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Role of Histone Kinases as Mediators of Corticotropin-Induced Steroidogenesis*

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In an attempt to determine the role of protein (histone) kinases as mediators of corticotropin-induced corticosterone formation, the ability of homogenates, prepared from adrenals treated with various doses of corticotropin to catalyse the phosphorylation of calf thymus histones was measured. Although corticotropin promoted an increase in histone kinase activity, much more of the hormone was required to induce this response than to stimulate steroidogenesis maximally. In addition, a derivative, nitrophenylsulphenyl-corticotropin, which inhibits the stimulatory effect of corticotropin on cyclic AMP accumulation, stimulated corticosterone synthesis without altering histone kinase activity. Very high doses of nitrophenylsulphenyl-corticotropin were capable of stimulating histone kinase activity. In contrast, when dibutyryl cyclic AMP was used to stimulate steroidogenesis under the same conditions, any dose of the nucleotide which increased adrenal corticosteroid content also increased histone kinase activity. Assuming that histones serve as useful substrates for measurement of total adrenal protein kinase activity, the role of protein kinases as mediators of steroidogenesis is not supported by these studies.

Corticotropin has been shown to stimulate adrenal steroidogenesis by enhancing the side-chain cleavage of cholesterol (Stone & Hechter, 1954). Although the mechanism by which this process occurs has been studied extensively, its nature is still elusive. The classic studies of Haynes (1957) and Haynes *et al.* (1959), which demonstrated that corticotropin stimulation promotes adrenal cyclic AMP accumulation and that cyclic AMP treatment stimulates steroidogenesis, suggested a role for cyclic AMP as a mediator of corticotropin-induced steroidogenesis. This concept was extended by Gill & Garren (1970) who found that cyclic AMP could bind to the regulatory subunit of an adrenal protein kinase which led to increased protein kinase activity. Consequently, the hypothesis that corticotropin mediates adrenal steroidogenesis by stimulating cyclic AMP accumulation, which in turn activates protein kinases leading to protein phosphorylation and subsequent cholesterol side-chain cleavage, has gained acceptance.

Although recent studies have not been able to disprove this concept, they have not supported it. For example, Moyle *et al.* (1973) found that little of the cyclic AMP synthesized in response to corticotropin is necessary for stimulation of steroidogenesis. Further, they found that chemical modification of the tryptophan residues of corticotropin drastically decreased its ability to stimulate adrenal cyclic AMP formation, but did not decrease its ability to stimulate steroidogenesis. Although this derivative, nitrophenylsulphenyl-corticotropin proved to be a potent inhibitor of corticotropin-induced cyclic AMP accumulation, it had no effect on corticotropin-stimulated steroidogenesis. These studies suggested that the functionally significant cyclic AMP must be compartmentalized. Thus measurements of total intracellular cyclic AMP seemed to be misleading. For this reason we attempted to measure functional cyclic AMP, assuming that it was bound to adrenal protein kinases. The results presented here indicate that measurement of histone kinase activity or bound cyclic AMP is not equivalent to measurement of functional cyclic AMP. Indeed, it is possible to measure a maximal stimulation of steroidogenesis without detecting stimulation of kinase activity or increased bound cyclic AMP after corticotropin stimulation, even though higher doses of the hormone will stimulate protein kinase activity and accumulation of cyclic AMP.

* Reprints will not be available.

Experimental

Chemicals

All non-radioactive chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.,

except for antiserum to corticosterone which was kindly supplied by Dr. G. Abraham, Harbor General Hospital, Torrance, CA, U.S.A. Pig corticotropin was purified on CM-cellulose columns by the procedure of Pickering *et al.* (1963), and had an activity of 180 i.u./mg. Unless noted, hormones were dissolved in 0.9% NaCl solution containing 1 mg of bovine serum albumin/ml. Nitrophenylsulphenyl-corticotropin was prepared by the procedure of Ramachandran & Lee (1970). Radiochemicals ([1,2,6,7-^{3}H]corticosterone, 80 Ci/mmol; cyclic [^{3}H]AMP, 32 Ci/mmol; [γ-^{32}P]ATP, 10 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A., and were used without further purification. Cellulose acetate filters were obtained from Millipore Corp., Bedford, MA, U.S.A.

