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Adenosine 3′:5′-Cyclic Monophosphate-Dependent Protein Kinase(s) of Rat Ovarian Cells

GONADOTROPIN REGULATION OF ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE-RECEPTOR ACTIVITY

By K. M. JAIRAM MENON and SALMAN AZHAR
Endocrine Laboratory, Departments of Obstetrics and Gynecology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109, *U.S.A.*

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Regulation of cyclic AMP-dependent protein kinase, cyclic AMP-receptor activity and intracellular cyclic AMP concentrations by choriogonadotropin was studied in ovarian cells prepared from 26-day-old rats. A close correlation was observed between phosphotransferase activity and cyclic AMP-receptor activity in 12000*g* supernatant fractions from rat ovarian homogenate. The apparent activation constant (K_a) and I_{50} (concentration required to produce 50% inhibition) of different cyclic nucleotides for phosphotransferase and cyclic AMP receptor activities respectively were also determined. Cyclic AMP and 8-bromo cyclic AMP were most effective, giving K_a values of 0.08 and 0.09 μM and I_{50} of 0.12 and 0.16 μM respectively. Other nucleotides were also effective, but required higher concentrations to give a comparable effect. An increased concentration of cyclic AMP produced by choriogonadotropin (1 μg/ml) treatment was accompanied by decreased cyclic AMP binding as early as 5 min after hormone addition. Choriogonadotropin also stimulated the protein kinase activity ratio (−cyclic AMP/+cyclic AMP) under identical experimental conditions. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine potentiated the action of choriogonadotropin on the three parameters measured in a dose- and time-dependent manner. The maximal cyclic AMP-binding capacity, as determined by cyclic AMP-exchange assay, remained unchanged before and after hormone addition. The endogenously bound cyclic AMP was determined from the difference between the maximal binding capacity and the exogenously bound cyclic AMP. With different choriogonadotropin concentrations, a quantitative correlation was established between maximal binding capacity, exogenous binding and endogenous binding activities. Approx. 60% of total binding sites were endogenously occupied in untreated cells, and choriogonadotropin (1 μg/ml) treatment fully saturated available binding sites with a parallel 10-fold increase in cellular cyclic AMP. The present results provide evidence for a probable intracellular compartmentalization of cyclic AMP in the ovarian cell, and suggest that in the unstimulated state all cyclic AMP present in the ovarian cell may not be available for protein kinase activation.

In the ovary, cyclic AMP is now believed to be an intracellular mediator in steroidogenesis regulated by gonadotropins (lutropin and choriogonadotropin). This subject has been reviewed (Menon & Gunaga, 1974; Marsh, 1976). The known mechanism by which cyclic AMP acts in eukaryotic cells is by activation of cyclic AMP-dependent protein kinases (Rubin & Rosen, 1975). Cyclic AMP binds to the regulatory subunit [R] of protein kinases with resultant dissociation and liberation of the catalytic subunit [C] in fully active form. The catalytic subunit then phosphorylates the appropriate substrate essential for the intracellular expression of the hormone's effects. The demonstration of hormone-stimulated protein kinases in a variety of hormone-specific target tissues further supports the above sequence of events (Corbin *et al.*, 1973; Richardson & Schulster, 1973; Soderling *et al.*, 1973; Walaas *et al.*, 1973; Means *et al.*, 1974; Field *et al.*, 1975; Keely *et al.*, 1975*a*,*b*; Spaulding & Burrow, 1975*a*,*b*; Vaitukaitis *et al.*, 1975; Byus *et al.*, 1976; Cooke *et al.*, 1976; Cooke & van der Kemp, 1976; Birnbaum & Fain, 1977; Skala & Knight, 1977; Ling & Marsh, 1977; Dufau *et al.*, 1977). Relatively fewer studies have been devoted to the quantitative relationship between the distribution of free and bound forms of cyclic AMP in relation to protein kinase activation.

From our laboratory, using ovarian tissue and cells, we have previously reported the properties of cyclic AMP-dependent protein kinases from the

cytoplasmic and particulate fractions (Menon, 1973; Azhar & Menon, 1975*a*,*b*) and their hormonal regulation (Azhar *et al.*, 1976; Clark *et al.*, 1976). In these earlier studies we have used an exogenous protein substrate for protein kinase to follow hormonal stimulation. In the present study we have utilized the above system to gain insight into the mechanism of regulation of cyclic AMP concentration by gonadotropin with respect to protein kinase activation and cyclic AMP receptor activity. Our results indicate that approx. 60% of the available sites on cyclic AMP-binding protein are occupied in the unstimulated state, whereas after stimulation with hormone the endogenous binding reached saturation with a 10-fold increase in the free cyclic AMP pool. Hormone treatment had no effect on the total binding capacity of the cyclic AMP-binding protein and no newly formed cyclic AMP was bound to the particulate fraction.

