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Modulation of the Response of Bovine Adrenocortical Adenylate Cyclase to Corticotropin

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An assessment was made of some of the basic parameters responsible for the modulation of adenylate cyclase activity in a bovine adrenocortical plasma-membrane preparation. When determined at 0.1 mM-ATP, basal adenylate cyclase activity increased with increasing $MgCl_2$ concentrations, whereas in the presence of corticotropin activity was essentially maximal at 10 mM-$MgCl_2$; high concentrations (25 mM) of $MgCl_2$ inhibited adenylate cyclase activity determined in the presence of both corticotropin and GTP. At all $MgCl_2$ concentrations, corticotropin and GTP activated the enzyme in a synergistic fashion. The magnitude of the stimulation of basal activity produced by corticotropin was a function of Mg^{2+} concentration, whereas that produced by GTP appeared largely independent of Mg^{2+} concentration. Adenylate cyclase activity in the bovine adrenal membrane was half-maximally stimulated by corticotropin concentrations in the range 0.3–1.0 nM. The concentration of corticotropin evoking half-maximum response was not significantly affected by raising the free Mg^{2+} concentration from 0.4 to 4.9 mM, nor by the presence of GTP. In the presence of GTP, high concentrations (over 1 μM) of corticotropin inhibited adenylate cyclase activity, although no inhibition was apparent in the absence of guanine nucleotide.

Investigations over the past 10 years have shown that corticotropin stimulates the activity of adenylate cyclase in the plasma membrane of adrenocortical cells, and the resultant increased concentration of cyclic AMP is considered to enhance the rate of corticosteroidogenesis (Grahame-Smith *et al.*, 1967; Mackie *et al.*, 1972; Seelig & Sayers, 1973).

Adenylate cyclases [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] are now recognized as membrane-bound oligomeric complexes which exhibit some features of soluble allosteric enzymes, their activity being regulated by a variety of effectors. Thus, although hormones act via interaction with a specific receptor on the exterior of the target cell, the catalytic activity may also be affected by the intracellular concentrations of Mg^{2+} and H^+, the various ionic forms of ATP ($MgATP^{2-}$ being presumed to be the substrate) and other purine nucleotides (see Hammes & Rodbell, 1976; Rodbell & Londos, 1976).

The present paper describes the modulation of the hormonal activation of adenylate cyclase in a bovine adrenocortical plasma-membrane preparation by some of the factors mentioned above.

Experimental

Materials

The corticotropin used in these studies was Synacthen [corticotropin-(1–24)-tetracosapeptide], which was kindly provided by CIBA, Horsham, Sussex, U.K. [α-^{32}P]ATP (10–12 Ci/mmol; sodium salt) and cyclic [8-^{3}H]AMP (27 Ci/mmol; ammonium salt) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Creatine kinase [rabbit muscle; 150 units (μmol/min)/mg], phosphocreatine (di-Tris salt), ATP (disodium salt), GTP (disodium salt), cyclic AMP (free acid), dithiothreitol, neutral alumina (WN-3) and bovine serum albumin (Cohn fraction V) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Dowex AG-50W (X4; 100–200 mesh; H^+ form) was supplied by Bio-Rad Laboratories, Richmond, CA, U.S.A., and poly(ethyleneimine)-cellulose plates were from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Tritosol (a xylene/Triton X-100-based scintillation 'cocktail') was prepared as described by Fricke (1975). Outdated human serum albumin was generously provided by the Blood Products Laboratory, Lister Institute, Elstree, Herts., U.K., and was extensively dialysed against water before use to remove loosely bound low-molecular-weight contaminants. All other reagents were of AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

Methods

Purified bovine adrenocortical plasma membranes were prepared essentially as described by Glossmann & Gips (1975). In brief, freshly excised bovine

adrenocortical material was homogenized (loose-fitting Dounce, 10–12 strokes) in 20mM-$NaHCO_3$ (4°C). Cell debris and nuclei were removed by a low-speed spin (1500*g*; 10min; 4°C) and the resultant supernatant was centrifuged (20000*g*; 30min; 4°C). The pellet was washed, resuspended in sucrose (0.25M) and layered on a discontinuous sucrose [45% (w/v), 5ml; 40%, 6ml; 35%, 7ml; 30%, 7ml; 25%, 5ml] gradient. All sucrose solutions were prepared in 10mM-Tris/HCl (pH7.6) containing 1mM-dithiothreitol (medium A). After centrifugation (Beckman SW27 rotor; 25000rev./min; 120min; 4°C), the material at the two upper interfaces was aspirated, diluted with medium A (4°C), and harvested by centrifugation (80000*g*; 30min; 4°C). The pellets were resuspended in medium A, quick-frozen and stored at −70°C.