Animal treatment and tissue preparation

Male Sprague–Dawley rats weighing 150–160 g, were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.) and maintained on Purina lab chow and water *ad lib.* Animals were hypophysectomized by the parapharyngeal approach at various intervals before corticotropin treatment. The success of hypophysectomy was determined by visual examination of the sella turcica after the experiment was completed. Results from animals not completely hypophysectomized were eliminated from the analysis. Hormone solutions (50 μl) were injected into the descending aorta of ether-anaesthetized hypophysectomized rats anterior to the adrenal arteries. At various intervals, a pair of adrenals was excised and homogenized in 500 μl of a solution containing 780 μg of KH_2PO_4 (pH 6.3), 1.68 mg of EDTA, 450 μg of theophylline, 840 μg of NaF and either 0 or 14.7 mg of NaCl. Portions of the homogenate were taken for protein determination (25 μl), total cyclic AMP determination (25 μl), bound cyclic AMP determination (100 μl), corticosterone determination (3 μl) and protein kinase activity (6 × 20 μl). Proteins were measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Determination of cyclic AMP

Total cyclic AMP was measured by a modification of a protein-binding procedure described previously (Moyle *et al.*, 1973). The portion was transferred to a glass tube (10 mm × 75 mm) and kept in a boiling-water bath for 15 min. The samples were cooled, diluted to 500 μl with water and portions analysed for cyclic AMP content without further treatment as described (Moyle *et al.*, 1973). Bound cyclic AMP was determined by adding the portion of homogenate to 1 ml of water and filtering this mixture through Millipore filters having a pore size of 0.45 μm. After being washed with an additional 1 ml of water, the filters were placed in 10 mm × 75 mm tubes containing 1 ml of water, and the tubes were kept in the boiling-water bath for 15 min. The filters were removed with forceps and the aqueous solution was freeze-dried. The samples were diluted to 100 μl with water and portions were analysed for cyclic AMP content without further treatment.

Determination of corticosterone

Corticosterone was analysed by radioimmunoassay by adding 3 μl of the homogenate to 12 mm × 75 mm tubes containing 0.8 pmol of [^{3}H]corticosterone and sufficient antiserum to bind 0.1 pmol of corticosterone in a total volume of 103 μl. This antiserum does not cross-react with other steroids made by the rat adrenal gland (G. E. Abraham, personal communication). By using the large amount of [^{3}H]corticosterone, the sensitivity of the assay was decreased to allow measurement of the large amount of corticosterone in the adrenal. Standard procedures for radioimmunoassay were used (Moyle *et al.*, 1975). All corticosterone values were determined from standard curves analysed simultaneously with each assay. Similar results were obtained when samples were assayed for corticosterone fluorimetrically as described previously (Peterson, 1957).

Determination of protein kinase activity

Protein kinase was assayed by a procedure involving histone phosphorylation similar to that of Miyamoto *et al.* (1969). Stock solutions of sodium α-glycerol phosphate (532 mg/10 ml, pH 6.5), magnesium acetate (257 mg/10 ml), ATP (17.1 mg/10 ml) and calf thymus histone (20 mg/ml) were stored frozen until use. On the day of the assay, protein kinase assay buffer was prepared by mixing the stock reagents in the proportions 2:1:2:5 (by vol.) and sufficient [γ-^{32}P]ATP was added to provide 2600000 d.p.m./50 μl. Then 5 ml of the resultant solution was added to 10 μl of cyclic AMP solution (3.5 mg of cyclic AMP/ml, neutralized with $NaHCO_3$). At 10 s intervals 20 μl of adrenal homogenate was added to 50 μl of the protein kinase assay buffer without cyclic AMP (three tubes) and with cyclic AMP (three tubes). At 1 min after the start of the assay procedure, 2 ml of ice-cold 20% (w/v) trichloroacetic acid was added at 10 s intervals to stop the reaction. After addition of 0.2 ml of 0.5% bovine serum albumin in water, the 10 mm × 75 mm glass tubes were centrifuged (1000 *g*, 10 min), and the supernatant was discarded. The pellets were redissolved in 0.2 ml of 1 M-NaOH and reprecipitated with 2 ml of 20% trichloroacetic acid. The precipitate was collected on Millipore filters and washed with 2 ml of 20% trichloroacetic acid. The filters were dissolved in 2.5 ml of scintillation fluid (Moyle *et al.*, 1975) and counted for radioactivity. Results were expressed as pmol of phosphate incorporated into protein. When the effect of corticotropin on protein kinase activation was studied, the ratio of the enzymic activity in the absence of cyclic

AMP was compared with that in the presence of exogenous cyclic AMP. One unit of protein kinase activity is defined as the transfer of 1 pmol of phosphate from ATP to proteins (histone)/min.

