Mineralocorticoid and Glucocorticoid Effects on 31,000- and 29,000-Dalton Proopiomelanocortin in Rat Anterior Pituitary and Neurointermediate Lobe

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ABSTRACT The effects of adrenal steroids on proopiomelanocortin (POMC) levels in rat pituitary have been studied by two-dimensional gel electrophoresis. In intact rats the relative abundance of POMC was much higher in the neurointermediate lobe (N-IL) than in anterior pituitary (AP); in both tissues the predominant species appeared to be of 29,000-dalton (29K) molecular mass, with lesser amounts of a 31K form. In both tissues, the 31K and 29K forms showed multiple spots, consistent with different degrees of sialoglycosylation. Adrenalectomy was followed by a marked increase in AP levels of POMC, and a marked decrease in N-IL levels. In adrenalectomized rats, dexamethasone administration did not affect N-IL levels of POMC, but suppressed ^{35}S incorporation into POMC in AP in a dose-related manner; deoxycorticosterone showed minimal effects on AP levels of POMC, but progressively elevated N-IL levels; 9α-fluorocortisol (9αfF) progressively both suppressed AP levels, and raised N-IL levels of POMC. Estimation of immunoreactive (ir) ACTH and ir-β-endorphin in parallel samples showed an elevation of N-IL levels in response to mineralocorticoids (deoxycorticosterone, 9αfF), and a paradoxical elevation of AP levels in response to glucocorticoids (dexamethasone, 9αfF) compared with oil-injected adrenalectomized controls. We conclude (*a*) that glucocorticoids suppress the secretion of ir-ACTH and ir-β-endorphin to a greater extent than they inhibit the synthesis of POMC; (*b*) that mineralocorticoids specifically elevate the N-IL levels of both POMC and its immunoreactive product (β-endorphin).

INTRODUCTION

There is now ample evidence that adrenocorticotropic hormone (ACTH),[1] β-lipotropin (βLPH), and β-endorphin (βEP) share a common precursor in both anterior pituitary (AP) and neurointermediate lobe (N-IL) (1–3). This precursor—termed proopiomelanocortin (POMC)—is processed differently in the AP and the N-IL (4). In the AP POMC is conservatively processed, with the C-terminal region yielding predominantly ACTH and βLPH. In the N-IL, however, POMC is processed further (5–9), to smaller peptides—α-melanocyte-stimulating hormone, (α-MSH), corticotropinlike intermediate lobe peptide, γLPH, β-MSH, βEP, C′ fragment (βLPH_{61-87}), *N*-acetyl βEP, and *N*-acetyl C′.

A second, salient difference between the AP and the N-IL lies in the factors regulating immunoreactive (ir-) ACTH and ir-βEP levels. Secretion of ACTH and ir-βEP is under glucocorticoid control in vivo (10). In vitro, the synthesis of POMC can, similarly, be suppressed by glucocorticoids in AtT20 cells, a mouse anterior pituitary cell line (11). In the N-IL, however, ir-βEP has been shown to be under tonic inhibitory dopaminergic control, with glucocorticoids having no demonstrable effect (12).

Previous in vivo studies from our laboratory have shown that the content of ir-ACTH and ir-βEP in the AP of the adrenalectomized rat is elevated by glucocorticoids, while mineralocorticoids specifically increase the content of ir-βEP in the N-IL (13). In this paper we have analyzed the effects of in vivo administration of glucocorticoids and mineralocorticoids on POMC synthesis in anterior and neurointermediate lobes of the rat pituitary gland, using two-dimensional (2-D) gel electrophoresis to complement the previous studies on immunoreactive content.

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[1] *Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; AP, anterior pituitary; 2-D gel electrophoresis, two-dimensional gel electrophoresis; DM, dexamethasone; DMEM, Dulbecco modified Eagle's medium; DOC, deoxycorticosterone; βEP, β-endorphin; 9αfF, 9α-fluorocortisol; ir, immunoreactive; 29K, 31K, 29,000-, 31,000-dalton proteins; N-IL, neurointermediate lobe of pituitary; PBS, phosphate-buffered saline; POMC, proopiomelanocortin.

METHODS

Chemicals and reagents. Dexamethasone (DM) was the gift of Merck Sharp & Dohme (Sydney, Australia) and 9α-fluorocortisol (9αfF) of E. R. Squibb & Sons, Inc. (Sydney, Australia). Deoxycorticosterone (DOC) was purchased from Steraloids, Inc. (Wilton, NH).

β-Mercaptoethanol, Tris-HCl, Trizma base and Nonidet P-40 were from Sigma Chemical Co. (St. Louis, MO); sodium dodecyl sulphate, of specially pure grade, from BDH Chemicals Ltd. (Poole, U. K.). Acrylamide (electrophoresis pure grade) was from Bio-Rad Laboratories (Richmond, CA). Pharmalyte pH 3–10 and pharmalyte pH 5–7 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Tetramethyl ethylene diamide (TEMED) and *N,N'*-methylene bis-acrylamide were from Eastman Kodak Co. (Rochester, NY). Fetal calf serum, Dulbecco modified Eagle's medium (DMEM), Dulbecco modified Eagle's medium (methionine free), sodium bicarbonate 2.8%, and glutamine 200 mM/ml were from Commonwealth Serum Laboratories (Melbourne, Australia). All other laboratory chemicals were from Ajax Chemicals (Sydney, Australia). [^{35}S]Methionine (>600 Ci/mmol) was obtained from Amersham Corp. (U. K.).

