The Role of Protein Kinase Activation in the Control of Steroidogenesis by Adrenocorticotrophic Hormone in the Adrenal Cortex

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A method has been developed for investigation of the effect of adrenocorticotrophic hormone (ACTH) on the state of activation of a cyclic AMP-dependent protein kinase within cells of the adrenal cortex. Enzyme activity was measured in terms of the quantity of ^{32}P transferred from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to histone under conditions in which bound cyclic AMP did not dissociate from the regulatory subunit of the protein kinase. ACTH (1×10^{-2} i.u./ml) caused a rapid and complete activation of the cyclic AMP-dependent protein kinase activity within 2 min of hormone addition to the isolated cells. In response to a range of ACTH concentrations a sigmoid log dose–response curve for protein kinase activation was obtained, with half-maximal stimulation attained at about 1×10^{-3} i.u./ml. However, some low doses of ACTH that elicited a marked (but sub-maximal) steroidogenic response failed to cause a clear stimulation of protein kinase activity in isolated adrenal cells. Theophylline (2 mM) potentiated the effect of ACTH on protein kinase activity. The results implicate an important role for protein kinase in ACTH action on the adrenocortical cell.

Gill & Garren (1970) reported the characteristics of a partially purified cyclic AMP-dependent protein kinase prepared from bovine adrenal cortical tissue. Cyclic AMP binds directly to a regulatory subunit of the protein kinase enzyme, causing this subunit to dissociate from a catalytic unit, which thereby becomes activated (Gill & Garren, 1971). It has been suggested that a rise in the concentration of adrenocortical cyclic AMP shown to occur in response to ACTH* (Grahame-Smith *et al.*, 1967) leads to an increase in activity of the cyclic nucleotide-dependent enzyme. Little evidence has been presented, however, concerning the state of activity of protein kinase within adrenocortical cells in relation to ACTH steroidogenic stimulation. Recent attention has focused on the biochemical significance of the very small (Mackie *et al.*, 1972*b*) or insignificant (Beall & Sayers, 1972; Moyle *et al.*, 1973) changes in cyclic AMP concentrations, in isolated adrenal cells stimulated with low but steroidogenically effective concentrations of ACTH. These studies emphasize the importance of the detailed relationship between protein kinase activity and the rate of steroidogenesis experienced after exposure of adrenocortical tissue to an extensive range of hormone treatments.

The present paper describes the measurement of protein kinase activity within isolated adrenal cells by a method similar to that developed for measure-

ment in islets of Langerhans (Montague & Howell, 1973). The characteristics of increases in protein kinase activity after stimulation of isolated adrenal cells with ACTH, are compared with the rates of steroidogenesis elicited by the hormone. A preliminary account of some of these findings has been published (Richardson *et al.*, 1973).

Experimental

Materials

The following reagents were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.: adenosine 5'-triphosphate, bovine serum albumin, EGTA [ethanedioxybis-(ethylamine)tetra-acetic acid], disodium β -glycerophosphate and calf thymus histone (type IIA). Collagenase was obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K., and Acthar corticotrophin (ACTH) from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. Human serum albumin was provided by the Lister Institute, Elstree, Herts., U.K. Theophylline was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and cyclic AMP from Calbiochem Ltd., London W.1, U.K. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity 500–3000 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was adjusted before use to the required specific radioactivity with unlabelled ATP.

*Abbreviation: ACTH, adrenocorticotrophic hormone.

Methods

Tissue preparation and incubations. Adrenals from female Sprague-Dawley rats (180–200 g) were decapsulated and quartered. Isolated adrenal cells were prepared from these quarters by digestion with collagenase by a method previously described (Richardson & Schulster, 1972). Electron-microscopic examination of the cell suspension revealed that over 90% of the prepared cells were those of the fasciculata-reticularis region of the adrenal cortex, there being a complete absence of cells either from the zona glomerulosa or adrenal medulla. Capillary cells and erythrocytes comprised the remaining 5–10% of the cell suspension.

