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REGULATION OF ENDORPHIN PRODUCTION BY GLUCOCORTICOIDS IN CULTURED PITUITARY TUMOR CELLS

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SUMMARY: When clonal AtT-20 mouse pituitary tumor cells were exposed in culture to physiological concentrations of glucocorticoids for 2 or more days, the intracellular endorphin content was reduced by 50-75%. The steroid specificity is consistent with a glucocorticoid-receptor mediated response. Gel filtration analysis indicates a reduction of both the major and the minor endorphin species present, which resemble β- and α-endorphin, respectively. The results suggest that endorphin synthesis, like corticotropin synthesis, is under negative regulatory control by glucocorticoids in AtT-20 cells and in the corticotropin/endorphin-producing cells of the adenohypophysis.

Clonal AtT-20/D1 mouse pituitary tumor cells, which synthesize corticotropin (ACTH[1]) (1), have been shown to synthesize also peptides with opiate activity (endorphins) (2). A common biosynthetic precursor peptide for ACTH, β-lipotropin (β-LPH), and β-endorphin ($\beta\text{-LPH}_{61-91}$) has been demonstrated in cultures of the variant AtT-20/D16v cell line (3,4). A similar peptide was found to be the product of cell-free translation of AtT-20/D16v mRNA (5).

Steroids of the glucocorticoid class inhibit secretion of ACTH and β-LPH or β-melanotropin ($\beta\text{-LPH}_{41-58}$) by the adenohypophysis (6-8). In addition, glucocorticoids reduce intra- and extracellular ACTH in AtT-20/D1 cultures (9) and reduce ACTH-precursor mRNA in pituitaries of adrenalectomized rats (10). The question arises as to whether β-endorphin synthesis and ACTH synthesis are coordinately regulated by glucocorticoids. This appears to be the case in rats, in which adrenalectomy elevates and dexamethasone administration reduces concentrations of both peptides in plasma and pituitary, determined by radioimmunoassay (11). In this communication, regulation of endorphin content of cultured AtT-20/D1 cells by glucocorticoids is demonstrated.

[1] Abbreviations: ACTH, corticotropin; β-LPH, β-lipotropin; mRNA, messenger RNA; PGE_1, prostaglandin E_1; cAMP, adenosine 3':5' cyclic monophosphate; BSA, bovine serum albumin.

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MATERIALS AND METHODS

Chemicals. Synthetic Met-enkephalin and human β-endorphin were from Peninsula Labs, naloxone from Endo, and Dextran T40 from Pharmacia. Steroids were from Sigma; concentrations of stock solutions were determined by ultraviolet absorbance in methanol (12).

Growth of cells. AtT-20/D1 cells, subculture 47-60, were grown in 10-ml or 500-ml cultures in 80 mm-diameter plastic Falcon dishes or 1-liter spinner bottles, respectively, as described (2), except for serum supplementation, which was 10% fetal bovine serum treated with charcoal (10 mg/ml Norit-A) and Dextran T40 (1 mg/ml) at 55° for 30 min to remove endogenous steroids (13). Steroids were added to culture medium by 1:1000 dilution of stock solutions in 50% ethanol; control cultures received an equal volume of 50% ethanol. Viability, determined by Trypan Blue exclusion, was 85-90%. NG108-15 hybrid cells were grown as described (2, 14).

Endorphin extraction. AtT-20/D1 cells were harvested by centrifugation at 160 x g for 3 min, resuspended and washed three times in phosphate-buffered saline (pH 7.4, adjusted to 340 mosm/kg with NaCl). The cell pellet from each dish or spinner bottle was suspended in 2 or 20 ml, respectively, of 2 M acetic acid at 85-95° and heated at 100° for 10 min. The suspension was subsequently dispersed with a narrow-bore pipette or homogenizer, and after removal of an aliquot for protein assay (15), centrifuged at 1500 x g for 7 min. The supernatant fraction was lyophilized and peptides were redissolved in 0.05 M Tris-HCl, pH 7.5 at 100° for 2 min. The supernatant fraction after a final centrifugation (1500 x g for 5 min) was assayed for opiate activity. The yield of endorphin by this extraction procedure was 2-3 times that obtained by boiling-water extraction (2).