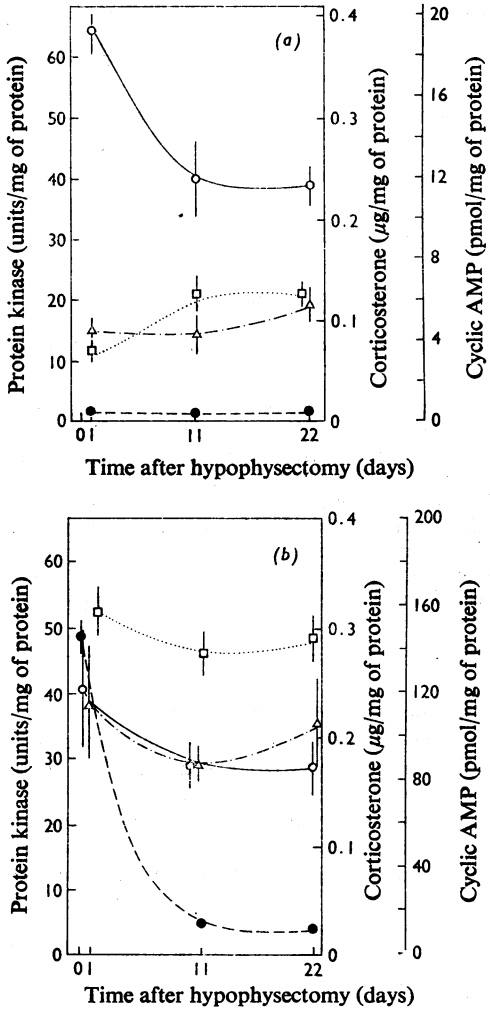

Fig. 1. *Responsiveness of the adrenal after hypophysectomy*

Adrenal protein kinase activity, measured in the absence (endogenous, △) and presence (total, ○) of added cyclic AMP, corticosterone content (●) and cyclic AMP concentrations (□) were measured as described in the text at 1, 11 and 22 days after hypophysectomy. (*a*) In the absence of exogenous corticotropin; (*b*) 5 min after corticotropin (1 μg/rat) injection. Values are the means ± S.D. of six measurements.

Results

The protein kinase assay used in these experiments was chosen because (1) it can be completed to the point of trichloroacetic acid addition within 2 min after homogenization of the adrenal, (2) it is linear with time for 1 min but not longer, (3) it is linear with adrenal protein concentration, (4) it provided sufficient ATP, cyclic AMP and histone and (5) it has a low blank (less than 0.01% of the radioactivity added is measured in the absence of the enzyme). To determine the appropriate time after hypophysectomy to perform these experiments, we studied the concentration of cyclic AMP, protein kinase activity and steroidogenesis at various periods after hypophysectomy. As shown in Figs. 1(*a*) and 1(*b*), corticotropin could stimulate all three parameters at all the intervals examined. In other experiments we noted that the adrenal contained large, often variable,

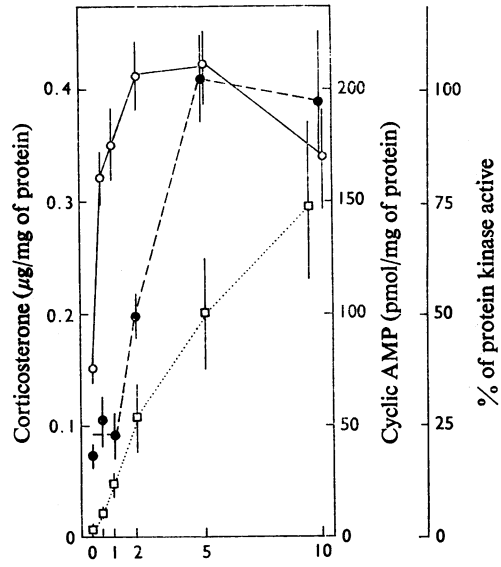

Fig. 2. *Changes in adrenal content after corticotropin treatment*

Adrenal protein kinase activity (ratio of the endogenous to total activity, ○), corticosterone content (●) and cyclic AMP concentrations (□) were measured, as described, 5 h after hypophysectomy at various times after corticotropin (1 μg) administration. Values are the means ± S.D. of six measurements. The change in the ratio of the endogenous to total kinase activity was significant at the earliest time measured (0.5 min) and corresponded to the time when the cyclic AMP content had increased. The first significant increase in corticosterone content occurred between 1 and 2 min after corticotropin treatment.

amounts of corticosterone in the absence of corticotropin treatment when measurement was made at 5h after hypophysectomy (results not shown). Since the largest stimulation of steroidogenesis and histone kinase activity was found 24h after hypophysectomy, most experiments were performed at this time.

High concentrations of corticotropin and nitrophenylsulphenyl-corticotropin increase protein kinase activity within seconds after injection (Figs. 2 and 3). This increase in activity might be responsible for the stimulation of corticosterone synthesis. Nonetheless, lower amounts of either corticotropin or nitrophenyl-sulphenyl-corticotropin administered to the rats stimulated steroidogenesis without altering the protein kinase activity (Figs. 4 and 5). The increase in kinase activity was closely associated with the increase in bound cyclic AMP and in most but not all experiments with total cyclic AMP. In the presence of NaCl in the homogenization buffer, administration of corticotropin but not nitrophenylsulphenyl-corticotropin tended to decrease transiently the total protein kinase activity measured after homogenization. In the absence of NaCl, total protein kinase activity often increased after corticotropin treatment. The biological significance of this NaCl effect is hard to determine, since it was variable and since it did not occur after nitrophenylsulphenyl-corticotropin treatment.