Suspensions of the isolated cells in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing glucose (0.2%, w/v) and human serum albumin (0.5%, w/v) were incubated at 37°C under an atmosphere of O₂+CO₂ (95:5) in polystyrene tubes. Compounds added to incubated cells were dissolved in 0.05 ml of 0.9% (w/v) NaCl containing 0.1% (w/v) human serum albumin. Incubates (total vol. 0.5 ml) contained cells isolated from the equivalent of one-quarter of an adrenal gland.

Corticosterone assay. Corticosterone in incubation samples (cells and medium) was assayed by a modified fluorimetric procedure (Sayers *et al.*, 1971).

Assay for protein kinase activity in the incubated cell suspension. After the initial incubation of the isolated cells, a volume (2 ml) of ice-cold Krebs–Ringer bicarbonate buffer (pH 7.4) with glucose (0.2%, w/v) was added and the tubes centrifuged (200 g, 5 min) at 4°C. The supernatant was decanted and ice-cold protein kinase assay buffer (0.5 ml) added to the cells. The protein kinase assay buffer (pH 6.0) was a modification of that used by Walsh *et al.* (1968) and comprised 50 mM-sodium β -glycerophosphate, 10 mM-magnesium acetate, 20 mM-NaF, 10 mM-theophylline, 0.3 mM-EGTA and 1 mg of histone/ml. The contents of each tube were sonicated for 6 s at 0°C and [γ -³²P]-ATP (50 nmol; 1 μ Ci) in protein kinase buffer (0.05 ml) was then added giving a final incubation volume of 0.55 ml. The phosphorylation incubation (5 min, 37°C) was started by warming the tubes to 37°C within 10 min of sonication. Compounds added directly to the reaction mixture for the enzyme assay were dissolved in protein kinase assay buffer. The reaction was terminated by the addition of 2 ml of 10% (w/v) trichloroacetic acid.

The precipitated proteins were collected by centrifugation, washed once with 2 ml of 10% (w/v) trichloroacetic acid and dissolved in 0.5 ml of 0.5 M-NaOH. The proteins were reprecipitated with 2 ml of 10% (w/v) trichloroacetic acid and washed once more with 2 ml of 10% (w/v) trichloroacetic acid. The final precipitate was dissolved in 3.0 ml of 0.5 M-NaOH and the ³²P content estimated by Čerenkov radiation

measured in a Packard 3320 liquid-scintillation spectrometer, the counting efficiency being approx. 25%. Except where indicated, ³²P incorporation into protein was calculated after correction for background ³²P present in trichloroacetic acid-precipitable protein from samples of protein kinase assay buffer incubated in the absence of sonicated adrenal cells. This background count (about 200–400 d.p.m.) usually represented less than 10% of radioactive counts incorporated into histone in the presence of sonicated cell preparations.

Preparation of bovine adrenal microsomal fractions. Two bovine adrenal cortices were homogenized with a Potter–Elvehjem homogenizer in 10 ml of 10 mM-sodium phosphate buffer (pH 6.5) containing 0.3 M-sucrose. The homogenate was centrifuged at 10000g for 10 min at 4°C. The resulting supernatant was centrifuged at 100 000g for 60 min at 4°C and the microsomal pellet obtained was suspended in 1 ml of protein kinase assay buffer.

Protein determinations. Adrenal cells were washed in Krebs–Ringer bicarbonate buffer and the protein content was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard.

Results and Discussion

Activation of protein kinase in cells exposed to ACTH

Addition of ACTH at a concentration (1×10^{-2} i.u./ml) in excess of that needed to stimulate maximum steroidogenesis in the adrenal cell suspension led to a very rapid increase in protein kinase activity (Fig. 1). Within 1 min of adding ACTH an increase of about 50% in protein kinase activity was observed and this stimulation was maximal within 2 min. This increased protein kinase activity was apparent well before the onset of steroidogenesis, since previous studies in this and other systems *in vitro* have shown that there is a delay of about 3 min after ACTH addition before any steroidogenic increase is discernible (Richardson & Schulster, 1972; Pearlmutter *et al.*, 1973). Increased protein kinase activity was observed even though the concentration of cyclic AMP in non-stimulated adrenal cells has been estimated to be 0.3 μ M (Richardson *et al.*, 1973); a concentration higher than that needed to activate fully partially purified adrenal cytosol protein kinase (Gill & Garren, 1970). This apparent anomaly may be due to extensive binding or compartmentalization of cyclic AMP leading to a decrease in the effective free concentration of the nucleotide. Alternatively, the characteristics of activation of protein kinase by cyclic AMP within the adrenocortical cell may be different from those determined in a cell-free system.

