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EFFECTS OF SEVERAL PRO-OPIOMELANOCORTIN DERIVED PEPTIDES ON STEROIDOGENESIS IN OVINE AND BOVINE ADRENAL CELLS

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Summary—The effects of several peptides derived from the amino-terminal end of proopiomelanocortin (*N*-POMC) alone or in combination with ACTH on ovine and bovine adrenal cell steroidogenesis have been studied. Neither porcine *N*-POMC$_{1-61}$ amide, nor γ_3-MSH, nor Lys-γ_3-MSH alone had any steroidogenic effect while porcine *N*-POMC$_{1-80}$ alone had a week but significant steroidogenic effect on isolated adrenal, the effect being more pronounced on cultural adrenal cells. The potentiation by *N*-POMC peptides of the steroidogenic action of ACTH was investigated using both freshly isolated and cultured adrenal cells. At 10^{-8}M *N*-POMC$_{1-61}$ amide, γ_3-MSH and Lys-γ_3-MSH did not modify the ACTH dose–response for steroids (gluco- and mineralocorticoids) and *c*AMP production. Likewise, the stimulatory effect of 10^{-12}M ACTH was not modified by increasing concentrations (10^{-11} to 10^{-8}M) of *N*-POMC$_{1-61}$ amide or γ_3-MSH. On the other hand, an additive effect of 10^{-8}M *N*-POMC$_{1-80}$ on the steroidogenic action of low concentration of ACTH was observed. This again was more pronounced in cultured adrenal cells. The same effects were observed when increasing concentrations of this peptide (10^{-9} and 10^{-8}M) were added together with 10^{-12}M ACTH. This result indicates that the potentiating effects of *N*-POMC derived peptides on the steroidogenic effect of ACTH which has been described on rat and human adrenal, is not a general phenomenon in mammals.

In addition to ACTH, several peptides from both the *C*-terminal [1–3] and the *N*-terminal [4–9] portions of pro-opiomelanocortin (POMC) have been reported to stimulate adrenal steroidogenesis. The steroidogenic action of the *N*-terminal glycopeptides seems to be related to the melanotropic (γ-MSH) sequence present in this portion of POMC [9]. The steroidogenic effect of the *N*-terminal POMC peptides alone is very weak, but they markedly potentiate the ACTH-induced secretion of glucocorticoids and mineralocorticoids in both rat and human adrenal cells [4–8]. However, neither the *N*-terminal glycopeptide nor γ_3-MSH have any steroidogenic effect, alone or associated with ACTH, in guinea pig adrenal cells [11]. An absence of effect of both the *N*-terminal glycopeptide and γ_3-MSH in normal isolated human adrenal cells has also been reported [9] but both peptides stimulated aldosterone secretion by human adrenal adenoma cells [9]. The discrepancy between species in the potentiating effects of *N*-POMC on ACTH-induced steroidogenesis raise the question whether this is a general phenomenon.

In the present work we have studied the steroidogenic effect of three *N*-terminal peptides of different lengths on isolated and cultured adrenal cells from two different species: bovine and ovine.

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EXPERIMENTAL

Materials

The POMC-derived peptides used were the following: porcine POMC *N*-terminal-(1–80) (*N*-POMC$_{1-80}$), amidated porcine POMC-*N*-terminal (*N*-POMC$_{1-61}$). These peptides were purified as described [12, 13]. γ_3-MSH and Lys-γ_3-MSH were synthesized [14] and provided by Drs N. Ling and R. Guillemin (Salk Institute, La Jolla, CA). Synthetic α-ACTH-(1–24) was supplied by Ciba-Geigy (Rueil-Malmaison, France), [^{3}H]corticosterone (sp. act. 50 Ci/mmol) and [^{3}H]aldosterone were obtained from the Radiochemical Centre (Amersham, England) and [^{3}H]cortisol (sp. act. 100 Ci/mmol) from CEA (France). Ham's F-10 and Ham's F-12 medium were obtained from Grand Island Biologicals Co. (New York). Ascorbic acid, transferrin, insulin and deoxyribonuclease type I were supplied by Sigma Chemicals (St Louis, MO). Collagenase was from Worthington (Flow Laboratories). [^{125}I]iodosuccinyl *c*AMP and *c*AMP antibodies were purchased from Institut Pasteur (Lyon, France).