Our inability to demonstrate an increase in histone kinase activity with steroidogenesis surprised us. As shown in Table 1, this dissociation was highly reproducible even though the dose–response curves varied slightly from one experiment to another, depending on the vehicle used to administer the

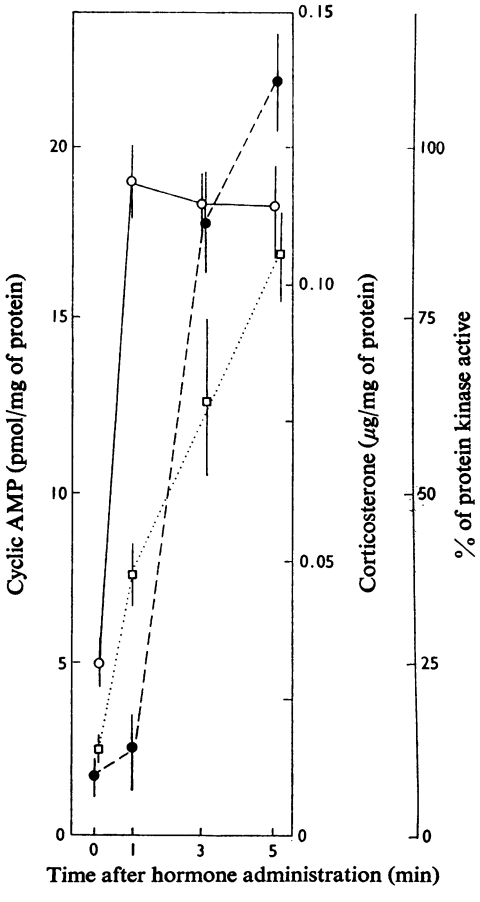

Fig. 3. *Changes in adrenal content after nitrophenylsulphenyl-corticotropin treatment*

Details were as for Fig. 2 except that nitrophenylsulphenyl-corticotropin (30μg) was injected into each rat. Protein kinase activity was maximal at the earliest period measured (1 min), whereas corticosterone synthesis did not increase until sometime between 1 and 3 min.

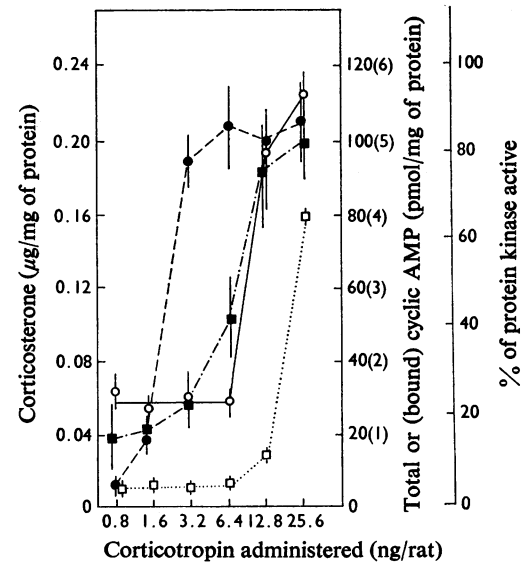

Fig. 4. *Changes in adrenal content as a function of the corticotropin dose*

At 18h after hypophysectomy, protein kinase activity (○), corticosterone content (●), total cyclic AMP content (□) and bound cyclic AMP content (■) were measured 5min after treatment of rats with graded doses of corticotropin. Values are the means ± S.D. of three determinations. Corticotropin at 1.6ng significantly increased the adrenal corticosterone content and at 3.2ng gave maximal stimulation, whereas protein kinase activity was not increased unless a dose between 6.4 and 12.8ng was given.