The amount of cyclic AMP formed by collagenase-dispersed adrenal cells in response to ACTH

Fig. 1. Time-course of the effect of ACTH (1×10^{-2} i.u./ml) on protein kinase activity of isolated adrenal cells

Isolated adrenal cells were incubated in the presence (●) or absence (▲) of ACTH added at zero time. At the times indicated, cells were rapidly cooled, centrifuged, sonicated and then assayed for protein kinase activity as described in the text, each value shown being the mean of estimates on duplicate incubations. For comparison, the effect of cyclic AMP ($10 \mu\text{M}$) added directly to sonicates from non-incubated tissue is shown by the vertical column. The error bar represents S.E.M. of estimates on quadruplicate incubations.

(1×10^{-2} i.u./ml) over a period of 2 min (Mackie *et al.*, 1972b) resulted in an increase in the concentration of the nucleotide of about 1.1 nM in the sonicates produced from those cells. This increase was much less than the concentration of cyclic AMP (more than 10 nM) needed to stimulate the protein kinase when the nucleotide was added directly to sonicates from non-incubated adrenal cells [the characteristics of activation of protein kinase by cyclic AMP (results not shown) being very similar to those obtained by Gill & Garren (1970)]. This demonstrates that the changes in the activity of protein kinase apparent after addition of ACTH to the adrenal cells were not due simply to activation of the enzyme by cyclic AMP released from the cells during the sonication procedure.

It appeared that hormonal effects on protein kinase activation were not being obscured by inactivation of the activated enzyme after dilution of intracellular cyclic AMP at sonication. Indeed, the following considerations suggest that the original activation state of the protein kinase enzyme within the adrenal cell was preserved after sonication.

(a) The rate of dissociation of cyclic AMP from the

regulatory subunit of the enzyme is very slow at $0-4^\circ\text{C}$ (Gilman, 1970; O'Dea *et al.*, 1971). Thus the amount of bound cyclic AMP that dissociated during the 10 min period at $0-4^\circ\text{C}$ before incubation with [$\gamma\text{-}^{32}\text{P}$]ATP was calculated to be less than 5% of the total bound, assuming that the time required for half dissociation of the cyclic AMP-regulatory subunit complex is about 150 min at 4°C (personal communication from Dr. B. L. Brown, Middlesex Hospital Medical School, London).

(b) Addition of ACTH (1×10^{-2} i.u./ml) to isolated adrenal cells elevated protein kinase activity to the value obtained by direct addition of cyclic AMP to sonicates at a concentration ($10 \mu\text{M}$) sufficient to activate fully adrenal protein kinase (Fig. 1). If inactivation of the protein kinase enzyme of the cells stimulated with ACTH had occurred at sonication, then the ACTH-stimulated value for protein kinase activity would have been less than the value obtained in the presence of cyclic AMP, which gives a measure of the total cyclic AMP-dependent protein kinase activity. These results also demonstrate that high concentrations of ACTH cause complete activation of total cyclic AMP-dependent protein kinase activity.

(c) Results presented in Table 1 provide direct experimental evidence against progressive inactivation of protein kinase kept at 0°C after dilution. Homogenates containing activated and non-activated protein kinase were diluted 120-fold so that the concentration of cyclic AMP decreased to a value (approx. 8 nM in the activated sample) less than that required to stimulate protein kinase directly. The diluted preparations were left at 0°C for various times and then assayed for protein kinase activity. It is notable that even after extended periods (up to 90 min) at 0°C the differential in protein kinase activities between the stimulated and non-stimulated preparations was maintained. It is consequently concluded that the measurements of protein kinase activity presented in this study represent the original states of activation of the enzyme within the intact adrenal cells.

