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Steroidogenesis in Isolated Adrenocortical Cells

CORRELATION WITH RECEPTOR-BOUND ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE

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Because several groups have recently questioned a mediating role for cyclic AMP in adrenocortical steroidogenesis, we analysed the problem in more detail by measuring three different cyclic AMP pools in cells isolated from decapsulated rat adrenals. Extracellular, total intracellular and bound intracellular cyclic AMP were determined by radioimmunoassay in comparison with corticosterone production induced by low corticotropin concentrations. The increase in extracellular and total intracellular cyclic AMP with low corticotropin concentrations was dependent on the presence of a phosphodiesterase inhibitor and short incubation times. Bound intracellular cyclic AMP was less dependent on these two parameters. In unstimulated cells cyclic AMP bound to its receptor represents only a small fraction of the total intracellular cyclic AMP. After stimulation by a concentration of corticotropin around the threshold for corticosterone production, an increase in bound cyclic AMP was observed which correlated very well with steroidogenesis both temporally and with respect to corticotropin concentration. This finding was complemented by measuring a concomitant decrease in free receptor sites. Full occupancy of the receptors was not necessary for maximal steroidogenesis. Binding kinetics of cyclic [^{3}H]AMP in concentrations equivalent to the intracellular cyclic AMP concentration suggest the presence of at least three different intracellular cyclic AMP pools. These observations are in agreement with a possible role for cyclic AMP as a mediator of acute steroidogenesis induced by low corticotropin concentrations.

Evidence has accumulated in numerous laboratories that the metabolic and physiological effects of cyclic AMP are mainly mediated by cyclic AMP-dependent protein kinases (cf. Rubin & Rosen, 1975). According to this concept cyclic AMP binds to the regulatory subunits of the inactive holoenzyme and thus releases active catalytic subunits. In many instances, e.g. acute adrenal steroidogenesis in isolated cells (Beall & Sayers, 1972; Nakamura *et al.*, 1972; Moyle *et al.*, 1973; Seelig & Sayers, 1973; Sharma *et al.*, 1974; Hudson & McMartin, 1975; Ramachandran & Moyle, 1977), no satisfactory correlation has been found between corticosterone stimulation by low corticotropin concentrations and cyclic AMP production. This might have been due to insensitivity of cyclic AMP assays or to the determination of an unrelated cyclic AMP pool. Such discrepancies were less marked or not apparent in studies with quartered rat adrenals (Haynes, 1958; Grahame-Smith *et al.*, 1967) or isolated adrenocortical cells (Mackie *et al.*, 1972), when the tissue was responsive to more than 20μ-i.u. of corticotropin/ml. It has been shown in testicular interstitial cells (Dufau *et al.*, 1977) and thyrocytes (Schumacher & Hilz, 1978) that the acute response of the corresponding trophic hormones correlates much better to the amount of intracellular cyclic AMP determined to be bound to the regulatory subunit of protein kinases than to total cyclic AMP concentrations. It was shown that both cyclic AMP bound to this subunit and receptor occupancy, i.e. occupied and free cyclic AMP-binding sites, corresponded very well to the hormonal effects (Podesta *et al.*, 1978). Paradoxically, both unstimulated and fully stimulated cells generally produce cyclic AMP concentrations which seem to be far in excess of that needed for complete kinase activation and full steroidogenesis.

Therefore it seemed essential to determine in isolated adrenocortical cells whether there is a real correlation between bound cyclic AMP and acute steroidogenesis stimulated by low concentrations of corticotropin. In addition we wished to determine in more detail the relationship between total intracellular and bound cyclic AMP, and to investigate whether the cyclic AMP-binding protein(s) have access to the total intracellular cyclic AMP.

Materials and Methods

Cyclic [^{3}H]AMP (52Ci/mmol), 2′-*O*-succinyl-[^{125}I]iodotyrosine methyl ester and cyclic AMP–antibody complex were purchased from New England

Nuclear, Boston, MA, U.S.A.; bovine serum albumin (fraction V) was from Fluka A.G., Buchs, Switzerland; trypsin no. 15330 was from Boehringer Mannheim G.m.b.H., Mannheim, Germany; lima-bean trypsin inhibitor and bacterial alkaline phosphatase no. 5130 BAPC (40units/mg) were from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; HAWP Millipore filters (pore size 0.45 μm) were from Millipore S.A., Molsheim, Switzerland; 3-isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; anion-exchange resin AG 1-X2, (200–400 mesh) was from Bio-Rad Laboratories, Richmond, CA, U.S.A., Instagel was from Packard, La Grange, IL, U.S.A.; corticotropin was corticotropin-(1–24)-tetracosapeptide (Synacthen; 1 fmol corresponds to 0.3 μ-i.u. of corticotropin) obtained from Dr. W. Rittel, Ciba–Geigy Ltd., Basel, Switzerland.