Materials and Methods

Female rats (26 days old) obtained from Spartan Farms, Haslett, MI, U.S.A. were used in the present experiments. The following chemicals were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A.: cyclic GMP, cyclic UMP, cyclic CMP, cyclic XMP, cyclic dAMP, cyclic IMP, cyclic dTMP, 8-bromo cyclic AMP, dibutyryl cyclic AMP, 5′-AMP, adenosine, mixed histone (type IIA) and bovine pancreas deoxyribonuclease I (EC 3.1.4.5). Cyclic [^{3}H]AMP (30Ci/mmol) and carrier-free [^{32}P]P_i were purchased from Schwarz–Mann, Orangeburg, NY, U.S.A. and ICN, Irvine, CA, U.S.A. respectively. Eagle's minimal essential medium with Earle's salts containing 10% foetal calf serum was obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Millipore filters (0.45μm pore size; HAWP 02500) and 3-isobutyl-1-methylxanthine were the products of Millipore Corp., Bedford, MA, U.S.A. and Aldrich Chemical Co., Milwaukee, WI, U.S.A. respectively. Collagenase (type I, 125–250units/mg of dry weight) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. [γ-^{32}P]-ATP was prepared as described by Glynn & Chappell (1964).

Preparation of ovarian cells

The isolation procedure used was that described earlier from this laboratory (Kawano *et al.*, 1975; Clark *et al.*, 1976): 30 rats (26 days old) were killed by decapitation and the ovaries were removed, trimmed of connective tissues and weighed. The ovaries were then cut into quarters in Eagle's minimal essential medium with Earle's salts in the presence of 500 units of collagenase (EC 3.4.24.3;1 unit equals 1μmol of amino acid liberated from collagen in 5h at 37°C; pH7.5)/ml and 0.4mg of deoxyribonuclease I/ml for 50mg of tissue/ml and digested at 37°C under O_2/CO_2 (19:1) with shaking for 2h. Mechanical dispersal was carried out by pipetting the ovarian-tissue suspension up and down 30 times in a 1ml plastic syringe at 30, 60 and 120min of incubation. After incubation, cells were collected by centrifugation (600*g*; 3min) and washed three times (4ml each) with fresh medium. Cells were finally resuspended in medium to a final concentration of 50mg of tissue/ml which gave approx. 3×10^8 cells/ml. Viability of the cells was determined by dye exclusion after staining in 0.02% Nigrosin. The viability of cells was over 90% and average yield was between 4×10^6 and 6×10^6 cells/mg of ovarian tissue. As reported earlier (Kawano *et al.*, 1975; Clark & Menon, 1976) and also tested during the present studies, treatment of these cells with choriogonadotropin (10ng/ml) gave at least a 40-fold increase in progesterone synthesis. DNA was assayed by the colorimetric procedure of Burton (1956).

Incubation conditions

Ovarian cells (equivalent to 40–80μg of DNA) were incubated in 1ml of Eagle's minimal essential medium containing 1μg of choriogonadotropin (where required)/ml in both the presence and the absence of 0.5mM 3-isobutyl-1-methylxanthine. After incubation at 37°C for 5min in an atmosphere of O_2/CO_2 (19:1), the cells were centrifuged at 600*g* for 3min. The sedimented fraction was then homogenized in 0.2ml of buffer [10mM-Tris/HCl (pH7.2)/1mM-EDTA / 0.5mM-3-isobutyl-1-methylxanthine] and the suspension was again centrifuged at 12000*g* for 20min. The clear supernatant was then used for the assay of phosphotransferase activity or cyclic AMP-receptor activity. Addition of 3-isobutyl-1-methylxanthine (0.5mM) in the homogenizing buffer caused almost 95% inhibition of cyclic AMP phosphodiesterase activities from both soluble and particulate fractions. Cyclic AMP phosphodiesterase was assayed by the procedure of Thompson & Appleman (1971) as modified by Boudreau & Drummond (1975).

Assay of protein kinase activity

Protein kinase (phosphotransferase) activity was assayed in duplicate on duplicate samples, in both the presence and absence of 2μM-cyclic AMP by the procedure of Corbin *et al.* (1973). The incubation medium in a final volume of 75μl contained 2μmol of NaF, 0.85μmol of potassium phosphate buffer, pH 6.8, 0.3μmol of $MgCl_2$, 16.5nmol of [γ-^{32}P]ATP (1.1×10^6–1.4×10^6 c.p.m.), 750μg of mixed histone, 20μl of enzyme extract and (where required) 150pmol of cyclic AMP. After incubation at 30°C for 10min, the tubes were processed for radioactivity determin-

ation by a modification of the procedure of Kuo & Greengard (1970) as adapted by Sanborn *et al.* (1973). Data are expressed either as pmol of acid-stable [^{32}P]P_i incorporated into histone/mg of homogenate protein over 10min incubation or as the activity of samples assayed in the absence of cyclic AMP divided by the activity in the presence of cyclic AMP. The latter is the protein kinase activity ratio (Corbin *et al.*, 1973).