Isolation and culture of adrenal cells

Bovine and ovine adrenal glands were collected from a local slaughterhouse, dissected, trimmed of fat and connective tissue and minced. Isolated adrenocortical cells were prepared as described [15]. After

dispersion the cell suspension was centrifuged at 100 *g* for 10 min, and washed twice. The cells were resuspended in Ham's F-10 medium supplemented with 5% fetal calf serum, antibiotics (penicillin 50 units/ml, streptomycin 25 μg/ml) and 20 mM Hepes pH 7.4.

When the hormone stimulation was carried out on freshly isolated cells, the cell suspensions were incubated for 2 h at 37°C with gentle shaking in the absence of hormones. At the end of the preincubation period, the cells were centrifuged and resuspended in the above medium containing 0.2 mM 3-isobutyl-methylxanthine, 2 mM glutamine, to achieve a final concentration at the time of incubation of about 1×10^5 cells/ml. ACTH-(1–24) and *N*-POMC peptides were added in 100 μl and the incubation (2 ml) was carried out at 37°C for 2 h in a shaking water bath. At the end of the incubation, 1 ml of the cell suspension was removed and quickly frozen at −20°C until assayed for steroids, while 0.9 ml of the remaining cell suspension was added to tubes containing 3 ml absolute ethanol precooled to −20°C for *c*AMP determination.

Cells were cultured in Ham's F-12 medium containing 20 mM Hepes, insulin (10 μg/ml), transferrin (10 μg/ml), ascorbic acid (100 μM), antibiotics (as above) and 2% horse serum. The plating density was 1.5 to 2×10^5 cells/cm^2. The cells were maintained in an humidified incubation at 37°C with 5% CO_2. The medium was changed daily. The day of the experiment the medium was removed and replaced with fresh medium. A lag period in the potentiating effects of POMC has been reported [6] and this has been related to the fact that the mechanism of action of these peptides required mRNA synthesis [7]. Therefore, when POMC peptides were associated to ACTH stimulation, the former were added 15 min before ACTH. At the end of the stimulation period, one aliquot was kept for steroid determination and another for *c*AMP.

cAMP and steroid determination

The ethanolic extracts of the incubation medium were evaporated to dryness and then dissolved in 50 mM sodium phosphate buffer, pH 6.2. After acetylation [16] *c*AMP was measured by radioimmunoassay [17]. Steroids present in incubation medium were assayed by radioimmunoassay using different antibodies. Corticosterone was assayed using an antibody (gift of Dr A. Kowarski) raised against corticosterone. The major cross-reacting steroids were deoxycorticosterone (61%), progesterone (35%), cortisol (1%) and aldosterone (0.4%). For cortisol determination, antibody raised against cortisol was used (Radioassay System Laboratories, Carson, CA, catalog number 1460). Only 21-deoxycorticosterone (11%) and corticosterone (1.4%) had a significant cross-reactivity. A very specific and sensitive antibody was used to assay aldosterone (gift of Dr Morel). The cross-reactivity of other steroids was negligible (corticosterone 0.01%, cortisol 0.00004%). All *c*AMP and steroid determinations were carried out in triplicate and each experiment was performed two to five times. Analysis of variance was used to evaluate the significance of difference between groups.

RESULTS

In the first series of experiments freshly isolated bovine adrenal cells were incubated for 2 h in the absence or presence of ACTH-(1–24), *N*-POMC-(1–80), *N*-POMC-(1–61) or γ_3-MSH at different concentrations (Table 1). Only ACTH and *N*-POMC-(1–80) were able to stimulate steroidogenesis. However, the concentrations of *N*-POMC-(1–80) required to induce an increase of corticosterone production were three orders of magnitude higher than that of ACTH. Similar results have been obtained using freshly isolated ovine adrenal cells (data not shown). In rat adrenal cells *N*-POMC peptides and γ_3-MSH alone have weak steroidogenic activity, but they potentiate the steroidogenic effect of ACTH [4–8]. Therefore, we have investigated such an effect using isolated ovine adrenal cells.

The addition of 10^{-8}M *N*-POMC-(1–61) or Lys-γ_3-MSH did not modify the effects of ACTH-(1–24) on cortisol production by isolated ovine adrenal cells (Fig. 1). On the other hand, *N*-POMC-(1–80) alone induced a small but significant ($P < 0.02$) increase of cortisol production but had no effect on *c*AMP production. The effect of *N*-POMC-(1–80) on corti-

Table 1. Effects of ACTH-(1–24), *N*-POMC-(1–80), *N*-POMC-(1–61) and γ_3-MSH on corticosterone production in freshly isolated bovine adrenal cells

	% Of control			
Peptide (M)	ACTH	POMC-(1–80)	POMC-(1–61)	γ_3-MSH
0	100 ± 7			
10^{-12}	303 ± 35*	NT	NT	NT
10^{-11}	883 ± 100*	94 ± 6	101 ± 6	105 ± 8
10^{-10}	1251 ± 140*	131 ± 17	97 ± 5	96 ± 3
10^{-9}	1540 ± 160*	169 ± 24*	88 ± 8	95 ± 2
10^{-8}	—	296 ± 26*	104 ± 6	105 ± 9

*$P < 0.05$ from control.

