GLUCAGON AND EPINEPHRINE STIMULATION OF ADENYL CYCLASE IN ISOLATED RAT LIVER PLASMA MEMBRANES*

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ABSTRACT

Adenyl cyclase has been shown to be specifically localized in the plasma membrane of rat liver. The activity of this enzyme is stimulated by glucagon and epinephrine in isolated plasma membrane systems. The stimulation by epinephrine has a lag period of about 10 minutes. Epinephrine shows selective binding to isolated plasma membranes. The results indicate that epinephrine binds to a receptor protein rather than interacting directly with the enzyme adenyl cyclase. Calcium ions also stimulate adenyl cyclase in isolated plasma membranes but the effect shows appreciable variability.

The biological significance of cyclic AMP (adenosine-3',5'-monophosphate) has been discussed by Sutherland and co-workers (1960, 1965, 1966, 1968). Cyclic AMP was discovered as a mediator of the glycogenolytic action of epinephrine and glucagon in liver systems by Rall et al (1956). Sutherland and co-workers postulated that cyclic AMP acts as a second messenger which mediates the effects of a number of hormones. Hormones such as glucagon and epinephrine are believed to stimulate adenyl cyclase activity in the cell membrane and the cyclic AMP then regulates the activity of a number of enzymes.

Sutherland and Rall, (1966) and Sutherland, Rall and Menon (1957) reported that adenyl cyclase activity was localized in the insoluble particulate fraction of the liver cell. Subsequently Devoren and Sutherland (1968) and Oye

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and Sutherland (1966) showed that adenyl cyclase was located in the avian erythrocyte cell membrane fraction. Rodbell (1967) has reported that adenyl cyclase occurs in fat cell ghosts. Recently McKeel and Jarett (1969) have reported that adenyl cyclase specific activity is 4-5 fold higher in the plasma membrane fraction of the fat cell as compared to fat cell ghosts.

The object of this research was to test the hypothesis that adenyl cyclase is localized specifically in the plasma membrane of the liver cell and is stimulated in vitro by hormones (glucagon and epinephrine) known to increase the level of cyclic AMP in several types of liver cell systems. Since our work began Pohl et al (1969) have reported the glucagon stimulation of adenyl cyclase in isolated rat liver plasma membranes. These workers found no stimulation by epinephrine.

MATERIALS AND METHODS

Rat liver plasma membranes were prepared by two methods: Method A utilized the procedure of Coleman et al (1967). Method B was a slightly modified procedure of Neville (1960) in which calcium ion at 0.5 mM is added to the homogenizing medium, and the homogenate is diluted 100 fold. The purity of the membrane preparation was checked by assay of marker enzymes and by electron microscopy.

Tritium labeled (^{3}H-(G)-ATP and ^{14}C-(U)-3',5' cyclic AMP and 7-^{3}H-DL-epinephrine were purchased from New England Nuclear Corp. Crystalline glucagon was a gift sample from the Lilly Research Lab. L-epinephrine was purchased from Nutritional Biochemicals. Other nucleotides were obtained from P-L Laboratories. The assay procedure for adenyl cyclase was as follows: After stopping the enzyme reaction by immersion in boiling water, carrier 3',5' cyclic AMP was added to each system to serve as a marker. 100 µl aliquots were then pipetted on 20 x 20 cm Whatman No. 3 MM filter paper and two dimensional chromatography was carried out. The first solvent was isopropanol-conc. NH_4OH-H_2O(7/2/1). The second solvent was isopropanol-conc. HCl-H_2O (65.0/16.7/18.3). The cyclic AMP spots were cut off and placed in counting vials. One ml of water was added to dissolve the

nucleotide, and then 15 ml of Bray's scintillation cocktail were added (Bray 1960). Radioactivity was determined with a Packard Tri Carb liquid scintillation counter. This two dimensional system gave a complete separation of cyclic AMP from interfering nucleotides, nucleosides and free bases. Supporting evidence that 3',5' cyclic AMP was formed in these membrane systems was obtained by use of brain 3',5' nucleotide phosphodiesterase (Cheung, 1967). The cyclic AMP formed in the system was hydrolyzed nearly to completion by brain phosphodiesterase.