Determination of exogenous cyclic AMP-binding activity

The assay for cyclic AMP-receptor activity *in vitro* was carried out by a slightly modified procedure of Do Khac *et al.* (1973). The incubation medium, in a final volume of 200μl, contained 20mM-Tris/HCl, pH7.5, 10mM-$MgCl_2$, 0.5mM-3-isobutyl-1-methylxanthine, 0.1μM-cyclic [^{3}H]AMP (16Ci/mmol), 0.1mM-cyclic AMP (where required) and 15–20μg of protein (cellular extract). After incubation at 0–4°C for 90min, the bound cyclic [^{3}H]AMP was separated from the unbound fraction by the Millipore-filtration technique of Gilman (1970). The data are expressed as pmol of cyclic AMP bound/mg of protein.

Determination of maximal cyclic AMP-binding capacity

Maximal cyclic AMP-binding capacity was determined by the total cyclic AMP-exchange method of Wilchek *et al.* (1971) as described by Do Khac *et al.* (1973), except that cyclic [^{3}H]AMP was not added to the homogenizing medium before homogenization. This modification was necessary, since in most cases the same cellular extracts were utilized to monitor both cyclic [^{3}H]AMP-receptor activity and cyclic AMP-dependent phosphotransferase activity. Initially, extracts prepared from both choriogonadotropin-treated and untreated cells were incubated with 100nM-cyclic [^{3}H]AMP at 0°C for 90min and then the tubes were brought to room temperature (23°C). After the addition of unlabelled 1μM-cyclic AMP containing 100μM-ATP and 10mM-$MgCl_2$, incubation was continued for the indicated period up to 150min. At each time point, samples were removed and the amount of cyclic [^{3}H]AMP that remained bound was determined by Millipore filtration (Gilman, 1970). Most of the cyclic [^{3}H]AMP bound was exchanged with unlabelled cyclic AMP within 60min of incubation (Fig. 1). By using this principle, maximum cyclic AMP-binding capacity was determined by reversing the experimental conditions. This was accomplished by first incubating the extract with 100nM-cyclic AMP for 90min at 0°C, and then 1μM-cyclic [^{3}H]AMP, 100μM-ATP and 10mM-$MgCl_2$ were added and the incubation was continued for a total period of 120min at 0°C. After this period

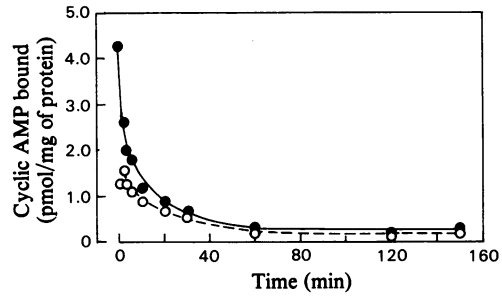

Fig. 1. *Exchange of prebound cyclic [^{3}H]AMP with unlabelled cyclic AMP*

Cellular extracts prepared from both choriogonadotropin-treated (1μg/ml, ○) and untreated (●) cells were incubated with 100nM-cyclic [^{3}H]AMP at 0°C for 90min. After treatment, 1μM-unlabelled cyclic AMP, 100μM-ATP and 10mM-$MgCl_2$ were added and the tubes were brought to room temperature (23°C), and incubation was continued for up to 150min. At the indicated times, samples were removed and the amount of cyclic AMP that remained bound was determined by Millipore filtration.

the temperature was raised to 23°C and the incubation continued for an additional 240min. At different time intervals, samples were removed and filtered through cellulose nitrate filters (0.45μm) and the radioactivity bound on the filters was determined (Gilman, 1970). The maximum radioactivity retained on the filters represented the maximum binding.

Since maximal cyclic AMP-binding capacities and exogenous cyclic AMP binding can be determined experimentally, the difference between these two should give the endogenously occupied or endogenous cyclic AMP-binding activity. However, since cyclic AMP exchange is a continuous process, attempts were first made to monitor the cyclic [^{3}H]AMP exchange both under binding conditions as well as under exchange conditions (Do Khac *et al.*, 1973). The difference between maximum binding capacity and exogenous binding was taken as endogenous binding.

Assay of cellular cyclic AMP

For the assay of cyclic AMP content, the ovarian cells were homogenized in 1ml of 5% (w/v) trichloroacetic acid, and the protein precipitate was removed by centrifugation (2000*g*; 10min) after the addition of 1M-HCl to give a final concentration of 0.1M. The samples were extracted with 5×5vol. of water-saturated ether and then the aqueous fractions were freeze-dried. The freeze-dried material was dissolved in 200μl of 50mM-sodium acetate, pH4.0. Samples (20 and 50μl) were then assayed for cyclic

AMP by the method of Gilman (1970). The data were expressed as pmol of cyclic AMP/μg of DNA.

