ACTIVATION OF RAT LIVER PLASMA MEMBRANE ADENYLATE CYCLASE BY A CYTOPLASMIC PROTEIN FACTOR

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Received 11 August 1977

1. Introduction

Studies of adenylate cyclase have tended to concentrate upon the mechanism of action of polypeptide hormones. These act by binding to a protein receptor on the outer surface of the plasma membrane. The hormone–receptor complex which is formed interacts with a catalytic unit of adenylate cyclase on the inner surface of the plasma membrane and modulates its activity [1].

There has been less interest in the possibility that cytoplasmic factors acting on the inner surface of the plasma membrane may also be important in regulating the activity of adenylate cyclase but there have been a number of observations which suggest that this may be the case. GTP and its analogue GMP–PNP have been shown to affect the activity of adenylate cyclase and to modulate the response of the enzyme to hormones [2] and it is likely that GTP exerts its effect on the inner surface of the cell membrane. The precise physiological function of this response is obscure since cytoplasmic concentrations of GTP are well in excess of the concentrations needed to produce a maximal response of adenylate cyclase activity.

Ho et al. [3] have shown that fat cells produce a factor in response to adrenaline that act as an antagonist of the hormone's lipolytic effect and have suggested that this factor is produced inside the cell and acts as a feed back inhibitor of adenylate cyclase.

Löw an Werner [4] have suggested that adenylate cyclase activity may be modulated by the redox state of the cell. Gill and King [5] have suggested that a cytoplasmic factor may be involved in the action of cholera toxin in stimulating adenylate cyclase.

Sanders et al. [6] have recently reported that the hormone responsiveness of adenylate cyclase in a particulate fraction from cardiac muscle is increased by addition of cytoplasm. Activation of liver plasma membrane adenylate cyclase by a protein factor has been demonstrated by Pecker and Hanoune [7].

Thus there is quite a large body of evidence to support the view that cytoplasmic factors may be important in modulating adenylate cyclase activity. In this report we describe a heat-stable, protease-sensitive factor from rat liver cytoplasm which activates adenylate cyclase in rat liver plasma membranes.

2. Materials and methods

Creatine phosphate, ATP, cyclic 3′5′-AMP and dithiothreitol were obtained from Sigma (London), Norbiton Station Yard, Kingston upon Thames, Surrey KT2 7BH. Creatine phosphokinase GTP and GMP-PNP were obtained from Boehringer (London) Bell Lane, Lewes East Sussex BN7 1LG. [α^{32}P]ATP was prepared by the method of Martin and Voorheis [8] using [^{32}P]P_i obtained from The Radiochemical Centre, Amersham, Bucks. Crystalline glucagon was the generous gift of Dr. E. W. Bromer, Eli Liley Corp. Indianapolis, IN. All other reagents were of analytical grade.

Rat liver plasma membranes and liver cytoplasm were prepared from the livers of Wistar rats in the weight range 120–150 g. Liver plasma membranes were prepared according to the method of Pilkis et al. [9].

Adenylate cyclase was assayed by the method of Salomon et al. [10]. Membrane protein, 0.01–0.04 mg,

was incubated for 10 min at 30°C in total vol. 0.1 ml containing 25 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM cyclic 3′5′-AMP, 0.5 ATP, 1 μCi [α^{32}-P] ATP, 10 mM creatine phosphate, and 50 U/ml creatine kinase. The reaction was stopped by adding 0.1 ml stopping solution (2% SDS, 40 mM ATP, 1.4 mM cyclic 3′5′-AMP).

2.1. *Preparation of liver cytosol*

Livers from two rats (15–20 g fresh wt) were homogenised by eight strokes of a Dounce homogeniser with a Teflon pestle (clearance 0.008) in 20 ml ice-cold buffer containing 20 mM Tris, pH 7.4 and 250 mM sucrose. The homogenate was centrifuged at 100 000 × *g* for 1 h and the clear supernatant dialysed for 18 h at 4°C against two changes of 1 litre 20 mM Tris, pH 7.4, then heated 95°C for 5 min. Denatured protein was removed by centrifugation.

