THE REQUIREMENT FOR A MEMBRANE COMPONENT TO DEMONSTRATE THE INHIBITION OF CELL-FREE PROTEIN SYNTHESIS BY CYCLIC AMP

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1. Introduction

Work from this laboratory has shown that under conditions of enhanced gluconeogenesis, cyclic AMP promotes a coordinated inhibition of hepatic anabolic pathways such as protein, cholesterol and fatty acid biosynthesis [1,2]. The mechanisms through which these effects of cyclic AMP are mediated has stimulated much recent interest [3–7].

Studies with rat liver slices have shown that cyclic AMP reduces protein synthesis by inhibiting peptide bond formation from ribosome bound aminoacyl t-RNA [2]. Similar results were also obtained with relatively crude cell-free systems from liver [2,8,9]. Inhibition, which was dependent upon the presence of ATP and cytosolic protein kinase, resulted in a stable modification of a component of the microsomal fraction [8].

The demonstration that purified ribosomal *subunits* could be phosphorylated in a cyclic AMP and protein kinase dependent reaction [10], appeared to provide a potential link between the control of protein synthesis and a cyclic AMP promoted modification of the synthetic apparatus. However, an exhaustive search failed to reveal any change in the synthetic capacity of ribosomes reconstituted from phosphorylated *subunits* [11]. On examination of the protein synthetic systems which responded to cyclic AMP, it soon became clear that inhibition was obtained only with cell-free preparations containing a membrane fraction (i.e. microsomes). This prompted us to investigate whether the presence of a membrane component is essential in the response to cyclic AMP. The studies presented in this communication indicate that this is the case.

2. Materials and methods

Conditions for the isolation of the post-mitochondrial supernatant (S_{10}), unwashed microsomes (M_1), washed microsomes (M_2) and cytosolic fraction (S_{100}) from Wistar albino rats have been described previously [8]. The only modification was that 25 mM potassium phosphate, pH 7.0, containing 25 mM KCl and 10 mM $MgCl_2$ was the preferred buffer for preparation of subcellular fractions. To prepare solubilised ribosomes, microsomes (M_1) were resuspended in the preparation buffer containing 0.25 M sucrose. Sodium deoxycholate (10%, w/v) in distilled water was then added to give a final concentration of 1.1%. After 10 min at 4°C, the suspension was centrifuged at 13 000 *g* for 15 min and 3.5 ml of the resulting supernatant was layered onto 3 ml of buffer containing 0.5 M sucrose. Ribosomes were collected by centrifugation at 105 000 *g* for 2.5 hr at 4°C. For protein synthesis measurements, incubations were supplemented with 5 μmol PEP, 3 μmol ATP and 2 μCi (46 Ci/nmol) L-[4,5-^{3}H]leucine for each 1 ml of reaction mixture. Incubations were for 15 min at 37°C. The incorporation of isotope into protein was measured by the procedure of Bloxham and Akhtar [2].

3. Results

Cell-free protein synthesis is frequently performed in Tris–HCl buffers (pH 7.4–7.8), however examination of the literature reveals that this buffer is not necessarily ideal for assaying cyclic AMP dependent protein kinase, e.g. [12]. Therefore, the first part of this investigation was to investigate whether the nature

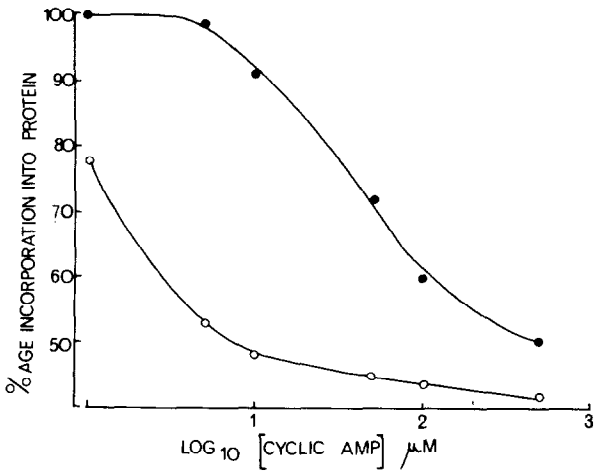

Fig.1. Influence of assay medium on the inhibitory effect of cyclic AMP. Protein synthesis was measured in post-mitochondrial supernatants prepared in either 25 mM potassium phosphate, pH 7.0, containing 25 mM KCl and 10 mM $MgCl_2$ (0-0) or 0.25 M sucrose containing 70 mM Tris–HCl, pH 7.8, 25 mM KCl and 10 mM KCl and 10 mM $MgCl_2$ (●—●). The control rates of incorporation were 2100 dpm/mg protein/15 min and 2800 dpm/mg protein/min respectively.

