THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 250, No. 4, Issue of February 25, pp. 1186-1192, 1975
Printed in U.S.A.

Adenylate Cyclase System of Bovine Adrenal Plasma Membranes*

(Received for publication, June 24, 1974)

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SUMMARY

The adenylate cyclase system present in a preparation enriched in plasma membranes derived from bovine adrenal cortex was investigated in considerable detail. This system is stimulated by adrenocorticotropic hormone (ACTH), by biologically active analogs of this hormone, and by fluoride ion. The preparation contains sodium-potassium- and magnesium-dependent ATPases that are markedly inhibited by 50 mM sodium fluoride. Incorporation of a pyruvate phosphokinase ATP generating system into the adenylate cyclase assay medium provided constant substrate levels. In the presence of the ATP generating system, the rate of cyclic AMP formation (basal, fluoride, and ACTH-activated) was proportional to enzyme concentration and was linear with time. Proportionality with respect to enzyme concentration as concerned the hormone-activated adenylate cyclase was achieved only when the ratio of hormone to enzyme protein was kept constant. The temperature optimum of the adenylate cyclase, basal or activated, was approximately 30°. Michaelis-Menten kinetics were observed when the ratio of Mg^{2+} to ATP was approximately 6:1. Both calcium and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid completely inhibited the adenylate cyclase system at concentrations of 5 and 0.5 mM, respectively. GTP was inhibitory at concentrations of 10^{-2} M but had little effect at lower concentrations.

Freezing in liquid nitrogen and storage at −60° exerted little effect on the fluoride-stimulated enzyme but lowered hormone stimulated activity. Preincubation in the presence of ACTH afforded a high degree of stabilization of the enzyme system while preincubation with a biologically inactive analog afforded no protection.

The earliest demonstration of the general nature of Sutherland's discovery (1) of the involvement of cyclic AMP[1] in peptide hormone action was provided by the finding of Haynes (2) that cyclic AMP accumulated in beef adrenal cortical slices in response to stimulation by natural ACTH. This observation was extended by Grahame-Smith *et al.* (3) who showed that formation of cyclic AMP preceded steroidogenesis in quartered rat adrenals and that cyclic AMP production could be enhanced by ACTH in rat adrenal homogenates. In the intervening years, a large body of evidence has accumulated in support of the view that cyclic AMP serves as the intracellular "second messenger" for many peptide hormones. In fact the first event between hormone binding to its receptor and physiological response is apparently activation of an adenylate cyclase system. Unfortunately, owing to the location of the mammalian enzyme in the plasma membrane and the problems encountered in attempts to solubilize adenylate cyclase, information regarding the relationship between hormone binding and adenylate cyclase activation is difficult to obtain. Investigations of the enzyme system located in the adrenal cortex have largely been confined to studies carried out with crude adrenal particulates (4, 5) and particulate fractions obtained from an ACTH-sensitive mouse adrenal tumor (6).

We have described (7) a subcellular fraction derived from bovine adrenal cortex shown by electron microscopy, electron microscopic cytochemistry, and marker enzyme assays to be enriched in plasma membranes. Structure-function studies have demonstrated that this preparation specifically binds ACTH and biologically active congeners and that these hormones activate a membrane-bound adenylate cyclase system. Furthermore, a number of ACTH analogs exhibiting low *in vivo* biological activity are capable of binding to the membrane preparation and acting as competitive inhibitors of the ACTH-sensitive adenylate cyclase system (8).

In assessing the relative abilities of various hormone analogues to stimulate adenylate cyclase, it is essential to establish conditions that result in linear reaction rates. The assay is complicated by the presence in the membrane preparation of enzymes that metabolize ATP and cyclic AMP. Although adenylate cyclases derived from various sources share many properties (9), significant differences in levels of associated ATPase and phosphodiesterase activities exist to merit a thorough investigation of these enzymes.

The present communication examines certain parameters of the bovine adrenocortical plasma membrane adenylate cyclase system and the effect of other related phosphate-metabolizing

* This work was supported by National Institutes of Health Grant AM 02811.

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[1] The abbreviations used are: cyclic AMP, cyclic adenosine 3′:5′-monophosphate; ACTH, adrenocorticotropic hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

enzymes present in the preparation on the levels of substrate and product.

EXPERIMENTAL PROCEDURE

Materials—Pyruvate phosphokinase (rabbit skeletal muscle, type II), P-enolpyruvate (trisodium salt, hydrate), dithioerythritol, dithiothreitol, EGTA, and ouabain were purchased from Sigma. Cyclic AMP, ATP (disodium salt), GTP (disodium salt), and [^{3}H]ATP (specific activity, 21 Ci per mmol) were obtained from Schwarz-Mann. Theophylline was purchased from Nutritional Biochemicals, cyclic [^{3}H]AMP (20.7 Ci per mmol) from Amersham-Searle, and AG 1-X2 (200 to 400 mesh) and AG 50W-X4 (200 to 400 mesh) from Bio-Rad. Corticotropin$_{1-24}$ (ACTH) was a gift from Dr. W. Rittel of Ciba-Geigy Corporation, Basel, Switzerland. *N* - Acetyl[Gln^5,*N*$^{\epsilon}$ - formyl - Lys$^{15, 16, 21}$]corticotropin$_{1-23}$ amide (protected ACTH) was prepared in this laboratory (10). [^{3}H]ATP was purified as described (7).