Variation of protein kinase activity with concentration of ACTH

The variation of protein kinase activity with ACTH concentration was studied after exposure of isolated adrenal cells to the hormone for both 2 min and 20 min (Fig. 2). For both time-periods of exposure the sigmoid dose-response curves for protein kinase activation were essentially the same, and similar to that curve obtained when cyclic AMP production in this system is plotted against ACTH concentration (Mackie *et al.*, 1972b). A concentration of ACTH just sufficient to elicit the maximum rate of production of corticosterone caused only a partial (30–40%) increase in protein kinase activity.

Table 1. *Effect of dilution on protein kinase activity*

Adrenal cells isolated from eight rat adrenals were homogenized in protein kinase assay buffer (0.5 ml). Portions (0.2 ml) were incubated (37°C, 5 min) in the absence or presence of cyclic AMP (1 μ M). Each sample was then diluted to 24 ml with the kinase assay buffer and stored at 0°C. At the times indicated, 1 ml samples were taken and incubated (5 min, 37°C) with [γ - 32 P]ATP (50 nmol; 1 μ Ci) and incorporation of 32 P into protein measured as described in the text. Results shown are means of assays on duplicate incubations.

Time after dilution (min)	Protein kinase activity		Ratio
	(pmol of ^{32}P incorporated/min per mg of protein)		
	−Cyclic AMP	+Cyclic AMP	
0	59.5	102.0	1.71
30	50.8	110.7	2.17
60	64.3	114.5	1.78
90	51.6	98.9	1.92

Fig. 2. *Variation of protein kinase activity with ACTH concentration in isolated adrenal cells*

Protein kinase activity was measured as described in the text in the sonicates derived from isolated adrenal cells that had been exposed to ACTH for 2 min (○) and 20 min (●). Corticosterone production (▲) was estimated after 20 min of exposure to the hormone. Where shown, vertical bars refer to S.E.M. of estimates on quadruplicate incubates. Other values shown are means of estimates on duplicate incubations.

It is of interest that some low concentrations of ACTH which caused substantial increases in steroidogenesis led to no discernible change in protein kinase activity. This relates to the insignificant (Beall & Sayers, 1972; Moyle *et al.*, 1973) or very small (Mackie *et al.*, 1972b) increases in cyclic AMP associated with such concentrations of ACTH in the adrenal cell suspension. The precise role of cyclic AMP at these particular submaximal doses of hormone remains very unclear. Several alternative suggestions, however, have been put forward recently by Moyle *et al.* (1973): (a) the steroidogenic effect of

ACTH is mediated by a small fraction of the total intracellular cyclic AMP which is in a separate compartment within the cell, or (b) the steroidogenic effect of ACTH is also mediated by an unknown factor other than cyclic AMP. Results presented here are consistent with these two suggestions but preclude a third possibility that very small changes in cyclic AMP concentrations could simply lead to a magnified signal in terms of overall protein kinase stimulation.

An implication arising from the first of the above suggestions involving compartmentalization of cyclic AMP would be that low concentrations of

Table 2. *Protein kinase activity in an adrenal homogenate: incorporation of ^{32}P into endogenous protein, histone and microsomal protein*

Two rat adrenals were homogenized in 20ml of protein kinase assay buffer minus histone and the homogenate preincubated at 37°C for 30min. Portions (0.4ml) of the homogenate were then incubated (37°C, 10 min) with [γ - ^{32}P]ATP in the presence or absence of cyclic AMP (20 μM). Where indicated, incubates (0.5 ml) contained additional substrate in the form of histone (0.4 mg) or bovine adrenal microsomal material (containing 0.5 mg of protein) inactivated by boiling. Incorporation of ^{32}P into protein was measured as described in the text and corrected for background incorporation into a control sample previously inactivated by boiling for 10min. Results are expressed as means of estimates on duplicate incubations.