Adenylate cyclase assay for opiate activity. PGE_1-stimulated adenylate cyclase activity of approximately 75 µg NG108-15 homogenate protein was determined as described previously (2). AtT-20 extracts were added in multiple concentrations to reaction mixtures and adenylate cyclase activity measured in duplicate 6 min incubations (37°) in the presence and absence of 100 µM naloxone, a specific opiate-receptor antagonist. The increase in activity, if any, due to naloxone was divided by the activity in the presence of naloxone and this fraction was compared to a standard concentration curve of inhibition due to Met-enkephalin (0.5-5000 nM) run with each assay. Half-maximal inhibition was obtained with approximately 10 nM Met-enkephalin or 75 nM synthetic human β-endorphin. For the work described in this paper, opiate activity was expressed as β-endorphin molar equivalents, which were calculated by multiplying the experimentally determined Met-enkephalin molar equivalents by 7.5. Statistical significance was estimated by the one-tailed Student's *t* test.

RESULTS

Linearity of assay for opiate activity. Opiate activity was routinely assayed by means of the opiate-receptor mediated inhibition of PGE_1-stimulated adenylate cyclase (E.C. 4.6.1.1) activity of homogenates of NG108-15 neuroblastoma x glioma hybrid cells (16, 2). The range in which this method could reliably assay opiate activity of crude AtT-20 extracts is shown in Fig. 1. The relationship between the amount of AtT-20/D1 extract protein tested and the amount of synthetic β-endorphin which resulted in equivalent naloxone-revers-

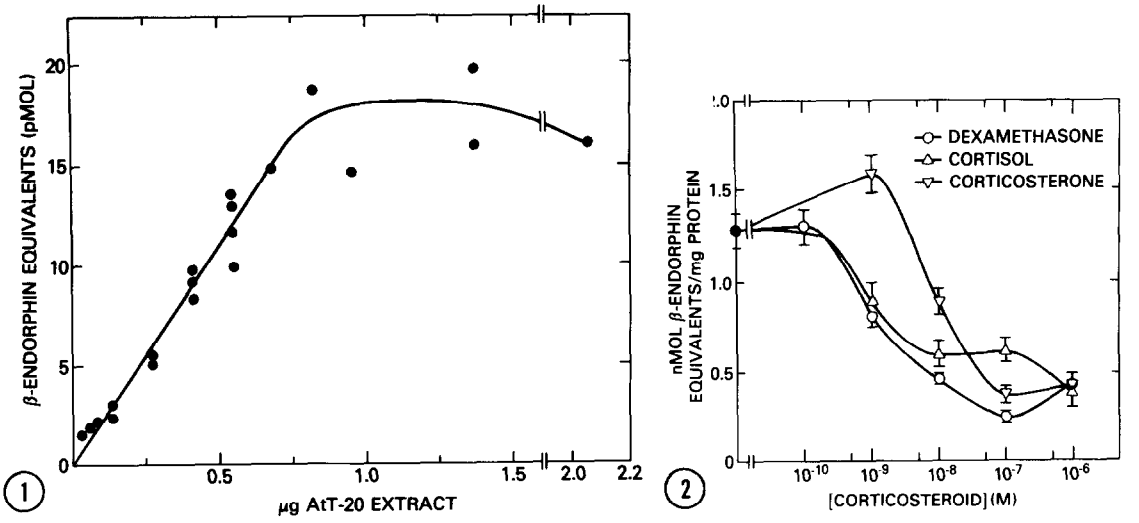

FIGURE 1: Relationship between AtT-20/D1 2 M acetic acid extract protein added to NG108-15 adenylate cyclase assay and amount of opiate activity measured. Results shown are from four separate experiments in which one extract was assayed.