Animal studies. Female Sprague-Dawley rats weighing 150–200 g, from a pathogen-free colony bred in the central animal house at Monash University, were used in all experiments. Bilateral adrenalectomy was performed by the dorsal midline approach under light ether anesthesia. Completeness of surgery was verified by inspection at the time of killing. Adrenalectomized rats were maintained on 0.9% saline and regular chow, six per cage (45 × 30 × 15 cm) in an air conditioned room, with a controlled 12-h light (0600–1800) and dark cycle. Each rat was numbered and weighed on the day of adrenalectomy, and reweighed on the day of killing. From day 1 postoperatively, 0.1 ml of vehicle (sterile maize oil), or steroids dissolved in maize oil, were administered intramuscularly daily, between 0930 and 1030 for 6 consecutive d. The rats were given steroids as follows: DM 2 μg, 20 μg, or 200 μg/d; 9αfF, 20 μg or 200 μg/d; DOC 60 μg or 600 μg/d. Sham-adrenalectomized rats were given 0.1 ml maize oil only.

4 h after the last injection, animals were killed by guillotine and the cranial vault opened. The AP and N-IL were removed separately under the dissecting microscope. Two glands from each group were analyzed by 2-D gel electrophoresis, and four were assayed for total ir-βEP and ir-ACTH. Details of both radioimmunoassays have been published (13). For ACTH radioimmunoassay, an antiserum (R72) raised against Synacthen ($ACTH_{1-24}$, Ciba-Geigy Corp., Lane Cove, Australia) was used; this antiserum recognizes Synacthen and human $ACTH_{1-39}$ (MRC 74/555) equally on a molar basis, but cross-reacts < 1% with $ACTH_{1-13}$, $ACTH_{18-39}$, and a variety of EP-related fragments. For βEP radioimmunoassay, an antiserum (R56) raised against synthetic ovine βEP (Peninsula Laboratories, Inc., San Carlos, CA) was used; this antiserum recognizes βEP, βLPH, *N*-acetyl βEP, βLPH_{61-87}, and *N*-acetyl βLPH_{61-87} equally on a molar basis, but cross-reacts < 1% with βLPH_{61-76}, βLPH_{61-77}, or a variety of ACTH-related fragments.

Processing of pituitary glands. Pituitary glands were individually placed in phosphate-buffered saline (PBS) at 37°C. Each gland was minced thoroughly with a sterile razor blade, and the tissue aspirated repeatedly up-and-down a siliconized, fine-bore Pasteur pipette. The dispersed tissue was incubated in 1 ml (AP) or 0.5 ml (N-IL) of methionine-free DMEM supplemented with 10% fetal calf serum, 2.8% bicarbonate (2%, vol/vol), 200 mM glutamine (1%), and 300 μCi [^{35}S]methionine/ml. Incubation was done in a plastic 24-well tissue culture plate, with each well of 16-mm Diam (Costar Data Packaging, Cambridge, MA) for 1 h at 37°C in a humidified atmosphere of 95% O_2/5% CO_2 using a Kevatron model 102 incubator. The tissue was then washed three times with 2 ml DMEM, the pellet lysed in 120 μl (AP) or 50 μl (N-IL) of lysis buffer (9.5 M urea, 2% Nonidet P-40, 1.6% ampholines pH 5–7, 0.4% ampholines pH 3–10, 5% β-mercaptoethanol), and an aliquot counted to determine the incorporation of [^{35}S]methionine.

2-D gel electrophoresis. The 2-D gel electrophoresis method of O'Farrell (14) was used for analysis of the newly synthesized cellular proteins, except that gels were dried between two sheets of thin cellulose acetate paper. To enable proper standardization of gels, ~25 μl of cell lysates containing ~0.5×10^6 cpm were applied to each tube gel. Autoradiographs of the gels were compared with each other by eye, using the actin spot as a marker protein. All gels were run in batches of 12, with the same samples run both concurrently and in separate runs, to monitor differences within and between runs. Such differences were found to be small; to minimize error due to between-run differences, all comparisons made are of gels in the same run.

Immunoprecipitation studies. Two methods were used to identify the 29K/31K spots on 2-D gels as POMC. AP from adrenalectomized rats were processed and labeled with [^{35}S]methionine as described above. At the end of the incubation, tissues were washed, lysed in PBS containing 0.1% Triton X-100, and part of this lysate kept as control. Aliquots (300 μl) of the immunoglobulin G fraction of anti-βEP (R56) immune serum were added to a Sepharose-protein A column (CL-4B, Pharmacia Fine Chemicals) in phosphate buffer, pH 8.0. Pooled AP lysates, pH 8.0, were then run through the column, followed by phosphate buffer. Both protein content and radioactivity were monitored in the fractions collected. The appropriate column fractions were lyophilized, dialyzed against distilled water, relyophilized, dissolved in 200 μl of lysis buffer, and analyzed by 2-D gel electrophoresis.

