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IN VITRO MATURATION OF OVINE FETAL ADRENAL CELLS ADENYLATE CYCLASE : CORTICOTROPIN-DEPENDENT AND INDEPENDENT DEVELOPMENT OF THE RESPONSE TO CORTICOTROPIN

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The present study investigated the corticotropin ($ACTH_{1-24}$) stimulation of cAMP production by fetal and newborn ovine adrenal cells maintained in culture for 6 days in absence or presence of $ACTH_{1-24}$. When fetal cells were exposed to $ACTH_{1-24}$ for 2 hours per day from day 1 the cAMP response to $ACTH_{1-24}$ increased during the course of the experiment to become on day 6, 40 fold higher than that observed on day 1. However, when $ACTH_{1-24}$ was continuously present in the culture medium the increase of the acute response to the hormone was weaker from day 4 onwards than in the case of cells exposed to $ACTH_{1-24}$ only for 2 hours per day. Moreover, the $ACTH_{1-24}$ stimulated cAMP production on day 6 of fetal cells cultured in ACTH-free medium for 5 days was 25 fold higher than the response obtained on day 1. When newborn adrenal cells were treated with $ACTH_{1-24}$ for 2 hours per day no significant change on $ACTH_{1-24}$ induced cAMP production during the first 4 days was observed, while continuous $ACTH_{1-24}$ treatment induced some decrease. Thereafter the hormonal responsiveness increased in both cases, but was lower in the case of continuous $ACTH_{1-24}$ treatment. Moreover, on the last day of experiment, the response of cells cultured without ACTH was 3 to 5 fold higher than that of cells treated with $ACTH_{1-24}$. These data and those reported previously (J. Steroid Biochem., 1981, 15, 445-448 and Endocrinology, 1981, 109, 2117-2123), suggest that during fetal life, maturation of ovine adrenal adenylate cyclase is inhibited by some factor(s). ACTH can overcome this inhibition and accelerate the spontaneous maturation of adenylate cyclase. They also suggest that the action of ACTH on both mature and fetal adrenal cells is a dichotomic process including both sensitization and desensitization, the latter being less marked in immature cells.

INTRODUCTION

In the ovine species, although newborn respond to ACTH [1] with an increase in corticosteroid plasma levels, the sensitivity of the fetal adrenal to this hormone remains very low during the last third of gestation (1). This poor response to ACTH seems due to partial defects of fetal adrenal cells in components of the ACTH sensitive adenylate cyclase system (2, 3) and to a low activity of several enzymes of the steroidogenic pathway (4, 5). However, perfusion of $ACTH_{1-24}$ to lamb fetuses for 2-5 days overcomes this refractoriness (6). Recently, we have shown that such a treatment

[1] The abbreviations used are : $ACTH_{1-24}$, corticotropin-(1-24)-tetracosapeptide ; MIX, 1-methyl-3-isobutylxanthine.

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induces development of most of these biochemical steps (7) which correlates closely with the changes which occur normally during late gestation. This suggests that ACTH is an important regulating hormone for the maturation of the fetal adrenal cells. However, it is not known whether this $ACTH_{1-24}$ induced maturation is due to a direct or to an indirect action of the hormone, and whether other hormone(s) or factor(s) are also involved in this development. In order to clarify these problems, and to gain some insight into the mechanism and the time course of action of ACTH on the adenylate cyclase development, we investigated in the present study the $ACTH_{1-24}$ stimulation of cAMP production by fetal and newborn ovine adrenal cells maintained in culture for 6 days in the absence or presence of $ACTH_{1-24}$.