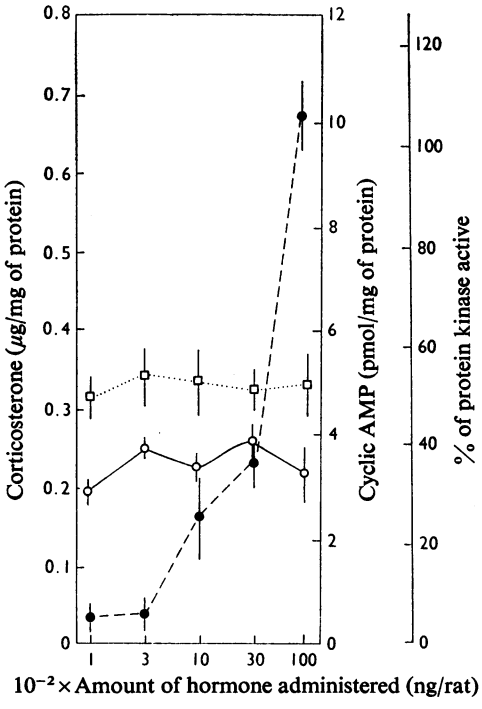

Fig. 5. *Changes in adrenal content as a function of the nitrophenylsulphenyl-corticotropin dose*

Details were as for Fig. 2 except that nitrophenylsulphenyl-corticotropin was substituted for corticotropin. Corticosterone content was increased when 1 μg of nitrophenylsulphenyl-corticotropin was administered, but sufficient amounts of the drug were not administered to elevate protein kinase activity. Other studies indicated that protein kinase activity was elevated by 30 μg of nitrophenylsulphenyl-corticotropin.

hormone. It seemed possible that the stimulation of protein kinase activity could have been transient at lower doses of corticotropin. To test this possibility we studied the temporal response to corticotropin doses which we knew from previous observation (made at 5 min) would stimulate steroidogenesis maximally but would have various effects on protein kinase activity. Fig. 6 shows that there is no transitory rise in histone kinase activity. Corticotropin is capable of stimulating steroidogenesis without first activating histone kinase activity.

In an attempt to determine the role of the adrenal medulla in the protein kinase response observed after corticotropin stimulation, we administered 200 μg of carbamoylcholine to the ether-anaesthetized rats. This drug at much lower doses is known to stimulate catecholamine secretion from the adrenal medulla (Feldberg, 1932). Carbamoylcholine had no effect on corticosterone synthesis and cyclic AMP content. It increased the ratio of endogenous/total histone kinase from 0.31 to 0.44 at 5 min after administration. Similar studies with 2 μg of corticotropin have yielded maximal increases in all three parameters.

If cyclic AMP was mediating corticotropin-induced steroidogenesis, it seemed likely that a similar pattern of histone kinase activation and corticosteroid accumulation should be observed for both corticotropin and dibutyryl cyclic AMP treatment. As seen in Fig. 7, the temporal response of the adrenal to dibutyryl cyclic AMP was similar to that of corticotropin. On the other hand, dose–response curves for dibutyryl cyclic AMP and corticotropin had diverging patterns. At all doses in which dibutyryl cyclic AMP stimulated steroidogenesis, it stimulated histone kinase activity (Fig. 8). Similar results were observed whether or not NaCl was present in the homogenization buffer. Further, when the adrenals were perfused with 0.9% NaCl to remove the residual vascular dibutyryl cyclic AMP before homogenization, the pattern of the dibutyryl cyclic AMP-induced response remained unchanged, indicating that the enzyme activity reflected intracellular activity rather than activation by residual extracellular dibutyryl cyclic AMP present in the homogenate. The effectiveness of the washing procedure was tested by injection of 10 μCi of $Na_2{}^{35}SO_4$ (used to measure extracellular space) at the same time as the dibutyryl cyclic AMP and measuring the ^{35}S remaining in the adrenal before and after perfusion. More than 50% of the ^{35}S was removed by the perfusion technique.

Discussion

We expected to find little correlation between the stimulation of adrenal steroidogenesis and measurable cyclic AMP accumulation in response to corticotropin and nitrophenylsulphenyl-corticotropin treatment, on the basis of previous studies (Moyle *et al.*, 1973). Knowing that cyclic AMP can stimulate adrenal protein kinase activity, we reasoned that if a small undetectable concentration of cyclic AMP was mediating the response to corticotropin we might be able to detect it by correlating steroidogenesis with adrenal protein kinase activity. The previous studies (Moyle *et al.*, 1973) indicated that if cyclic AMP is the physiological mediator of steroidogenesis, then only a small fraction of the total cellular cyclic AMP (compartmentalized or functional cyclic AMP) is actually mediating the corticotropin effect. Apart from the obvious structural barriers to diffusion, such as membranes, binding of cyclic AMP to its receptors may represent such a compartment. Assuming that protein kinases are the class of receptors responsible for compart-

Table 1. *Summary of dose–response relationships*

Various amounts of corticotropin, nitrophenylsulphenyl-corticotropin or dibutyryl cyclic AMP were injected into hypophysectomized rats. Corticosterone, protein kinase activity and cyclic AMP were measured 5 min later as described in the text. Values are the least detectable dose (ng of hormones; mg of dibutyryl cyclic AMP) (LDD) required to stimulate protein kinase activity or cyclic AMP accumulation or are those which stimulate corticosterone synthesis and protein kinase activity maximally (Max.). –, Not assayed.