The second method of demonstrating that the protein spots seen on the 2-D gels were immunologically equivalent to ir-βEP was that of Crine et al. (15). AP were labeled with [^{35}S]methionine in methionine-free DMEM, as described. The labeled tissues were lysed in PBS with 0.025 M sodium EDTA and 2% Triton X-100, centrifuged to remove non-dissolved tissues, and the supernatant collected. Specific rabbit anti-βEP antiserum was then added, incubated for 16 h at 4°C, and second antibody (goat anti-rabbit) added with further incubation for 16 h. The incubation mixture was layered on top of 1 M sucrose in PBS, and centrifuged at 10,000 *g* for 5 min. The pellet of antigen-antibody complexes was washed two times, dissolved in 200 μl of lysis buffer, and the lysate analyzed by 2-D gel electrophoresis, in the same run as the untreated tissue sample and that passed down the affinity column.

RESULTS

Differences in POMC. As can be clearly seen in Fig. 1, there are distinct differences in the region corresponding with the putative POMC spots in the AP and N-IL. First, there is a greater abundance of POMC in the N-IL compared with the AP, even when levels in the latter tissue are increased postadrenalectomy. Secondly, the POMC proteins in the AP are almost

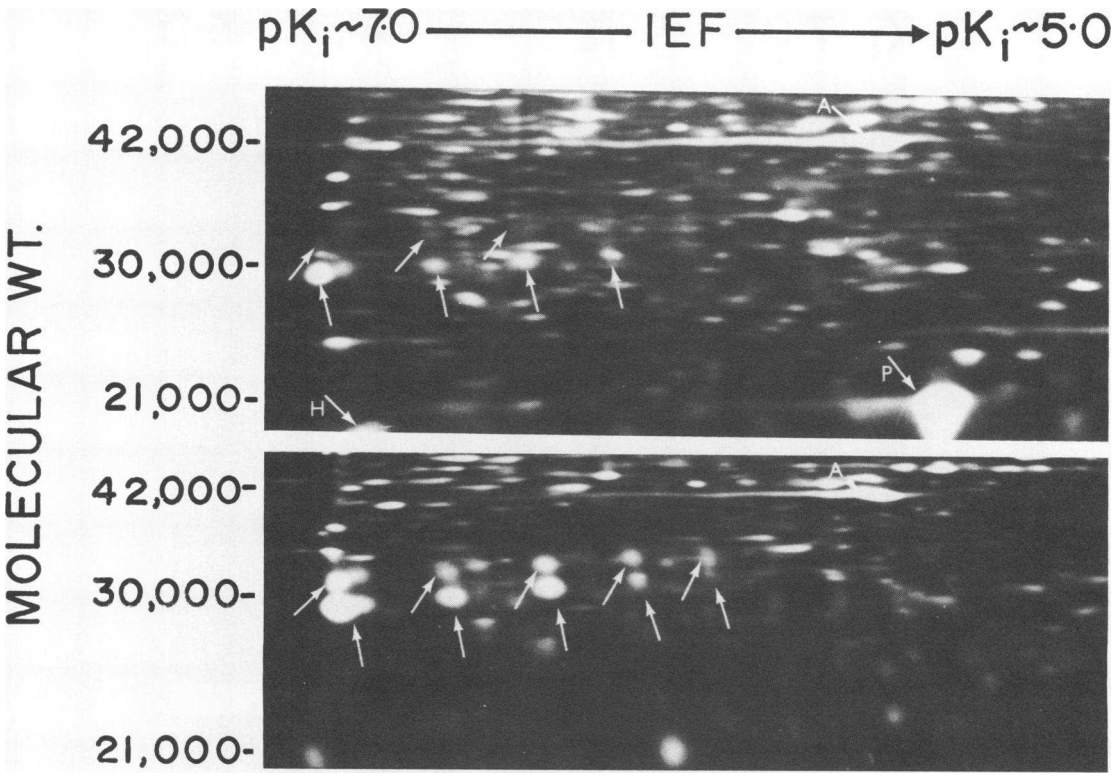

FIGURE 1 2-D gel autoradiograph of AP from a 6-d adrenalectomized, oil-injected rat (upper panel), and of N-IL from a sham-adrenalectomized, oil-injected rat (lower panel). Actin (mol wt 42,000, $pK_i \sim 5.4$) is designated A, prolactin P (mol wt 21,000, $pK_i \sim 5.2$), and growth hormone (mol wt 20,000, $pK_i \sim 6.8$) H. The putative 29K and 31K POMC spots are shown by unlabeled arrows.

exclusively of ~29K molecular mass, with trace levels of a species with higher molecular mass (~31K). In the N-IL, even though the predominant protein species is the 29K, there is still clearly more of the 31K species than in the AP. Both the 29K and 31K species include 4–5 different forms, with a range of isoelectric points (pK_i) presumably reflecting differences in sialic acid content of differentially glycosylated forms (16). The position of the proteins (31K, 29K), and their differentially glycosylated forms on 2-D gel electrophoresis, are essentially identical to the 32K and 29K POMC previously demonstrated in mouse pituitary tumor cells (11). Similarly, Crine et al. (15) were also able to demonstrate different molecular mass species ranging from 36 to 32K.