Protein determinations

Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Effect of cyclic nucleotides on phosphotransferase and cyclic AMP-receptor activities

Since we are dealing with an ovarian extract and not a purified protein kinase system, we first established that both phosphotransferase and cyclic AMP-receptor activities are related to each other. This was accomplished by comparing the effect of different cyclic nucleotides on both activities. Cyclic AMP at 0.5–1 μM fully stimulated protein kinase activity. The apparent activation constant determined by a Lineweaver–Burk plot (Lineweaver & Burk, 1934) was 71 nM. Fig. 2 shows the effect of nucleotides on cyclic [^{3}H]AMP-receptor activity. A comparison of the concentration of different nucleotides needed to cause a 50% inhibition of cyclic [^{3}H]AMP binding revealed that cyclic AMP was the most effective competitor with a half-maximal inhibition produced at 0.12 μM concentration. Other cyclic nucleotides were also effective, but required much higher concentrations to elicit a 50% inhibition (results not shown).

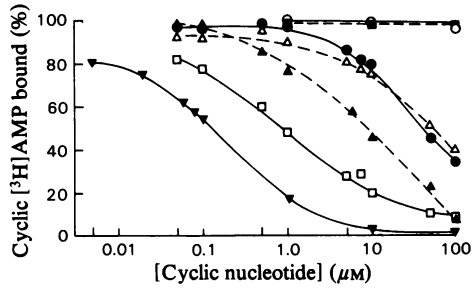

Fig. 2. *Effects of increasing concentrations of various cyclic nucleotides on cyclic AMP-receptor activity*
The incubation medium (final volume of 200 μl) contained 4 μmol of Tris/HCl, pH 7.5; 2 μmol of $MgCl_2$, 0.1 μmol of 3-isobutyl-1-methylxanthine, 20 pmol of cyclic [^{3}H]AMP (16 C/mmol), 40 μg of protein (cellular extract) and indicated concentrations of different cyclic nucleotides. After incubation at 0°C for 90 min the bound cyclic AMP was determined as described in the Materials and Methods section. ▼, Cyclic AMP; □, cyclic IMP; ●, cyclic UMP; ■, 5′-AMP; △, cyclic GMP; ▲, cyclic CMP; ○, adenosine.

Similarly a comparison of the activation constant (K_a) and I_{50} (concentration that causes a 50% inhibition in the extent of cyclic [^{3}H]AMP binding) of different cyclic nucleotides for phosphotransferase and cyclic AMP-receptor activities respectively revealed that cyclic AMP and 8-bromo cyclic AMP were most effective, with K_a values of 0.08 μM and 0.09 μM and I_{50} values of 0.12 μM and 0.16 μM respectively. Other nucleotides were also effective, but required higher concentrations to elicit comparable effects. From these results it was inferred that both phosphotransferase and cyclic AMP-receptor activities are closely related.

Cyclic AMP synthesis by ovarian cells

The effect of choriogonadotropin and 3-isobutyl-1-methylxanthine on cyclic AMP synthesis by rat ovarian cells is shown in Table 1. Choriogonadotropin (1 μg/ml) enhanced cyclic AMP synthesis more than 10-fold. 3-Isobutyl-1-methylxanthine, although showing no effect on cyclic AMP concentration, potentiated the stimulatory effect of choriogonadotropin. The results presented in Fig. 3 show the effect of increasing concentrations of choriogonadotropin on cyclic AMP synthesis. Concentrations of choriogonadotropin as low as 10 ng/ml significantly stimulated cyclic AMP synthesis, and the extent of stimulation was increased with increasing concentrations of choriogonadotropin used.

Correlation between protein kinase activation, cyclic AMP-receptor activity and cyclic AMP synthesis in situ under the influence of choriogonadotropin

Since it has been shown previously in this laboratory that protein kinase activity in ovarian cells is stimulated by choriogonadotropin (Azhar *et al.*, 1976; Clark *et al.*, 1976), we decided to see if the increased concentrations of cyclic AMP could be

Table 1. *Effect of choriogonadotropin and 3-isobutyl-1-methylxanthine on cyclic AMP synthesis by rat ovarian cells*
Cells (70 μg of DNA) were incubated with test substances for 10 min at 37°C in O_2/CO_2 (19:1). After incubation, the cells were processed for cyclic AMP determination as described in the Materials and Methods section. Results are means ± S.E.M. of three experiments.

Addition to incubation medium	Cyclic AMP (pmol/μg of DNA)
None	0.32 ± 0.02
3-Isobutyl-1-methylxanthine (0.5 mM)	0.38 ± 0.06
Choriogonadotropin (1.0 μg/ml)	3.80 ± 0.30
3-Isobutyl-1-methylxanthine (0.5 mM) + choriogonadotropin (1.0 μg/ml)	5.50 ± 0.20

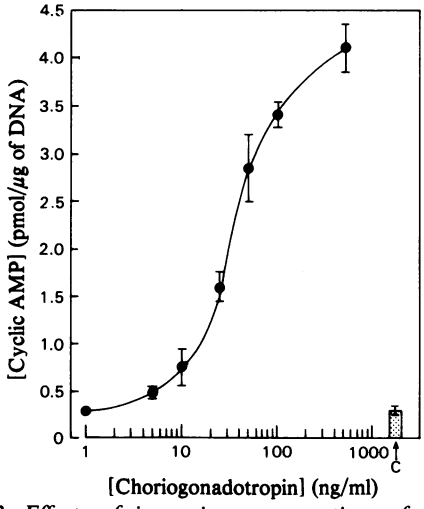

Fig. 3. *Effects of increasing concentrations of choriogonadotropin on cyclic AMP synthesis by ovarian cells*
Ovarian cells (60μg of DNA) were incubated with concentrations of choriogonadotropin and 0.5mM-3-isobutyl-1-methylxanthine in a final volume of 1.0ml. After incubation at 37°C for 10min in O_2/CO_2 (19:1), the cells were separated from the medium by centrifugation and processed for cyclic AMP assay as described in the Materials and Methods section. C denotes control, i.e. cyclic AMP concentration in cells incubated without choriogonadotropin. Bars indicate ±S.E.M. of three experiments.