The results are the means ± SD of three to five different adrenal preparations. For each preparation the incubations were performed in triplicate. NT: not tested.

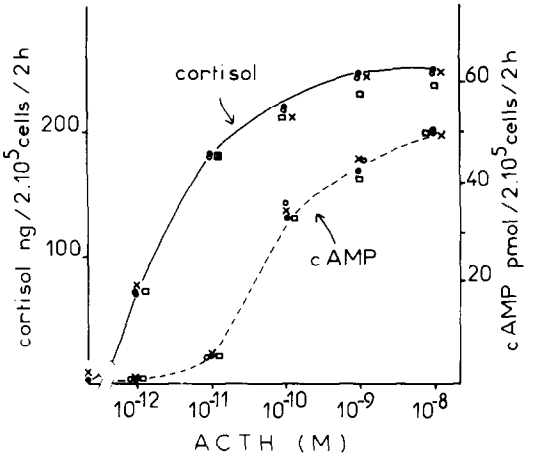

Fig. 1. ACTH dose–response for production of cortisol (——) and *c*AMP (– – –), by isolated ovine adrenal cells, in the absence (●) or presence of *N*-POMC-(1–80) (×), *N*-POMC-(1–61) (○) or Lys-γ_3-MSH (□) all at 10^{-8}M. POMC peptides were added 15 min before ACTH. The results are the means of triplicate determinations.

sol was additive to that of 10^{-12}M ACTH, but this effect was not seen at higher concentrations of ACTH. In three independent experiments *N*-POMC-(1–80) did not modify the ED_{50} of ACTH activation steroidogenesis (7.6 ± 2.10^{-12}M and $6.5 \pm 3 \times 10^{-12}$M) in the absence or presence of 10^{-8}M *N*-POMC-(1–80). Likewise, in the same experiment, this peptide did not modify the ACTH-(1–24) dose–response for *c*AMP production.

It has been reported that in both human and rat adrenal cells [6] and in human adenomas [9], *N*-POMC peptides stimulate aldosterone secretion, suggesting that perhaps these peptides stimulate preferentially glomerulosa cells. We have investigated the effects of *N*-POMC peptides and γ_3-MSH on corticosterone and aldosterone secretion by isolated ovine adrenal cells. The results (Fig. 2) show that neither *N*-POMC-(1–61) nor γ_3-MSH modified the ACTH dose–response for corticosterone and aldosterone productions. Again, *N*-POMC-(1–80) alone has a weak although significant stimulatory effect and the effect was additive to that of low concentrations of ACTH-(1–24). Similar results were obtained in a second experiment.

The absence of action of *N*-POMC-(1–61), γ_3-MSH and Lys-γ_3-MSH on freshly isolated cells could be due to damage of the receptor for these peptides occurring during the isolation procedure. Therefore, the cells were cultured for 4 days and their response to ACTH in the absence or presence of *N*-POMC peptides was studied. In these experiments the steroidogenic response was evaluated by measuring corticosterone, since we have shown [18 and unpublished results] that after 5 days in culture in the absence of ACTH, the main steroid secreted is corticosterone rather than cortisol, due to a loss of 17α-hydroxylase activity. The results (Fig. 3) show that neither *N*-POMC-(1–61) nor γ_3-MSH had any effect on the ACTH dose–response for production of *c*AMP and corticosterone. *N*-POMC-(1–80) alone at 10^{-8}M had a significant steroidogenic effect (similar to the effect of 10^{-11}M ACTH), but no synergistic effect between *N*-POMC-(1–80) and ACTH was observed. In addition, *N*-POMC-(1–80) at 10^{-8}M induced a small but significant increase in *c*AMP production (11.2 ± 0.7 pmol/2 × 10^5 cells/2 h vs 6.4 ± 0.8 in control) close to the effect of 10^{-12}M ACTH. Similar results to those presented in Fig. 3 have been obtained in 2 experiments using cultured bovine adrenal cells.