Methods—The bovine adrenocortical plasma membrane preparation was obtained as described (7). Protein was determined according to the method of Lowry *et al.* (11) using bovine serum albumin as standard. Plasma membranes were solubilized in 0.1 N sodium hydroxide containing sodium lauryl sulfate (0.75 mg per ml).

Cyclic AMP was determined by a modification (7) of the method of Krishna *et al.* (12). Cyclic AMP and AMP are simultaneously eluted with 0.1 N H_3PO_4 from the AG 1-X2 columns. Further development of the columns with 0.01 N HCl-0.01 M NaCl (50 ml) allows recovery of ADP and finally ATP is obtained by elution with 0.1 N HCl-0.05 M NaCl (50 ml).

Sodium-potassium- and magnesium-dependent ATPases were determined by the method of Uesugi *et al.* (13). Total ATPase activity was measured in a medium containing NaCl (142 mM), KCl (20 mM), $MgCl_2$ (4 mM), ATP disodium salt (4 mM), and pH 7.5 imidazole-HCl buffer (30 mM). Magnesium-dependent ATPase activity was measured by including ouabain (0.1 mM) in the incubation medium. Reagents were preincubated at room temperatures for 15 min and plasma membrane suspensions containing 0.1 to 0.4 mg of protein were added to initiate the reaction. Total reaction volume was 1.2 ml. Following incubation for 15 min at 37°, the reaction was terminated by addition of ice-cold 5% trichloroacetic acid in chloroform-methanol (1:1, v/v). Following centrifugation, an aliquot of upper phase was withdrawn for determination of inorganic phosphate by the method of Bartlett (14). Sodium-potassium ATPase activity is defined as the difference between total ATPase and magnesium-dependent ATPase activity. Results are expressed in micromoles of P_i released per mg of protein per hour. Enzyme activity was linear with respect to protein concentration and incubation time.

Adenylate cyclase was determined as described (7) except that 0.01 ml of dithiothreitol or dithioerythritol (0.8 M) was added to the membrane suspensions. ATP reaction mixture (0.6 ml) consisted of [^{3}H]ATP (0.5 to 0.6 mM), 0.04 M Tris-HCl buffer (pH 7.3), 0.01 M theophylline, and 4.4 mM $MgSO_4$ (4.4 mM $MgCl_2$ was substituted in experiments dealing with the effects of Ca^{2+} or EGTA). Sodium fluoride, when used, was present at a concentration of 10^{-2} M. ATP generating system contained pyruvate phosphokinase (0.01 ml) and an aqueous solution (50 mg per ml) of P-enolpyruvate (0.1 ml). Final volume of the assay solution was 0.82 ml.

The Ca^{2+} content of the membrane preparation was determined by the method of Diehl and Ellingboe (15) using a Corning model 940 calcium analyzer.

RESULTS

The high ATPase activity of plasma membranes is well documented (16). Indeed, it was in an effort to inhibit these enzymes that the effect of fluoride ion on hormone-sensitive adenylate cyclases was actually discovered (17). Bovine adrenal plasma membranes contain both sodium-potassium- and magnesium-activated ATPases. The specific activities of the two enzymes measured in a series of experiments were 3.15 ± 0.27 μmol of P_i per mg of protein per hour for the sodium potassium and 7.52 ± 1.14 μmol of P_i per mg of protein per hour for the magnesium-dependent enzyme. The decline in enzyme activity with time is illustrated in Fig. 1.

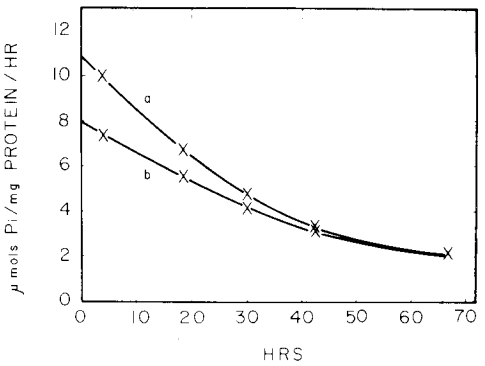


FIG. 1. Plasma membrane ATPase activity. *Curve a*, total ATPase activity; *Curve b*, enzyme activity in the presence of 0.1 mM ouabain (Mg^{2+}-dependent activity). The level of sodium-potassium ATPase activity is obtained by subtracting the values presented in *Curve b* from those in *Curve a*. Membranes were stored at 4° in 1 mM bicarbonate buffer, pH 7.5, and aliquots were withdrawn for assay at the times indicated. The zero hour point refers to the level of enzyme activity measured 30-hour postmortem on purified plasma membranes (see text).