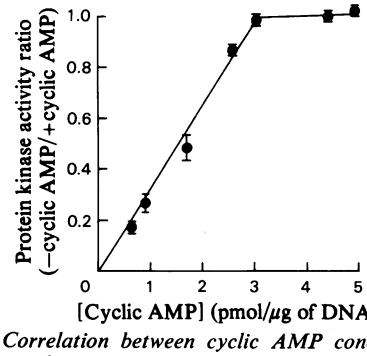

Fig. 4. *Correlation between cyclic AMP concentrations and protein kinase activation in ovarian cells under the influence of choriogonadotropin*
Two sets of ovarian cells (80μg of DNA), each in triplicate, were incubated with increasing concentrations of choriogonadotropin (1, 2.5, 10, 25, 100, 500 and 1000ng/ml) in the presence of 0.5mM-3-isobutyl-1-methylxanthine for 10min at 37°C in O_2/CO_2 (19:1). After incubation, cells from the first set were processed for the determination of protein kinase activity ratio and those from second set for cyclic AMP content. Other details were as described in the Materials and Methods section. A protein kinase activity ratio of 1.0 represents full activation of protein kinase. Bars indicate±S.E.M. of three experiments.

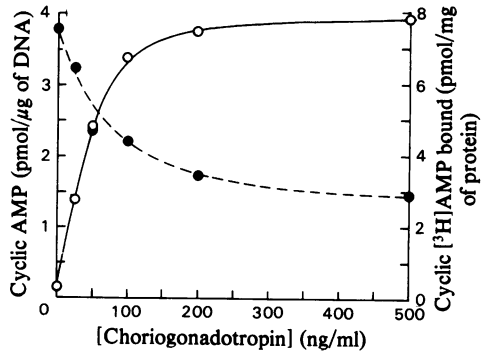

Fig. 5. *Effects of increasing concentrations of choriogonadotropin on cyclic [³H]AMP-receptor activity and cyclic AMP concentrations in ovarian cells*
Incubation conditions were the same as described in Fig. 4. Cyclic [³H]AMP binding was carried out as described in the Materials and Methods section; ○, cyclic AMP concentration; ●, cyclic [³H]AMP bound.

correlated with activation of phosphotransferase activity with concomitant inhibition of exogenous cyclic [³H]AMP-receptor activity (due to occupancy *in situ*). This relationship was studied by incubating ovarian cells with different concentrations of choriogonadotropin in the presence of 3-isobutyl-1-methylxanthine. The protein kinase activity ratio in the tissue from each incubation point was plotted against the corresponding concentrations of cyclic AMP. The results presented in Fig. 4 show that an increase in the tissue content of cyclic AMP under the influence of different concentrations of choriogonadotropin resulted in a parallel increase in protein kinase activity ratio. Similarly, as shown in Fig. 5, inhibition of cyclic [³H]AMP-binding activity was proportional to the increase in cyclic AMP within the cells. When cyclic AMP was increased to 3.8pmol/μg of DNA, it inhibited almost 50% of cyclic AMP-binding activity. It can be concluded from these experiments that protein kinase stimulation by choriogonadotropin can be demonstrated by assaying either the phosphotransferase activity or the cyclic AMP-receptor activity.

Effect of incubation time and protein concentration on exogenous cyclic AMP binding to extracts prepared from the cells pretreated with or without choriogonadotropin

The exogenous cyclic AMP binding to ovarian cells was very rapid during the first 20min of incubation in extracts prepared from both choriogonadotropin-treated and untreated cells (Fig. 6). However, after this period, binding reached saturation in

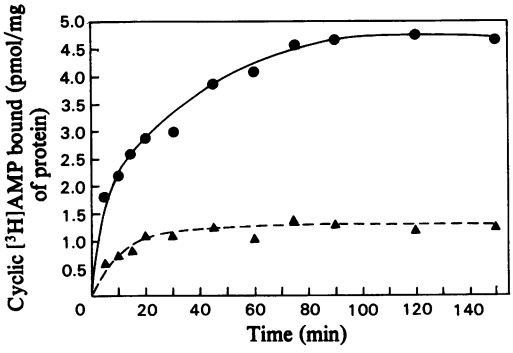

Fig. 6. *Effects of incubation time on cyclic [^{3}H]AMP binding to cellular extract pretreated with and without cyclic AMP*

Cyclic [^{3}H]AMP binding was carried out as described in Fig. 1, except that unlabelled cyclic AMP was not included in the incubation medium. After incubation at 0°C for the indicated time interval, the samples were processed for cyclic [^{3}H]AMP binding as described in the Materials and Methods section. ●, Control, ▲; choriogonadotropin (1 μg/ml)-treated cells.