The effects of several concentrations of *N*-POMC peptides and γ_3-MSH alone or in the presence of 10^{-12}M ACTH-(1–24) are shown in Fig. 4. *N*-

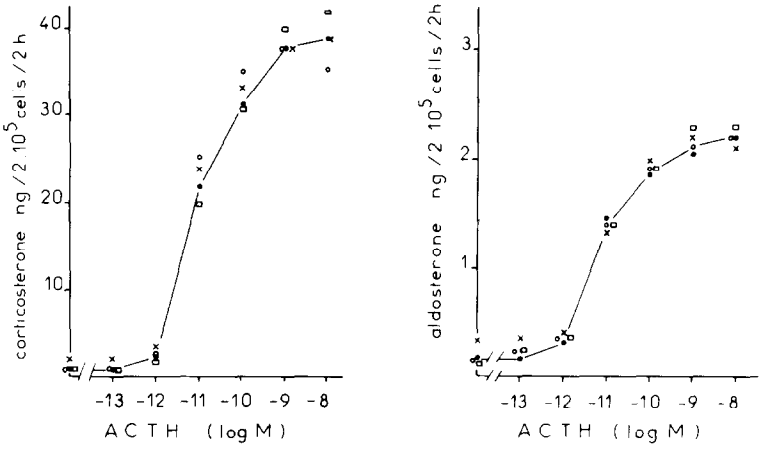

Fig. 2. ACTH dose–response for production of corticosterone (left panel) and aldosterone (right panel) by isolated ovine adrenal cells in the absence (●) or presence of *N*-POMC-(1–80) (×), *N*-POMC-(1–61) (○) or γ_3-MSH (□) all at 10^{-8}M. POMC peptides were added 15 min before ACTH. The results are the mean of triplicate determination.

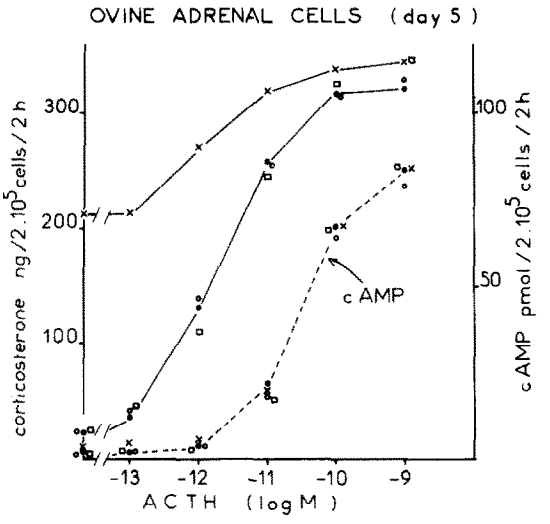

Fig. 3. ACTH dose–response for production of corticosterone (——) and *c*AMP (– – –) by cultured ovine adrenal cells in the absence (●) or presence of *N*-POMC-(1–80) (×), *N*-POMC-(1–61) (○) or γ_3-MSH (□) all at 10^{-8}M. The cells were cultured for 5 days in medium alone. The results are the mean of triplicate determinations.

POMC-(1–80) stimulated corticosterone (at 10^{-9} and 10^{-8}M) and *c*AMP (10^{-8}M) production. These effects are additive to those produced by 10^{-12}M ACTH. On the other hand, neither POMC-(1–61) nor γ_3-MSH had any effect whether alone or in the presence of ACTH.

DISCUSSION

The work demonstrates the lack of steroidogenic effect of porcine amidated *N*-POMC-(1–61) and γ_3-MSH on bovine and ovine adrenal cells. Our results confirm the very weak or absence of steroidogenic effect of *N*-POMC peptides in rat adrenal, both *in vivo* and *in vitro* [4–8]. In contrast, porcine *N*-POMC-(1–80) alone had a weak but significant steroidogenic effect in both bovine and ovine adrenal cells, the effect being more pronounced in cultured than in freshly isolated cells. It is unlikely that the action of this peptide was due to a contamination by ACTH, since the preparation we used had no detectable ACTH immunoreactivity at 10^{-5}M [9] and since its steroidogenic action is higher than its potency in stimulating *c*AMP production when compared to ACTH. The difference between *N*-POMC-(1–80) and *N*-POMC-(1–61) and γ_3-MSH could be due either to the *C*-terminal sequence in *N*-POMC-(1–80) and/or to the presence of carbohydrate on residue 65 of the former. This does not, however, explain discrepancies between our results and those of Al-Dujaili *et al.*[6] showing the steroidogenic effect of human *N*-POMC-(1–76) in both human and rat adrenal cells. It is interesting to note that, using cultured fetal ovine cells, we have shown that the effects of *N*-POMC-(1–81) on fetal adrenal maturation are different from those of *N*-POMC-(1–61) and γ_3-MSH [19].