TABLE I

Effect of sodium fluoride on ATPase activity

Additions	ATPase activity at NaF[a] concentrations:		
	0	10 mM	50 mM
	μmol P_i released/mg protein/hr		
None	9.16	4.71	1.79
Ouabain (0.1 mM)	7.85	3.87	0.83

[a] The final concentration of sodium ion was adjusted to 146 mM (see "Experimental Procedure"). In each assay 0.3 mg of membrane protein was used.

As might be expected (18, 19) both types of ATPase activity are inhibited by fluoride ion (Table I). It should be noted that the fluoride ion concentration necessary for full activation of the adenylate cyclase system (10 mM) (8) does not completely inhibit either ouabain-sensitive or ouabain-insensitive activity. The degree of inhibition observed even with 50 mM fluoride ranged from 81 to 94% in various membrane preparations examined.

The efficiency of the pyruvate phosphokinase ATP generating system in maintaining ATP concentration is illustrated in Fig. 2. The generating system preserves substrate both in the presence and absence of sodium fluoride throughout the course of the assay.

ACTH has no detectable effect on the ATPase activity of the adrenocortical plasma membrane preparation. Neither total ATPase nor magnesium-dependent ATPase activity is affected when tested at a hormone to membrane-protein ratio that elicits full adenylate cyclase activity (Table II).

The relation between enzyme concentration (expressed as milligrams of membrane protein) and the rate of cyclic AMP formation was investigated with the unstimulated, as well as the ACTH- and fluoride ion-stimulated enzyme (Fig. 3). All three rates were proportional to enzyme concentration. In the case of the ACTH-sensitive enzyme, a constant ratio between hormone and membrane protein had to be maintained in order to achieve linearity. The rate of product formation was also linear throughout the time course investigated (25 min) both as concerns ACTH- and fluoride ion-stimulated enzyme (Fig. 4).

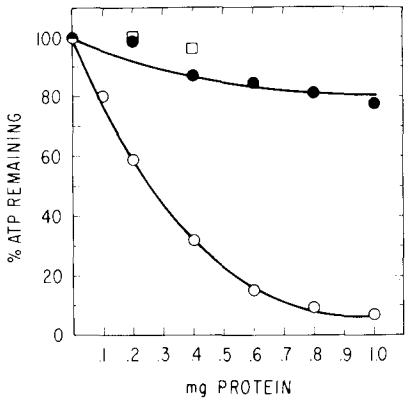


FIG. 2. Influence of ATP generating system on substrate levels of adenylate cyclase assays. ○, incubation in the absence of generating system; □, incubation in the presence of generating system; ●, incubation in the presence of sodium fluoride (10^{-2} M) and generating system. For details see text.

TABLE II

Effect of ACTH on ATPase activity

Additions	ATPase activity	
	Total ATPase	Magnesium ATPase[a]
	μmol P_i released/mg protein/hr	
None	7.97	4.67
ACTH (3.98 μM)	7.93	4.75

[a] Magnesium-dependent ATPase is the enzyme activity remaining after addition of ouabain (see text for details). In each assay, 0.3 mg of membrane protein was used.

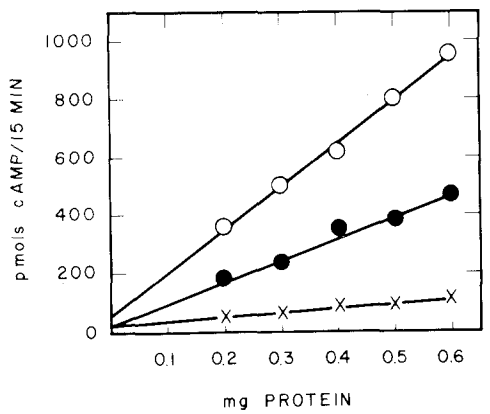


FIG. 3. Cyclic AMP production as a function of plasma membrane protein concentration. ○, fluoride-stimulated adenylate cyclase; incubation solution contained sodium fluoride (10^{-2} M). ●, ACTH-stimulated adenylate cyclase; a constant ratio of hormone to membrane protein (8.13 nmol of ACTH per mg of membrane protein) was employed. This concentration of ACTH is sufficient to activate the adenylate cyclase system fully under the conditions of the assay. ×, unstimulated adenylate cyclase.

Routinely a 15-min incubation time was employed. The ratio of fluoride ion to ACTH stimulation varied considerably from one membrane preparation to another; however, in most instances fluoride stimulation markedly exceeded that observed with the hormone.