		Dose required to stimulate				
			Protein kinase activity		Cyclic AMP accumulation†	
Expt. no.	Concn. of NaCl* (M)	Corticosterone synthesis (Max.)	(LDD)	(Max.)	Total (LDD)	Bound (LDD)
Stimulation by corticotropin						
1	0.5	3	10	100	30	–
2	0.5	3–10	10	100	100	–
3	0.5	3	10	100	30	–
4	0.5	2	–	–	20	20
5	0.5	2	6	60	20	6
6	0.5	10	30	100	100	100
7	0.5	2	4	32	>32‡	8
8	0.5	3.2	12.8	35.6	25.6	6.4
9	0	6	6	20	–	–
10	0	3	10	–	–	–
Stimulation by nitrophenylsulphenyl-corticotropin						
11	0.5	§	1000	10000‖	10000	–
12	0.5	§	300	10000‖	10000‡	–
13	0.5	300	–	–	10000‡	–
14	0.5	10000	¶	¶	¶	¶
Stimulation by dibutyryl cyclic AMP						
15	0.5	8	2	16	–	–
16	0.5	8	1	16	–	–
17	0.5	8–16	2	16	–	–

* Adrenals from these assays were homogenized in the presence or absence of 0.5 M-NaCl. Except for the differences in total kinase activity seen after corticotropin stimulation mentioned in the text, the dose–response curves for both conditions were indistinguishable.

† The corticotropin dose required to stimulate total cyclic AMP accumulation maximally was always more than 200 ng/rat.

‡ Marginal increases were found relative to the control value ($P < 0.01$) when assayed by the Student's t test. This was the highest dose tested.

§ Corticosterone concentrations were maximal in the absence of hormone stimulation for unknown reasons. No activation of protein kinase activity in the absence of hormone injection was ever seen.

‖ Protein kinase activity was not completely activated by this dose.

¶ Protein kinase activity and cyclic AMP concentrations were not increased at the highest dose used (10000 ng) in this experiment.

mentalizing cyclic AMP, measurement of cyclic AMP binding or kinase activity should be an index of the amount of compartmentalized cyclic AMP. The results presented in this paper suggest that binding of cyclic AMP to protein kinases is not responsible for corticotropin-induced steroidogenesis. Of course, binding of cyclic AMP to a small fraction of the total adrenal protein kinases would not be detected in these studies. The importance of binding of cyclic AMP to membrane proteins can be questioned in that direct measurements of bound cyclic AMP (which would have included membrane-bound cyclic AMP) paralleled protein kinase activity. Hence, we still have no indication of what the cyclic AMP compartment is, if it exists at all.

One alternative explanation of the results presented here might be that the histone kinase assay used did not reflect protein kinase activity accurately. It is doubtful that histones are involved in the mechanism by which corticotropin stimulates steroidogenesis. Nonetheless, we used histones as our substrate for protein kinase activity for two reasons. First, protein kinases do not appear to have tremendous protein substrate specificity (Miyamoto