A 4–6-fold enrichment of the specific activity of adenylate cyclase in the purified preparation relative to the homogenate was observed, and the distribution of standard marker enzymes (succinate–cytochrome *c* reductase, acid and alkaline phosphatases and glucose 6-phosphatase) in the various fractions agreed with the findings of Glossmann & Gips (1975).

Adenylate cyclase

Activity was determined at 30°C, essentially as described by Londos & Rodbell (1975), in a 50μl incubation volume that contained [α-^{32}P]ATP (0.6–1.2μCi), 1mM-cyclic AMP, cyclic [^{3}H]AMP (approx. 0.025μCi), human serum albumin (0.2%, w/v), 1mM-dithiothreitol and 25mM-Tris/HCl (pH7.6). The concentrations of membrane protein, ATP, $MgCl_2$, components of the ATP-regenerating system and other agents used are listed in the Figure legends. Unless stated otherwise incubations were initiated by the addition of membrane protein and terminated after 15min by the addition of 'stopping solution' [100μl; sodium dodecyl sulphate (2%, w/v) containing 40mM-ATP and 12.5mM-cyclic AMP, pH7.5]. Reaction rates were linear with respect to both time and protein concentration throughout the ranges used.

Cyclic [^{32}P]AMP was isolated by sequential Dowex–alumina chromatography as described by Salomon *et al.* (1974), incorporating the modifications to the Dowex-column stage proposed by Birnbaumer & Yang (1974). The Dowex columns (1.5ml) were subjected to a rigorous regeneration between experiments (sequential washing with 20ml of water, 10ml of 2M-NaOH, 20ml of water, 10ml of 2M-HCl and 60ml of water). Fresh alumina columns (0.6g) were prepared for each experiment. By this protocol, 5′-AMP, the major contaminant of the isolated cyclic AMP, was removed and radioactivity of reaction blanks was not greater than the counter's background. The cyclic AMP eluted finally from the alumina columns with 0.1M-imidazole/HCl, pH7.5 (recovery 65–80%), was collected directly into counting vials containing 15ml of Tritosol scintillant. Scintillation counting was performed in a Packard Tri-Carb liquid-scintillation spectrometer model 3320 with channel setting as proposed by Rodbell (1972). Unless stated otherwise, results shown are the means of duplicate determinations which were within ±7% of the mean and they represent at least two such experiments. Maintenance of substrate concentrations was assessed by chromatography of portions of the reaction mixture [after removal of protein by precipitation with 50% (v/v) ethanol] on poly(ethyleneimine)-cellulose plates developed with 1M-LiCl as described by Rodbell *et al.* (1971). Nucleotide spots were located under u.v. light, cut out and counted for radioactivity as above. At the ATP concentrations used as a routine, at least 90% of the original substrate remained unchanged after the incubation.

The membrane-protein concentration used in each experiment was determined by the method of Lowry *et al.* (1951), after the addition of trichloroacetic acid (final concn. 7.5%, w/v) to precipitate protein, centrifugation (1500*g*; 10min), and aspiration of the supernatant, which contained dithiothreitol. The pellet was resuspended by heating (30min; 90°C) in 0.1M-NaOH containing sodium dodecyl sulphate (0.1%). Crystalline bovine serum albumin was used as a standard.

Estimation of kinetic parameters

Adenylate cyclase activity (v) can be related to hormone concentration ([H]) by the expression:

$$v - v_0 = \frac{(V_{max.} - v_0)[H]^h}{(K_{0.5})^h + [H]^h}$$

(after Hill, 1913) where v_0 is basal adenylate cyclase activity, $V_{max.}$ is the activity in the presence of saturating hormone concentrations [H], $K_{0.5}$ is the amount of hormone producing a half-maximal stimulation of enzyme activity, and h is the Hill coefficient.

Where indicated in the text, data were fitted to this model by the use of an iterative non-linearizing least-squares analysis. The programme is written in ALGOL 68 for an ICL 1904A computer. Copies of this programme are available from D. M. F. C. on request.