FIGURE 2: Concentration dependence of glucocorticoid-mediated inhibition of AtT-20 endorphin content. Replicate dishes were cultured for 5 days in medium (changed on the 3rd day) containing 10% fetal bovine serum and steroid as indicated. Average cell protein/dish was as follows: initial 0.33 mg; final 3.0 and 2.1 mg for control and 1 μM-glucocorticoid-treated cultures, respectively. Each point represents the mean ± SE of 3, or for control 6, identical dishes.

ible inhibition of cAMP synthesis was linear between 0-0.75 μg extract protein/reaction mixture. Extracts were routinely assayed within this range.

Effect of steroids on endorphin content. AtT-20/D1 cells were cultured for 60 hr in complete growth medium in the presence of steroids (1 μM) and intracellular opiate activity determined (Table I). Treatment with steroids possessing potent glucocorticoid activity, i.e., dexamethasone, cortisol, and corticosterone, reduced endorphin content to 48-53% of that of controls. Deoxycorticosterone and aldosterone, mineralocorticoids which have relatively weak glucocorticoid activity (17), evoked smaller reductions to 68-76% of controls. Testosterone, progesterone, or 17β-estradiol had no effect. Thus, reduction of AtT-20 endorphin content is a glucocorticoid-specific phenomenon.

TABLE I: Effect of steroid treatment on AtT-20 intracellular endorphin content

Addition to culture medium	nmole β-endorphin equivalents/mg protein ± SE (n=3)	% of control
None	1.37 ± 0.12 (n=6)	(100)
Dexamethasone	0.65 ± 0.04**	47 ± 3
Cortisol	0.73 ± 0.06**	53 ± 4
Corticosterone	0.72 ± 0.09**	53 ± 7
11-Deoxycorticosterone	1.04 ± 0.08	76 ± 6
d-Aldosterone	0.93 ± 0.09*	68 ± 7
Testosterone	1.57 ± 0.17	115 ± 12
Progesterone	1.34 ± 0.10	98 ± 7
17β-Estradiol	1.52 ± 0.12	111 ± 9

Replicate dishes were cultured for 60 hr in medium containing 10% untreated fetal bovine serum and 1 μM steroid. Estimated initial and average final values of cell protein per dish were 1.2 and 4.5 mg, respectively. *Significantly different from control, $P<0.05$; ** $P<0.005$.

The half-maximally effective concentrations were found to be 1 nM dexamethasone, 2 nM cortisol, and 15 nM corticosterone (Fig. 2).

The time course of the effect of 0.1 μM dexamethasone is shown in Fig. 3. Endorphin content of treated cells relative to control cells declined during the first 3 days with an apparent half-life of approximately 2 days, but between 3 and 5 days endorphin content declined at a slower rate, to 36% of the control level (panels A, B). During the 5-day period cells multiplied with a doubling time of protein/dish of 1.6 day with or without dexamethasone (panel C). In other experiments (not shown) treatment with dexamethasone for 8 days resulted in reductions of endorphin content to 25-35% of controls. The reduction was slower in slowly growing cultures; for example, in the absence of serum, exposure to dexamethasone for 48 hr reduced endorphin content to 75% of controls.

To determine whether glucocorticoid treatment altered the species of intracellular endorphin peptides, extracts from control and treated cells were fractionated by gel filtration on a Bio-Gel P-4 column (2). Most of the opiate activity from control cells (Fig. 4A) was distributed between two species, I

