 1 mM EDTA, 1 mM theophylline, 5 mM $MgSO_4$, and an ATP-regenerating system of 7.4 mg/ml creatine phosphate and 1 mg/ml creatine kinase. Reactions were initiated by addition of neutralised ATP to a final concentration of 1.5 mM. The assay pH was adjusted to pH 7.4 in a final assay volume of 100 μl. The concentration of ligands in the assay medium were glucagon (10^{-6} M), fluoride ($1.5 \cdot 10^{-2}$ M) and GMP-P(NH)P (10^{-4} M). After the appropriate incubation period, the assays were terminated by placing the tubes for 3 min in a water bath at 90 °C. The tubes were allowed to cool to room temperature and 100 μl of an alumina slurry (0.45 gm added to 1 ml in 50 mM triethanolamine/HCl buffer pH 7.6) added, followed by vortexing and centrifugation. Aliquots (50 μl) of the supernatant were then taken for assay of cyclic AMP by the competition assay of Brown et al. [11]. A standard curve was constructed for every assay set by "spiking" assay cocktails (containing either no membranes or heat denatured membranes) with known amounts of cyclic AMP, and carrying them through the above procedure.

The alumina did not remove any of the cyclic AMP from solution, but did remove residual ATP and other nucleotides which had significant effects on the competition assay, markedly reducing its sensitivity. The addition of the ligands used did not affect the standard curve. Assays were carried out over 5 time points to assess linearity. Reaction velocity was linear with respect to protein concentration over the ranges used in this study. All temperature-activity studies were carried out over similar ranges from about 10–40 °C.

Protein determinations were carried out as described previously [12]. Des-His-glucagon was a generous gift from Eli Lilly and Co. GMP-P(NH)P, ATP, triethanolamine/HCl, creatine phosphate and creatine kinase were obtained from Boehringer Corp. Glucagon, theophylline, and neutral alumina type WN3 were obtained from Sigma.

RESULTS

(1) *The temperature versus activity profiles of adenylate cyclase stimulated by different ligands*

The adenylate cyclase activity of rat liver plasma membranes was stimulated at least 37 fold by glucagon, 6.5 fold by fluoride and 10 fold by GMP-P(NH)P over basal activity when assayed at 30 °C. The stimulation factor for glucagon is high compared with some previous results (e.g. see refs. 1, 2, 5 and 14) but similar to the 30-fold stimulation observed by Johnson et al. [13]. The specific activity of adenylate cyclase stimulated by glucagon in our preparations was of the same order as the values reported previously, and the reason for the relatively high stimulation factor was the low basal activity achieved by the repeated washing of the membranes, which

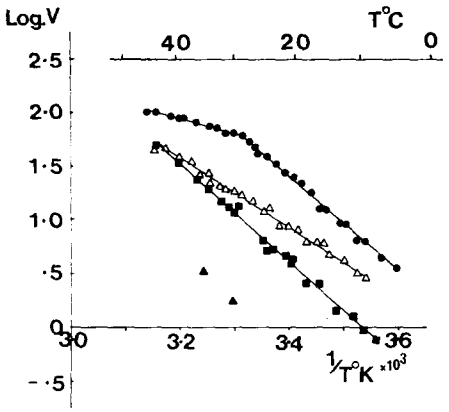

Fig. 1. Arrhenius plots of adenylate cyclase activity stimulated by different ligands, 10^{-6} M glucagon (●); $1.5 \cdot 10^{-2}$ M fluoride (■); 10^{-4} M GMP-P(NH)P (△); and no added ligand (▲). Velocities are expressed throughout in μ units/mg protein.

TABLE I

INFLECTION TEMPERATURES AND ACTIVATION ENERGIES FROM ARRHENIUS PLOTS OF ADENYLATE CYCLASE ACTIVITY STIMULATED BY VARIOUS LIGANDS

Errors are given as S.D., with number of determinations in brackets

Ligands	Inflection (°C)	Activation energy (kcal · mol^{-1})	
		Above break	Below break
Glucagon	28.6±1 (9)	6±2	20±3
Fluoride	– (6)	21±2	
+glucagon	28.5±0.5 (6)	6±2	22±3
+des-His-glucagon	29.0±0.4 (6)	5±2	22±3
GMP-P(NH)P	– (3)	15±4	
+glucagon	29.0±0.5 (3)	5±2	21±2
+des-His-glucagon	28.8±0.6 (3)	3±1	29±4

was always less than 1.6 pmol/min/mg protein. (No detailed study of basal activities was undertaken because of the uncertainty about its origin, see e.g. refs. 1 and 2). The Arrhenius plots for adenylate cyclase activity stimulated by glucagon, fluoride and GMP-P(NH)P are compared in Fig. 1. Both fluoride and GMP-P(NH)P stimulated-activities yielded linear plots, whereas the glucagon stimulated-activity showed a well-defined break at 28.5 °C. Activation energies differed markedly (Table I).