The temperature profiles for basal, ACTH-, and fluoride ion-stimulated adenylate cyclase coincide and results from several experiments indicate that the temperature optimum is about 30° (Fig. 5).

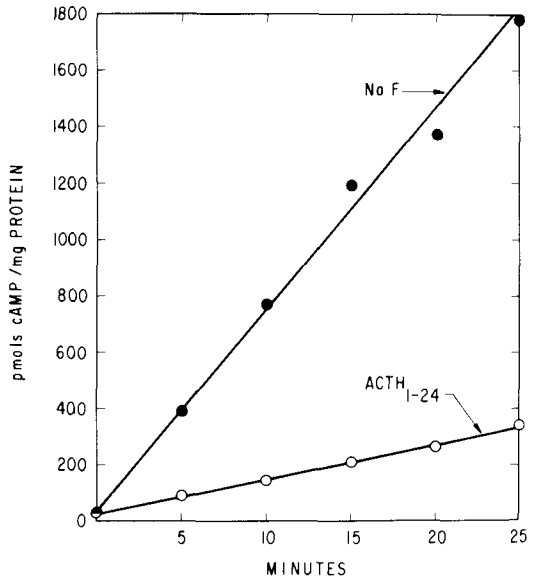


FIG. 4. Formation of cyclic AMP as a function of time. ●, incubation solution contained sodium fluoride (10^{-2} M); ○, incubation solution contained ACTH (3.98 μM). For details of assay see text.

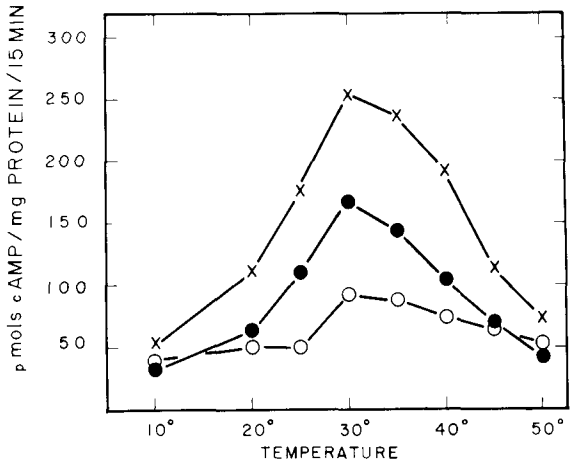


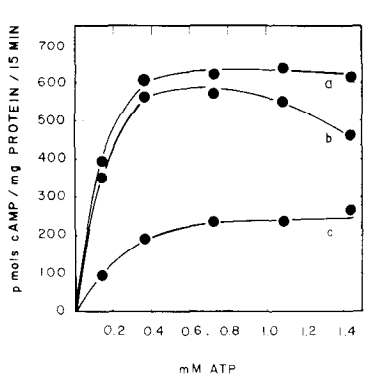
FIG. 5. Adenylate cyclase activity as a function of temperature. ×, fluoride-stimulated (10^{-2} M); ●, ACTH-stimulated (3.98 μM); ○, basal activity.

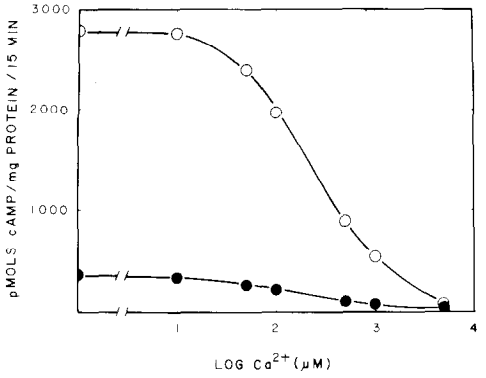
The relationship between ATP concentration and adenylate cyclase activity at constant and varying concentrations of added $MgSO_4$ is shown in Fig. 6.

The effect of added $CaCl_2$ on membrane adenylate cyclase is shown in Fig. 7. A significant decrease in the activity of ACTH-stimulated cyclase occurred in the presence of 50 μM Ca^{2+} and complete inhibition was observed when the calcium concentration reached 5 mM. Basal activity also was abolished at this concentration.

Addition of the calcium complexing agent, EGTA, had a similar inhibitory effect on the activity of the hormone sensitive cyclase (Fig. 8). However, basal activity was somewhat elevated by the chelating agent. The two activity curves converged at an EGTA concentration of approximately 500 μM with the greatest changes in both activities occurring when the EGTA concentration was between 10 and 100 μM.

The calcium content of several membrane preparations ranged from 0.02 to 0.06 μmol per mg of protein. Thus, using 0.4 mg of membrane protein for adenylate cyclase assay, the total calcium added with the membranes did not exceed 10 to 29 μM.