RESULTS AND DISCUSSION

The data in Table 1 show that adenyl cyclase activity is localized in the

TABLE 1

CELLULAR LOCALIZATION OF ADENYL CYCLASE

System	Enzyme Activity
Experiment 1*	nmoles/mg protein/hr
Plasma membranes	24
Total cell particulates	0.2
Experiment 2*	
Plasma membranes	102
Mitochondria + microsomes	1.2
Nuclei	0.0
Total particulates	1.2

* The incubation system was as follows: plasma membranes (70-135 µg protein), 40 mM Tris-HCl buffer pH 7.4, 3.3 mM $MgCl_2$, 2 mM ATP (containing 5-10 µC H^3-ATP), 1 mM EDTA, 6.7 mM caffeine. In addition, exper. 1 contained 10 mM NaF. The final volume was 0.5 ml. Incubation was carried out at 37° C for 15 min. The reaction was stopped by placing the tubes in boiling water for 3 min. The subcellular fractions were prepared by the method of Coleman et al (1967) and washed in 0.04 M Tris buffer pH 7.4.

liver cell plasma membrane since the specific activity of this enzyme is 100-140 fold higher in the plasma membrane as compared to the other cellular membrane fractions. The small activity observed in the mitochondria-microsomal fraction may be due to incomplete removal of plasma membranes from this fraction.

The stimulation of adenyl cyclase activity by glucagon and epinephrine is shown in Table 2. The degree of stimulation by glucagon varied from one prepara-

TABLE 2

SIMULATION OF MEMBRANE BOUND ADENYL CYCLASE BY GLUCAGON AND EPINEPHRINE*

System	Enzyme Activity nmoles/mg protein/hr.	
I. Control	39.5 ± 4.4	(6)
plus glucagon (10^{-5}M - 2 x 10^{-6}M)	75.3 ± 8.5	(6)
II. Control	71.4 ± 11.0	(5)
plus epinephrine (10^{-4}M - 10^{-5}M)	118.2 ± 11.8	(5)

* The incubation system contained 40 mM Tris-HCl buffer pH 7.4, 3.3 mM $MgCl_2$, 2 mM ATP (containing 5 μC of ^{3}H-ATP), and 100-160 μg of membrane protein. In experiments in group I, 6.7 mM caffeine was added. The results are given as the mean ± the standard deviation. The number in parenthesis represents the number of experiments in each group. The hormones were used in the concentration range indicated. Membranes were prepared by method B. The membrane preparations used in I were different from those used in II (membranes were prepared on different days). Caffeine had no appreciable effect on adenyl cyclase activity.

tion to another and was dependent on the hormone concentration as seen in Figure 1. At least 5 x 10^{-6} M glucagon and 10^{-5} M epinephrine were required for stimulation. In Figure 1, the various glucagon concentrations were done on one membrane preparation and the various epinephrine concentrations were done on a different