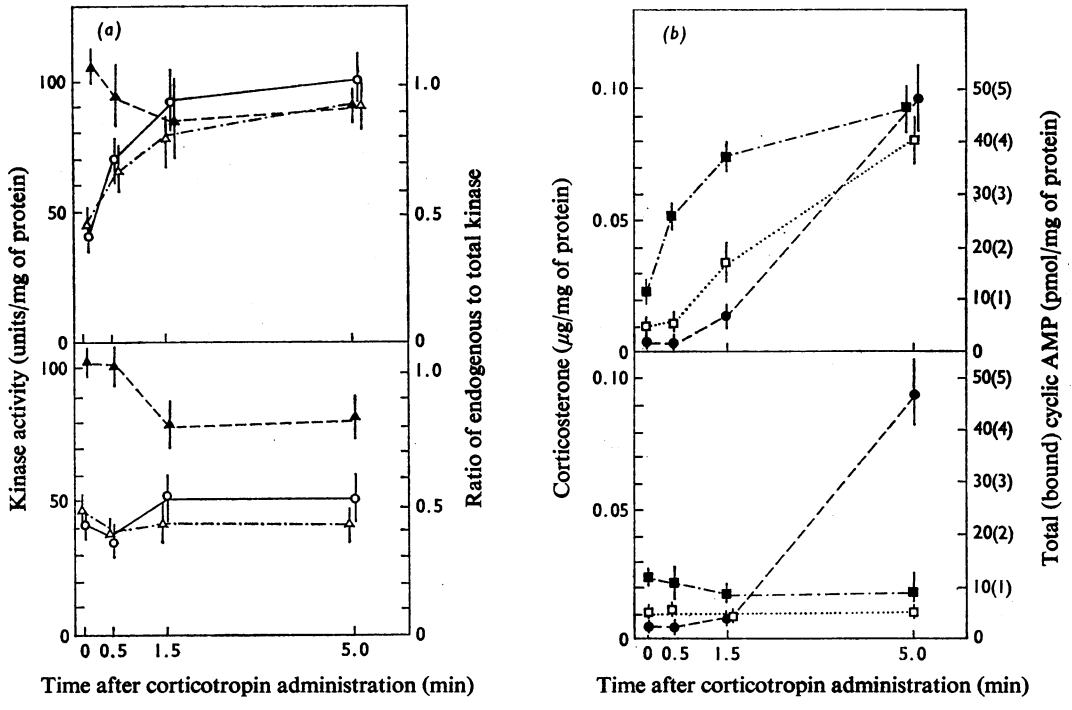

Fig. 6. *Temporal changes in adrenal activities after low doses of corticotropin*

(*a*) Changes in protein kinase activity (total, ▲; endogenous, △; ratio of endogenous to total, ○); (*b*) alteration in corticosterone (●) and cyclic AMP (total, □; bound, ■) concentrations; after injection of 3 or 15ng of corticotropin (lower and upper panels respectively) 18h after hypophysectomy. Values are the means ± S.D. of three determinations. The dose of 3ng of corticotropin significantly altered corticosterone synthesis, whereas kinase activity was not altered unless 15ng of the hormone was administered.

et al., 1969; Langan, 1973). Histone is an excellent protein substrate for kinases from several tissues. Secondly, we do not know the physiological protein substrate(s) phosphorylated in response to small doses of corticotropin, and consequently, have no alternative but to use a substrate which is available. It seems likely that a ribosomal protein (Walton *et al.*, 1971) or a membrane protein might have been a better choice. Future studies in which phosphorylation of proteins with labelled phosphorus is measured should resolve these difficulties. Nonetheless, the apparent lack of specificity shown by the cyclic AMP-activated protein kinases suggests that the results obtained in the present studies are true indications that the bulk of the adrenal protein kinase activity is not involved in steroidogenesis. Perhaps the adrenal has compartmentalized protein kinases, as might be suggested by the tendency that some protein kinases are associated with membranes (Walton *et al.*, 1971), including the plasmalemma of some cells (Rubin *et al.*, 1972).

Adrenal protein kinase binds cyclic AMP and reversibly dissociates into two subunits, one of which has catalytic activity (Gill & Garren, 1970). It is conceivable that we failed to observe protein kinase activation because homogenization of the adrenal causes at least a 20-fold dilution of cyclic AMP concentration, which allowed the regulatory subunit to reassociate with and inactivate the catalytic subunit. This theory is untenable, since doses of corticotropin which had little effect (less than a twofold increase) on total cyclic AMP concentration completely activated protein kinase activity. Thus the homogenization procedure would have diluted the cyclic AMP concentration more than 10-fold relative to that in the intact untreated adrenal. Further, the assay conditions (high salt concentration) were chosen to minimize any subunit recombination (Corbin *et al.*, 1973). When the period between homogenization and assay was increased from a few seconds to several minutes at 0°C, enzyme activity was not altered. In addition similar results were found when the adrenal was frozen before homogenization (results not shown). On the other hand the

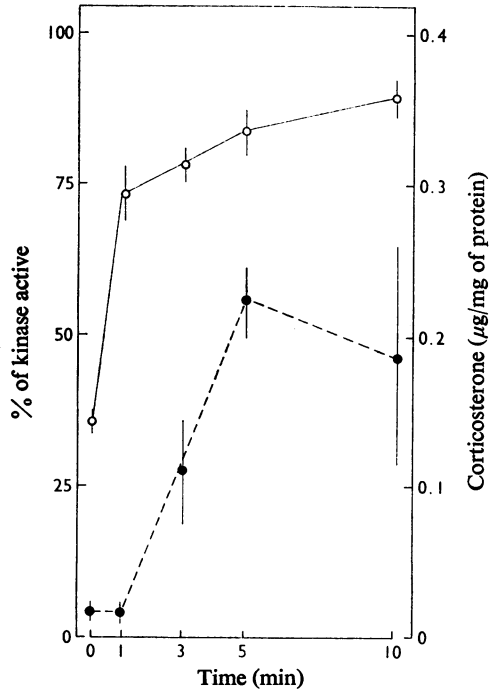

Fig. 7. *Kinetics of dibutyryl cyclic AMP stimulation*

Dibutyryl cyclic AMP (8mg) was injected into hypophysectomized rats. Adrenal protein kinase activity (○) and corticosterone content (●) were measured at the periods indicated on the abscissa as described in the text. Vertical bars extend to the limits of the S.D.

protein kinase responsible for the corticotropin effect might have been activated by 0.5M-NaCl. Such an enzyme has been found in heart muscle (Corbin *et al.*, 1975). Because the pattern of histone kinase activity seen after corticotropin treatment was the same in the presence or absence of salt, we can discount this possibility.