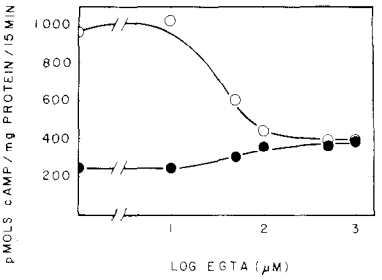


FIG. 6 (*left*). Effect of substrate and Mg^{2+} concentration on adenylate cyclase activity. *Curve a*, ACTH-stimulated, ratio of Mg^{2+}:ATP was 6:1. *Curve b*, ACTH-stimulated, Mg^{2+} concentration was 4.4 mM. *Curve c*, unstimulated, Mg^{2+} concentration was 4.4 mM. ACTH when present was 3.98 μM.

FIG. 7 (*center*). Effect of added Ca^{2+} on adenylate cyclase activity. ○, ACTH stimulated; incubation solutions contained ACTH (3.98 μM). ●, unstimulated. The *points* on the ordinate represent the adenylate cyclase activity in the absence of added Ca^{2+}.

FIG. 8 (*right*). Effect of EGTA on adenylate cyclase activity. ○, ACTH stimulated; incubation solutions contained ACTH (3.98 μM). ●, unstimulated. The *points* on the ordinate represent the adenylate cyclase activity in the absence of EGTA.

TABLE III

Phosphodiesterase activity of plasma membranes

Plasma membrane pellets containing 0.4 mg of protein were prepared in the usual manner. To each pellet was added 0.01 ml of dithioerythritol (0.8 M), 0.01 ml of pyruvate kinase, 0.1 ml of P-enolpyruvate (50 mg per ml), and 0.1 ml of 0.1% bovine serum albumin (pH 7.5) in the order given. The reaction was initiated by addition of 0.6 ml of a solution containing pH 7.3 Tris-Cl buffer (0.04 M), $MgSO_4$ (4.4 mM), 0.06 μCi of cyclic [^{3}H]AMP, and cyclic AMP as shown. Total volume of reaction mixture was 0.82 ml. Recovery of cyclic AMP (usually 90%) was determined using boiled plasma membranes.

Cyclic AMP added	Theophylline (10^{-2} M)	Cyclic AMP remaining
nmol		*nmol*
0.17	+	0.14
0.17	−	0.05
1.70	+	1.63
1.70	−	1.20
17.00	+	16.90
17.00	−	12.90

Phosphodiesterase, an enzyme originally considered to be located exclusively (20) in the cytosol, recently has been shown to exist in at least two kinetically distinguishable forms (21), one of them associated with plasma membranes (22). The phosphodiesterase activity of the bovine adrenal plasma membrane preparation was examined at several cyclic AMP levels (Table III). At concentrations in the range normally produced in our adenylate cyclase assay (0.17 nmol) a significant proportion of the cyclic AMP was destroyed when theophylline was omitted from the incubation medium. Even in the presence of the inhibitor, complete recovery of added cyclic [^{3}H]AMP was not achieved when the nucleotide was present in low concentrations.

Additions of various amounts of GTP to the bovine adrenocortical plasma membrane preparation failed to alter basal or hormone-stimulated activity significantly until the guanine triphosphate concentration was 10- to 30-fold in excess of the ATP concentration (Table IV). At GTP concentrations of 10^{-2} M complete inhibition occurred.

The stability of the adenylate cyclase system was investigated under a variety of conditions. Gradual inactivation resulting in total loss of activity occurred in every instance when the membranes were stored at 4°. Storage at −15° produced immediate (upon freezing) inactivation. Optimal stability resulted when the membranes were frozen rapidly in liquid nitrogen and stored at −60° (Table V). Under these conditions fluoride-stimulated activity was practically quantitatively retained while basal and, to a larger extent, ACTH-stimulated activities decreased.

TABLE IV

Effect of GTP on adenylate cyclase activity

The assay solution contained in 0.82 ml: 0.4 mM [^{3}H]ATP, 0.03 M Tris-Cl (pH 7.3), 0.01 M theophilline, 3.2 mM $MgSO_4$, 0.01 M dithiothreitol, 0.01 ml of pyruvate kinase, 0.1 ml of P-enolpyruvate (50 mg per ml), and GTP (disodium salt) at the concentrations indicated. The reaction was initiated by addition of plasma membranes (0.4 mg of protein in 0.3 ml of 1 mM sodium bicarbonate, pH 7.5).

GTP molarity	Cyclic AMP	
	− ACTH	+ ACTH[a]
	pmol/mg protein/15 min	
0	229 (±16)	422 (±20)
10^{-8}	239 (±7)	398 (±26)
10^{-6}	267 (±13)	507 (±11)
10^{-4}	290 (±4)	548 (±31)
10^{-2}	30 (±4)	34 (±6)

[a] ACTH concentration, 3.98 μM.