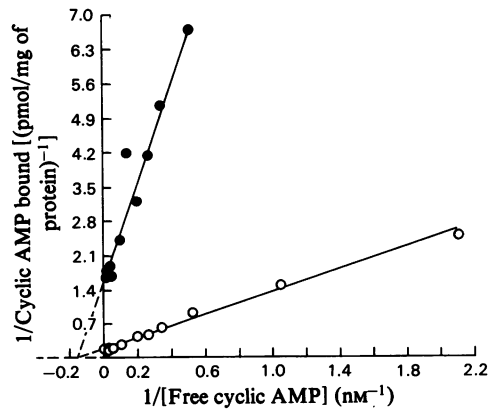

Fig. 7. *Determination of dissociation constant (K_d) for exogenous cyclic [^{3}H]AMP binding in cellular extracts pretreated with and without choriogonadotropin*

Cellular extracts (40 μg of protein) pretreated with (●) and without (○) choriogonadotropin (1 μg/ml) as described under Fig. 6 were incubated with increasing concentrations of cyclic [^{3}H]AMP. Other details were as described for Figs. 5 and 6. Results were then plotted according to the Klotz (1953) equation.

choriogonadotropin-treated cells, whereas in the untreated cells it continued to increase and required 90min of incubation to reach a saturation state.

Similarly the binding was linear with protein concentration up to 50 μg of protein in both choriogonadotropin-treated and untreated cells.

Effect of increasing concentrations of cyclic AMP

Incubation of cellular extracts with increasing concentrations of cyclic [^{3}H]AMP resulted in increased cyclic [^{3}]AMP-binding activity. When cyclic AMP concentration was raised to 100nM, the binding reaction was fully saturated. The apparent K_d, determined by plotting 1/[free cyclic AMP] against 1/[bound cyclic AMP] by the procedure of Klotz (1953), was 6.5nM. The K_d was unaltered by pretreatment of the ovarian cells with choriogonadotropin (Fig. 7).

Cyclic AMP binding to particulate and soluble fractions

Results presented in Table 2 show the binding of cyclic [^{3}H]AMP to particulate and soluble fractions of cellular extract. Although a significant amount of cyclic [^{3}H]AMP binding occurred in the particulate fraction after hormonal stimulation, there was no decrease in cyclic [^{3}H]AMP binding to the particulate fraction, suggesting that the newly formed cyclic AMP was not bound to the particulate fraction (12000*g* sediment).

Table 2. *Cyclic AMP binding to cytosol and particulate fractions of rat ovarian cells pretreated with choriogonadotropin*

Cells (40 μg of DNA) were incubated with and without choriogonadotropin (1 μg/ml) in the presence of 0.5mM-3-isobutyl-1-methylxanthine for 5min at 37°C. After incubation, the cells were centrifuged (600*g*; 3min) and homogenized in 200 μl of buffer [10mM-Tris/HCl (pH7.5)/1mM-EDTA/0.5mM-3-isobutyl-1-methylxanthine] and centrifuged at 12000*g* for 20min at 0°C. The supernatant from each set was kept and the sediment was rinsed twice with buffer and finally suspended in 200 μl of buffer. Both the soluble and particulate fractions were then used for cyclic [^{3}H]AMP-binding assay. Results are means ± S.E.M. of three experiments.

Treatment	Cyclic [^{3}H]AMP bound (pmol/mg of protein)	
	Supernatant	Sediment
Untreated cells	3.93 ± 0.41	0.87 ± 0.06
Choriogonadotropin-treated cells (1 μg/ml)	1.40 ± 0.06	1.07 ± 0.08

Determination of maximal binding capacities

Maximal cyclic AMP-binding capacity was calculated by the total cyclic AMP-exchange assay described by Wilchek *et al.* (1971) and as described in the Materials and Methods section.

The results presented in Table 3 show the exchange of cyclic [^{3}H]AMP under binding conditions as well

Table 3. *Effect of incubation time on cyclic AMP exchange under binding and exchange conditions*

The cellular extracts from choriogonadotropin-treated and untreated cells were incubated with 100nM-cyclic AMP for 90min at 0°C, and then 1 μM-cyclic [^{3}H]AMP, 100μM-ATP and 10mM-$MgCl_2$ were added and the incubation was continued for a further period of 120min at 0°C. After this period the temperature was raised to 23°C and incubation continued for a further period of 120min. At the indicated time intervals, samples were removed and filtered through cellulose nitrate filters (0.45μm), and the radioactivity associated with the filters was determined by the procedure described in the Materials and Methods section. Condition A was cyclic AMP binding under binding conditions, in which cyclic [^{3}H]AMP and ATP were added at 0min and incubated at 0°C for the different time intervals up to 120min; Condition B was cyclic AMP binding under exchange conditions; after incubation at 0°C for 120min (condition A) the temperature was raised to 23°C and incubation continued for the different time periods up to 240min.