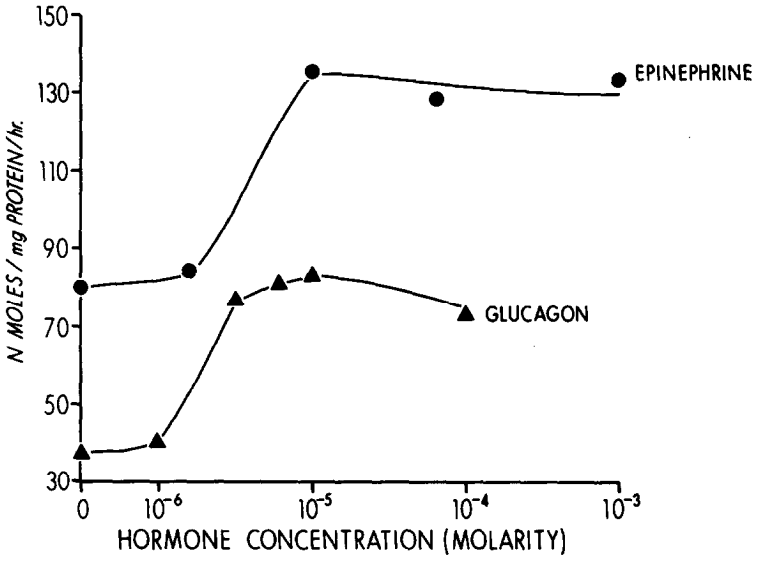

Figure 1. Effect of Hormone Concentration on Adenyl Cyclase Activity

Membranes were incubated for 15 min at 37° C with glucagon or L-epinephrine at the concentrations given. The incubation medium and assay conditions are given in the text and in Table 2.

membrane preparation. The different control values are due to the different membrane preparation and different incubation medium.

The effect of calcium ion is seen in Table 3. The stimulation by calcium

TABLE 3

THE EFFECT OF CALCIUM ION ON ADENYL CYCLASE ACTIVITY*

	Adenyl cyclase activity nmoles/mg protein/hr	
Control	59.6 ± 10.2	(5)
plus Ca^{++}(1-4 mM)	79.0 ± 20.3	(5)

* The data are given as the mean ± std. deviation. The number of experiments are given in parentheses. In every experiment the enzyme activity with calcium present was higher than the control. Membranes were prepared by method B. The incubation medium was the same as that given in Table 2.

ion had a fairly large standard deviation due mainly to the fact that calcium is added to the homogenizing medium during preparation of the membranes and different amounts will remain bound from one preparation to another. However in every experiment the adenyl cyclase activity with calcium was higher than the control without calcium. It was found necessary to add calcium ions to the homogenate during the preparation of the membranes in order to stabilize them and increase their yield.

TABLE 4.

BINDING OF ^{3}H-EPINEPHRINE TO RAT LIVER CELL FRACTIONS**

Cell Fraction	Bound epinephrine p moles/mg protein/hr.	
	Ave.	Range
Nuclear	6.8	(2.6-12.5)
Mitochondria	3.0	(0-8.9)
Microsomes	15.6	(5.3-26)
Plasma membrane	70.9	(55.8-82.5)
Cell sap	5.5*	
Total Homogenate	2.0*	

** 0.6 µC of ^{3}H-epinephrine was incubated at 37° for 10 min. with 0.2-1.0 mg of cell fraction protein in a final volume of 0.5 ml containing 80 mM Tris HCl buffer pH 7.5 and 100 µg of albumin. To each tube were then added 3.0 ml of 0.1 N acetic acid (or 3 ml of 5% TCA). The protein fractions were then centrifuged and washed three more times with the same solution. The last wash was found to contain no significant amount of radioactivity. The protein pellets were dissolved in 0.3 ml of Nuclear-Chicago NCS reagent and then 15 ml of Bray's scintillation cocktail were added.

"Blanks" were also carried out at 0° C. These values (which were about 10% the value of the experimental values at 37°) were subtracted from the experimental values. Similar results were obtained by dialyzing the incubated samples against several changes of 0.1 N acetic acid. Membranes were prepared by method B.

* only one value represented here. The others represent the average of 3 experiments.

The binding of epinephrine to the different cell fractions is given in Table 4. The results show a marked affinity of epinephrine for the plasma membrane. This finding is in agreement with a hypothesis that the hormone action is at the plasma membrane level. One would like to determine whether the hormone binds to a specific receptor protein in the membrane or whether it interacts directly with the membrane-bound enzyme adenyl cyclase. The results shown in Figure 2 bear on this point. The initial (0-10 min) binding of epinephrine occurs at a time when the hormone does not increase the activity of adenyl cyclase activity. Moreover, the epinephrine stimulation of adenyl cyclase is seen only after a certain period of preincubation (9-12 min). These results indicate that epinephrine binds to a receptor protein rather than to the enzyme directly. Further data in support of this idea comes from studies with heat treatment and