Rats were male outbred Wistar strain Tif:RAIF (SPF); they were kept and fed as described earlier (Neher & Milani, 1978) and supplied with 10 μg of dexamethasone/ml of drinking water *ad libitum*.

Preparation and incubation of adrenocortical cells of zona fasciculata reticularis type

The digestion of decapsulated adrenals followed published procedures (Swallow & Sayers, 1969; Lowry *et al.*, 1973; Neher & Milani, 1978). Incubations (37°C) were done in triplicate or quadruplicate in 12mm × 75mm Falcon plastic tubes under O_2/CO_2 (19:1) with shaking (100 cycles/min) for different periods of time. Each tube contained 0.5ml of a suspension of about 10^5 cells (150 μg of protein) in Krebs–Ringer bicarbonate solution, pH 7.4 (Krebs & Henseleit, 1932), containing 0.01 M-glucose and 0.5% (w/v) albumin and 0.5ml of the above mixture, pH 7.4, if necessary containing 3-isobutyl-1-methylxanthine and corticotropin. The incubation was stopped by cooling the tubes in ice/water and all further processing was done at 0–4°C.

Steroid assay

The cells were sedimented at 500*g* for 5 min, and samples of the supernatants were assayed for corticosterone and cyclic AMP released into the medium (extracellular cyclic AMP). Corticosterone was determined by fluorimetry (Silber *et al.*, 1958; Neher & Milani, 1978).

Extracellular cyclic AMP analysis

A 700 μl sample of the supernatant was transferred to a glass tube, heated for 10 min in a boiling-water bath, cooled and centrifuged at 1700*g* for 20 min; the supernatant was kept frozen or used immediately for assay as described below.

Total intracellular cyclic AMP and intracellular protein-bound cyclic AMP

The assay followed published procedures (Dufau *et al.*, 1977) with slight modifications. After incubation, cells corresponding to half and one adrenal were used for intracellular and protein-bound cyclic AMP. Cell pellets were washed once with 2 ml of Krebs–Ringer bicarbonate buffer containing 0.1 mM-3-isobutyl-1-methylxanthine, centrifuged at 500*g* for 5 min and resuspended in 0.7 and 1 ml of 20 mM-Tris/HCl buffer, pH 7.4, containing 0.1 mM-3-isobutyl-1-methylxanthine and 0.1 mM-2-mercaptoethanol (buffer TIM) for intracellular and bound cyclic AMP, respectively. The suspensions were sonicated at 100W for 15s with a Labsonic 1510 sonicator with a micro-tip adaptor. The samples for intracellular cyclic AMP were transferred to glass tubes and heated in a boiling-water bath for 10 min, transferred to small conical plastic tubes (10mm × 35mm), centrifuged at 7300*g* for 15 min, and the supernatants frozen. The sonicated samples for bound cyclic AMP were immediately centrifuged for 1 min up to 10000*g*, the supernatants were transferred to plastic tubes (12mm × 75mm), diluted with 2 ml each of buffer TIM and filtered immediately through Millipore filters, which adsorbed the receptor protein and cyclic AMP bound to it (Tao *et al.*, 1970; Dufau *et al.*, 1977). The tubes were rinsed with 2 ml of buffer TIM, the washings filtered again and the filter was washed with 2 × 2 ml of buffer TIM. After drying for a short time under suction, the filters were transferred to 12mm × 75mm glass tubes, and 700 μl of buffer TIM was added. After heating in boiling water for 10 min the eluate was frozen.

Control experiments for bound cyclic AMP determinations

These were carried out to determine whether unbound cyclic AMP could associate with free receptor protein during the procedure used for isolating bound cyclic AMP described above. For this purpose cell pellets were suspended in 1 ml of buffer TIM containing 25, 250 and 2500 fmol of cyclic [^{3}H]AMP per 10^5 cells and processed as described for bound cyclic AMP. The filters were transferred to glass scintillation vials, dried for 1 h at 37°C and dissolved in 2 ml of 2-methoxyethanol during 1 h at 20°C; after addition of 10 ml of scintillation cocktail [5g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 litre of toluene], ^{3}H radioactivity was counted in a liquid-scintillation counter with an efficiency of 25%.

Radioimmunoassay of cyclic AMP

This was performed in the three different sample preparations described above by the method of

Steiner *et al.* (1972), including acetylation as described by Harper & Brooker (1975). This procedure increased the assay sensitivity to a detection limit of 10fmol of cyclic AMP. Standards were always prepared in the same buffer as used for the samples. Up to 100μl samples were used. Cyclic AMP tracer was diluted with 1% normal rabbit serum in 0.05M-sodium acetate buffer, pH6.2; cyclic AMP–antibody complex was diluted in the same buffer; 100μl portions of each tracer and antibody solution were used.