Incubation (min)	Cyclic [^{3}H]AMP bound (pmol/mg of protein) Control	Choriogonadotropin-treated (1μg/ml)
Condition A		
10	1.10	2.61
20	1.71	2.80
30	1.85	3.00
45	2.11	3.21
60	2.52	3.31
90	3.01	3.40
120	3.61	4.50
Condition B		
150	6.62	7.21
180	7.06	7.10
240	7.13	7.18

as under exchange conditions. Extracts of ovarian cells pretreated with or without choriogonadotropin in the presence of 3-isobutyl-1-methylxanthine were first incubated with unlabelled 100nM-cyclic AMP for 90min at 0°C, and then with 1 μM-cyclic [^{3}H]AMP, 100μM-ATP and 10mM-$MgCl_2$ were added and incubation was continued for a total period of 120min at 0°C. After this period the temperature was raised to 23°C and incubation continued for 240min. At the indicated time intervals (Table 3) samples were removed, filtered through cellulose nitrate filters (0.45μm pore size) and radioactivity retained on the filters was determined (Gilman, 1970). As shown in Table 3, 33–40% of cyclic AMP was exchanged under the binding conditions, and thus a correction was applied to calculate the endogenous cyclic AMP-binding activity as shown in Table 4.

Correlation between intracellular cyclic AMP concentration and endogenous cyclic AMP-binding activity in cells pretreated with different concentrations of choriogonadotropin

The results presented in Table 4 show that incubation of ovarian cells with 2.5–1000ng of choriogonadotropin/ml resulted in an increase of cellular cyclic AMP. This increase was accompanied by a decrease in exogenous cyclic [^{3}H]AMP-binding activity, with a concomitant increase in endogenous cyclic AMP binding.

Since the cyclic [^{3}H]AMP-exchange procedure described allows one to determine the amounts of endogenously bound and free cyclic AMP, cells were incubated with different concentrations of choriogonadotropin ranging from 2.5–1000ng/ml and the corresponding amounts of endogenous bound and free forms of cyclic AMP were determined. From these data 1/[endogenously bound cyclic AMP] was plotted against 1/[free cyclic AMP] (Klotz, 1953), which gave a dissociation constant (K_d) of 63nM (Fig. 8). This K_d for endogenous cyclic AMP binding, however, was 10-fold higher than the K_d (6.5nM, Fig. 7) for exogenous cyclic AMP binding.

Discussion

In the present studies we have attempted to correlate the relationship between endogenous binding of cyclic AMP to the regulatory subunit of cyclic AMP-dependent protein kinase with that of phosphotransferase activity in 12000*g* extract from ovarian cell suspension. Evidence presented here suggests that both phosphotransferase and cyclic AMP-receptor activities are closely related to each other and not significantly altered during the tissue homogenization and subsequent preparation of the homogenate for assay. The effect of various cyclic nucleotides on these two activities suggests that close relationship between these two activities. The correlation between increase in cyclic AMP with the activation of phosphotransferase activity and inhibition of cyclic AMP-receptor activity by choriogonadotropin further supports the suggestion that these two properties are controlled by similar mechanism(s). We have also demonstrated that incubation of cells with choriogonadotropin resulted in an increase in protein kinase activity ratio with a concomitant increase in endogenous binding of cyclic AMP and resultant decrease in exogenous cyclic [^{3}H]AMP binding. Choriogonadotropin treatment did not increase the maximal binding capacity of the regulatory subunit of protein kinase, but increased the occupation of the available receptor sites. In these studies we have used the indirect procedure described by Wilchek *et al.* (1971) and Do Khac *et al.* (1973). Although protein kinases are also

Table 4. *Relationship between intracellular cyclic AMP concentrations and cyclic AMP binding in ovarian cell suspension incubated with choriogonadotropin*

Two sets of ovarian cells (each 80μg of DNA) in triplicate were incubated with indicated concentrations of choriogonadotropin for 10min at 37°C of O_2/CO_2 (19:1). After incubation, one set was processed for cyclic AMP determination and other was used to prepare cellular extract. The cellular extract were then used to determine maximal binding capacity and exogenous binding as described in the Materials and Methods section. Results are means ±S.E.M. of three experiments.

Choriogonadotropin (ng/ml)	Cyclic AMP (pmol/μg of DNA)	Cyclic AMP bound (pmol/mg of protein)			
		Maximal binding capacity (a)	Exogenous binding (b)	Endogenous binding (a−b)	Endogenous binding corrected*
No addition	0.29±0.01	6.7±0.1	4.0±0.3	2.7	3.8
2.5	0.44±0.01	6.9±0.3	3.8±0.1	3.1	4.1
10	0.76±0.19	6.8±0.6	3.0±0.1	3.8	5.1
50	2.46±0.02	6.7±0.1	2.6±0.1	4.4	5.9
100	3.40±0.14	6.8±0.3	2.0±0.1	4.8	6.4
500	4.10±0.25	6.8±0.1	2.0±0.2	4.8	6·4
1000	4.10±0.30	6.8±0.2	1.9±0.2	4.9	6.5

* Calculated by the procedure described in Table 3 and also in the Materials and Methods section.