That these proteins represent authentic POMC is attested to by their selective removal by prior immunoabsorption with antisera directed against βEP (Fig. 2, center panel). When the immunoabsorbed material was run on 2-D gel electrophoresis, a single spot (29K mol mass, $pK_i \sim 6.8$) consistent with POMC was seen (Fig. 2C).

Effects of adrenalectomy on POMC levels. 6 d after adrenalectomy, the total level of POMC in the AP was markedly increased. Fig. 3 shows that all four forms, with their different pK_i, appear increased. In contrast, after adrenalectomy, there is a marked decrease in the levels of the various forms of 31K and 29K proteins found in N-IL from the same sham and adrenalectomized animals as used for AP studies (Fig. 4).

There were also several other protein changes seen in both the AP and N-IL after adrenalectomy; these, however, were minor and not consistently seen. Not surprisingly, given the molecular mass range of the proteins analyzed (~20K–~100K), the lower molecular mass products of POMC in both N-IL and AP were not detected.

Steroid effects on the N-IL. After six daily doses of DOC (60 and 600 μg), there was a marked increase in the POMC in the N-IL, the increase being especially prominent in the 29K species (Fig. 5). Similarly, with 9αfF (20 and 200 μg), there was also a marked increase in both the 29K and 31K species in the N-IL (Fig. 6).

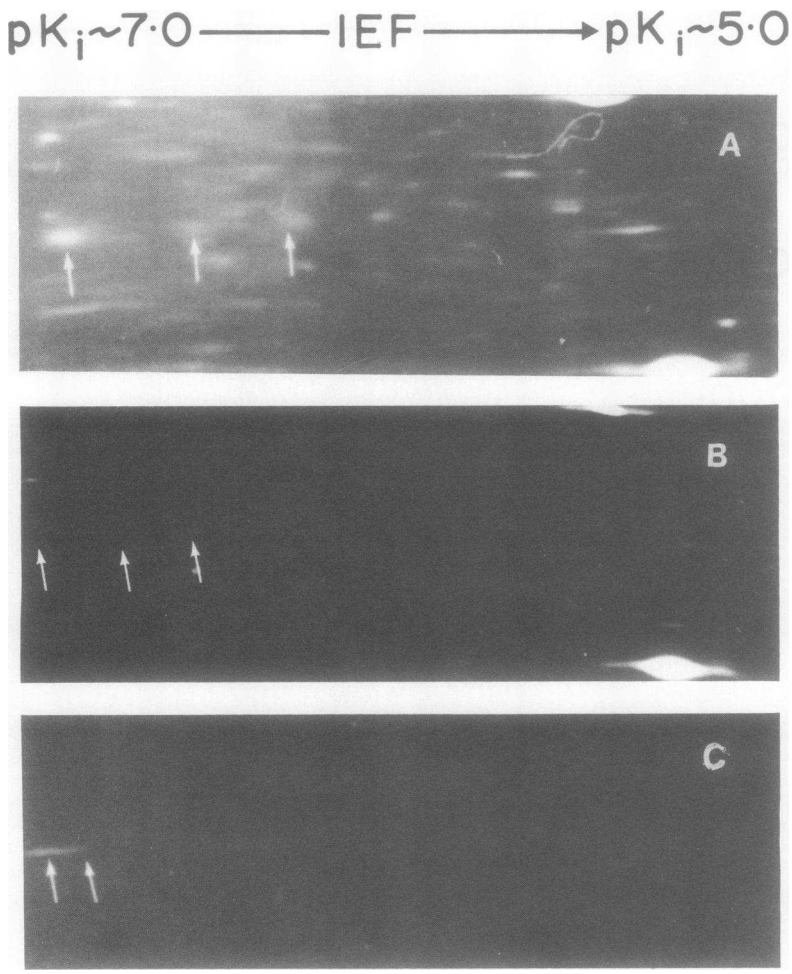

FIGURE 2 2-D gel autoradiograph of AP from a 6-d adrenalectomized oil-injected rat (A), of the same preparation after immunoadsorption with anti-βEP antiserum (B), and of the immunoadsorbed material (C). Arrows show position of POMC in each frame.

In contrast, there was no effect seen in the N-IL with doses of DM up to 200 μg/d (Fig. 6A).

Steroid effects on AP. DM, a potent glucocorticoid, caused a significant, dose-related decrease in abundance of the 29K/31K protein in the AP (Fig. 7). With high doses of DM there appears to be complete suppression of the 29K/31K POMC protein spots. 9αfF, also a potent glucocorticoid, caused a decrease in 29K/31K POMC in AP (Fig. 8). In contrast, DOC up to 600 μg/d showed less marked effects on the intensity of the POMC spots in AP, consistent with its predominant glucocorticoid antagonist activity (Fig. 8D).