(2) *The effects of glucagon or des-His-glucagon on fluoride or GMP-P(NH)P adenylate cyclase activity*

Des-His-glucagon has been shown to be a reversible competitive inhibitor of the binding of glucagon to its receptor and also of the stimulation by glucagon of adenylate cyclase activity in plasma membranes from rat liver and other sources, whilst having no effect itself on basal activity [2, 8, 9]. We have been able to confirm

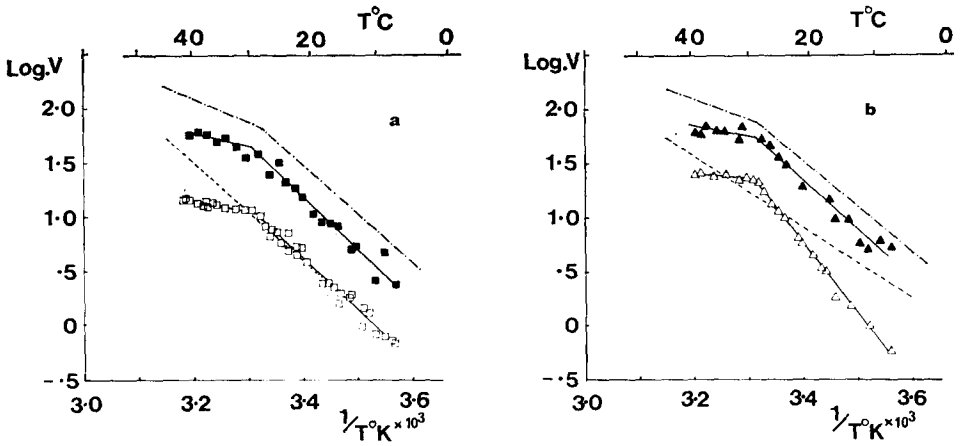

Fig. 2. Arrhenius plots demonstrating the effect of des-His-glucagon or glucagon on fluoride or GMP-P(NH)P stimulated adenylate cyclase activity (a) in the presence of 10^{-6} M glucagon and $1.5 \cdot 10^{-2}$ M fluoride (■); 10^{-6} M des-His-glucagon and $1.5 \cdot 10^{-2}$ M fluoride (□). Adenylate cyclase activity in the presence of $1.5 \cdot 10^{-2}$ M fluoride alone is shown (– – – –); and the calculated sum of the separate glucagon and fluoride-stimulated activities (– · – · –). The combination 10^{-7} M des-His-glucagon and $1.5 \cdot 10^{-2}$ M fluoride gave an identical result to that obtained with 10^{-6} M des-His-glucagon and $1.5 \cdot 10^{-2}$ M fluoride. (b) In the presence of 10^{-6} M glucagon and 10^{-4} M GMP-P(NH)P (▲); 10^{-6} M des-His-glucagon and 10^{-4} M GMP-P(NH)P (△). Adenylate cyclase activity in the presence of GMP-P(NH)P alone is shown (– – – – –), and the calculated sum of the separate glucagon and GMP-P(NH)P stimulated activities (– · – · –).

these observations in the present work. Stimulation by K_a concentrations of glucagon ($4 \cdot 10^{-9}$ M) can be completely abolished by 10^{-6} M des-His-glucagon, and reduced by 60 % with 10^{-7} M des-His-glucagon.

The effect of glucagon or des-His-glucagon on the activity of fluoride or GMP-P(NH)P-stimulated adenylate cyclase were compared. The Arrhenius plots for all these combinations of ligands are shown in Fig. 2a and b. The plots are all biphasic, showing similar breakpoints and activation energies to those obtained in the presence of glucagon alone (Table I).

The addition of des-His-glucagon to the fluoride stimulated enzyme (Fig. 2a) yielded a biphasic Arrhenius plot with a break at 29 °C, below 29 °C the activity was similar to the activity of the enzyme in the presence of fluoride alone, but was relatively inhibited at higher temperatures. The plot obtained in the presence of both fluoride and glucagon (Fig. 2a) was very similar in form to that obtained with glucagon alone, although the specific activity was significantly lower at all temperatures than when stimulated by glucagon alone. Glucagon and des-His-glucagon therefore produced very similar changes in the form of the Arrhenius plots, with breaks at the same temperature and similar activation energies for the two temperature ranges (Table I).