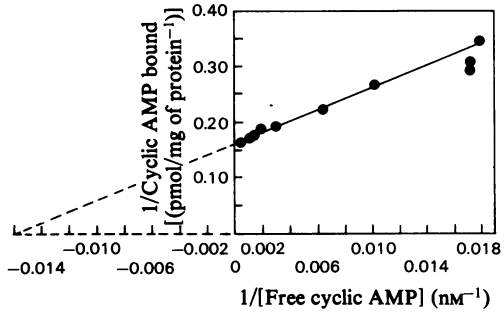

Fig. 8. *Determination of dissociation constant (K_d) for endogenous cyclic AMP binding*

Ovarian cells (60μg of DNA) were incubated with different concentrations of choriogonadotropin (2.5–1000ng/ml) for 10min. The cellular extracts were then used for the determination of endogenously bound cyclic AMP as described in Table 4. The data were then plotted according to the Klotz (1953) equation to determine the endogenous K_d. Free cyclic AMP concentrations were determined by the procedure of Gilman (1970) as described in the Materials and Methods section.

known to be associated with the particulate fractions (Rubin *et al.*, 1972; Menon, 1973; Azhar & Menon, 1975*a*,*b*; Corbin *et al.*, 1977), the present studies were carried out with 12000*g* supernatant fraction mainly because changes in cyclic [^{3}H]AMP-receptor activity after choriogonadotropin treatment were observed only in this fraction. Consequently hormonal activation of cyclic AMP-dependent protein kinase in this system was also performed with this fraction.

Under the present experimental conditions, about 40% of total available receptor sites were not occupied in the unstimulated state, suggesting compartmentalization of cyclic AMP in this system. Choriogonadotropin caused a 2-fold stimulation of endogenous cyclic AMP binding, but the total cyclic AMP concentration, on the other hand, was increased approx. 11-fold. The particulate fraction, when tested for the exogenous cyclic [^{3}H]AMP binding, showed very little change after hormonal stimulation under the experimental conditions used. However, the particulate fraction did have cyclic AMP-binding sites approximately equivalent to 25% of those of the supernatant fraction. But under hormonal stimulation very little of the newly formed cyclic AMP appeared to bind to the particulate fraction (Table 2).

In the present studies, although the apparent K_d for endogenous cyclic AMP binding to protein kinase was approx. 10-fold higher than the K_d for exogenous cyclic AMP binding, there are several other factors, such as protein kinase concentrations (Beavo *et al.*, 1974), endogenous protein inhibitor of protein kinase (Appleman *et al.*, 1966; Ashby & Walsh, 1972), the influence of molecules such as MgATP (Haddox *et al.*, 1972) and the readily dissociable nature of protein kinase by cyclic AMP (Swillens *et al.*, 1974), which may complicate the measurement of a true dissociation constant (K_d) for protein kinase. Thus no conclusions can be drawn from this difference between endogenous and exogenous dissociation constants.

In the hormone-sensitive systems, several attempts have been made to correlate the dose–response relationship between hormone binding, cyclic AMP stimulation, activation of protein kinase *in situ* and

finally the physiological end point (Kawano *et al.*, 1975; Clark *et al.*, 1976; Cooke *et al.*, 1976; Ling & Marsh, 1977). Cooke *et al.* (1976) have demonstrated a correlation of protein kinase activation and testosterone production in Leydig cells after lutropin treatment. However, in their system maximum testosterone production was attained with a lower amount of lutropin than that required to stimulate protein kinase maximally. Similar results have also been reported by Ling & Marsh (1977) for protein kinase stimulation and progesterone synthesis in bovine corpus-luteum slices under the influence of lutropin. We have previously reported that in rat ovarian interstitial cells, progesterone production was stimulated by doses of choriogonadotropin that did not result in detectable stimulation of cyclic AMP production (Clark & Menon, 1976). Similarly, in agreement with the above observation, we noticed a correlation between protein kinase activation and progesterone synthesis when rat ovarian cells were treated with choriogonadotropin (S. Azhar & K. M. J. Menon, unpublished work). However, in our system for maximal protein kinase activation a higher concentration of choriogonadotropin was required than the dose required for maximum production of progesterone.

In the absence of a detectable increase in cyclic AMP production, the above observations raise the possibility that steroidogenesis is activated by extremely low concentrations of cyclic AMP, possibly by translocation of cyclic AMP within the intracellular pool. Thus the complexity of the intracellular compartmentalization of cyclic AMP, as suggested in the present studies as well as those reported by others (Reporter, 1972; Do Khac *et al.*, 1973; Corbin *et al.*, 1977; Terasaki & Brooker, 1977), further suggests that the regulation of intracellular processes by cylic AMP is complex and that multiple compartments of cyclic AMP might be responsible for regulating specific intracellular processes in hormone-sensitive target cells.

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