Additional substrate	Protein kinase activity		
	(pmol of ^{32}P incorporated/min per mg of protein)		Ratio $\frac{+\text{cyclic AMP}}{-\text{cyclic AMP}}$
	-Cyclic AMP	+Cyclic AMP	
—	5.35	5.28	0.99
Histone	95.2	218.0	2.29
Inactivated microsomal material	12.1	21.0	1.74

ACTH cause localized accumulation of cyclic AMP leading only to the increased activity of a particular species of protein kinase, sufficient to lead to stimulation of steroidogenesis but insufficient to increase overall protein kinase activity as measured in this study. It is of interest that cyclic AMP-dependent protein kinase activity has been demonstrated in a range of cell membrane preparations (Rodnight & Weller, 1972; Guthrow *et al.*, 1972; Johnson *et al.*, 1972; Rubin *et al.*, 1972) and that recent observations in this laboratory (unpublished work in collaboration with R. A. J. McIlhinney) have confirmed the presence of such activity in adrenal cell membranes prepared by the method of Hofmann *et al.* (1970). This raises the possibility that local changes in cyclic AMP concentrations in the vicinity of the adrenal cell membrane specifically alter the activity of membrane-bound species of protein kinase.

Protein kinase activity in the presence and absence of histone

Measurement of protein kinase activity in the presence and absence of histone (Table 2) showed that incorporation of ^{32}P into endogenous protein was very low in comparison with the degree of incorporation experienced in the presence of calf thymus histone. Moreover, the limited incorporation apparent in the absence of additional substrate was not susceptible to stimulation by cyclic AMP. These observations may indicate that in the absence of histone, substrate availability becomes rate-limiting, obscuring changes in protein kinase activity. The lack of effect of cyclic AMP on the rate of phosphorylation

of endogenous proteins may be compared with the lack of stimulation by ACTH (10 munits/ml) or cyclic AMP of the incorporation of [^{32}P]phosphate into cellular protein of intact isolated adrenal cells (Mackie *et al.*, 1972a). It thus appears that even when protein kinase is stimulated in the adrenal cell, the amount of overall protein phosphorylation may not be affected. Intracellular organization may thus impose some specificity on the protein phosphorylation machinery.

Addition of histone to the adrenal homogenate increased ^{32}P incorporation into protein both in the presence and absence of cyclic AMP (Table 2). A smaller increase was seen on addition to the homogenate of adrenal microsomal protein, components of which may act as intracellular substrates for protein kinase (Walton *et al.*, 1971). As such, these substrates would not be expected to cause a dramatic change in the equilibrium between the catalytic and regulatory subunits of the protein kinase enzyme. The fact that the (cyclic AMP-stimulated)/(non-stimulated) ratio for the protein kinase was marginally higher in the presence of histone than with microsomal protein as substrate suggests that histone itself was not causing marked dissociation of the catalytic and regulatory subunits of protein kinase. This process would have raised activity in the absence of cyclic AMP and hence would have obscured the cyclic AMP-dependent increase in activity.

Effect of theophylline on protein kinase activity

The effect of theophylline, an inhibitor of phosphodiesterase, on protein kinase activity in isolated adrenal cells is shown in Fig. 3. This methylxanthine

Fig. 3. Effect of theophylline on protein kinase activity in isolated adrenal cells

Isolated adrenal cells were prepared and incubated as described in the text. Incubations (20 min, 37°C) were carried out in the presence (●) and absence (○) of theophylline (2 mM) over a range of ACTH concentrations. Protein kinase activity was then assayed in the incubated cells as outlined in the text. Values shown are means of estimates on duplicate incubates.

potentiated the effect of ACTH on protein kinase activity in isolated adrenal cells over a range (1×10^{-4} – 3×10^{-3} i.u./ml) of ACTH concentrations. Within the same concentration range both cyclic AMP concentrations and rates of steroidogenesis increase in the presence of theophylline (Mackie & Schulster, 1973). This is an important example of a situation where an increase in protein kinase activity correlates with an elevated rate of steroidogenesis and, as such, lends support to the view that protein kinase stimulation is an important process in the chain of events leading to hormone-induced steroidogenesis.

Concluding remarks

The results presented suggest an important role for cyclic AMP-dependent protein phosphorylation in the regulation of adrenal metabolism by ACTH, since changes in intracellular cyclic AMP concentration induced by the hormone lead to rapid and marked modification of overall adrenal protein kinase activity. Further clarification is needed, however, concerning the role of cyclic AMP and cyclic AMP-dependent protein kinase at certain low, submaximal doses of hormone. The methodology described provides a novel approach for studies on the regulation

of protein kinase activity and may have wide application for the study of cyclic AMP-mediated effects in other hormone-sensitive tissues.

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