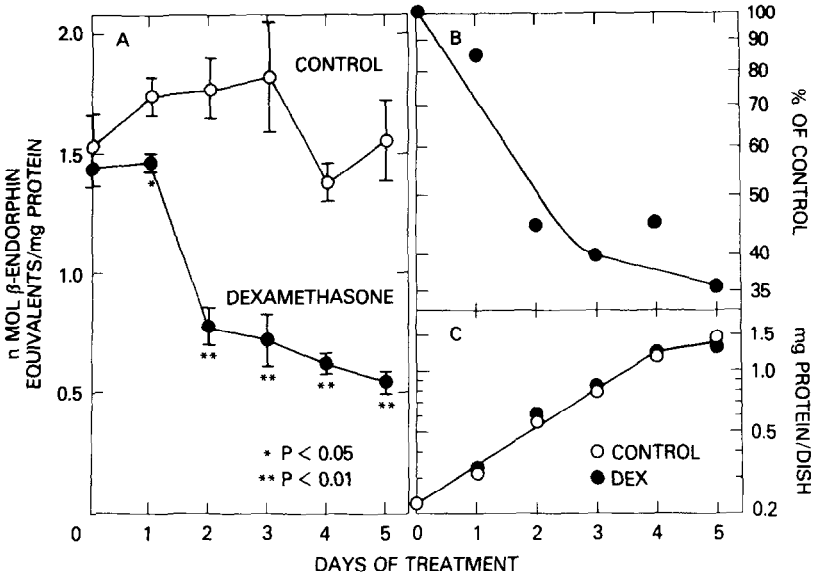

FIGURE 3: AtT-20 endorphin content as a function of duration of treatment with 0.1 μM dexamethasone. At zero time, there were 2.4 x 10^6 cells/dish, plated 24 hr previously. A: Endorphin content. Zero-time dishes were harvested 10 min after dexamethasone addition. Each point represents mean ± SE of triplicate dishes. B: Replotted data of panel A showing endorphin content of dexamethasone-treated cells expressed as % of content of control cells at each time. Zero-time control and treated cells were assumed to have equal endorphin content. C: Cell protein per dish for cultures used for panels A, B.

and II, comprising 72% and 21% of the recovered activity, respectively. Endorphin I is similar to β-endorphin with respect to mobility on Bio-Gel P-4 columns (2) and amino acid composition after purification (S. Sabol, manuscript in preparation), while endorphin II resembles α- or γ-endorphin in mobility (2). Analysis of dexamethasone-treated cells, from which 31% as much endorphin activity per mg cellular protein was extracted, revealed (Fig. 4B) a similar chromatographic pattern of endorphin activity with, however, 27% and 40% as much of I and II per mg cellular protein, respectively, as that found in control cells.[2] This is consistent with a quantitative rather than qualitative effect of glucocorticoids on AtT-20/D1 endorphins.

[2]Dexamethasone treatment also reduced, to 35% of the control, the amount of presumed endorphin precursor(s) eluting in the P-4 void volume and detected by the opiate activity generated upon trypsinization (data not shown).

