EFFECTS OF PEPTIDES OF PITUITARY ORIGIN ON THE FORMATION OF C_{21} STEROID BY FETAL CALF ADRENAL CELLS IN CULTURE

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SUMMARY

Corticotrophic activity of opiate-like peptides was assessed by their ability to stimulate the formation of C_{21} steroids from [^{3}H] progesterone by three-day old cultures of fetal calf adrenal cells. $ACTH_{1-39}$, $ACTH_{\alpha 1-24}$ and a purified preparation of pituitary ovine β-endorphin caused a marked increase in 17α and 21-hydroxylation while a preparation of pure synthetic porcine β-endorphin gave a minimal stimulation. The activity of the purified ovine β-endorphin preparation could not be accounted for by contamination by ACTH or by a synergistic action between the two peptides. The novel pituitary factor described here may be due to a contaminant of the β-endorphin peak which is different from $ACTH_{1-39}$.

INTRODUCTION

The fetal adrenal plays a major role in both maturation of certain fetal tissues (1,2) and the initiation of parturition (3). In the sheep fetus the increase in glucocorticoid secretion occurs prior to any substantial increase in fetal plasma ACTH (4). This suggests that either the fetal adrenal sensitivity to ACTH changes late in gestation (5), or that another fetal pituitary trophic factor exists (6). Silman et al (7) detected several peptides in extracts of human fetal pituitaries. Since it has been well established that the fetal pituitary controls growth and function of the fetal adrenal (8) it seems reasonable to propose that certain peptides derived from the pituitary may replace ACTH as the fetal adrenal trophic factor. One of these peptides, β-LPH, is of particular interest. Since β-endorphin has opiate-like activity (9) and ACTH

Abbreviations: β-LPH, β-lipotrophic hormone; F, cortisol; B, corticosterone; S, 11-deoxycortisol; DOC, 11-deoxycorticosterone; 17-OH-P, 17α-hydroxyprogesterone; P, progesterone; TLC, thin layer chromatography.

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has been shown to interact with the opiate receptor (10), we investigated the possible reciprocal action of β-endorphin on an ACTH target gland. Moreover, Mains, Eipper and Ling (11) showed that β-LPH has a common biosynthetic origin with ACTH and Guillemin et al (12) have demonstrated the β-LPH is secreted simultaneously with ACTH.

MATERIALS AND METHODS

[^{14}C] and [^{3}H] labeled steroids were purchased from New England Nuclear, Dorval, Quebec. All enzymes and unlabeled steroids were obtained from Sigma Chemical Co., St. Louis, Missouri. Synthetic $ACTH_{1-24}$ was obtained from Organon, Dorval, Quebec, Canada. Human $ACTH_{1-39}$ was obtained from the National Pituitary Agency of the NIH. The preparation of synthetic porcine β-endorphin[1] used was obtained in the highest possible purity (11) and a purified preparation of pituitary ovine β-endorphin was prepared by methods described (14,15). All peptides and enzymes were sterilized by passage through a Millipore filter (0.22 μm) before use. The culture medium was a modified Ham's F-12 (Flow Laboratories, Missisauga, Ontario), supplemented with 17% fetal calf serum, penicillin, (50 U/ml) and streptomycin (50 μg/ml).

Bovine fetuses (130-200 days gestation) were collected from a local abbatoir and the adrenals were pooled and cut into small pieces. These were then digested twice with 10 ml of collagenase type I (1.2 mg/ml), hyaluronidase type III (1.2 mg/ml) and DNase I (0.1 mg/ml) in the culture medium for 15 min. The disaggregated cell suspension was filtered through sterile gauze and centrifuged at 2,000 rpm for 10 min. The cell pellet was washed twice with culture medium and 900,000 cells were aliquoted per Petri dish. A plating period of 24 hrs was allowed for cell attachment and then the cells were washed with Hank's Basal Salt Solution (HBSS) (16) containing penicillin (200 U/ml) and streptomycin (200 μg/ml) and incubated with 2 ml of fresh medium with or without hormone. The medium was replenished every 24 hours. The cells were exposed to the various peptides for a total of 48 hours, and then detached from the Petri dish and homogenized in 1.5 ml Kreb's Ringer bicarbonate buffer (KRB). The homogenate was incubated with 2 μCi of [1,2 ^{3}H] progesterone in 2 ml KRB, supplemented with NADPH generating system (1.2 mg glucose-6-phosphate, 0.8 mg NADPH and 1 U of glucose-6-phosphate-dehydrogenase) at 37° for 30 minutes. The incubations were stopped by the addition at 4°C of a methanol solution containing 50 μg of unlabeled and known amounts of [^{14}C]-labeled cortisol (F), corticosterone (B), 11-deoxycortisol (S), 11-deoxycorticosterone (DOC), 17α-hydroxyprogesterone (17OH-P) and progesterone (P). These mixtures were extracted twice with ten volumes of methylene chloride and the organic phase was evaporated *in vacuo*.