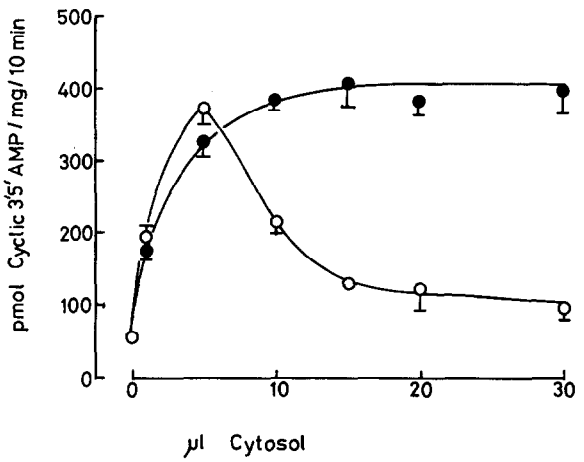

Fig.1. Varying amounts of cytosol containing 12.6 mg/ml protein (o——o) Or heated cytosol containing 1.4 mg/ml protein (•——•) were added. Results are mean ± SEM of three parallel incubations.

3. Results and discussion

It was found that the addition of a dialysed cytosol fraction from rat liver to rat liver plasma membranes increased the activity of adenylate cyclase. It seemed likely that this factor had a relatively high molecular weight since it was retained on dialysis. This conclusion was reinforced by the observation that the activating factor was excluded by Sephadex G 25 implying a molecular weight greater than 3000.

Figure 1 shows the variation of adenylate cyclase activity with increasing concentrations of cytosol. The activity was first markedly increased, passed through a maximum and was then reduced. If the cytosol was heated at 95°C for 5 min the activation was not affected but the subsequent reduction in activity did not occur. There was a considerable purification of the activating factor, the amount of protein required for half-maximal activation being reduced from 284–42 μg/ml. All subsequent experiments employed heated cytosol so as to avoid the complications inherent in attempting to characterise two effects which to some extent appear to overlap. We have not attempted in this study to establish the nature of the inhibition which may result from the presence of proteases in the cytosol preparation or may perhaps be caused by a physiologically important factor.

Table 1 shows the results of experiments undertaken to determine whether or not the activating factor was a protein. The activity was only marginally reduced by incubation of the cytoplasm fraction with trypsin. The proteases pepsin, pronase and nagarse however all completely abolished the capacity of the cytosol preparation to activate adenylate cyclase leading to the conclusion that the activating factor contains protein as an essential component.

The effect of the cytosol factor in the presence of maximally-activating concentrations of other activators of adenylate cyclase was examined (table 2). There was no additional activation in the presence of GTP and the activation produced by the cytosol factor is closely comparable to that produced by GTP. In the presence of GMP–PNP the cytosol factor caused a reduction in adenylate cyclase activity and in the presence of glucagon an increase. In the presence of glucagon and either GTP or GMP–PNP there was no effect and there was little effect in the presence of F^-. The pattern of the response to the cytosol factor was very similar to the pattern of the response of adenylate cyclase to GTP. The factor is ineffective in the presence of GTP and increases the activity in the presence of glucagon. In view of this similarity the possibility that the factor might be GTP was considered. This seemed unlikely for a number of reasons. The cytosol factor is excluded by

Table 1
Effect of cytosol on adenylate cyclase activity

Additions	Adenylate cyclase (pmol/10 min/mg membrane protein)	%
1. None	125 ± 5	100
10 μl Cytosol	375 ± 18	300
10 μl Cytosol pretreated with trypsin	326 ± 10	261
Glucagon 10^{-7} M	332 ± 6	265
Glucagon 10^{-7} M pretreated with trypsin	108 ± 10	86.4
2. None	232 ± 13	100
10 μl Cytosol	721 ± 27	311
10 μl Cytosol pretreated with pepsin	226 ± 13	97
10 μl Cytosol pretreated with pronase	223 ± 20	96
10 μl Cytosol pretreated with nagarse	235 ± 10	104
3. None	87 ± 5	100
10 μl Cytosol	214 ± 12.5	246
10 μl Cytosol preincubated	171 ± 11	196
10 μl Cytosol preincubated with pepsin	74 ± 7	85
10^{-6} M GTP	263 ± 11	302
10^{-6} M GTP preincubated with pepsin	262 + 33	301

Cytosol was prepared as described in the Materials and methods section. Cytosol, 0.01 ml, containing 0.014 mg protein was added. GTP, glucagon and cytosol were preincubated with 1 mg/ml trypsin, nagarse, pronase or pepsin for 15 min at 30°C. In the case of pepsin the buffer was acetate pH 2 and the solution was brought to pH 7.4 at the end of the incubation. For the other three proteases the incubation was in 25 mM Tris, pH 7.4. The reaction was stopped by heating at 95°C for 5 min. Results are mean ± SEM of three observations.