Results and Discussion

Basal and Synacthen-stimulated adenylate cyclase activities were stable during storage (in 10mM-Tris/HCl, pH7.6, containing 1mM-dithiothreitol) at −70°C for up to 2 months. However, the initial

process of freezing and subsequently thawing the membranes caused a loss of 25–40% of basal and hormone-stimulated activities compared with the activities in freshly prepared membranes. The latter could not be used as a routine for adenylate cyclase assays because of the 8h preparation times. Some inter-batch variation, with respect to both absolute adenylate cyclase activities and the magnitude of the maximum stimulation by Synacthen, was noted. Adrenal adenylate cyclase activity, in both the presence and absence of Synacthen, was linear with respect to membrane-protein concentrations, which were several times those used as a routine in the assays. Activity was also essentially linear with time for at least 25min (results not shown).

To find optimum assay conditions for adenylate cyclase activity, the effects of various phosphocreatine concentrations were investigated (ATP concentration of 0.1mM). A minimum phosphocreatine concentration was required, since at concentrations less than 2mM activity was diminished owing to depletion of substrate. At 20mM-phosphocreatine, adenylate cyclase activity was again decreased relative to that observed at 2mM. Both phosphocreatine/creatine kinase (Birnbaumer & Yang, 1974; Londos & Rodbell, 1975) and phosphoenolpyruvate/pyruvate kinase (Garbers & Johnson, 1975) ATP-regenerating systems have been shown to affect adenylate cyclase activity in other mammalian membrane preparations. By the use of the minimum concentration of phosphocreatine required to maintain substrate concentrations we consider that the potential for the appearance of artifacts due to this compound has been minimized.

The activity of adrenal adenylate cyclase as a function of total ATP concentration in the presence of different concentrations of $MgCl_2$ is shown in Fig. 1. [Notwithstanding our observations on the inhibitory nature of high concentrations of phosphocreatine, it was considered necessary to use 20mM-phosphocreatine in this experiment because of the wide range (0.05–5mM) of ATP concentrations involved.] At all ATP and $MgCl_2$ concentrations tested, Synacthen increased basal activity, and this stimulation was itself invariably enhanced by GTP. It should be noted, however, that hormone-stimulated activity was decreased on raising the concentration of $MgCl_2$ from 5 to 25mM, and this effect was even more pronounced in the presence of GTP. Similar inhibitory effects of high Mg^{2+} concentrations on adrenal adenylate cyclase have been observed by Londos & Rodbell (1975) and Glossmann & Gips (1975).

When ATP concentrations substantially exceeded those of $MgCl_2$, adenylate cyclase activity declined (Fig. 1). In the absence of Synacthen adenylate

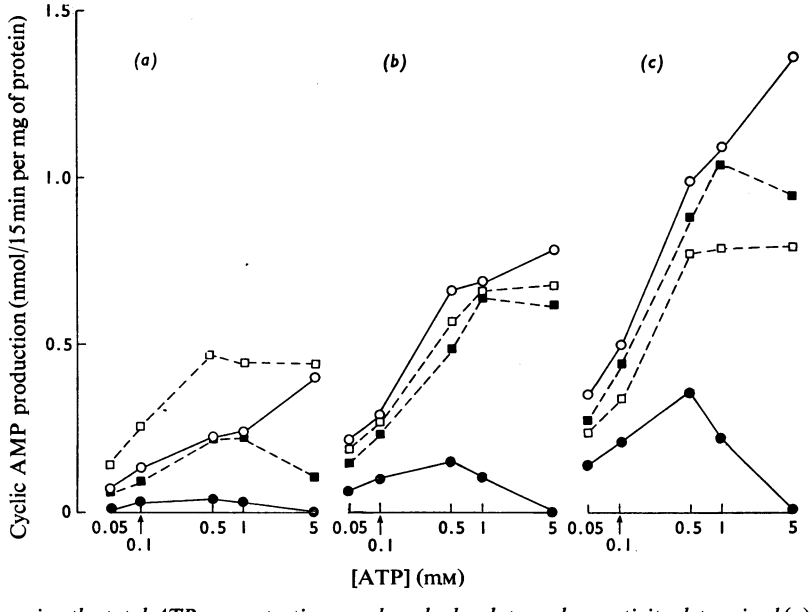

Fig. 1. *Effects of varying the total ATP concentration on adrenal adenylate cyclase activity determined (a) in the absence and (b) in the presence of Synacthen and (c) in the presence of both Synacthen and GTP*

Concentrations of the assay components were: $MgCl_2$ (●, 0.5mM; ■, 2.5mM; ○, 5mM; □, 25mM), phosphocreatine (20mM), creatine kinase (80 units/ml) and, when present, Synacthen (1μM) and GTP (10μM). Membrane protein was 30μg per assay. Other conditions were as described under 'Methods'.