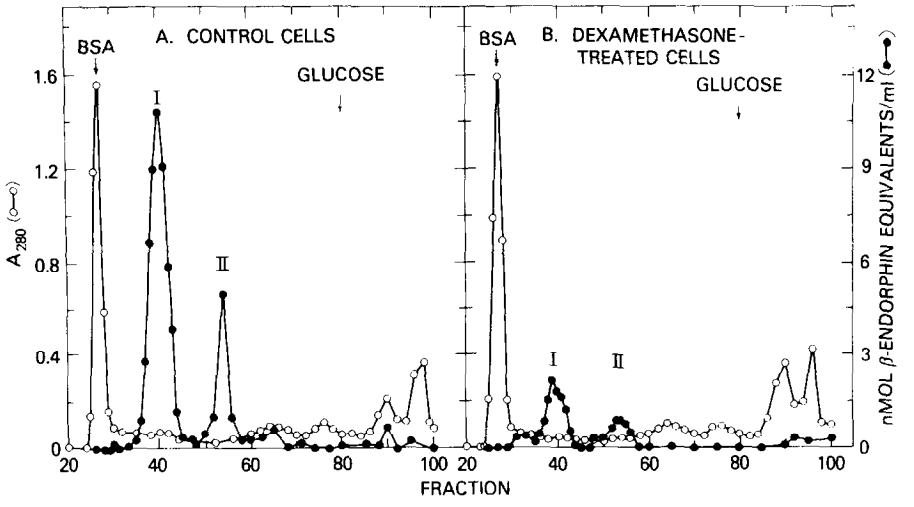

FIGURE 4: Gel filtration of intracellular endorphins from cells cultured with or without 0.1 μM dexamethasone for 5 days. Cell protein per spinner culture was initially 32 mg and, at time of harvest 102 and 77 mg for control and treated cultures, respectively. Acetic acid extracts were lyophilized, suspended in 1.5 ml of 0.1 M ammonium acetate (pH 7), and 1.4 ml applied to a Bio-Gel P-4 (200-400 mesh) column, 1.5 x 97 cm, equilibrated with the same buffer at 0° C. Fractions (1.45 ml) were collected in plastic tubes and assayed for absorbance at 280 nm (○) and opiate activity (●). Arrows indicate elution positions of BSA and glucose. Distributions of endorphins (nmole β-endorphin equivalents) were as follows: control culture: 226 extracted, 211 applied to column, 104 recovered, 74.6 in I, 22.2 in II; dexamethasone-treated culture: 50.9 extracted, 47.5 applied to column, 26.8 recovered, 15.2 in I, 6.6 in II.

The endorphin content of AtT-20/D16v cells was found to be similarly reduced from 1.58 to 0.34 nmole β-endorphin equivalents/mg cell protein by 5-day treatment with 0.1 μM dexamethasone.

Secretion of endorphin by AtT-20 cells. Opiate activity in AtT-20/D1 culture medium was found to be unstable and thus did not reflect the amount of secreted endorphin. Synthetic β-endorphin (0.75 μM) was rapidly inactivated in conditioned medium (95% loss in 12 hr at 37°) and less rapidly in unconditioned medium (42% loss in 12 hr). Thus AtT-20/D1 cells apparently release one or more enzymes which inactivate β-endorphin in culture medium.

DISCUSSION

The present investigation has demonstrated that the intracellular endorphin content of AtT-20/D1 cells is under negative regulatory control by

glucocorticoids. These mouse pituitary tumor cells, as well as cells of the variant line AtT-20/D16v, functionally resemble corticotrophs of the adenohypophysis in that they synthesize and secrete molecules resembling ACTH (1, 3), β-LPH (3), and β-endorphin (2, 3). Their cytoplasm, like that of corticotrophs, contains densely-staining "secretory" granules approximately 200 nm in diameter which may contain endorphins (2, A. Ling, M. Daniels, and S. Sabol, unpublished). Furthermore, AtT-20/D1 cells possess glucocorticoid receptors (18) which bind steroids with a specificity which correlates well with the specificity of steroids in reducing ACTH synthesis (9) and endorphin content (Table I, Fig. 2).

The reduction of intracellular endorphin by glucocorticoids may be due to one or more of the following processes, among which the present study does not distinguish: a reduced rate of biosynthesis at the level of transcription, translation, or post-translational processing; an increased rate of secretion; or an increased rate of catabolism. Other workers have shown that glucocorticoids reduce the concentration of ACTH mRNA of the adenohypophysis of adrenalectomized rats (10) and that this mRNA codes for a presumed prohormone of molecular weight about 28,500 which contains amino acid sequences of ACTH and β-endorphin (5). Thus, a reasonable working hypothesis is that glucocorticoids, through a receptor-mediated process, inhibit the synthesis of ACTH-endorphin precursor mRNA and thus elicit a coordinate reduction of endorphin and ACTH biosynthesis, which becomes manifest slowly as the concentration of pre-existing ACTH-endorphin mRNA declines.

Although the physiological role(s) of pituitary β-endorphin and the negative regulation of its production by glucocorticoids are presently unclear, the mechanism(s) of this regulation are amenable to study in AtT-20 cells.

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