Measurement of free and total cyclic AMP-receptor sites

Cell pellets corresponding to one adrenal were processed as described above for bound cyclic AMP samples. After sonication, 200μl portions were incubated with 300μl of 80mM-potassium phosphate buffer, pH6.5, containing 10mM-magnesium acetate, 1mM-2-mercaptoethanol, 0.1mM-3-isobutyl-1-methylxanthine and either 1nM-cyclic [^{3}H]AMP (30min at 2–4°C for free receptor sites; near non-exchange conditions) or 0.1μM-cyclic [^{3}H]AMP (1h at 37°C for total cyclic AMP-receptor sites; exchange conditions). After incubation, cyclic AMP-receptor protein was isolated by Millipore filtration and ^{3}H radioactivity counted as described above for control experiments. Non-specific binding was determined in parallel incubations in the presence of 10μM unlabelled cyclic AMP and the values were corrected accordingly. Non-specific binding was less than 2fmol per tube when up to 100nmol of cyclic [^{3}H]AMP was used.

Cyclic nucleotide phosphodiesterase assay

Cell incubations corresponding to 0.5 adrenal each and cell suspensions without incubation were cooled to 4°C and centrifuged at 500*g* for 5min. The pellets were resuspended in 500μl of 0.4M-Tris/HCl buffer, pH8.0, containing 50mM-$MgCl_2$, $6H_2O$ and 40mM-2-mercaptoethanol and sonicated for 15s (25W); 100μl portions were used for the phosphodiesterase assay based on a modified procedure of Thompson & Appleman (1971). The assay was run in a total volume of 400μl at substrate concentrations of 0.1mM- and 0.1μM-cyclic AMP and in the presence of 10nM-cyclic [^{3}H]AMP for 10min at 30°C. The reaction was stopped by boiling for 2min, samples were cooled and 0.3 unit of bacterial phosphatase (Worthington) in 100μl of water was added. The incubation was continued for 10min at 30°C and stopped by cooling. The products were poured on columns with 1ml of purified resin AG 1X-2 (Cl^- form) suspended in 60% (v/v) methanol acidified with acetic acid to pH3.2. The eluate and 3×1ml washings with acidified 60% (v/v) methanol were collected directly in scintillation vials and counted for radioactivity after addition of 10ml of Instagel to each. Total radioactivity and non-specific background were determined in parallel.

Results and Discussion

Fig. 1(*b*) shows the corticosterone production of cells isolated from decapsulated rat adrenals in response to submaximal corticotropin concentrations. In the presence of 0.1mM-3-isobutyl-1-methylxanthine, a cyclic nucleotide phosphodiesterase inhibitor, the threshold concentration of corticotropin was less than 1pM. The sensitivity of the response was increased in a parallel fashion compared with that in the absence of 3-isobutyl-1-methylxanthine.

In the same experiment (Fig. 1*a*) extracellular and total intracellular cyclic AMP were determined in response to the same range of corticotropin concentration. In the presence of 0.1mM-3-isobutyl-1-methylxanthine the sensitivity of this response was higher

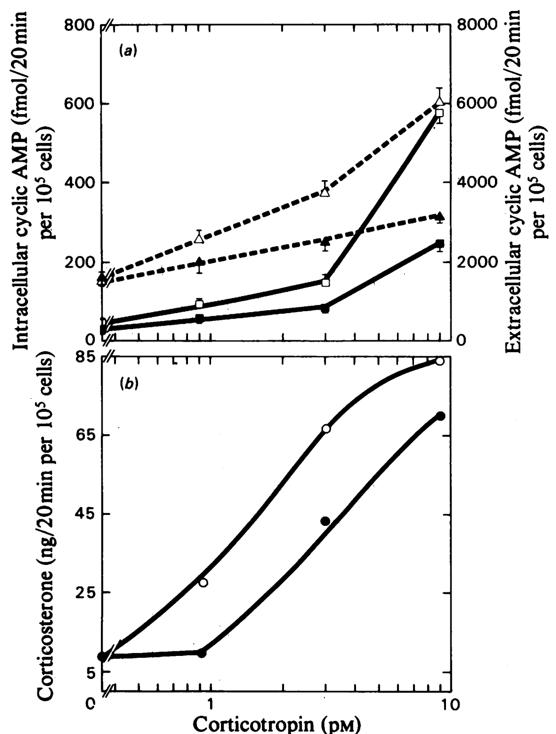

Fig. 1. *Corticotropin dose–response curve*
(*a*): Intracellular (△, ▲) and extracellular (□, ■) cyclic AMP in isolated rat adrenocortical cells after incubation for 20min in the presence (△, □) or absence (▲, ■) of 0.1mM-3-isobutyl-1-methylxanthine with 0–9pM-corticotropin. Results are mean values ± S.D., $n = 4$. (*b*): Concomitant corticosterone release into medium in presence (○) or absence (●) of 0.1mM-3-isobutyl-1-methylxanthine.

than that without 3-isobutyl-1-methylxanthine. After incubation of isolated cells for 20min the initial increase of both extracellular and intracellular cyclic AMP was observed at the same corticotropin concentration that produced an initial rise in corticosterone production. At near-maximal corticotropin concentration for corticosterone production, extracellular cyclic AMP was up to 10 times higher than intracellular cyclic AMP.

