THE MODE OF ACTION OF CYCLIC AMP IN THE RAT ANTERIOR PITUITARY

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1. Introduction

Several of the cell types present in the rat anterior pituitary increase their rates of hormone secretion in conditions likely to be associated with raised intracellular concentrations of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) [1–4]. Furthermore, Steiner and co-authors [5] have shown directly that pituitary levels of cyclic AMP are elevated under conditions of accelerated growth hormone and thyrotropin release; intracellular levels of cyclic AMP therefore appear to be the principal factor in the regulation of secretion in these cells. Since the effects of cyclic AMP in a variety of tissues appear to be related to the binding of this nucleotide to a specific receptor protein which may lead directly to the activation of a protein phosphokinase [6–8], we have investigated the occurrence of a comparable cyclic AMP-binding protein in extracts of rat anterior pituitary and its relationship to protein kinase activity extracted from the same tissue.

In this study a protein has been demonstrated in a 100,000 *g* supernatant prepared from rat anterior pituitary which, after purification by polyacrylamide gel electrophoresis, appeared as a single component possessing both cyclic AMP-binding and protein kinase activity. The cyclic AMP-stimulated kinase was capable of phosphorylating not only exogenous histone but also endogenous pituitary proteins, suggesting a possible role of the receptor/kinase complex in the stimulation of hormone secretion by cyclic AMP.

2. Methods

2.1. *Extraction procedures*

Anterior pituitary tissue obtained from male Sprague-Dawley rats weighing 300–400 g was utilized throughout. In most experiments fragments of tissue were homogenised at 4° in 10 mM sodium phosphate buffer (pH 6.5) containing 0.3 M sucrose, particulate material was removed by centrifugation of the homogenate at 100,000 *g* for 40 min in a Spinco Model L2 centrifuge, the supernatant being decanted and treated immediately with 325 mg ammonium sulphate per ml solution. The resultant precipitate was centrifuged for 15 min under the same conditions as before, the supernatant being discarded. The precipitate was redissolved in buffer containing 10 mM Tris (pH 7.4) and 6 mM mercaptoethanol and dialysed against the same buffer. The extract remaining after dialysis was stored at −20° until required.

Further purification of the crude extract was achieved by polyacrylamide gel electrophoresis in the system of Davis [9], without the use of sample or spacer gels. A final acrylamide concentration of 7% was utilized, electrophoresis at 4 mA per tube being employed to provide a standard 4 cm migration of a bromophenol blue tracker dye. Cyclic AMP binding or protein phosphokinase activities were determined directly in 2 mm segments of the gel.

2.2. *Assay for cyclic AMP binding protein*

The binding of cyclic AMP to tissue proteins was determined in homogenates, soluble extracts or column eluates by the method of Walton and Garren [10]. Distribution of cyclic AMP-binding activity in polyacrylamide gels was determined by soaking the gels for 4 hr at 4° in 50 mM Tris buffer (pH 7.4) containing 8 mM theophylline and 1×10^{-7} M ^{3}H-labelled cyclic AMP (specific activity 6.5 Ci/mM; Radiochemical Centre, Amersham, England); free cyclic AMP was eluted from the gels by washing in water at 4° for 16 hr. The gels were then sliced into 2 mm segments be-

fore dissolving in hydrogen peroxide at 60° overnight; radioactivity in each segment was determined in a liquid scintillation spectrometer after addition of Triton:toluene:PPO scintillant (700:300:5; v/v/w). A similar method of assessing the distribution of cyclic AMP-binding protein in polyacrylamide gels has recently been described by Gill and Garren [11].