Still another explanation for our failure to find that adrenal protein kinase activity is activated by doses of corticotropin just sufficient to activate steroidogenesis might be that some cyclic AMP-dependent protein kinase activity is present in the medulla (Shima *et al.*, 1974; Guidotti *et al.*, 1975). Three observations argue against this possibility. First, corticotropin stimulates the adrenal cortex, which has a larger amount of cyclic AMP-dependent protein kinase than the medulla (Shima *et al.*, 1974). Secondly, carbamoylcholine stimulation (which promotes adrenaline secretion) had little effect on protein kinase activity and none on steroidogenesis. Thirdly, protein kinase activity was completely stimulated by doses of corticotropin which had no effect on total adrenal cyclic AMP concentration.

The observation that a much larger amount of corticotropin or nitrophenylsulphenyl-corticotropin was required to stimulate protein kinase activity than steroidogenesis implies that activation of protein kinases may not be essential to stimulate steroidogenesis. Further, our failure to find an increase in bound or total cyclic AMP concentrations throughout the dose range in which corticosterone production is stimulated submaximally suggests that elevation of cyclic AMP concentration is not required for augmented steroidogenesis. Only by increasing the

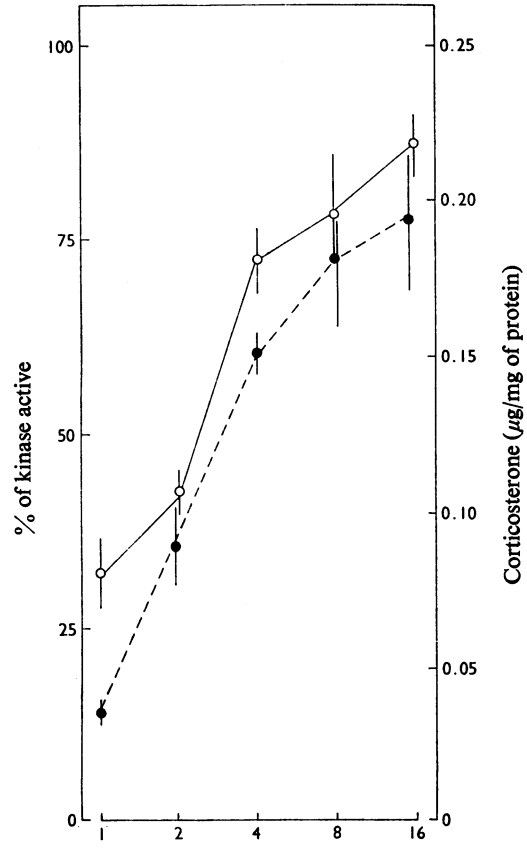

Fig. 8. *Changes in adrenal content as a function of dibutyryl cyclic AMP dose*

Various amounts of dibutyryl cyclic AMP were injected into hypophysectomized rats as listed on the abscissa. Adrenal protein kinase activity (○) and corticosterone content (●) were measured after perfusion of the adrenals with 0.9% NaCl solution. Values are means ± S.D. for three animals.

complexity of the classical second-messenger hypothesis (Sutherland & Robison, 1966), for example to include protein kinases having differing affinities for cyclic AMP, can one argue that activation of adenylate cyclase is responsible for mediating the corticotropin effect. We consider that simpler hypotheses which continue to account for the presence of cyclic AMP and protein kinases can be constructed to explain the results presented in the present paper. One that we favour postulates that cyclic AMP and protein kinases are major cofactors and coenzymes necessary for the corticotropin stimulation of steroidogenesis but which are not directly responsible for the effect. Another, yet unidentified, factor, which requires the presence of cyclic AMP already synthesized, is thought to mediate steroidogenesis in response to low doses of corticotropin. In this idea, changes in cyclic AMP concentrations and protein kinase activity would promote steroidogenesis, but would not necessarily be required for a hormonal effect. We consider that studies similar to those in the present paper using cholera toxin, a protein known to activate adrenal adenylate cyclase (Haksai *et al.*, 1975), or cyclic nucleotides other than dibutyryl cyclic AMP, to stimulate steroidogenesis may help to resolve these hypotheses. In addition, studies suggesting a possible role for 3′:5′-cyclic GMP in adrenal steroidogenesis (Sharma *et al.*, 1974) may provide a clue as to the mechanism of action of corticotropin.

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