Effect of steroids on the content of ir-ACTH and ir-βEP in the pituitary. The differential effects of glucocorticoids and mineralocorticoids on the content of ir-ACTH and ir-βEP in the AP and N-IL have been presented elsewhere (13). These findings were confirmed in the present study, the results of which are shown in Table I. Adrenalectomy was followed by a significant increase (2.5-fold) in the content of ir-βEP and ir-ACTH in AP, with no significant change in N-IL levels. DM caused a dose-dependent increase in the content of ir-ACTH and ir-βEP in the AP, again with no detectable changes in the N-IL. DOC increased the content of ir-βEP in the N-IL, but had no effect on the AP. 9αfF, which is a potent glucocorticoid and a potent mineralocorticoid, increased the ir-βEP and ACTH in the AP as well as the ir-βEP in the N-IL.

DISCUSSION

The finding of changes in biosynthetic profiles of the 29K and 31K POMC in AP after adrenalectomy and

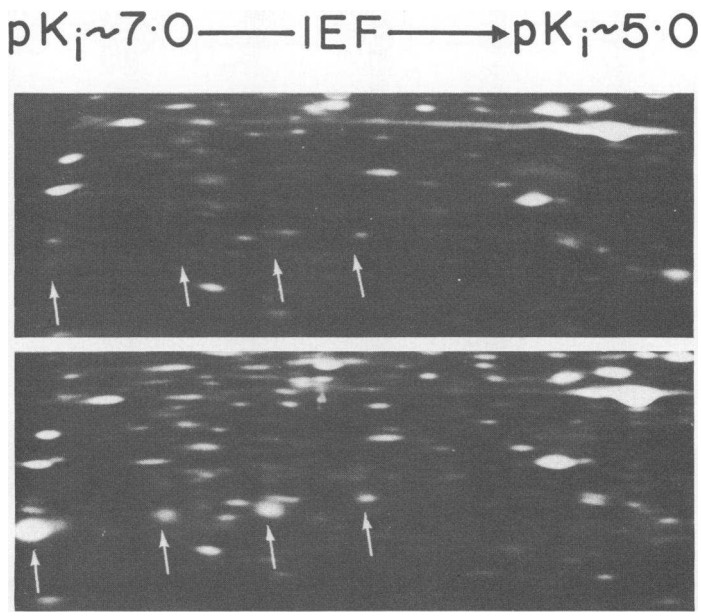

FIGURE 3 Effect of 6-d adrenalectomy on POMC levels in AP. Upper panel: sham-adrenalectomized; lower panel: adrenalectomized. All animals received daily injections of oil.

glucocorticoid administration in vivo confirms and extends the previous reports from in vitro studies on pituitary tumor cells (3, 11). A novel finding was the decrease, after adrenalectomy, of 31K and 29K protein spots in the N-IL, and the marked increase following mineralocorticoid treatment, with glucocorticoids having no appreciable effect. These findings appear worthy of discussion under several headings.

The first area for discussion is that of the effect of glucocorticoids on the pituitary gland. We have re-

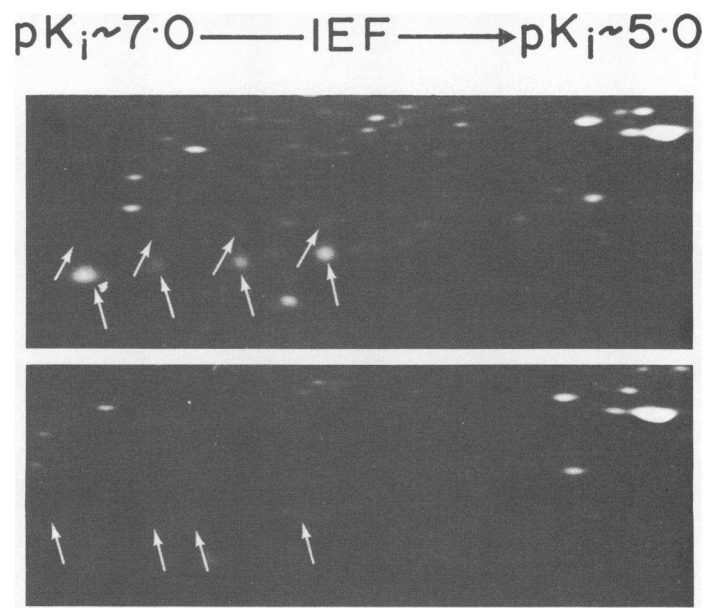

FIGURE 4 Effect of 6-d adrenalectomy on POMC levels in N-IL. Upper panel: sham-adrenalectomized; lower panel: adrenalectomized. All animals received daily injections of oil.