2.3. *Protein phosphokinase assay*

Protein phosphokinase activity was measured at pH 6.5 in 0.2 ml of an assay mixture containing the following: 50 mM sodium-DL-β-glycerophosphate, 20 mM potassium fluoride, 0.3 mM ethylene glycol-bis-(β-aminoethylether)*N*,*N*′-tetra-acetic acid, 5 mM theophylline, 3 mg/ml histone (Type IIA Sigma), 0.2 mM γ-^{32}P-adenosine triphosphate (ATP) ($1-3 \times 10^6$ cpm), 10 mM magnesium acetate, and tissue extract with or without 1 μM cyclic AMP. The reaction was started by the addition of enzyme and after 60 min incubation at 37° it was stopped by the addition of 4 ml of 10% w/v trichloroacetic acid (TCA). Carrier protein (0.1 ml of a 5 mg/ml solution of bovine serum albumin) was added to each tube and the precipitated proteins were collected by centrifugation, washed once with 4 ml of TCA, and redissolved in 0.2 ml 2 N sodium hydroxide. The proteins were reprecipitated with TCA and washed once more with TCA. The final precipitate was counted in a Beckman liquid scintillation spectrometer, without the addition of scintillator, by the use of Cherenkov radiation. In order to determine the distribution of enzyme activity in the polyacrylamide gels, 2 mm segments of the gel were added directly to the assay mixture: a similar method of assay of protein phosphokinase activity has recently been described [11].

Protein concentrations in tissue extracts were estimated by the method of Lowry et al. [12] utilizing crystalline albumin standards.

3. Results

Estimations of levels of cyclic AMP-binding protein and of cyclic AMP-dependant protein phosphokinase in homogenates and extracts showed that over 80% of both activities were present in the 100,000 *g* supernatant from tissue homogenates. Centrifugation alone achieved a two-fold purification in binding and protein kinase activity per unit protein. Ammonium sulphate precipitation provided a further enrichment of both activities to four times the levels found in the original homogenates. Considerable further purification was achieved after polyacrylamide gel electrophoresis, the binding and cyclic AMP-stimualted protein kinase activities possessing identical mobilities in the gel system employed (fig. 1).

Material purified by polyacrylamide gel electrophoresis was used in analyses of the characteristics of the binding protein and protein kinase, utilizing histone as substrate for the protein kinase. It was found that the cyclic AMP-binding protein possessed an apparent binding affinity constant for cyclic AMP of 1×10^{-8} M while the cyclic AMP-dependent protein kinase had an activation constant for cyclic AMP of 1×10^{-8} M and a pH optimum at 6.0.

It seemed of some interest to determine whether

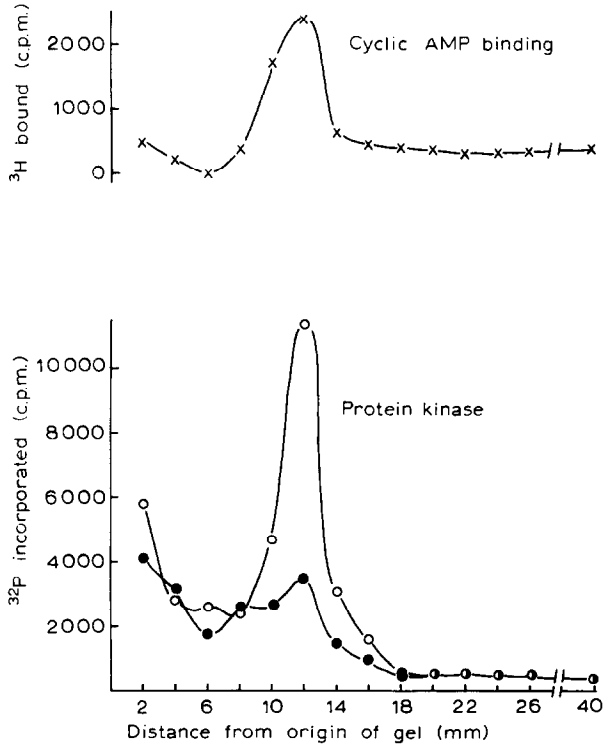

Fig. 1. Distribution of cyclic AMP binding (upper panel) and protein kinase activities of pituitary extracts after purification by polyacrylamide gel electrophoresis. Protein kinase activity was determined in the absence (•) or presence (o) of 1 μM cyclic AMP.