The total intracellular cyclic AMP pool increased from about 150fmol/10^5cells in unstimulated cells up to 600fmol/10^5 cells at 9pM-corticotropin, a near-maximal hormone concentration for steroidogenesis in our isolated cell preparation. Since a correlation was observed between corticotropin-induced corticosterone production and intracellular cyclic AMP accumulation (Fig. 1), we investigated whether this increase in total intracellular cyclic AMP was also paralleled by an increase in the so-called active intracellular cyclic AMP pool as represented by cyclic AMP bound to a receptor protein (cf. Dufau *et al.*, 1977). For this purpose the bound cyclic AMP pool was isolated by Millipore filtration from adrenocortical cells incubated with corticotropin (0–10pM) for different periods of time, and cyclic AMP eluted from the filters was determined by radioimmunoassay (Fig. 2). Bound cyclic AMP rose within 2min with 1pM- and 10pM-corticotropin ($P<0.05$ and <0.005 for 1pM versus control and 10pM versus control, respectively, by Student's *t*-test analysis of four individual incubations in four separate experiments, one of them being illustrated in Fig. 2), and maximal concentrations were reached by 5min. Corticotropin concentrations as low as 0.1pM were able to increase bound cyclic AMP significantly ($P<0.02$) after 10min incubation.

To determine whether free intracellular cyclic AMP would associate with the free receptor protein during the isolation procedure, control experiments were carried out (see the Materials and Methods section). When 25, 250 and 2500fmol of cyclic [^{3}H]-AMP/10^5 broken cells were added, 0.84 ± 0.009, 6.08 ± 0.13 and 16.7 ± 1.15fmol (mean values ± S.D., $n=3$) were bound respectively, representing less than 0.5% of the total intracellular cyclic AMP and 1–3% of the bound cyclic AMP in stimulated and unstimulated cells. This contribution could be neglected.

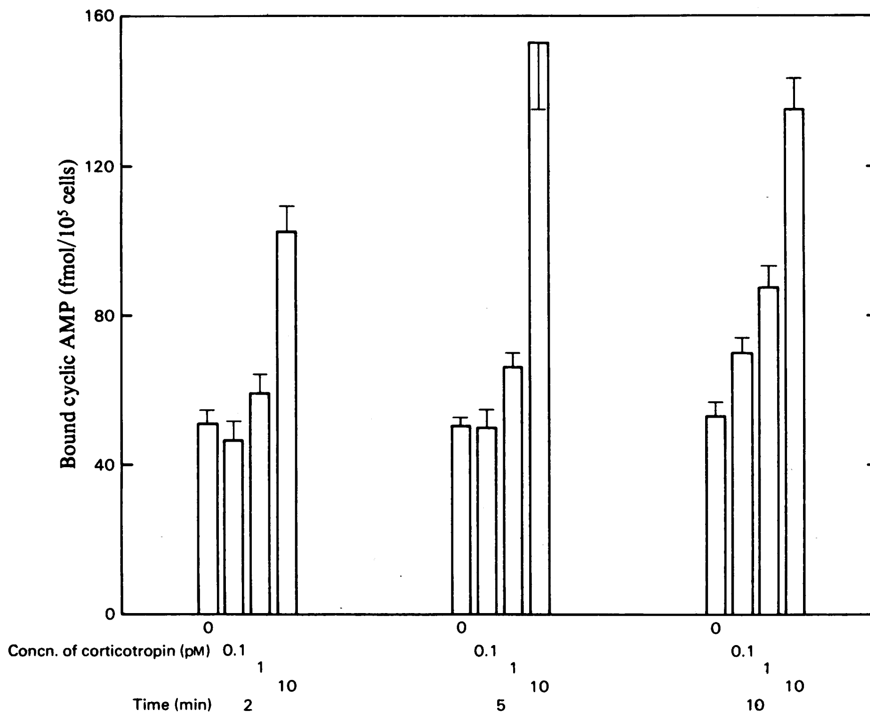

Fig. 2. *Time course and corticotropin dose–response curve*

Cells were incubated with the indicated concentration of corticotropin in the presence of 0.3mM-3-isobutyl-1-methylxanthine. Protein-bound cyclic AMP was isolated by Millipore filtration (for details see the Materials and Methods section). Results are mean values ± S.D. (bars), $n=4$.