MATERIAL AND METHODS

Cross breed (Ile de France x Romanov-Ile de France) fetuses (115-120 days old) and newborn lambs (< 0.5 day old) were used in these experiments. The normal gestation period of this breed is 145 ± 1 days. Fetuses were obtained by caesarean section under general anesthesia of the mother (fluothane/O_2). Animals were killed by decapitation and pairs of adrenals were quickly removed and kept in physiological saline until processed. Isolated adrenocortical cells were prepared as described elsewhere (8). The cell pellet was suspended in Ham's F_{12} medium (Gibco, Glasgow, Scotland) pH 7.4 supplemented with gentalline, 2 % horse serum, 10 mM Hepes, 10 µg/ml porcine insulin (Sigma), 10 µg/ml transferrin (Sigma), 40 ng/ml fibroblast growth factor (Collaborative Research Inc., Weltham, Mass.) and 100 µM ascorbic acid (solution I). An aliquot of the suspension was taken for counting using a hemocytometer ; cell viability was assessed by trypan blue exclusion. Aliquots of the cell suspension (2 ml, ≃ 8×10^5 cells) were seeded in 25 cm^2 sterile culture flasks (Falcon Plastics, CA) which were placed in a humidified incubator at 37°C. The gas phase was air, containing 5 % CO_2. One day after the beginning of culture (day 1) $ACTH_{1-24}$ (Synacthen, Ciba, Rueil Malmaison, France) was added in the appropriate flasks to achieve a final concentration of 10^{-8} M. At selected times during culture, the medium was aspirated and replaced by 3 ml of solution I containing 0.5 mM of MIX with or without $ACTH_{1-24}$ (10^{-8} M). Culture flasks were then placed in the incubator at 37°C. Aliquots (0.5 ml) of this medium were withdrawn at 30, 60, 90 and 120 minutes, or at 120 minutes only and added to tubes containing 3 ml of absolute ethanol precooled at - 20°C. These tubes were centrifuged (1500 x g for 10 minutes) and the supernatant(s) kept at – 20°C until assayed for cAMP content. At the end of culture, cells were detached from the culture dishes in NaCl 0.9 %, 1 mM EDTA pH 7.4 containing 1 mg/ml trypsin (Worthington, NJ) and counted. Measurement of cAMP was performed by radioimmunoassay (9). In order to determine whether ACTH present in horse serum could interfere in the experiments, 4 ml of horse serum were extracted with silicic acid (10) ; the extracted ACTH was resuspended in 0.5 ml of 0.02 M veronal buffer, pH 8.4, and assayed with a CEA RIA kit (Saclay, France). ACTH concentration was found to be less than 15 pg/ml of buffer giving a final ACTH concentration in the culture medium of less than $7.5 \cdot 10^{-15}$ M. Alternatively the ACTH level in horse plasma was $1.2 \cdot 10^{-11}$ M.

RESULTS

Under the culture conditions used in these studies, the plating efficiency was always higher than 90 % and the number of cells remained constant ($8.9 \pm 0.4 \times 10^5$ cells, mean ± SD, n = 20) throughout the culture period. Although no specific study was performed to identify the cells at the beginning and at the end of culture, the above results suggest that the type of cells was probably similar throughout the experiment. Under basal conditions, cells formed a flattened epithelial-like monolayer. Under phase

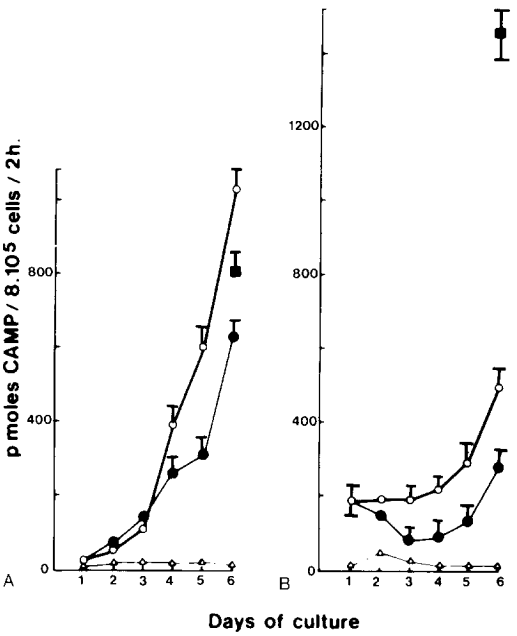

Figure 1. cAMP production by cultured adrenal cells from fetuses (A) and newborn lambs (B). Cells were cultured in the absence (Δ) or presence of $ACTH_{1-24}$ either 24 hours (●) or 2 hours (O) per day. Basal or $ACTH_{1-24}$ (10^{-8} M) stimulated cAMP productions during 2 hours were assessed daily. The response to $ACTH_{1-24}$ (10^{-8} M) on the last day of the experiment by cells cultured during five days in ACTH-free medium is also indicated (■). The results represent the mean ± SD of triplicate determinations of two different experiments.

contrast microscopic examination, a single nucleus was visible, surrounded by a broad expanse of cytoplasm. Following addition of $ACTH_{1-24}$ to the culture medium, most of the cells retracted and rounded-up slightly. This effect of $ACTH_{1-24}$ was observed at any time during the culture period but became more marked in cells cultured for several days (in the absence or presence of $ACTH_{1-24}$) (data not shown).