Table 1
Incorporation of γ-^{32}P-ATP into endogenous pituitary soluble proteins in the presence of enzyme purified by polyacrylamide gel electrophoresis.

Additions to assay mixture	cpm ^{32}P incorporated/hr	
	−cyclic AMP	+cyclic AMP
None	124 ± 61 (6)	309 ± 52 (6)*
$CaCl_2$ (1×10^{-4} M)	143 ± 30 (12)	225 ± 21 (12)*
Colchicine (5×10^{-4} M)	113 ± 46 (6)	256 ± 42 (6)*

* Indicates values significantly different ($p < 0.05$) from controls incubated in absence of cyclic AMP, although not significantly different from each other at the $p < 0.05$ level.

the protein kinase present in anterior pituitary homogenates was capable of phosphorylating endogenous substrates as well as the histone used in the experiments already described. For this purpose, pituitary fragments were incubated for 90 min at 37° to permit decay of endogenous cyclic AMP, after which they were homogenized and centrifuged as before for 45 min, aliquots of the supernatant being utilized in place of histone in the protein kinase assay already described. The results obtained in these experiments are shown in table 1. It is clear that there was some phosphorylation of endogenous protein even in the absence of added cyclic AMP, the level being significantly increased in the presence of the nucleotide. Addition of calcium (1 mM) or of colchicine (5×10^{-4} M) was without significant effect on either the basal or cyclic AMP-stimulated protein phosphokinase activity under these conditions (table 1).

4. Discussion

The results reported here provide clear evidence for the presence in the rat anterior pituitary of a soluble cyclic AMP-binding protein associated with a cyclic AMP-dependent protein kinase. Comparable associations between these two activities have been reported in a number of other mammalian tissues, and the characteristics of the binding protein/protein phosphokinase complex of the anterior pituitary appear to be like those of similar complexes derived from brain [6], adrenal cortex [7] or skeletal muscle [8]. We have not so far been able to achieve dissociation of binding protein from phosphokinase activities by polyacrylamide gel electrophoresis following incubation of the complex with 10^{-6} M cyclic AMP, in contrast to the findings of Gill and Garren [11]. This difference might be a consequence of phosphodiesterase activity remaining in our tissue extracts since preliminary results suggest that such a separation may be achieved by gel filtration of the complex on Sephadex G-200 columns eluted in the presence of cyclic AMP [13].

The demonstration of a cyclic AMP-binding protein/phosphokinase complex in the anterior pituitary is of interest in view of the known role of cyclic AMP in the regulation of hormone secretion from this gland. Furthermore, the observation that the phosphokinase can utilize endogenous soluble pituitary protein as substrate suggests that one result of an increase in intracellular levels of cyclic AMP *in vivo* may be activation of the protein kinase; indeed, such an activation has been proposed to account for many of the known effects of cyclic AMP in metabolism [8].

Calcium, which is essential for cyclic AMP-stimulated hormone secretion by the pituitary, but which is without effect on pituitary adenyl cyclase [5], was ineffective in altering the *in vitro* phosphorylation of endogenous substrates. Similarly, colchicine, which induces disaggregation of the microtubular protein and which has been shown to play a role in the secretory process in the pancreatic β-cell [14] and in the thyroid [15], was without effect on the rate of phosphorylation of endogenous substrate; this finding is consistent with the reported lack of effect of colchicine on growth hormone secretion *in vitro* [16].

The secretion of hormones by the cells of the anterior pituitary involves the migration of hormone storage granules to the plasma membrane with subsequent fusion of the granule and plasma membranes and liberation of the granule contents into the extracellular space. The relationship between the elevation of intracellular cyclic AMP levels and these secretory processes is unknown; it will therefore be of some interest to investigate the nature of the endogenous substrates of the cyclic AMP-dependant phosphokinase and their possible roles as activators or even effectors of the secretory mechanism.

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