Initial thin layer chromatography in methylene chloride:acetone:ethyl acetate:cyclohexane:ethanol (40:10:45:45:10) separated F, B, S, DOC, 17OH-P and P into distinct bands which could be visualized under UV light. The individual bands were removed from the plate by suction and the steroids eluted with methylene chloride:methanol (9:1). Aliquots were taken for counting and the remainder was further purified by TLC in the appropriate solvent systems (16) to obtain a constant $^{3}H/^{14}C$ ratio. The percent conversion from progesterone was calculated from this ratio.

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[1] In these studies ovineβ-endorphin refers to a purified peptide which was isolated from ovine pituitaries. Synthetic β-endorphin refers to a highly purified synthetic peptide corresponding to the sequence of porcine β-endorphin.

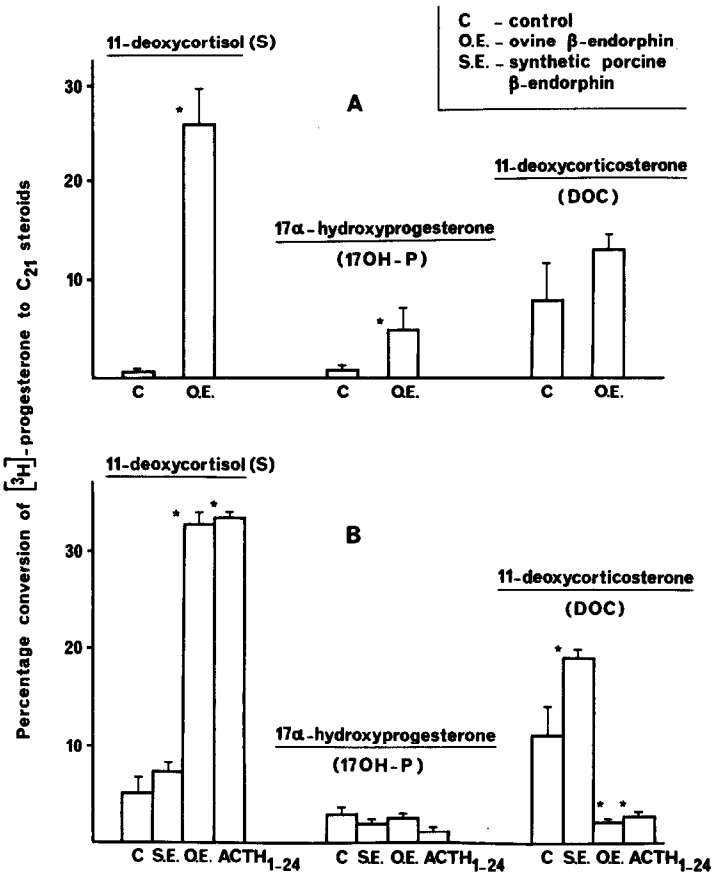

Fig. 1A and 1B: Effect of peptides on the conversion of [^{3}H] progesterone to C_{21} steroids by fetal calf adrenal cells in culture. Each bar represents the mean percentage conversion, ± S.D. of 4 determinations. *Signifies statistical significance at $p < 0.05$ or less as compared to controls. The peptides used were: synthetic β-endorphin (S.E.), a preparation of ovine β-endorphin (O.E.) and $ACTH_{\alpha 1-24}$ added in the amount of 2 μg per Petri dish daily for two days.

RESULTS

Initial data obtained demonstrated that throughout gestation the fetal calf adrenal homogenates elaborate mainly corticosterone from [^{3}H]-progesterone. After three days in culture, fetal calf adrenal cells lose most of their capacity to hydroxylate at C-11 while maintaining a strong capacity for C-17 and C-21 hydroxylation. This leads to an accumulation of the C-17 and C-21 hydroxylated intermediates. In our first studies a preparation of ovine β-endorphin, markedly

TABLE 1. Effect of $ACTH_{\alpha 1-24}$ and Synthetic Porcine β-Endorphin on the Formation of C_{21} Steroids from [^{3}H]-progesterone by Fetal Calf Adrenal Cells in Culture.