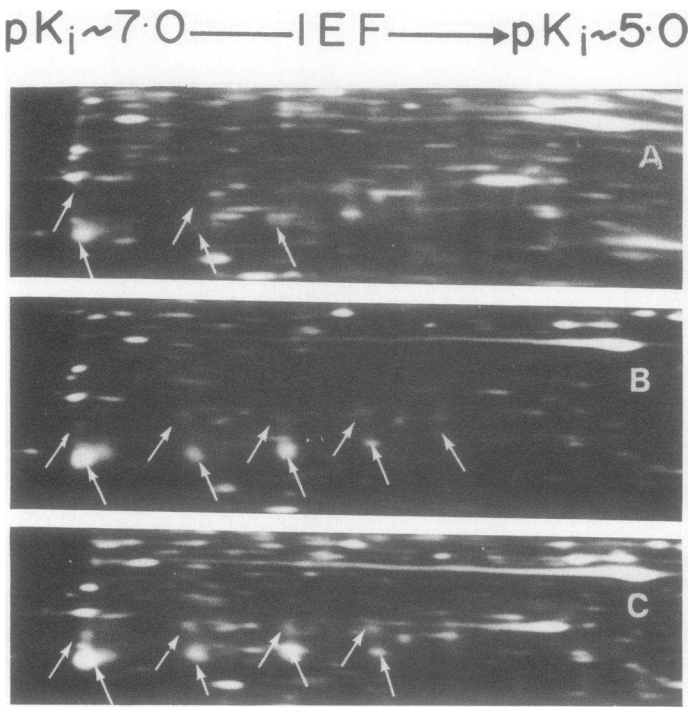

FIGURE 5 Effect of DOC for 6 d on POMC levels in N-IL from adrenalectomized rats. A, upper panel: oil-injected control; B, center panel: DOC, 60 μg/d; C, lower panel: DOC, 600 μg/d.

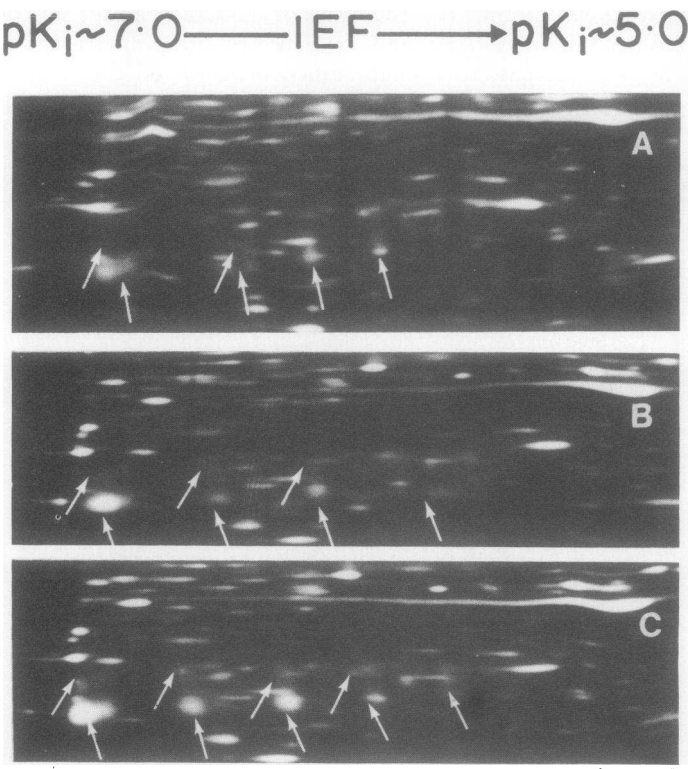

FIGURE 6 Effect of DM and 9αfF for 6 d on levels of POMC in N-IL from adrenalectomized rats. A: DM, 200 μg/d; B: 9αfF, 20 μg/d; C: 9αfF, 200 μg/d.