In the first series of experiments the ability of cultured adrenal cells to produce cAMP was investigated daily. Basal cAMP output by cells from fetuses remained low and constant throughout the 6 days of culture (20.3 ± 3.3 pmoles/8.10^5 cells/2 h mean ± SE) (Fig. 1a). The cAMP production stimulated by $ACTH_{1-24}$ on day 1 was very low (33.8 ± 2.5 pmoles) but significantly higher than basal value ($p < 0.01$) which agrees with the response of freshly isolated adrenal cells from fetuses of the same age (8). When cells were exposed to $ACTH_{1-24}$ for 2 hours per day, from day 1 onwards, the response to this hormone increased in a striking way during the course of the experiment, and the production of cAMP on day 6 (1040 ± 60 pmoles) was near 40 fold higher than that observed on day 1. However, when $ACTH_{1-24}$ was continuously present in the culture medium from day 4 onwards, the acute response to the hormone was weaker (610 ± 30 pmoles on day 6) than in the case of cells exposed to $ACTH_{1-24}$ for only 2 hours per day. An unexpected finding of this study was the high production of cAMP in response to $ACTH_{1-24}$ on day 6 (810 ± 40 pmoles), by fetal cells cultured in

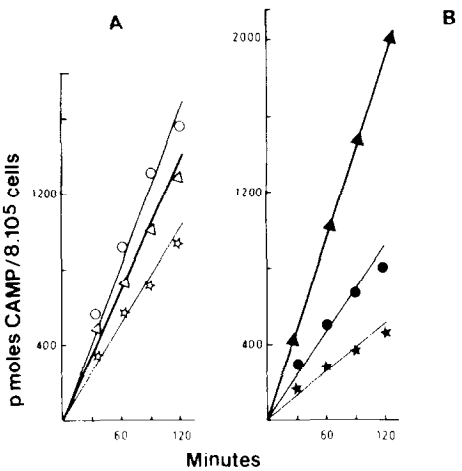

Figure 2. Time course of $ACTH_{1-24}$ stimulated cAMP production by cultured adrenal cells from fetuses (A) and newborn (B) lambs. Cells were maintained for 5 days in the absence (△, ▲) or in the presence of $ACTH_{1-24}$, either 24 hours (☆ , ★) or 2 hours (○, ●) per day. On day 6, cells were incubated with 10^{-8} M $ACTH_{1-24}$ in presence of 0.5 M MIX. At indicated times, aliquots (0.5 ml) of medium were withdrawn and cAMP contents were assessed as described under Materials and Methods. The results represent the mean of triplicate determinations for two different experiments.

absence of this hormone for 5 days, which was about 25 fold higher than the response obtained on day 1 (Fig. 1a).

Basal cAMP output by cultured adrenal cells from newborn lambs remained low and roughly stable throughout the course of the experiment (23.5 ± 5.5 pmoles/8.10^5 cells/2 h) (Fig. 1b). On day 1 of culture, the $ACTH_{1-24}$-stimulated cAMP production (193.2 ± 23.7 pmoles) was significantly higher ($p < 0.001$) than that of cells from fetuses, which agrees with data obtained from freshly isolated ovine adrenal cells (8). When cells from newborn lambs were cultured in presence of $ACTH_{1-24}$ for 2 hours per day, the acute cAMP response to this hormone remained unchanged during the first 4 days of the experiment, then doubled between day 4 and day 6. It must however be underlined that the value reached on day 6 (510 ± 30 pmoles) was only one half of that observed with fetal adrenal cells similarly treated. The continuous presence of $ACTH_{1-24}$ in the culture medium resulted in a progressive decrease of the acute response to the hormone during the first 3 days of treatment, followed by a slight increase until day 6. However, the response obtained under these conditions was always lower than that of cells exposed to $ACTH_{1-24}$ for only 2 hours per day. Alternatively, the absence of $ACTH_{1-24}$ in the culture medium for the first 5 days of culture resulted on day 6 in a cAMP response to $ACTH_{1-24}$ which was very high (1480 ± 110 pmoles), 5 and 3 fold higher than the production achieved by cells treated with $ACTH_{1-24}$ continuously or 2 hours per day respectively (Fig. 1b).