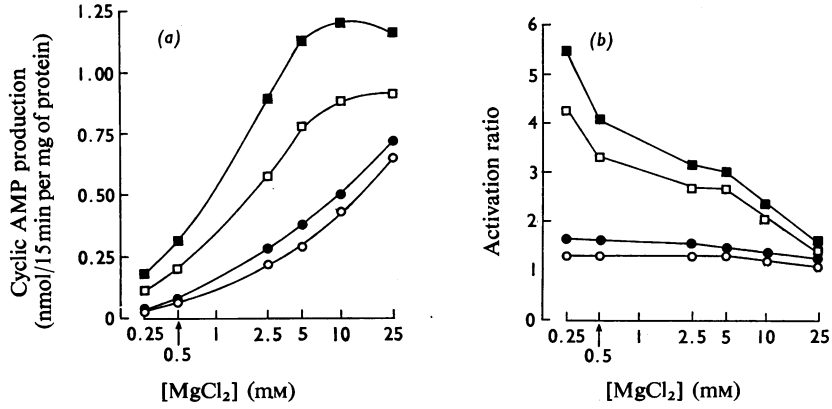

Fig. 2. *Effect of variation of $MgCl_2$ concentration on adrenal adenylate cyclase activity*

(*a*) Concentrations of assay components were: ATP (0.1 mM), phosphocreatine (2 mM), creatine kinase (25 units/ml), membrane protein (19 μg per assay) and, when present, Synacthen (1 μM) and GTP (10 μM). Other conditions were as described under 'Methods'. ○, Basal activity; ●, activity in the presence of GTP; □, activity in the presence of Synacthen; ■, activity in the presence of GTP and Synacthen. (*b*) Data from (*a*) replotted to show Synacthen- and GTP-elicited adenylate cyclase activation ratios as a function of $MgCl_2$. (Activation ratio is the stimulated activity expressed relative to activity in the absence of the effector.) □, ■, Hormonal activation ratio in the absence (□) and presence (■) of GTP; ○, ●, GTP activation ratio in the absence (○) and presence (●) of Synacthen.

cyclase activity (determined at 0.1 mM-ATP) increased as $MgCl_2$ concentration was raised from 0.25 to 25 mM (Fig. 2*a*). In contrast, hormone-stimulated activity was essentially maximal at 10 mM-$MgCl_2$. Increasing $MgCl_2$ concentrations (at 0.1 mM-ATP) caused a relatively greater increase in basal enzyme activity than in Synacthen-stimulated activity, as reflected by the dependence of the hormonal activation ratio on Mg^{2+} concentration (Fig. 2*b*). [The activation ratio is defined as: adenylate cyclase activity in the presence of one particular effector relative to activity (1.0) in the absence of that effector. Thus, for example, the hormonal activation ratio in the presence of GTP is: activity in the presence of both hormone and GTP/activity in the presence of GTP alone.] These observations may indicate either an inhibitory role of unchelated uncomplexed forms of ATP (De Haen, 1974; Rendell *et al.*, 1975) or an activatory function of Mg^{2+} (Garbers & Johnson, 1975).