Experiments designed to determine whether proteolysis plays a major role in terminating the interaction between ACTH and bovine adrenocortical plasma membranes led to unsuspected findings. The results presented in Table VI indicate that a marked deactivation of adenylate cyclase occurred when the membrane preparation was preincubated in the presence of ATP generating system, dithiothreitol, and bovine serum albumin for 45 min at 30°. Inclusion of ACTH in the preincubation medium afforded significant protection of the enzyme system. Protected ACTH, a biologically inert peptide that does not bind to the membranes (23), failed to stabilize the enzyme.

DISCUSSION

The ACTH-sensitive adenylate cyclase system of the adrenal cortex has been studied in crude systems by many investigators (4–6). Scant information is available pertaining to the validity

TABLE V

Effect of freezing on adenylate cyclase activity

Control samples were assayed in the usual manner. Samples for freezing were prepared as follows. An aliquot (0.4 ml) of plasma membranes suspended in 1 mM sodium bicarbonate buffer, pH 7.5 (1 mg of protein per ml), was mixed with 0.15 ml of a buffer containing pH 7.3 Tris-Cl (0.12 M), theophylline (0.03 M), and $MgSO_4$ (13.2 mM). To this was added 0.01 ml of dithiothreitol (0.8 M) and either 0.1 ml of 0.1% bovine serum albumin (pH 7.5), albumin containing 3.25 nmol of ACTH, or albumin containing sodium fluoride (82 mM). The suspensions were rapidly frozen in liquid nitrogen and stored at −60°. After 1 week, the frozen suspensions were thawed at 37° and 0.05 ml of the above buffer containing [^{3}H]ATP (5.89 mM) and ATP generating system (0.11 ml) was added. Adenylate cyclase activity was measured as described in the text. Figures in parentheses refer to per cent of initial activity.

Additions	Cyclic AMP[a]			
	Experiment 1		Experiment 2	
	Control	Frozen	Control	Frozen
None	137	114 (83%)	196	163 (83%)
ACTH	318	207 (65%)	568	385 (68%)
NaF	773	755 (98%)	1536	1485 (97%)

[a] Measured in picomoles of cyclic AMP per mg of protein per 15 min.

TABLE VI

Stabilization of adenylate cyclase by ACTH

Membrane pellets (0.4 mg of protein each), prepared in the usual manner, were preincubated at 30° for 45 min in a solution containing ATP generating system (0.11 ml), 0.8 M dithiothreitol (0.01 ml), and 0.1% bovine serum albumin, pH 7.5 (0.1 ml). Where indicated, ACTH (3.25 nmol), protected ACTH (3.25 nmol), or sodium fluoride (8.2 μmol) in 0.1% albumin (0.1 ml) replaced the albumin solution. After preincubation a second addition of the above solutions of either albumin the peptides or NaF was made as shown. Adenylate cyclase assays were performed as described. Final volume was 0.82 ml.

Additions to preincubation medium	Additions to assay medium			
	None	ACTH	Protected ACTH	NaF
	pmol cyclic AMP/mg protein/15 min			
Control[a]	231	1568 1578[b]	260	1415 1224[c]
None	192 (83%)	689 (44%)		1151 (81%)
ACTH	1536 (98%)	1534 (97%)		
Protected ACTH	205 (79%)	806 (51%)		1100 (78%)
NaF	1268 (90%)			962 (79%)

[a] No preincubation.

[b] An additional portion of ACTH (3.25 nmol) was added.

[c] An additional portion of NaF (8.2 μmol) was added. Figures in parentheses refer to per cent of the appropriate control value.

of adenylate cyclase assays that are performed with such preparations. The present investigation defines apparently optimal conditions for assay of ACTH- and fluoride ion-sensitive adenylate cyclase in a bovine adrenocortical preparation enriched in plasma membranes. Information pertaining to substrate levels, proportionality of enzyme concentration and rate, as well as linearity of the reaction with time is critical for the development of an adenylate cyclase assay, particularly since the adrenal membranes contain ATPases and phosphodiesterase that influence the levels of substrate and product.

The specific activities of sodium-potassium- and magnesium-dependent ATPases in adrenocortical plasma membranes are somewhat lower than is observed in plasma membranes from other sources (24–26). This may be attributed to inactivation during the isolation procedure employed for obtaining purified membranes. When the membrane preparation is stored at 4° in 1 mM bicarbonate buffer, pH 7.5, the decrease in enzymic activity follows first order kinetics with the rate of inactivation of the ouabain-sensitive enzyme being higher (Fig. 1). It is reasonable to assume therefore, that both the specific activities of the enzymes and the per cent contribution to activity of the sodium-potassium ATPase are lower than would be observed with fresh plasma membranes. The point designated as zero time in Fig. 1 corresponds to the time when purified plasma membranes are collected from the zonal rotor and is actually obtained 30 hours postmortem: unfortunately, extrapolation of the first order activity-decay curve cannot be used for assessing the actual ATPase content since the tissue is not maintained at the same conditions during the isolation procedure.