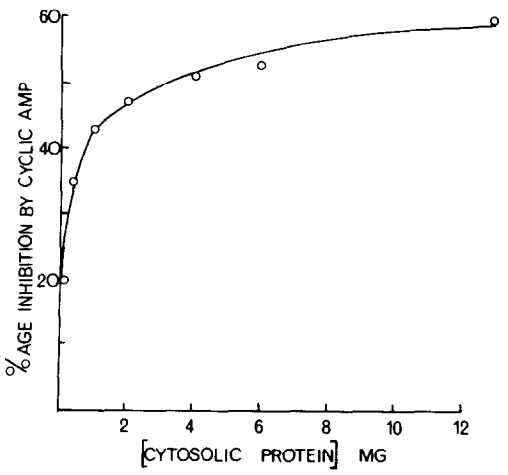

Fig.2. Enhancement of cyclic AMP dependent inhibition of microsomal protein synthesis by the cytosolic fraction. Microsomal protein (2 mg) was incubated with various amounts of cytosolic protein either in the presence or absence of cyclic AMP (100 μM). The percentage inhibition by cyclic AMP was measured relative to the control. Since unwashed microsomes (M_1) were used in this experiment, a basal rate of protein synthesis occurs in the absence of cytosolic protein.

of the incubation buffer would influence the response of the protein synthesising system to cyclic AMP. After testing a variety of buffers we found that potassium phosphate buffered solutions, pH 7.0, provided the optimal system which possessed good synthetic activity and responded to cyclic AMP. Fig.1 illustrates the improvement in the inhibitory response to cyclic AMP in this buffer compared with that in a more coventional medium for protein synthesis (0.25 M sucrose containing 70 mM Tris–HCl, pH 7.8, 25 mM KCl and 10 mM $MgCl_2$). An important consequence of changing the assay buffer is that there is an increase in the sensitivity to cyclic AMP so that the effective concentrations of the nucleotide now approach the expected physiological concentration.

The site of inhibition of protein synthesis by cyclic AMP lies in the microsomal fraction [8]. Since hepatic protein kinase is located predominantly in the cytosol, it follows that a cyclic AMP sensitive system should be reconstituted by mixing together appropriate quantities of microsomal and cytosolic protein. Fig.2 shows that the percentage inhibition due to cyclic AMP increases progressively as the concentration of cytosolic protein is increased. When 12 mg of cytosolic protein was added back to the microsomes the post-mitochondrial supernatant was effectively completely reconstituted and in this case the inhibition (60%) was the same as might be expected in a directly prepared post mitochondrial supernatant. The maximum rate of protein synthesis was obtained with 4 mg of cytosolic protein. Since this system also gave a 45% inhibition of protein synthesis in the presence of cyclic AMP we chose this concentration of cytosolic protein in subsequent experiments.

Having shown that the addition of cytosolic proteins to microsomes enhances the inhibitory response to cyclic AMP, we were able to examine whether a similar effect could be obtained when deoxycholate solubilised (membrane-free) ribosomes were substituted for microsomes. The results of a typical experiment are shown in table1. It must be emphasised that in this experiment all fractions were obtained from a homogenate prepared from a single rat liver. It is immediately apparent that whereas the postmitochondrial supernatant and the microsomal system both respond to cyclic AMP, the ribosomes were unaffected by cyclic

Table 1
Cyclic AMP inhibition of protein synthesis in various cell free systems

Protein synthetic system	Protein synthesis dpm/mg protein/15 min	% inhibition by cyclic AMP
S_{10}	1600	–
S_{10} + cyclic AMP	720	55%
M_1 + S_{100}	1050	–
M_1 + S_{100} + cyclic AMP	620	40%
Ribosomes + S_{100}	850	–
Ribosomes + S_{100} + cyclic AMP	880	0%

Post mitochondrial supernatant (S_{10}, 15 mg protein), microsomes (M_1, 2.5 mg protein), ribosomes (0.6 mg protein) and cytosolic protein (S_{100}, 4 mg protein) were incubated in the appropriate combinations either in the presence or absence of cyclic AMP (100 μM). This experiment was repeated on six separate occasions.

AMP despite the presence of the cytosolic fraction. Since the ribosomes retain a high proportion of their synthetic capacity this result conclusively demonstrates the obligatory requirement for the presence of a membrane component in the development of the observed inhibition of protein synthesis by cyclic AMP.

4. Discussion

The inhibition of hepatic protein synthesis by cyclic AMP provides an interesting example of a system by which protein synthesis could be controlled at the translational level. Previous studies with cell free systems had demonstrated that inhibition was directed at the ribosomal level and resulted in a stable modification of a component of the microsomal system [8,9]. Obviously the most likely candidate for a cyclic AMP promoted chemical modification is a phosphorylation reaction. Thus the report that phosphorylated ribosomes were unchanged in regard to their synthetic capacity [11] was initially disappointing until it was appreciated that this might be an indication that the site of modification might lie elsewhere i.e. a membrane component. We have now shown systematically that such an explanation could account for the observed results. With cell, various fractions prepared sequentially from a single animal in the inhibitory response is lost once the ribosomes are solubilised from the membrane.

This report does not specify the role of the membrane in the inhibitory response to cyclic AMP, however two mechanistic possibilities can be readily envisaged. First, phosphorylation of ribosomes may only be reflected in altered synthetic activity provided the ribosome is attached to a membrane. Second, the covalent modification may occur on a membrane protein which then regulates the synthetic acitivity of the ribosomes. Currently a number of factors point towards the latter theory. Thus ribosome phosphorylation only occurs readily with subunits rather than intact ribosomes [13] and a number of instances of cyclic AMP stimulated phosphorylation of regulatory membrane proteins have now come to light [14–16].

In conclusion, it is shown that the inhibition of hepatic protein synthesis by cyclic AMP is dependent upon the presence of a membrane component. It is worthwhile noting that although our understanding of the precise mechanism of protein synthesis has depended upon the ability to resolve and purify individual components of the synthetic process, it is possible that a similar approach might tend to obscure important regulatory phenomena which are only maintained while the synthetic system retains its integrity.

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