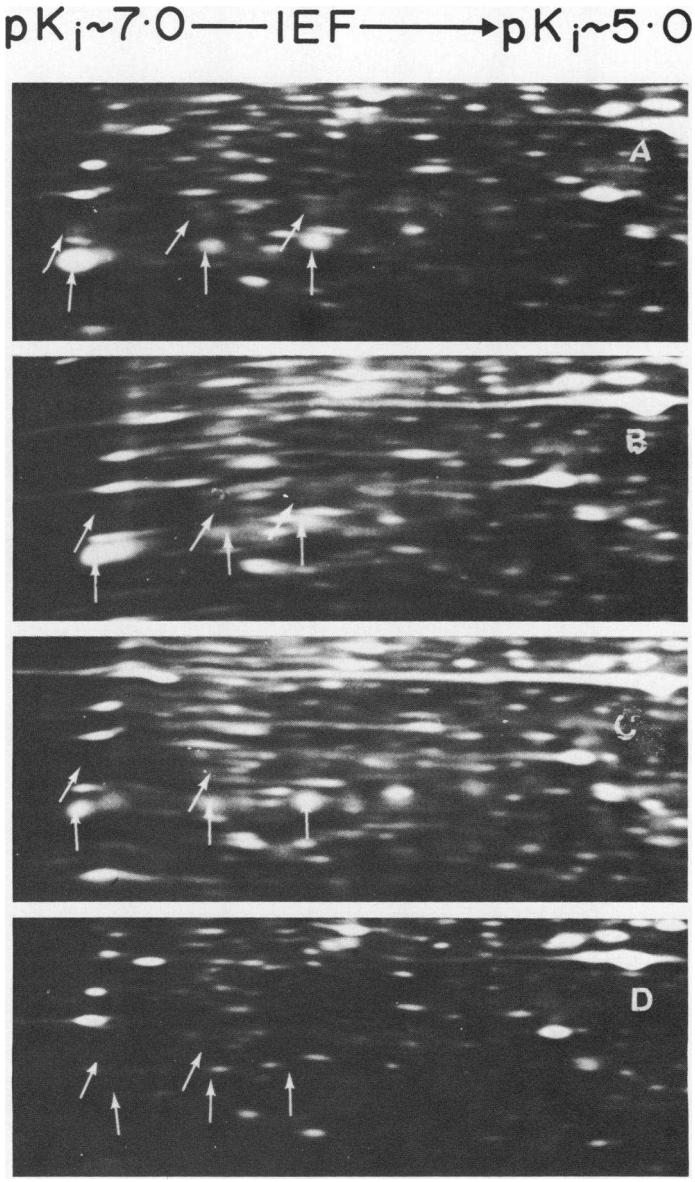

FIGURE 7 Effect of DM for 6 d on levels of POMC in AP from adrenalectomized rats. A: oil-injected control; B: DM, 2 μg/d; C: DM, 20 μg/d; D: DM 200 μg/d.

cently demonstrated a consistent, stepwise increase in the content of ir-ACTH and ir-βEP in the AP of adrenalectomized rats given increasing doses of glucocorticoids (13). This finding was confirmed in the present study by immunoassay and biosynthetic data and extends our understanding regarding the mechanisms involved. The lesser amounts of 31K proteins in the 2-D gel autoradiographs after DM treatment is consistent with previous in vitro studies (3, 11) which showed that glucocorticoids suppress the synthesis of POMC in AtT20 mouse pituitary tumor cells. The concurrent increases in ir-EP and ir-ACTH levels after glucocorticoid administration in vivo must, thus, reflect a greater degree of inhibition of ir-βEP and ir-ACTH release from the AP cells than suppression of POMC synthesis. Such differential inhibition may reflect the combination of a direct effect of glucocorticoids on the corticotropes, together with glucocorticoid-induced suppression of hypothalamic release of corticotropin-releasing factor; consistent with this hypothesis, glucocorticoid receptors have been demonstrated in both AP and hypothalamus (17, 18).

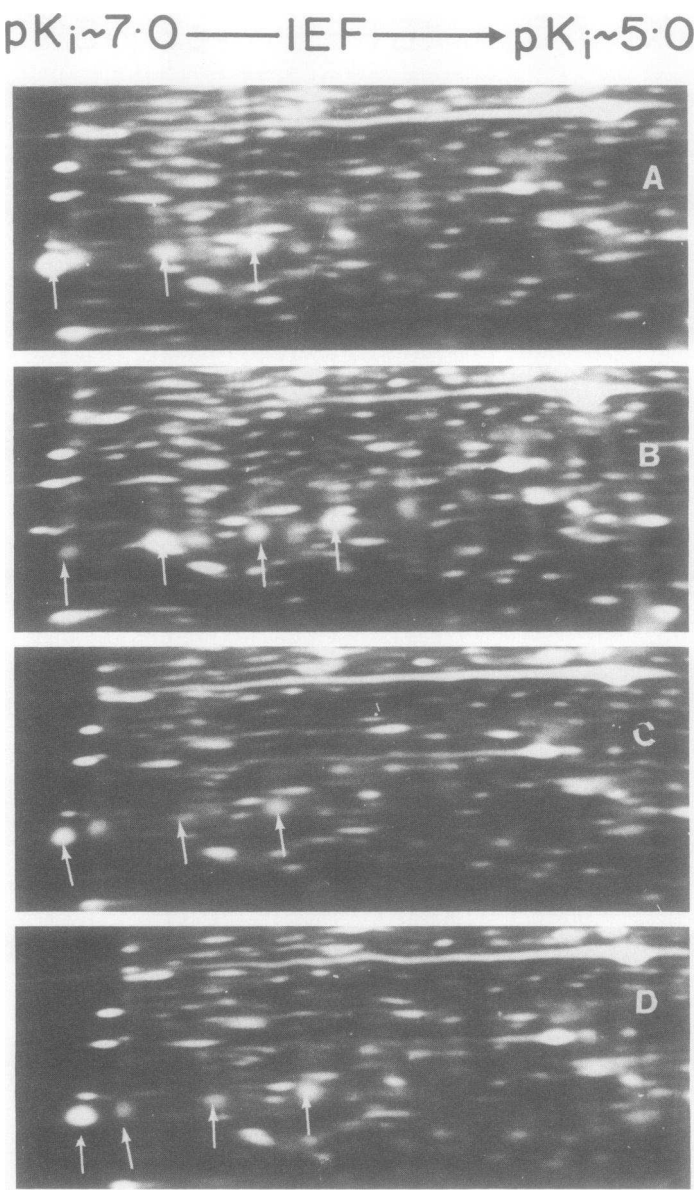

FIGURE 8 Effect of 9αfF and DOC for 6 d on levels of POMC in AP from adrenalectomized rats. Top: oil-injected control; second: 9αfF, 20 μg/d; third: 9αfF, 200 μg/d; bottom: DOC, 600 μg/d.

The effects of 9αfF on the AP and N-IL are presumably explained by the fact that 9αfF is a potent glucocorticoid in addition to being a potent mineralocorticoid. DOC, however, is a predominant glucocorticoid antagonist in most classical glucocorticoid systems (19); its minimal effect on the AP content of ir-ACTH and ir-βEP, and POMC proteins, is presumably a reflection of this predominant antagonist activity.