The absence of potentiating effects of *N*-POMC peptides and γ_3-MSH on ACTH-induced steroidogenesis in both bovine and ovine adrenal cells are in contrast with the potentiation of the steroidogenic effect of ACTH by several *N*-POMC peptides: i.e. mouse trypsin-treated 16K fragment [4, 5], γ_3-MSH [5] and Lys. γ_3-MSH [8] in rat adrenal and human *N*-POMC-(1–76) in both rat and human adrenal cells [6]. Several hypotheses can be postulated

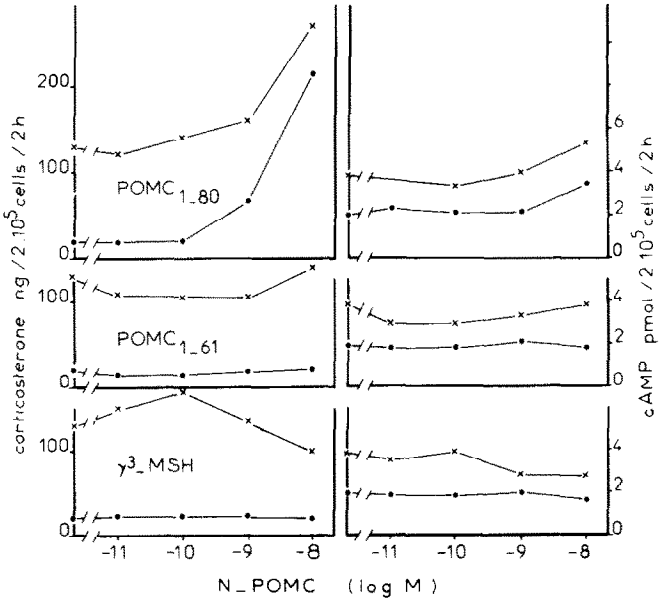

Fig. 4. *N*-POMC dose–response for production of corticosterone (left panel) and *c*AMP (right panel) by cultured ovine adrenal cells in the absence (●) or presence (×) of 10^{-12}M ACTH. The cells were cultured as indicated in Fig. 3. The results are the mean of triplicate determinations.

to explain the differences between our results and those reported previously: (a) Loss for unknown reasons of the biological activity of the peptide used in the present work. This seems unlikely since the same preparation used at the same time has been shown to be active in inducing ovine fetal adrenal cell maturation [19]; (b) different origins of the peptides: porcine pituitary in our work, mouse pituitary tumor in Pedersen's work [4, 5] and human pituitary in Al-Dujaili's work [6,7]. This cannot, however, be applied to γ_3-MSH and Lys-γ_3-MSH used by Pedersen *et al.*[5] and Farese *et al.*[8] respectively. These peptides (bovine sequence) like those used in the present work, were synthesized by Dr N. Ling[14]; (c) Damage to the γ_3-MSH receptor [20] in adrenal cells during the isolation procedure. This seems also unlikely since our method is similar to that used by Farese *et al.*[8] and our preparation remains sensitive to several stimuli including ACTH, angiotensin [21] and *N*-POMC-(1–80). Moreover, the increased responsiveness of cultured adrenal cells to several stimulatory agents, including *N*-POMC-(1–80), did not conform to the appearance of the potentiating effect of POMC peptides on ACTH action; (d) difference between species concerning the specificity of *N*-POMC peptides towards adrenal cells and the sensitivity of these cells from different species to *N*-POMC peptides. As an example, the amino-terminal end of human POMC potentiates the steroidogenic effect of ACTH in both human and rat adrenal cells [6, 7] but not in guinea pig adrenal cells [11]. Likewise γ_3-MSH potentiates the ACTH effect in rat adrenal cells [5, 8] but has no action in guinea pig [11], bovine and ovine (present work) adrenal cells. Moreover, the small potentiating effect of these peptides on the steroidogenic response to ACTH of fetal ovine adrenal cells was seen only after 3 days in culture [19]. Discrepancy between species in the response of an endocrine cell to several stimuli has been well established, e.g. LHRH and estradiol modified the steroidogenic activity of rat Leydig cells [22, 23], while both hormones are inactive in both mouse [24, 25] and porcine [26, 27] Leydig cells.

In summary, our results indicate that the pronounced potentiating effects of *N*-POMC peptides on the steroidogenic effects of ACTH observed in rat and human adrenal seem not to be present in other species (adult ovine and bovine). Therefore, the potential physiological role of these peptides in mammalian adrenal regulation requires further studies.

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