The validity of assaying cAMP in the incubation medium of 2 hours as a good evaluation of the cAMP production of the cells is demonstrated by the results of fig. 2 which shows the time course response in cAMP by $ACTH_{1-24}$ stimulated cultured

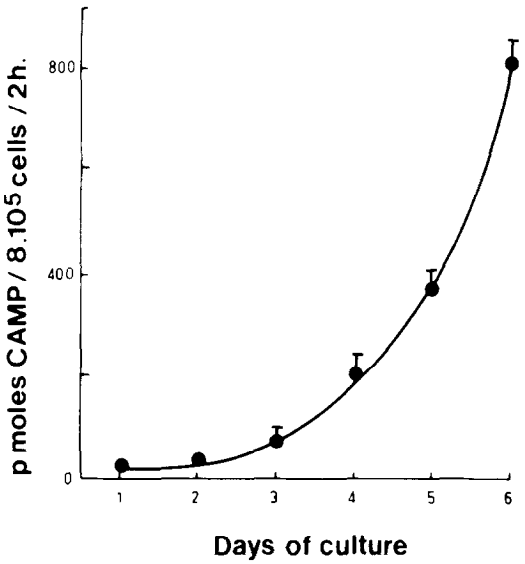

Figure 3. Development of the cAMP response to acute $ACTH_{1-24}$ stimulation of ovine fetal adrenal cells cultured in the absence of ACTH. Cells were maintained in ACTH free medium from day 1 to day 6 ; each day $ACTH_{1-24}$ (10^{-8} M) was added to two flasks, and the cAMP production of 2 hours was determined. Each point represents the mean ± SD of triplicate determinations for two different experiments.

adrenal cells from ovine fetuses and newborn lambs. For both fetal (Fig. 2a) and neonatal (Fig. 2b) cells, cAMP production increased linearly between 0 and 120 minutes. Moreover, at two hours, the intracellular cAMP content represented less than 8 % of the extracellular cAMP (data not shown).

In order to specify the time-course of the enhanced response to $ACTH_{1-24}$ of fetal adrenal cells cultured in the absence of this hormone, the acute effect of $ACTH_{1-24}$ on cAMP production was investigated daily in cells maintained in ACTH-free media from day 1 to day 6. The typical results depicted in fig. 3 show that the response remained nearly constant during the first 2 days, then roughly doubled every day until the end of culture.

DISCUSSION

Results from several laboratories have shown that in late gestation the ovine fetal adrenal undergoes fundamental alterations resulting in an enhanced capacity of cells to produce both cAMP and corticosteroids (8, 11, 12), and that this development can be induced prematurely by perfusing lamb fetuses *in utero* with $ACTH_{1-24}$ (6, 8). The present data demonstrate that some of the *in vivo* $ACTH_{1-24}$ induced maturational changes can be reproduced *in vitro*. Five days exposure to $ACTH_{1-24}$ of cultured adrenal cells from 115 days old ovine fetuses resulted in increasing response to $ACTH_{1-24}$ in terms of cAMP which become even higher than that of cells isolated from newborn lambs and treated similarly.