Although the ATPases display only a fraction of their original activity at the time the purified plasma membranes are harvested, the residual activity is sufficient to warrant inclusion of an ATP generating system in the adenylate cyclase assay medium to maintain approximately constant substrate levels (Fig. 2). In the absence of a generating system, less than 40% of the substrate is available to the cyclase after 15 min of incubation using 0.4 mg of membrane protein. The pyruvate phosphokinase ATP generating system effectively maintains a constant level of substrate both in the presence and absence of fluoride ion. Over 95% of added ATP is preserved throughout the incubation period using standard conditions (see text) for adenylate cyclase assay. The proportion of substrate converted to cyclic AMP is approxiately 0.5% of the total ATP added.

Hadden *et al.* (27) have reported that both norepinephrine and insulin stimulate human lymphocyte plasma membrane sodium-potassium ATPase activity. Stimulation of plasma membrane ATPases by ACTH was not observed in the present study (Table II).

The relationship between enzyme activity and ATP concentration is a complex one depending, as well, on magnesium concentration. Taunton *et al.* (6) working with a particulate preparation derived from an ACTH-sensitive mouse adrenal tumor, reported that no adenylate cyclase activity could be detected in the absence of Mg^{2+}; however, these authors observed a decrease in ACTH and sodium fluoride-activated enzymic activity when Mg^{2+} was present in concentrations exceeding that of ATP and concluded that free ATP was the true substrate. In other tissues (9, 28) where the relationship between Mg^{2+} and ATP has been studied, quite the opposite has been found, namely that enzyme activity is inhibited when ATP concentration exceeds that of Mg^{2+}. Consequently, a Mg^{2+}-ATP complex is now considered to be the substrate for most of the adenylate cyclases investigated. The data obtained with ACTH-stimulated adrenocortical adenylate cyclase (Fig. 6) are in agreement with this postulate. At a fixed level of Mg^{2+} inhibition occurs even before ATP concentration exceeds that of Mg^{2+} whereas, when a constant molar ratio of Mg^{2+}:ATP (6:1) is maintained, a typical Michaelis-Menten relationship between reaction velocity and substrate concentration is obtained. The apparent K_m for ATP measured with a constant Mg^{2+}:ATP ratio (Fig. 6, *Curve a*) is

approximately 0.1 mM for the adrenal system. The apparent K_m for ATP of other adenylate cyclases is reported to be in the range of 0.08 to 0.5 mM (9).

The stimulatory effect of calcium on steroidogenesis in adrenal tissue preparations is well recognized (29, 30). More recently Sayers *et al.* (31) have shown with rat adrenal cells that both steroidogenesis and cyclic AMP formation are enhanced when the cells are incubated in the presence of 7.65 mM calcium. The cation produces quite a different effect on the adenylate cyclase system of isolated adrenal plasma membranes. The addition of calcium at concentrations as low as 50 μM causes inhibition of cyclic AMP formation (Fig. 7), although some calcium must indeed be necessary for adenylate cyclase activity since the same concentrations, *i.e.* 50 μM of the calcium chelating agent, EGTA, significantly inhibits ACTH activated cyclase (Fig. 8). Since the membrane preparation contained approximately 0.06 μmol of Ca^{2+} per mg of protein, the basal concentration of the cation in the assay medium was 30 μM. Lefkowitz *et al.* (32) found that EGTA added either as the magnesium salt or the free acid inhibited adenylate cyclase of an adrenal particulate preparation from an ACTH-sensitive tumor while the Ca^{2+} complex of EGTA showed no inhibition. We have confirmed this latter observation.

Although the results reported in this communication with the isolated adrenocortical plasma membranes would seem to be contradictory to those obtained with viable adrenal cells, direct comparisons between the two may not be valid for several reasons. The concentration of calcium in an intact cell is considered to be regulated by an active transport system pumping the calcium out of the cell as well as by the actions of the mitochondria and microsomes both of which possess the ability to accumulate calcium. Thus the level of calcium in the surrounding medium may have little bearing on the actual intracellular concentration. Furthermore, as Sayers *et al.* (31) have suggested, compartmentalization of enzymes in the intact cell may render them inaccessible to added calcium.

The fact that cyclic AMP is an intermediate rather than an end product in cellular metabolism introduces still another complication into adenylate cyclase assays. Conversion of cyclic AMP to AMP by phosphodiesterase can presumably occur even when adenylate cyclase assays are performed with purified plasma membrane preparations owing to the presence in membranes of a low K_m type of phosphodiesterase (22). Addition of theophylline to the incubation medium was not sufficient to inhibit completely cyclic AMP destruction, particularly when the nucleotide was present in low concentrations (Table III). It is not clear whether this is due to incomplete inhibition of phosphodiesterase activity or whether some other enzymes catalyze cyclic AMP destruction.