When the sensitivity to hormonal stimulation of cyclic AMP production in intact adrenal cells (Mackie *et al.*, 1972; Seelig & Sayers, 1973; Peytremann *et al.*, 1973; Finn *et al.*, 1976) is compared with that of the adenylate cyclase activity in membrane preparations from adrenal glands of the same species (Hoffmann *et al.*, 1974; Londos & Rodbell, 1975; Dazord *et al.*, 1975; Glossmann & Gips, 1975), the former system is commonly more sensitive. However, we have consistently found a $K_{0.5}$ value in the range 0.3–1.0 nM for all batches of bovine adrenal membranes. This compares favourably with values determined for half-maximal activation of cyclic AMP production in isolated bovine adrenal cells (approx. 4 nM-Synacthen; Peytremann *et al.*, 1973; Finn *et al.*, 1976). Glossmann & Struck (1976), using essentially the same system as us, reported that when Synacthen was directly included in the adenylate cyclase assay (15 min) the $K_{0.5}$ value was approx. 20 nM, but that if adrenal membranes were preincubated (10 min; 4°C) with the hormone before the assay half-maximal activation was observed at 1.2 nM-Synacthen. Thus the time required to attain a steady-state rate of cyclic AMP production is inversely related to hormone concentration, ranging from rather more than 3 min at 10 pM to approx. 1 min at 1 μM-Synacthen (Fig. 3). When these data are replotted (not shown) to relate hormone concentration to adenylate cyclase activity over the various incubation periods, it is evident that the concentration of Synacthen required to elicit a half-maximal activation of adenylate cyclase decreases from 1 nM at 2 min to a stable value, approx. 0.3 nM, at 5 min. These results underline the importance of equilibration of the system with hormone on the $K_{0.5}$ value that is observed. Bockaert *et al.* (1973) have also noted substantial changes in the values of $K_{0.5}$ and h for pig kidney adenylate cyclase before the establishment of equilibrium between the hormone and the enzyme.

Adrenal adenylate cyclase activity, determined in the presence of either 0.5 or 5 mM-$MgCl_2$, was increasingly stimulated by Synacthen concentrations over the range 10 pM–0.1 μM (Fig. 4). Although absolute activities, at all hormone concentrations,

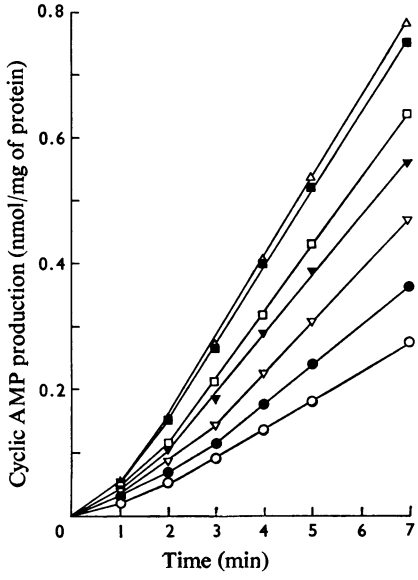

Fig. 3. *Time course of adenylate cyclase activity at various Synacthen concentrations*

Components of the adenylate reaction mixture were preincubated (30°C; 4min). Reactions were initiated by the addition of prewarmed (30°C; 4min) adrenal membranes to produce a final reaction volume of 0.4ml, in which the concentrations of the assay components were: ATP (0.1mM), $MgCl_2$ (5mM), phosphocreatine (2mM), creatine kinase (25 units/ml), membrane protein (1.23mg/ml), Synacthen at various concentrations (○, absent; ●, 10pM; ▽, 0.1nM; ▼, 1nM; □, 10nM; ■, 0.1μM; △, 1μM) and other components as described under 'Methods'. At the indicated times, samples (50μl) were transferred into 'stopping solution' (100μl) for subsequent isolation of cyclic [^{32}P]AMP. Points shown represent the values of individual determinations.

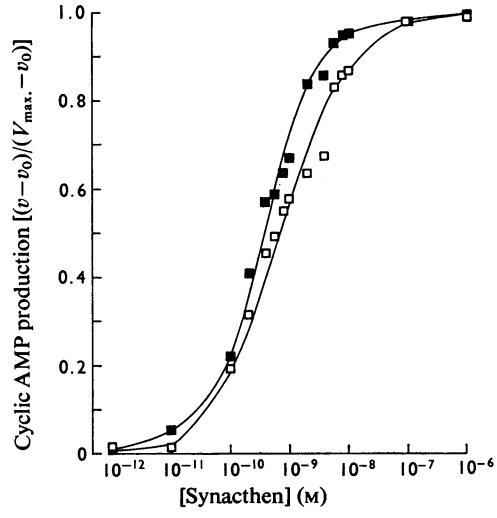

Fig. 4. *Adrenal adenylate cyclase activity as a function of Synacthen concentrations: effect of $MgCl_2$*

Concentrations of assay components were: ATP (0.1mM), phosphocreatine (2mM), creatine kinase (25 units/ml), membrane protein (18μg/assay) and $MgCl_2$ (■, 0.5mM; □, 5mM). Other conditions were as under 'Methods'. Data are expressed as $(v-v_0)/(V_{max.}-v_0)$, and adenylate cyclase activities under these conditions were: ■, $v_0 = 48$, $V_{max.} = 292$; □, $v_0 = 156$, $V_{max.} = 656$pmol of cyclic AMP/15min per mg of protein respectively. Kinetic parameters ($K_{0.5}$ and h) were determined by the procedure described under 'Methods'.