Peptide Added	Steroids formed expressed as percentage conversion from [^{3}H]-progesterone ± S.D.		
	11-deoxycortisol	17α-hydroxy progesterone	11-deoxycorticosterone
Control #	1.4 ± 0.9	1.6 ± 0.2	16.1 ± 8.6
$ACTH_{\alpha 1-24}$ (2 µg)*	11.0 ± 4.8 p < 0.002**	12.5 ± 1.6 p < 0.001	10.2 ± 2.3 N.S.
Synthetic β-endorphin (2 µg)	3.6 ± 1.0 p < 0.05	2.0 ± 0.4 N.S.	24.7 ± 5.0 N.S.
Synthetic β-endorphin (0.1 µg)	2.9 ± 1.3 N.S.	2.3 ± 0.8 N.S.	20.7 ± 6.0 N.S.
Synthetic β-endorphin (0.002 µg)	2.6 ± 0.9 N.S.	1.8 ± 0.4 N.S.	22.6 ± 5.9 N.S.

There were 4 determinations in each experiment shown.
* amount of peptide added to the cells every 24 hours
** the p was calculated using the Student's t test
N.S. signifies that the p was greater than 0.05.

stimulated the formation of S and 17 OH-P but had little effect on the formation of DOC (Fig 1A).

In a second study, both the native ovine β-endorphin preparation and $ACTH_{\alpha 1-24}$ increased S, decreased DOC and had no significant effect on the formation of 17OH-P (Fig 1B). Pure synthetic porcine β-endorphin had no effect on the formation of S and 17OH-P but did, in this instance, increase DOC. The dose response data for the synthetic β-endorphin, presented in Table 1, shows that a small stimulation of S formation only occurs at the 2 µg level.

Radioimmunoassay of the native ovine β-endorphin preparation by Dr. Dorothy Krieger showed that there was less than 10 pg of ACTH per 100 ng of the peptide (0.01% possible contamination). On this basis a dose response curve of the

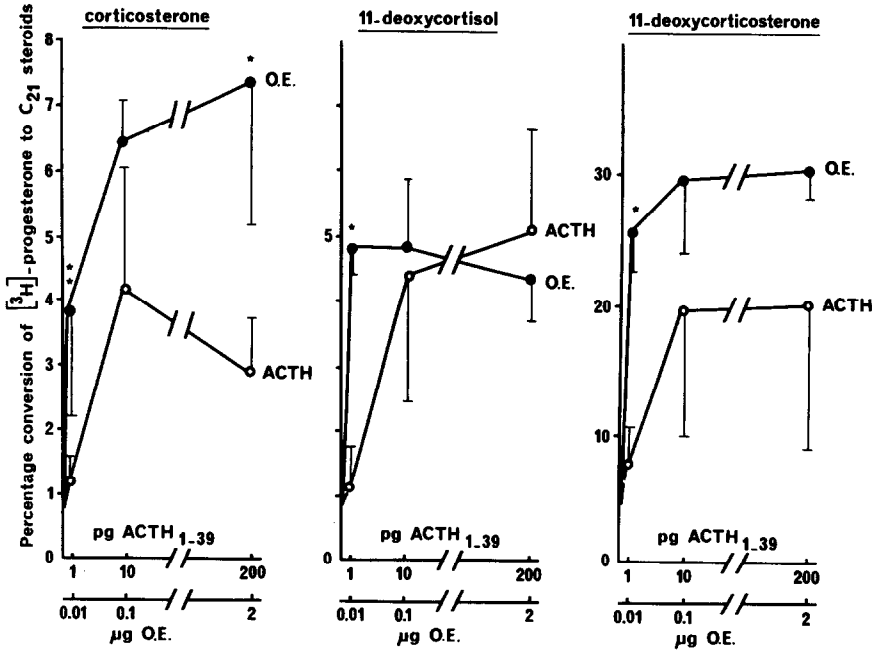

Fig. 2: Effect of a preparation of ovine β-endorphin and $ACTH_{1-39}$ on the conversion of $[^3H]$-progesterone to C_{21} steroids by fetal calf adrenal cells in culture. The peptides were added daily for 2 days. ACTH was used at concentrations 10,000 times lower than ovine β-endorphin preparation to simulate the possible 0.01% level of contamination. *Statistically significant difference at $p < 0.05$ or less. **Statistically significant difference at $p < 0.1$. Each point is the mean of 4 determinations.

pituitary ovine β-endorphin preparation was compared to that of ACTH at 0.01% contaminating levels. The results presented in Fig 2 demonstrate that a concentration of $ACTH_{1-39}$ of 10 pg/2 ml or greater, gives an activity which is not statistically different from that found for 2 µg of the native ovine β-endorphin preparation. The 1 pg/2ml concentration of ACTH does not account for the activity found in 0.01 µg of the preparation of ovine β-endorphin. Conversion to 17OH-P is not shown in Fig 2 because, in this set of experiments, this steroid was not elevated above control values by ACTH, and was depressed by the ovine β-endorphin preparation with a concommitant increase in S, an effect not observed for ACTH.