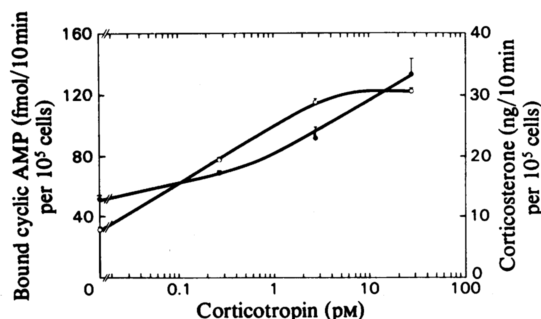

Fig. 3. *Correlation of protein-bound cyclic AMP and corticosterone production*

Cells were stimulated by corticotropin in the range 0.1pM–0.1nM in 10min incubations in the presence of 0.3mM-3-isobutyl-1-methylxanthine. ●, Bound cyclic AMP/10^5 cells; ○, corticosterone/10^5 cells. Results are mean values ± S.D., $n = 4$. Statistical comparisons: bound cyclic AMP with 0.3, 3 and 30pM-corticotropin versus controls, $P<0.02,<0.05,<0.005$, respectively; corticosterone with 0.3, 3 and 30pM-corticotropin versus controls, $P<0.005$.

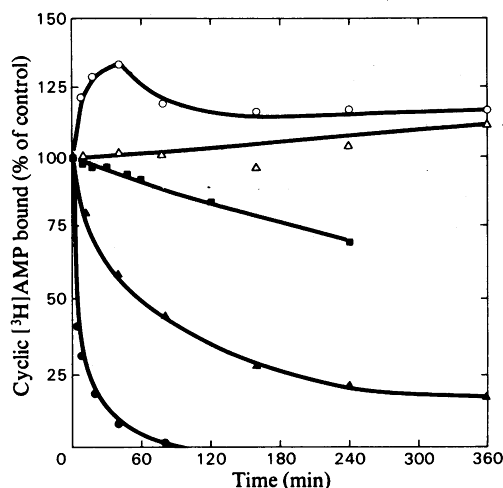

Fig. 4. *Time course of the dissociation of cyclic AMP*

The dissociation of 1nM-cyclic [^{3}H]AMP bound to its receptor (by preincubation for 120min at 4°C) in sonicated adrenocortical cells was followed at 4°C (■), 20°C (▲) and 37°C (●) during 360min in presence of 0.1μM unlabelled cyclic AMP. The decrease in bound cyclic [^{3}H]AMP is expressed as percentage of binding at zero time. The stability of cyclic AMP receptor (open symbols) in sonicated cells was checked as follows. A preincubation in the absence of cyclic [^{3}H]AMP for 0–360min at 20°C (△) or 37°C (○) was followed by a second incubation in the presence of 1nM-cyclic [^{3}H]AMP for 120min at 4°C, the binding without preincubation being taken as 100%.

A comparison between receptor-bound cyclic AMP and corticosterone production is shown in Fig. 3. A good correlation between these two parameters was observed within the steroidogenic range of hormone concentration (0.3–2.5pM-corticotropin) for a 10min incubation period. When corticotropin was increased to 30pM, a further increase of bound cyclic AMP was observed which was not associated with a further rise in corticosterone production.

When the pool of cyclic AMP bound to the protein receptor (occupied sites) is increased during stimulation of cells by corticotropin, the amount of free receptor sites (available sites) should decrease concomitantly. To check this assumption and to measure both free and total receptor sites, it was essential to study the dissociation rate of cyclic AMP from its receptor, and both the optimal binding of cyclic [^{3}H]AMP to the available sites under near non-exchange conditions ('free sites') and to total sites under exchange conditions ('free and occupied sites'). Under non-exchange conditions there should be no dissociation of endogenous cyclic AMP bound to its receptor during the assay procedure. Under exchange conditions exogenous cyclic [^{3}H]AMP should both bind to free sites and replace cyclic AMP bound to occupied sites. Total release of cyclic [^{3}H]AMP was observed (Fig. 4) after incubation for about 60min at 37°C. In addition, some exchange between exogenous and endogenous bound cyclic AMP occurred at 37°C, producing an increase of bound cyclic [^{3}H]AMP over control values when the stability of the receptor was studied (Fig. 4, uppermost curve).