However, the development observed seems to involve combination of at least 3 phenomena : (i) increased cAMP response to $ACTH_{1-24}$ when cells are cultured in the absence of this hormone for several days, (ii) $ACTH_{1-24}$ induced enhancement of cAMP production when $ACTH_{1-24}$ is given 2 hours per day, (iii) desensitization to $ACTH_{1-24}$ which occurs when the hormone is continuously present in the culture medium for more than 48 hours. The precise causes of the inversed response to $ACTH_{1-24}$ of fetal adrenal cells cultured without this hormone are unknown. Changes in phosphodiesterase activity seem unlikely since similar differences in cAMP production between days 1 and 6 were observed when $ACTH_{1-24}$ stimulation was performed in the absence (data not shown) or presence of phosphodiesterase inhibitor (MIX 0.5 mM). Moreover the linear production of cAMP between 0 and 120 minutes with cells cultured in the absence and in the presence of $ACTH_{1-24}$ does not favour this hypothesis. An alternative explanation might be that this increase expresses progressive recovery of membrane structure(s) destroyed during the isolation procedure. However, it has been shown that such damages, if any, are reversed after 48 hours of culture (13), while the phenomenon described here takes place later. Hence it seems reasonable to postulate the existence in the ovine fetus of factor(s) which inhibit the "spontaneous" development in vivo of some component(s) of the $ACTH_{1-24}$-sensitive adenylate cyclase, which would be absent in the culture medium. The action of this inhibiting factor can be overcome by perfusing lamb fetuses in utero with rather high doses of $ACTH_{1-24}$, since such a treatment increases the response of adrenal membrane adenylate cyclase to $ACTH_{1-24}$, Gpp(NH)p and NaF as well as the number of $ACTH_{1-24}$ binding sites, resulting in an enhanced capacity of isolated adrenal cells to produce cAMP as compared to control fetuses (7, 8). Moreover, our results indicate that $ACTH_{1-24}$ can accelerate directly the maturation of the receptor adenylate cyclase system, since the cAMP response to the hormone of cells pretreated with $ACTH_{1-24}$ was higher than that of non $ACTH_{1-24}$ treated cells during the first four days of culture. Thereafter the hormonal responsiveness of cells treated continuously with $ACTH_{1-24}$ becomes lower than that of non treated cells, indicating that continuous $ACTH_{1-24}$ treatment induces some desensitization of the adenylate cyclase system.

The results obtained with newborn lamb cultured adrenal cells were somewhat different. $ACTH_{1-24}$ treatment two hours per day does not produce significant changes on $ACTH_{1-24}$-induced cAMP production during the first four days, while continuous $ACTH_{1-24}$ treatment induces some desensitization. Thereafter, the hormonal responsiveness increases in both cases, but the recovery is lower in the case of continuous ACTH treatment. Moreover, on the last day of the experiment, the response to $ACTH_{1-24}$ of cells cultured without this hormone is several times higher than that of cells treated with ACTH. These in vitro results appear similar to those obtained using an in vivo - in vitro model (14, 15) in which it has been shown that adrenals from both 7 days hypophysectomized rats (14, 15) and 5 days $ACTH_{1-24}$ treated rats (15), produce in vitro more cAMP in response to ACTH, than adrenals from control rats. Since it

would seem improbable that both a decreased and an increased level of trophic hormone results in the same regulatory mechanism, these apparent contradictory findings might be explained as follows : in vivo administration of $ACTH_{1-24}$ for several days increases the number of its own receptors (7, 16) and probably of adenylate cyclase (7). However, short term exposure (less than 24 hrs) of adrenal cells or quarters to ACTH induce a decrease in cAMP formation (15, 17-19) and adenylate cyclase activity (17, 18) in response to further stimulation by $ACTH_{1-24}$ which seems result from a defect in the coupling between hormone binding sites and the adenylate cyclase. Thus, according to the intensity of each process the result would be either sensitization or desensitization of adrenal cell adenylate cyclase to further $ACTH_{1-24}$ stimulation.

All the data presented so far can be reconciled by postulating that "in vivo" under physiological conditions, the ACTH-sensitive adrenal adenylate cyclase system of mature cells functions in a state of partial "uncoupling", due either to some pituitary factor(s) or more likely to ACTH itself. This state can be reversed "in vivo" by hypophysectomy or "in vitro" in an ACTH free medium. On the contrary an excess of ACTH might enhance the "decoupling" but later on this inhibitory effect is overcome by the positive effect of ACTH on its own receptors and adenylate cyclase. These regulating mechanisms do not seem to be completely operative in the ovine fetal adrenal probably because the immaturity of the receptor-adenylate cyclase system (3). In fetal adrenal the first measurable effect of $ACTH_{1-24}$ is the development of the ACTH-receptor-adenylate cyclase system. Only when the system is developed it becomes sensitive to the desensitizing effect of the hormone.

In conclusion these data, together with preceding results (3, 7, 16-18) suggest that under both in vivo and in vitro conditions, the action of ACTH on adrenal cells is a dichotomic process including both sensitization and desensitization of the ACTH sensitive adenylate cyclase, the latter being much less marked if any in immature cells. They also favour the hypothesis of the existence, during fetal life, of some factor(s) inhibiting the maturation of the adrenal adenylate cyclase. Further investigations of these mechanisms may clarify some aspects of the regulation of adrenal cell activity by ACTH.

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