To investigate the possibility that the activity observed with the ovine

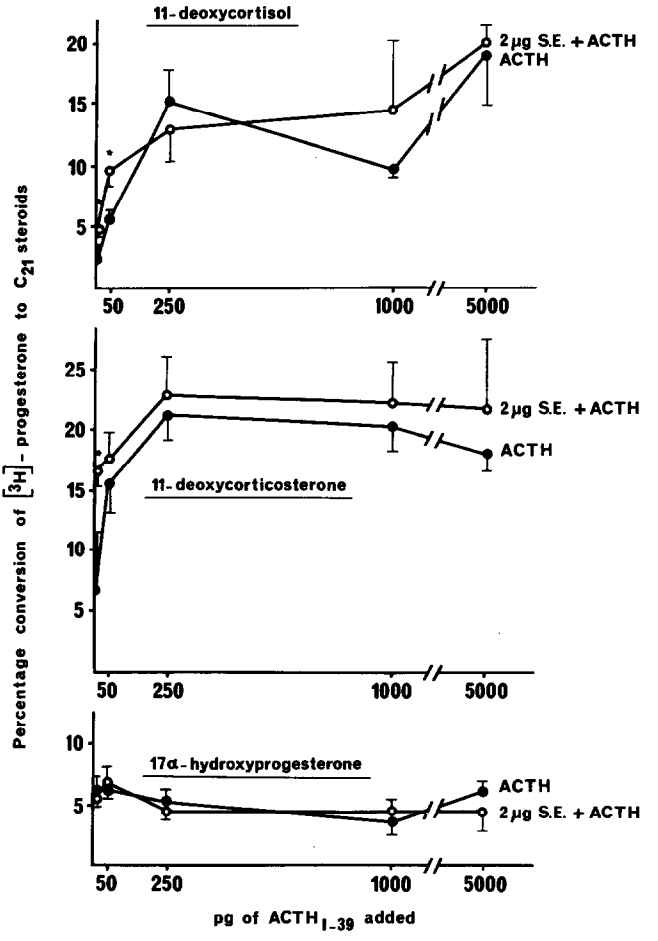

Fig. 3: Possible synergistic effects of 2 µg synthetic porcine β-endorphin (S.E.) with varying amounts of $ACTH_{1-39}$ on conversion of $[^3H]$-progesterone to C_{21} steroids in fetal calf adrenal cell in culture. The peptides were added every 24 hours for 2 days before incubation with $[^3H]$-progesterone. *Statistically significant at $p < 0.05$ between the ACTH treated and the synthetic endorphin plus ACTH treated cultures. Each point is the mean of 4 determinations.

β-endorphin preparation was due to a synergism with ACTH, we determined the effect of a mixture of both peptides on steroid formation. The data shown in Fig 3 demonstrates that when $ACTH_{1-39}$, in concentration of 50 to 5,000 pg, per 2 ml are mixed with 2 µg of synthetic porcine β-endorphin there are no statistical differences on steroid formation from ACTH added alone. The only difference observed was with 2 µg of synthetic endorphin added alone on DOC formation, as

well as with 50 pg of ACTH compared to the mixture of 50 pg of ACTH plus 2 μg of synthetic endorphin on S formation (Fig 3).

DISCUSSION

The results presented here demonstrate that the ovine pituitary β-endorphin preparation had a marked corticotrophic effect on the fetal calf adrenal cells. In contrast, pure synthetic porcine β-endorphin had only a slight stimulatory effect (Table 1 and Figs 1B and 3). ACTH contamination of the native ovine β-endorphin preparation was less than 0.01%. This concentration of ACTH did not account for the activity of the native β-endorphin preparation observed at the 0.01 μg level (Fig 2). Since no synergistic effect was observed between ACTH and the synthetic β-endorphin (Fig 3), it seems reasonable to suggest that the effect of the native ovine β-endorphin preparation is not due to an enhancement by ACTH contamination. Data from one of us (R.G.) indicates that the preparation of ovine β-endorphin used here when examined by high pressure liquid chromatography contains an additional peptide accounting for ca. 10% of the weight of the β-endorphin peak. Thus the corticotrophic-like activity of the native ovine preparation might be due to this contaminant.

$ACTH_{1-24}$, $ACTH_{1-39}$ and ovine β-endorphin all stimulate the conversion of [^{3}H]-progesterone to 11-deoxycortisol but the stimulation of 17α-hydroxyprogesterone and 11-deoxycorticosterone formation is variable. A possible explanation for these discrepancies is the variability in different cell preparations.

The identification of a new corticotrophic factor which is not ACTH or β-endorphin from adult sheep pituitaries raises several questions as to its possible role in the control of both adult and fetal adrenal glands. Investigations are in progress to demonstrate the presence of such peptides in fetal bovine pituitaries. It is possible, but not yet proven, that such a pituitary factor(s) stimulates the fetal adrenal at a time when corticosteroid production is high but the adrenal is refractory to ACTH (6).

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