At our working temperature of 0–4°C the total amount of free receptor sites was only saturated after an incubation period of 6h (results not shown), when already approx. 30% of cyclic [^{3}H]AMP bound to the receptor was dissociated. Therefore the conditions for the measurement of free sites had to be such that no appreciable dissociation occurred, i.e. 30min at 0–4°C (20% saturation of free cyclic AMP-receptor sites). Using these conditions we measured free receptor sites in adrenocortical cells incubated for 30min in the absence or presence of 2.7pM–0.14nM-corticotropin (Table 1). A gradual decrease of free cyclic AMP receptor and an increase of corticosterone production was observed with increasing corticotropin concentration. At the lowest corticotropin concentration, which produces a 16-fold increase of corticosterone, there was only a very small decrease in free cyclic AMP-receptor sites, as opposed to a clear increase of bound cyclic AMP after 10min (Fig. 2). This small decrease is probably due to the fact that under the assay conditions used only about 20% of free receptor sites were saturated, thus limiting the assay sensitivity. The measurement of bound cyclic AMP (Figs. 2 and 3) proved to be more

Table 1. *Measurement of free cyclic AMP-receptor sites and corticosterone in rat adrenal-cortical cells*

Rat adrenocortical cells were incubated with corticotropin for 30min in the presence of 0.1mM-3-isobutyl-1-methylxanthine. After incubation the cells were sonicated. Samples were used for the cyclic [^{3}H]AMP-binding assay under near-non-exchange conditions. Receptor-bound cyclic [^{3}H]AMP was isolated by Millipore filtration. Results are mean values of duplicate incubations and duplicate binding assays. Free cyclic AMP receptor is expressed as percentage of cyclic [^{3}H]AMP binding (fmol/10^5 cells); 100% represented the values in non-stimulated cells.

Concn. of corticotropin (pM)	Free cyclic AMP receptor (%)	Corticosterone (fold increase)
0	100.0	1.0
2.73	96.8	16.1
27.3	86.2	33.9
136	49.9	39.7

sensitive. Even so, we were able to show a decrease in free receptor sites with 27pM-corticotropin, a concentration which was not yet maximal for corticosterone production.

Furthermore, we studied more closely the dependence of cyclic AMP and corticosterone concentrations on 3-isobutyl-1-methylxanthine. The time course of formation of intracellular cyclic AMP and corticosterone is shown in Figs. 5(*a*) and 5(*b*) respectively. Without corticotropin no change of intracellular cyclic AMP and corticosterone was observed during 30min whether or not 3-isobutyl-1-methylxanthine was present.

In the presence of corticotropin intracellular cyclic AMP increased significantly within 2min, with 3-isobutyl-1-methylxanthine slightly enhancing this effect. The maximal concentration of intracellular cyclic AMP was reached between 10 and 20min. During this period the cyclic AMP was increased markedly by 3-isobutyl-1-methylxanthine. After 30min the concentration decreased in the absence or presence of 0.1mM-3-isobutyl-1-methylxanthine, whereas 1mM-3-isobutyl-1-methylxanthine was able to maintain the maximal value. Corticosterone production (Fig. 5*b*) increased after 5min whether 3-isobutyl-1-methylxanthine was present or not. 3-Isobutyl-1-methylxanthine at 0.1mM slightly enhanced corticosterone production after 20min, whereas 1mM-3-isobutyl-1-methylxanthine inhibited at times after 5min.

These results show that the initial increase of intracellular cyclic AMP precedes that of corticosterone. The supportive effect of 0.1mM-3-isobutyl-1-methylxanthine on the intracellular cyclic AMP concentration was seen after 2min, whereas that on corticosterone production was seen only at times

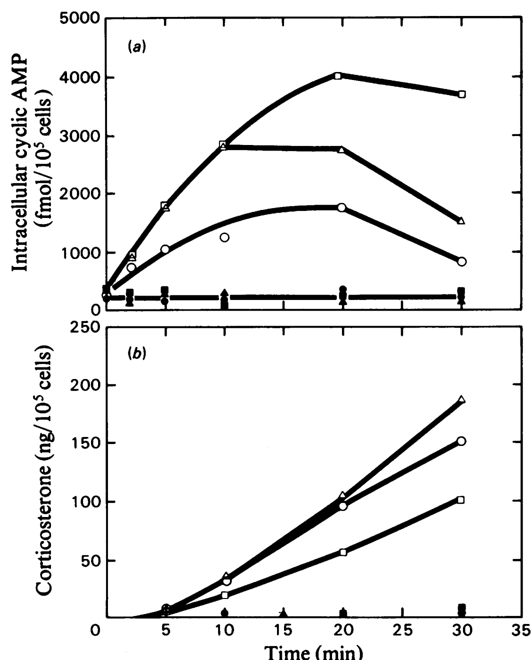

Fig. 5. *Time course of intracellular cyclic AMP* (*a*) *and corticosterone production* (*b*)