The combination of either glucagon or des-His-glucagon with GMP-P(NH)P produced qualitatively similar changes to those described for the fluoride-stimulated enzyme (Fig. 2b, Table I). Both Arrhenius plots show a break at about 29 °C, and have activation energies similar to those obtained with glucagon alone. Des-His-glucagon inhibited GMP-P(NH)P stimulated activity below 25 °C and above 33 °C, although it caused a slight activation between these two temperatures. The activity of adenylate

cyclase stimulated by both GMP-P(NH)P and glucagon was similar to that when glucagon was present alone. We note that Saloman et al. [18] observed a further increase in adenylate cyclase activity when 5 μm GMP-P(NH)P was added with glucagon at 10^{-9} M, which is about the K_a value for glucagon, and we can confirm at about K_a concentrations of glucagon, GMP·P(NH)P causes additional stimulation. However in the present experiments where glucagon was added at saturating concentrations ($\approx 100 \times K_a$), the addition of GMP-P(NH)P has little effect on adenylate cyclase activity.

A potential complication in the use of GMP-P(NH)P stimulated activities to construct Arrhenius plots is that there is a lag period of about 20–30 s before maximum activity develops in rat liver plasma membranes [18]. However, the time course of all the adenylate cyclase activities we observed showed no lag phase significantly longer than 30 s, and the activities were then constant for at least 10–20 min at all temperatures. These constant activities were used to construct Arrhenius plots of adenylate cyclase activity stimulated by all of the ligands used.

DISCUSSION

Both fluoride and GMP-P(NH)P produced linear Arrhenius plots of adenylate cyclase activity and both of these ligands activate the enzyme independently of the glucagon receptor. Glucagon activation, either alone or in combination with fluoride or GMP-P(NH)P, resulted in biphasic Arrhenius plots with a well-defined break at about 28.5 °C. Des-His-glucagon prevents glucagon stimulation of adenylate cyclase activity by competing for the receptor and it has no significant activating effect on basal activity [2, 8, 9]. Nevertheless, it produced a very similar change to glucagon in the form of the Arrhenius plots for adenylate cyclase activity stimulated by either fluoride or GMP-P(NH)P. Thus occupancy of the glucagon receptor has an overriding effect on the form of the Arrhenius plots of adenylate cyclase activity. The biphasic curves for the Arrhenius plots with des-His-glucagon and either fluoride or GMP-P(NH)P strongly suggest that des-His-glucagon must either induce a structural interaction between the glucagon receptor and the catalytic unit, or cause a change in the preexisting interaction. This structural coupling is distinguished from that produced by glucagon in that it causes only small changes in adenylate cyclase activity, from which it follows that the interaction of the glucagon receptor with the catalytic unit need not cause activation of adenylate cyclase.

The Arrhenius plots of fluoride and glucagon-stimulated adenylate cyclase activities reported here are similar to those described by Kreiner et al. [15], who obtained a linear plot in the presence of fluoride and a biphasic plot for glucagon-stimulated activity. However in their work the break occurred at 32 °C and the activation energy increased above this temperature. These differences may be due to the source of membranes, or the assay conditions used. We have only attempted in this study to define the major break at 28–29 °C in the Arrhenius plots of adenylate cyclase activity, but not any minor inflections which may be due to lipid phase separations in the two halves of the bilayer (see e.g. refs. 16 and 17).

The results described for des-His-glucagon are compatible with both of the current models of receptor-adenylate cyclase interaction. If the receptor is always associated with the enzyme as a multicomponent entity, then the data suggests that

des-His-glucagon and glucagon cause different changes in the interaction between receptor and catalytic units. The more interesting possibility is that the receptor migrates independently in the outer half of the bilayer until glucagon provides the signal for a locking interaction with the adenylate cyclase in the inner half of the bilayer. The effect of des-His-glucagon would then imply that it can cause a locking interaction which does not lead to the marked activation displayed by the action of glucagon on the receptor.

In the following paper we show that the biphasic Arrhenius plots observed with glucagon and des-His-glucagon can be attributed to the response of the receptor to its lipid environment.

ACKNOWLEDGEMENTS

M.D.H. is an ICI Postdoctoral Fellow and a Research Fellow of Queens' College, Cambridge; G.B.W. is the Stothert Research Fellow of the Royal Society. This work was supported by a grant from the MRC to J.C.M.

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