The second finding reported in this paper is the specific effect of mineralocorticoids on the N-IL synthesis of POMC.

Our earlier studies (13) showed a consistent and significant increase in the level of ir-βEP content in the N-IL in rats given DOC and 9αfF. The present study confirms this finding, and similarly shows that the effect is seen at DOC doses of 60 μg/d or 9αfF of 200 μg/d. An increase in ir-βEP can be ascribed to a va-

TABLE I

Effect of Corticosteroids on Pituitary ir-ACTH and ir-βEP

Treatment	AP ir-ACTH	AP ir-βEP	N-IL ir-βEP
		μg/tissue	
Sham			
+ oil	0.39±0.09*	0.44±0.11*	0.94±0.15
Adrenalectomy			
+ oil	1.05±0.14	1.12±0.19	0.98±0.12
+ DM 2 μg	1.91±0.15*	2.05±0.16*	1.15±0.11
+ DM 20 μg	3.04±0.18‡	3.12±0.21‡	0.88±0.09
+ DM 200 μg	3.38±0.24‡	3.54±0.29‡	1.02±0.14
+ DOC 60 μg	1.20±0.17	0.98±0.20	1.72±0.16*
+ DOC 600 μg	1.21±0.14	1.17±0.16	1.86±0.14*
+ 9αfF 20 μg	1.78±0.1*	1.98±0.18*	1.29±0.12
+ 9αfF 200 μg	2.90±0.23‡	3.03±0.23‡	1.79±0.14*

Data represent mean±SEM; $n = 4$.

* $P < 0.05$ cf adrenalectomy + oil.

‡ $P < 0.01$ cf adrenalectomy + oil.

riety of mechanisms—a decrease in βEP release, a decrease in βEP degradation, an increase in precursor synthesis, or a combination of some or all of the above. The 2-D gel electrophoresis clearly shows a consistent increase in the 31K and 29K precursor. The increase in ir-βEP in the N-IL thus may be, in large part, due to an increase in precursor synthesis, given the time course of the study (1-h pulse with [^{35}S]methionine).

For the N-IL, as for the AP, there appear to be differential effects on POMC synthesis and release of ir-βEP. In this, and the previous study, we found minimal differences between intact and oil-injected adrenalectomized rats in terms of N-IL ir-βEP; we consistently found, however, clear differences in POMC level, suggesting parallel changes in synthesis and release postadrenalectomy. In contrast, mineralocorticoid administration is followed by more marked changes in apparent POMC abundance than in levels of N-IL ir-βEP, suggesting a specific stimulation of release as well as synthesis.

Whether such changes are direct genomic effects of mineralocorticoids on N-IL cells cannot be answered at present. Currently, we cannot demonstrate N-IL mineralocorticoid receptors,[2] even using tissue amounts equivalent to those used for the demonstration (20) of AP mineralocorticoid receptors. It is possible that the changes are indirect, secondary to hypothalamic effects of mineralocorticoids, or even more indirect via systemic effects of mineralocorticoid administration. Other studies from our laboratory have shown significant changes in the ir-βEP in the N-IL with manipulation of salt balance in the rat (21). If the changes are indirect, sodium balance would thus appear to be a likely stimulus for the changes in the 31K/29K precursor in the N-IL. The extent to which such an effect is direct, and/or via the hypothalamus, is yet to be elucidated.

The third area of discussion is that of the differences in the POMC proteins in AP and N-IL. The 31K POMC can be demonstrated in both the AP and N-IL; it appears, however, to be a very minor protein in the AP, the 29K proteins being much more abundant. In the N-IL, the 31K proteins are more prominent than in the AP, although still less than the 29K proteins. This 29K protein has exactly the same four different glycosylated species, with differing pK_i, as the 31K protein. There are recent reports of different species of POMC in the N-IL of the rat (15), and in AtT20 mouse pituitary tumor cell lines (11). Our findings are in close agreement with those of Roberts et al. (11), in terms of two molecular mass species of POMC (29K, 31K), and of 4–5 different pK_i for both species of POMC, attributable to differences in glycosylation. Crine et al. (15) obtained protein species of higher molecular mass—36, 34, and 32K—from the N-IL, and showed that these higher molecular mass forms were glycosylated precursors of βEP and ACTH. The differences between their results and ours may reflect differences in handling, [^{35}S]methionine labeling, and/or processing of the tissues.

The difference in relative abundance of the 31K species between AP and N-IL is striking. One possible explanation is the rapid processing in the AP of the 31K to the 29K species, with quite different processing in the N-IL, and the substantial production of lower molecular mass peptides. An alternative, less plausible explanation, is that of a second gene in the N-IL responsible for the 31K/29K proteins. If this is the case, differential effects of steroids at the genome level could explain our findings of the differences in steroid control mechanisms between the two lobes—the AP being under negative feedback glucocorticoid control, and in the N-IL being stimulated, directly or indirectly, by mineralocorticoids.

[2] Krozowski, Z., and J. W. Funder. Unpublished observations.

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