Adrenocortical cells were incubated without corticotropin (closed symbols) and with 27pM-corticotropin (open symbols) in the absence (●, ○) or presence of (▲, △) 0.1mM- and (■, □) 1mM-3-isobutyl-1-methylxanthine. After incubation and during processing 0.1mM-3-isobutyl-1-methylxanthine was always present (see the Materials and Methods section).

after 20min. Therefore the concentration of cyclic AMP in the absence of 3-isobutyl-1-methylxanthine seems to be sufficient for the maximal corticosterone production induced by near-maximal 27pM-corticotropin for up to 20min. The effects obtained with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine are consistent with the high phosphodiesterase activity found in our adrenocortical-cell preparation; 3300 and 16pmol of cyclic AMP were hydrolysed/10min per 10^5 cells at substrate concentrations of 0.1mM and 0.1μM respectively, a 90% inhibition being obtained by 1mM-3-isobutyl-1-methylxanthine at substrate concentrations between 10 and 0.1μM.

Since cyclic AMP bound to its receptor protein is known to be resistant to phosphodiesterase (Brostrom *et al.*, 1971; O'Dea *et al.*, 1971), we studied the time course of bound cyclic AMP formation in response to the submaximal concentration of 2.7pM-corticotropin with and without 3-isobutyl-1-methylxanthine (Fig. 6). Bound cyclic AMP increased continuously during the whole incubation period of 30min even

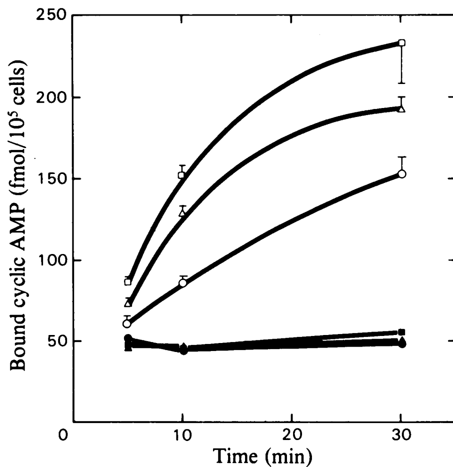

Fig. 6. *Time course of bound cyclic AMP*
Adrenocortical cells were incubated without (closed symbols) or with 2.7pM-corticotropin (open symbols) in the absence (●, ○) or presence of (▲, △) 0.1mM- and (■, □) 0.3mM-3-isobutyl-1-methylxanthine. After incubation and during processing 0.1mM-3-isobutyl-1-methylxanthine was always present (see the Materials and Methods section).

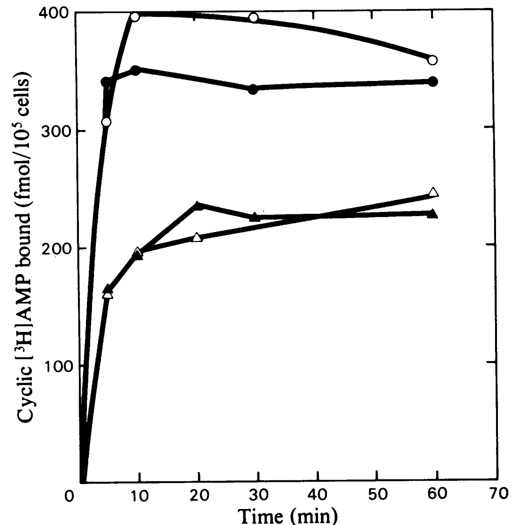

Fig. 7. *Cyclic [^{3}H]AMP-binding studies*
Binding of (▲, △) 0.1μM- and (●, ○) 1μM-cyclic [^{3}H]AMP to sonicated adrenocortical cells in the absence (closed symbols) or presence of 0.3mM-3-isobutyl-1-methylxanthine (open symbols) for 0–60min at 37°C. Bound cyclic AMP was isolated by Millipore filtration (see the Materials and Methods section).

in the absence of 3-isobutyl-1-methylxanthine; the amounts were higher in presence of 0.1 and 0.3mM-3-isobutyl-1-methylxanthine In contrast with the observation with intracellular cyclic AMP (Fig 5), bound cyclic AMP did not decrease between 10 and 30min.