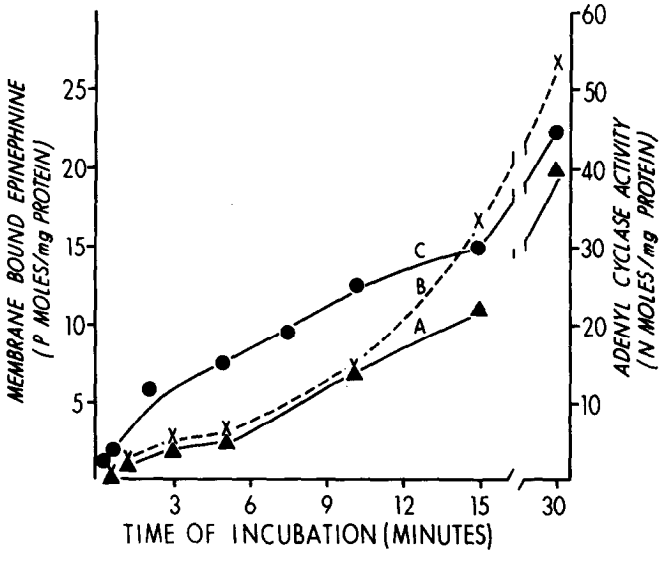

Figure 2. Relationship between epinephrine binding to isolated membranes and epinephrine stimulation of adenyl cyclase at different time intervals of incubation.

0.62 μC of ^{3}H-epinephrine were used in the binding studies and 10^{-5} M L-epinephrine was used in the experiments for the assay of adenyl cyclase activity. The experimental conditions of the system are given in the text and in Table 2. Curve A- adenyl cyclase activity without added epinephrine, curve B- adenyl cyclase activity with 10^{-5} M epinephrine, curve C- amount of ^{3}H-epinephrine bound to the membrane.

with the effect of p-CMB. Heating the membrane preparation for 1 minute at 90° abolishes or greatly inhibits the enzyme activity without affecting the binding of epinephrine whereas 10^{-4} M p-CMB increases the enzyme activity but decreases epinephrine binding. Bitensky et al (1968) have postulated a similar mechanism for epinephrine action. By use of Sephadex column chromatography a protein fraction containing bound epinephrine has now been isolated. A study of this protein fraction and the nature of the binding is now underway. Glucagon also binds to the plasma membrane. The binding of glucagon is complete within 2-3 minutes whereas epinephrine binding continues for 30 minutes.

The failure of Pohl et al to observe epinephrine stimulation of adenyl cyclase may be due to either their use of 30° C for incubation (versus our 37° C) or to their using a 10 min incubation time whereas we use a 15 min. incubation. Of course, other explanations are possible. We found that a minimum concentration of 10^{-5} M Ca^{++} was necessary for the stimulation of adenyl cyclase by epinephrine whereas the stimulation by glucagon is reduced by added Ca^{++}. These findings were obtained in membranes prepared in the absence of Ca^{++} or in membranes in which Ca^{++} was removed by washing with EDTA. The main point to consider is that both epinephrine and glucagon should be expected to stimulate adenyl cyclase activity (Sutherland et al 1968).

We have also observed that (a) fluoride at a concentration of 1-10 mM inhibits adenyl cyclase activity in our systems (b) treatment of plasma membranes with the detergent sodium dodecyl sulfate "solubilizes" the membrane but has little effect on the adenyl cyclase activity (c) freezing and thawing of membranes leads to a loss of adenyl cyclase activity.

By use of ^{14}C-3,5'-cyclic AMP we were able to demonstrate very little cyclic 3',5' nucleotide phosphodiesterase activity in the isolated membranes. Only 5-8% of the added cyclic AMP was hydrolyzed under the same conditions used to assay for adenyl cyclase. This finding is in agreement with the results of Pohl et al.

This paper demonstrates the specific localization of adenyl cyclase in the

liver cell plasma membrane and provides evidence for the action of glucagon and epinephrine at the membrane level in a cell free system.

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