A stimulating effect of GTP on liver plasma membrane adenylate cyclase at low ATP concentrations (0.5 mM or less) was first reported by Rodbell *et al.* (33). The initial observation was confirmed for adenylate cyclases derived from other tissues as well (34–36). The significance of the GTP stimulation is somewhat difficult to interpret since this nucleotide triphosphate apparently also enhances (in the case of glucagon stimulation of liver plasma membrane-adenylate cyclase) dissociation of the hormone from its receptor.

In contrast to these findings are the more recent observations (37) concerning the effect of GTP on fat cell adenylate cyclase. In every case the activity of the enzyme was inhibited by the presence of GTP, although hormone- and fluoride-stimulated activities were somewhat less affected than basal activity. Under the conditions employed addition of GTP to the adrenal membranes (Table IV) had little effect on either basal or ACTH-stimulated adenylate cyclase until a GTP concentration of 10^{-2} M was reached. The reason for the marked inhibition in the presence of large amounts of GTP is unknown but does not appear to have physiological significance. Rodbell *et al.* (33) have postulated that GTP binds at a regulatory site independent of the glucagon binding site. Such a regulatory action for GTP is not apparent from the results presented in Table IV.

Attempts to solubilize and isolate membrane-bound adenylate cyclase thus far have resulted in only modest successes (9). Instability of the enzyme, as reflected in large losses in specific activity during storage, has been a major drawback. The problem is particularly acute with the enzyme derived from adrenal plasma membranes. Thus far activity has been preserved only by rapid freezing in liquid nitrogen at $-60°$ (Table V). Under these conditions fluoride ion-sensitive activity can be virtually quantitatively retained. In general fluoride-stimulated activity is less sensitive to conditions that modify membranes, *i.e.* detergents and phospholipase, than is hormone-stimulated activity (38). Fluoride ion is considered to interact with the enzyme at a site different from that influenced by hormones (28, 39).

The possibility that plasma membranes contain proteolytic enzymes capable of destroying the peptide hormones that specifically activate adenylate cyclases has been suggested by the results of Pohl *et al.* (40). Glucagon (41) is rapidly inactivated when incubated with liver membranes. Nearly 50% of the hormone is destroyed within the first 2 min of incubation. Furthermore, the presence of high concentrations of several other peptide hormones did not protect glucagon from inactivation. When experiments designed to determine whether proteolysis plays a major role in terminating the interaction between ACTH and its plasma membrane receptor were carried out, very little evidence for ACTH destruction was found (Table VI). The levels of both basal and ACTH-sensitive activity were markedly decreased when the membranes were kept at 30° for 45 min prior to assay. Surprisingly the presence of ACTH in the preincubation medium afforded a high degree of stabilization for the system. This result suggests that the membrane preparation does not contain significant proteolytic activity. That binding of the hormone to the membranes is necessary for stabilization to occur is evident from results obtained when protected ACTH previously shown (23) to be incapable of binding to the membranes was added to the preincubation medium. Virtually the same degree of enzyme inactivation occurred whether this peptide was present or absent. Fluoride activation is not as sensitive to preincubation as is hormone stimulation. Reactivation of preincubated membrane adenylate cyclase by fluoride ion is approximately 80%.

It appears unlikely that these results can be explained in terms of stabilization of the receptor. The ability of the receptor to bind ACTH is less sensitive to inactivation than is the complete adenylate cyclase system; for example, repeated freezing and thawing of the membrane preparation does not affect the capacity of the receptor to bind hormone (7) while the same treatment totally inactivates the cyclase. At the present state of knowledge of hormone-sensitive adenylate cyclase systems it is difficult to interpret these observations. Since both hormone-receptor interaction and adenylate cyclase activation by fluoride ion are more stable than hormone activation of adenylate cyclase, it may be tentatively concluded that inactivation during preincubation results from disruption, in some manner, of the communication between receptor and cyclase.

The adenylate cyclase system of bovine adrenal cortical plasma membranes exhibits many of the same characteristics of cyclases derived from other tissues. Because the enzymes are located in the plasma membrane they are associated with ATPase and phosphodiesterase activities. Provided that proper precautions are taken, however, linear reaction rates can be obtained. In agreement with previous reports the substrate appears to be an ATP-magnesium complex.

Cyclase hormonal control is the most sensitive component of the adrenocorticotropic plasma membrane adenylate cyclase system. In contrast to other reports the hormone effect is not associated with stimulation of ATPase activity, nor does GTP appear to modulate the cyclase system.

Acknowledgments—The authors wish to express their appreciation to Mr. I. Rosen of Western Packers for his cooperation in supplying the bovine adrenal glands. They also wish to thank Dr. Warren Diven for determining the Ca^{2+} content of the membrane preparation.

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