Intracellular cyclic AMP concentrations in either non-stimulated or stimulated cells in the order of 1–10μM (cf. Fig. 5) appear to be far in excess of what is required for full occupancy of cyclic AMP-receptor sites. However, Fig. 6 shows that in intact non-stimulated cells, without or with 3-isobutyl-1-methylxanthine, only about 20% of the receptor sites are occupied, and in intact stimulated cells, with or without 3-isobutyl-1-methylxanthine, the receptor sites are not fully occupied before 30min. To check the assumption that all receptor sites are occupied at 1μM total intracellular cyclic AMP we studied the kinetics of binding of 0.1–1μM-cyclic [^{3}H]-AMP in broken cell preparations in the absence and presence of 3-isobutyl-1-methylxanthine (Fig. 7). With 1μM-cyclic AMP full occupancy was obtained within 10min. With 0.1μM-cyclic AMP the rate of occupancy was slower. These results show that, at the total intracellular cyclic AMP concentration of 1–10μM as mentioned above, full occupancy should be reached within 10min if total intracellular cyclic AMP was in the same pool as the cyclic AMP receptor. These findings and those of Fig. 6 suggest the presence of at least two intracellular cyclic AMP pools, with only the smaller pool being in equilibrium with the cyclic AMP receptor protein.

In recent years many authors pointed to the fact that in isolated adrenocortical cells the ED_{50} of corticotropin for corticosterone production was lower than that for cyclic AMP formation (Beall & Sayers, 1972; Mackie *et al.*, 1972; Nakamura *et al.*, 1972; Moyle *et al.*, 1973; Seelig & Sayers, 1973; Sharma *et al.*, 1974; Hudson & McMartin, 1975; Ramachandran & Moyle, 1977). We investigated whether this apparent lack of correlation between cyclic AMP formation and steroidogenesis was due to one or more of the following reasons: insensitivity of the cyclic AMP assay, rapid degradation of cyclic AMP formed, compartmentalization of cyclic AMP and assay of the most relevant pool, and choice of the correct time for the assay after stimulation. Our results suggest that probably all of these points are critical for a successful analysis of cyclic AMP in isolated rat adrenocortical cells, as has been shown with Leydig cells (Dufau *et al.*, 1977). The radioimmunoassay used by us was based on the modification of Harper & Brooker (1975) and allowed the reproducible determination of as little as 10fmol of cyclic AMP per sample. Cyclic AMP once formed intracellularly may be degraded rapidly. In order to study the role of degradation of cyclic AMP we

did our experiments in both the absence and the presence of 3-isobutyl-1-methylxanthine, a cyclic phosphodiesterase inhibitor. The findings of Peytreman *et al.* (1973) and Mackie & Schulster (1973) and our own investigations show a high phosphodiesterase activity in rat adrenocortical cells and a potentiation of corticotropin-induced cyclic AMP and corticosterone production by 3-isobutyl-1-methylxanthine. The inhibition of the phosphodiesterase activity was nearly complete by 1mM-3-isobutyl-1-methylxanthine, a concentration that began to inhibit steroidogenesis. For this reason, we had to compromise and used for the most part a concentration of 0.3M-3-isobutyl-1-methylxanthine, which was able to preserve most of the cyclic AMP formed during the incubations with corticotropin. Thus we found a much better correlation between intracellular cyclic AMP and corticosterone production, using 3-isobutyl-1-methylxanthine and choosing a limited period of time. Bound cyclic AMP, i.e. a small fraction of total intracellular cyclic AMP, was less susceptible to the action of phosphodiesterase and was increased by low corticotropin concentrations for about 30min even in the absence of 3-isobutyl-1-methylxanthine (Fig. 6), correlating very well with corticosterone production (Fig. 2). These results are in complete agreement with the findings by Saez *et al.* (1978), demonstrating a good correlation between cortisol production and protein kinase activation, but not with total cyclic AMP concentrations in human adrenocortical cells.

Our results show that cyclic AMP is highly compartmentalized. When cyclic AMP is formed intracellularly, part is transported passively or actively (cf. Rindler *et al.*, 1978) into the extracellular space as soon as a minimal intracellular concentration is reached (Fig. 1). The total intracellular cyclic AMP pool was also found to be compartmentalized. Most of this pool was susceptible to degradation by phosphodiesterase and a small fraction was in equilibrium with the cyclic AMP-receptor pool (bound cyclic AMP, Figs. 5 and 6). These conclusions are in line with the compartment guidance concept of Schwyzer (1974, 1978) and with the suggestion of Menon & Azhar (1978) that all cyclic AMP present in rat ovarian cells may not be available to the cyclic AMP receptor.

The increase in bound cyclic AMP correlated very well with acute adrenocortical steroidogenesis at the threshold concentration of corticotropin, in agreement with findings of Hayashi & Sala (1978). Full occupancy of the available receptor sites was not necessary for maximal steroid production. Similar findings were reported by Saez *et al.* (1978) concerning cortisol production and kinase activation in human adrenocortical cells.

The discrepancy between the dose of corticotropin to induce corticosterone and that to produce cyclic AMP, as mentioned above, could be resolved when receptor-bound cyclic AMP, a very small fraction of the total cyclic AMP, was measured to evaluate the corticotropin-induced formation of receptor-bound cyclic AMP. Further studies are needed to show the functional role of receptor-bound cyclic AMP on steroidogenesis.

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