Table 2
Adenylate cyclase activity

Additions	No cytosol (pmol/mg membrane protein/10 min)	10 μl Cytosol (pmol/mg membrane protein/10 min)
None	93 ± 3	278 ± 14
GTP 10^{-5} M	307 ± 12	323 ± 23
GMP–PNP 10^{-5} M	736 ± 73	506 ± 17
Glucagon 5×10^{-6} M	257 ± 16	443 ± 38
Glucagon 5×10^{-6} M, GTP 10^{-5} M	596 ± 23	576 ± 32
Glucagon 5×10^{-6} M, GMP–PNP 10^{-5} M	780 ± 27	849 ± 50
NaF 10^{-2} M	507 ± 10	414 ± 20

Cytosol, 0.01 ml, containing 0.019 mg protein was added. Further additions were as in the table. Results are mean ± SEM of three observations.

Sephadex G-25 and is not dialysable. No steps were taken in the preparation of the cytosol factor to prevent the enzymic hydrolysis of GTP. Incubation of GTP with pepsin under the same conditions which resulted in the complete loss of the ability of the cytosolic factor to activate adenylate cyclase had no effect on the ability of GTP to activate adenylate cyclase (table 1).

The pattern of activation of adenylate cyclase by the cytosol factor is different from the pattern of activation with glucagon and the ability of glucagon to activate adenylate cyclase is abolished by trypsin while the cytosol factor was not affected. We conclude therefore that the activating factor is neither glucagon nor GTP both of which might be expected to be possible contaminants of the preparation.

The time course of the activation is shown in fig.2. There was no lag in the response. The activation was also unaffected by the removal of cyclic 3′5′-AMP from the assay system or by the addition of Ca^{2+} or by the removal of trace Ca^{2+} by the addition of EGTA. If the activation was the result of phosphorylation or dephosphorylation of the adenylate cyclase or of some other type of covalent modification catalysed by an enzyme in the cytosol a lag in the response would be expected. Alterations in the cyclic 3′5′-AMP and Ca^{2+} concentrations would be expected to affect the rate of a phosphorylation or dephosphorylation process. It seems likely therefore that the activation is caused by a direct interaction of the factor with adenylate cyclase or an associated protein rather than by a covalent modification of the enzyme.

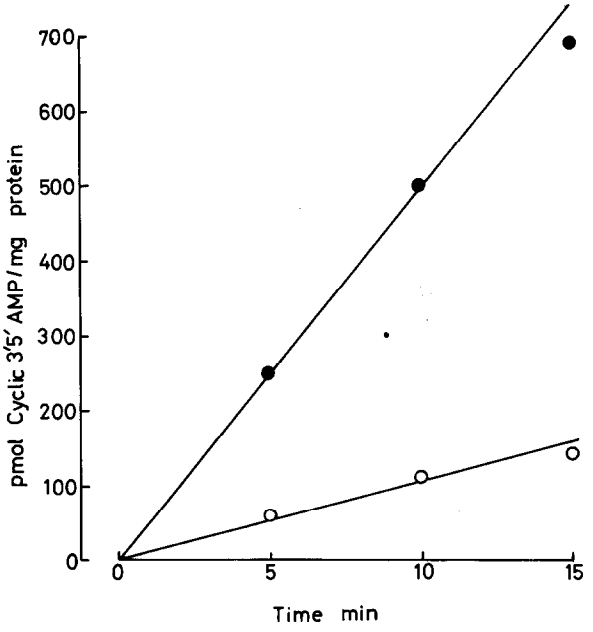

Fig.2. Rat liver plasma membranes (0.5 mg protein) were incubated in 1 ml adenylate cyclase assay medium containing no cytosol (○——○) or 0.15 mg cytosol (●——●). Samples 0.1 ml were removed at timed intervals and mixed with 0.1 ml of stopping solution. Results are means of two parallel incubations.

We have established the existence of a high molecular weight, heat-stable factor in liver cytosol which activates adenylate cyclase and has a protein as an essential component. The pattern of the response shows a marked similarity to the response to GTP suggesting that the factor may act through the same component of the adenylate cyclase system as GTP. In this respect it seems to be similar to the factors described by Sanders et al. [6] and Pecker and Hanoune [7]. The factor described by Pecker and Hanoune [7] is also heat stable but the factor described by Sanders et al. appears to be inactivated by heating. This difference may reflect the different methods used. Sanders et al. [6] used a 10 000 × *g* supernatant from muscle which was not dialysed while we used a dialysed 100 000 × *g* supernatant from liver.

There have been a number of reports of heat-stable cytosolic proteins which are important as factors regulating a variety of cellular processes, notably by controlling the enzymes of glycogen metabolism [11,12]. It is tempting to suggest that the factor described here is of a similar nature but it will be necessary to purify and further characterise the protein in order to determine whether or not this is the case.

Acknowledgement

We are grateful to the Medical Research Council for financial support.

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