were greater in the presence of 5mM-$MgCl_2$, only relatively minor differences were observed in the hormonal $K_{0.5}$ value and the Hill coefficient (h) at the two $MgCl_2$ concentrations. Thus at 0.5mM-$MgCl_2$ $K_{0.5} = 0.35$nM-Synacthen and $h = 0.84$, whereas at 5mM-$MgCl_2$ $K_{0.5} = 0.66$nM-Synacthen and $h = 0.64$. Birnbaumer *et al.* (1976) have reported a similar lack of effect of $MgCl_2$ concentration on these kinetic parameters for activation of adenylate cyclase from rabbit corpus luteum as a function of lutropin concentrations. In contrast, Roy (1976) has reported that the $K_{0.5}$ value for vasopressin in the activation of kidney adenylate cyclase was raised by an order of magnitude as $MgCl_2$ concentration in the assay medium was lowered from 2.0 to 0.25mM (at 0.2mM-ATP).

GTP (10μM) had no significant effect on the concentration of Synacthen required to stimulate adrenal adenylate cyclase activity half-maximally, although in its presence, activity was enhanced at all hormone concentrations up to 1μM (Fig. 5). An analogous lack of effect of the guanine nucleotide has been described for other adenylate cyclase systems (Birnbaumer & Yang, 1974; Birnbaumer *et al.*, 1976; Hanoune *et al.*, 1975). However, Rodbell *et al.* (1974) found that GTP caused a 10-fold decrease in the $K_{0.5}$ value for glucagon in activating hepatic adenylate cyclase. Interestingly, Glossmann & Struck (1976) have shown that, although the GTP analogue guanyl-5′-yl imidodiphosphate has little effect on the concentration of Synacthen required for half-maximal activation of bovine adrenal adenylate cyclase, the nucleotide caused a 20-fold decrease in the hormonal $K_{0.5}$ value for the rat adrenal enzyme.

One feature of the hormonal dose–response curve that we observed in the presence of GTP, which was not markedly apparent in its absence, was the inhibition of adrenal adenylate cyclase activity by very high concentrations (over 1μM) of Synacthen (Fig. 5). The recent report that catecholamines stimulate a specific guanosine triphosphatase activity in turkey erythrocyte membranes (Cassel & Selinger,

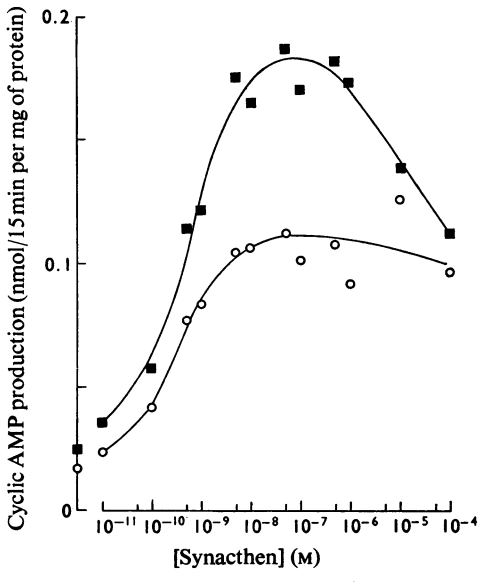

Fig. 5. *Adrenal adenylate cyclase activity as a function of Synacthen: effect of GTP*

Concentrations of assay components were: $MgCl_2$ (0.5mM), membrane protein (29μg/assay) and, when present (■), GTP (10μM) (○, no GTP). Other conditions were as in Fig. 4.

1976) may bear some relevance to this observation, in that, by analogy, high concentrations of hormone in our system may stimulate a guanosine triphosphatase, resulting in lowered GTP concentration and consequently a decrease in the synergism observed between Synacthen and GTP.

In conclusion, the present paper describes an assessment of basic assay parameters for bovine adrenal adenylate cyclase. The particulate enzyme responds sensitively to Synacthen, and this response may be modulated by varying the $MgCl_2$ concentration and by GTP. The system thus seems useful for further studies on the regulation of adrenocortical adenylate cyclase activity.

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