GLUCOCORTICOIDS ELEVATE THE LEVEL OF ENKEPHALIN-LIKE PEPTIDES IN NEUROBLASTOMA × GLIOMA HYBRID CELLS

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1. Introduction

Met- and Leu-enkephalin first isolated from pig brain as endogenous ligands for opioid receptors [1], have later been shown to occur also in peripheral nervous tissue such as the plexus of the gastrointestinal tract of some species [2] and the bovine adrenal medulla [3,4]. Peptides probably identical with Met- and Leu-enkephalin are also present in neuroblastoma × glioma hybrid cells 108CC15, as determined by 3 independent assays [5,6]. These hybrid cells express many properties characteristic of neurons. One is their production of acetylcholine, another their response to several neurohormones. The elevation by prostaglandin E_1 (PGE_1) of the intracellular concentration of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) is inhibited by opioids. The inhibition is prevented by the opioid antagonist naloxone (review [7]).

Little is known about the regulatory mechanisms, which control the neuronal concentration of the enkephalins [8–12]. Here we report that in the hybrid cells 108CC15 glucocorticoids specifically increase the intracellular concentration of opioid peptides.

2. Materials and methods

Dexamethasone (Dex), progesterone, corticosterone and testosterone were from Sigma (München); aldosterone from Serva (Heidelberg); diazepam from Hoffman-La Roche (Grenzach); naloxone from Endo-Labs. (Garden City NY). PGE_1 from Upjohn Co. (Kalamazoo MI); Leu- and Met-enkephalin (Leu- and Met-Enk) were gifts from Drs L. Moroder and E. Wünsch (Martinsried); [^{3}H]Leu-enkephalin (64 Ci/mmol) and [^{3}H]Met-enkephalin (36 Ci/mmol) were purchased from Amersham Buchler (Braunschweig).

Culturing of cells in Petri dishes (145 mm diam.) and multiwell trays containing 24 wells (16 mm diam.), as well as the preparation and purification of extracts have been described [5,6]. Briefly, cells were extracted by 1 M acetic acid. After addition of 6.5×10^4 dpm of [^{3}H]Leu-Enk, and sometimes also of [^{3}H]Met-Enk, for determination of the recovery, the extracts were purified by adsorption chromatography on polystyrene beads and subsequent chromatography on Sephadex LH-20 [6]. These extracts, finally dissolved in 200 μl 0.05 M Tris–HCl, pH 7.4 (25°C) will be referred to as partially purified extracts. In some instances partially purified extracts were further purified by high performance liquid chromatography (HPLC) [6]. Briefly, partially purified extracts were isocratically chromatographed on an analytical C_{18}-reversed-phase column at a flow rate of 1.5 ml/min. The solvent system was 59% 0.001 M ammonium acetate (pH 4.5) and 41% methanol. Fractions were collected according to a calibration of the column with synthetic Met- and Leu-Enk. Evaporated fractions were dissolved in 200 μl 0.05 M Tris–HCl, pH 7.4 (25°C). Hormones were added to the growth medium as 50 μM solutions in Dulbecco's modified Eagle's medium and, unless indicated otherwise, were present during the whole culture period.

Opioid activity in the extracts was determined by 2 independent assays:

(i) A radioimmunoassay with an antiserum against Leu-Enk was performed by a standard procedure [5,13]. The antiserum was specific for the two enkephalins; none of the many other peptides tested, including the endorphins, crossreacted [13,14].

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(ii) Hybrid cells were incubated (10 min, 37°C) with PGE_1 and Leu-Enk or extract fractions before the concentration of cyclic AMP was determined as in [14,15].

In the protein assay [16] bovine serum albumin was used as a standard.

3. Results

In hybrid cell extracts, opioid activity is equally enhanced (3–4-fold) after incubation of the cells with Dex and corticosterone (table 1). But in most other experiments corticosterone was somewhat less potent than Dex. The stimulation over the control by Dex varies mostly between 3–5-fold. The effect is specific for glucocorticoids. Of the other steroid hormones tested only aldosterone exerts a small stimulatory effect. Sometimes opioid activity is detected in C6-BU-1 glioma cells [6]. However, this activity cannot be increased by treatment with Dex (not shown).

Half-maximal elevation of opioid activity in hybrid cells is achieved at 50 nM (fig.1). At higher concentrations the intracellular level of opioids decreases again. The maximal cellular content of opioids is generally obtained already after a 1 day incubation with Dex (fig.2). Only occasionally, a somewhat longer period (1.5–2 days) is necessary. The initial decrease of opioid activity after 6 h incubation is unexplained. Dex increases the content in the hybrid cells of Met- and Leu-Enk-like material (fig.3). In order to demonstrate this, partially purified extracts were further purified by HPLC. The fractions with the retention times of Met- and Leu-Enk were assayed for their capacity to inhibit the PGE_1-evoked increase in the level of cyclic AMP. For comparison, the inhibitory action of Leu-Enk is shown in fig.3A (curve a). The Met-Enk-like (fig.3B,D, curves a) and the Leu-Enk-like fractions (fig.3C,E, curves a) also inhibit the formation of cyclic AMP. After treatment of hybrid cells with Dex, lower volumes of both the Met- and Leu-Enk-like fractions (fig.3D,E, curves a) are neces-

Table 1
Effect of several steroid hormones and dexamethasone on the opioid activity of hybrid cells

Addition to the growth medium (1 μM)	Protein/dish (mg)	Leu-Enk-equiv. (pmol/mg protein)
–	6.8	0.05
Dexamethasone	6.9	0.18
Corticosterone	8.2	0.17
Aldosterone	7.2	0.10
Testosterone	8.6	0.04
Progesterone	7.4	0.05

After a culture period of 4 days each extract was prepared from cells (passage no. 20 = P 20) of 12–14 Petri dishes. All extracts were partially purified. Opioid activity was determined by radioimmunoassay

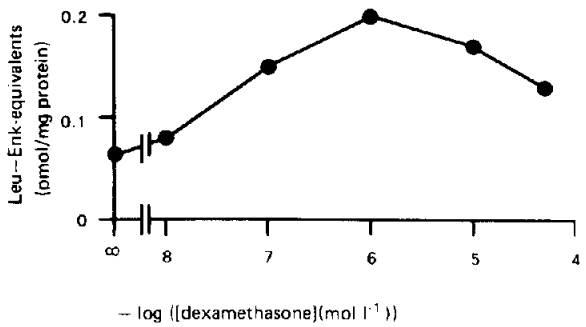

Fig.1. Effect of the concentration of Dex on the level of opioids in hybrid cells. Each of the partially purified extracts was prepared from cells (P 20) of 11–15 Petri dishes. Their content of opioids was determined by radioimmunoassay.

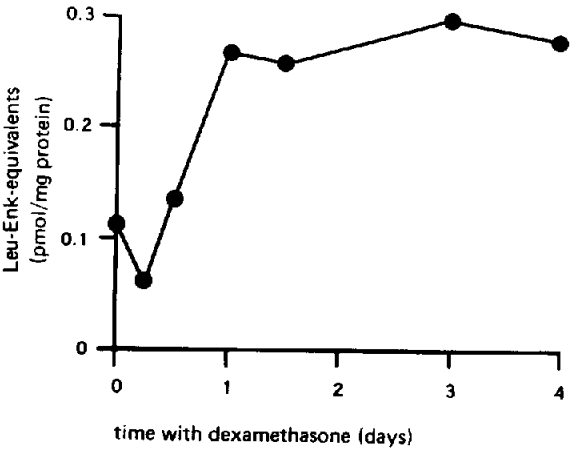

Fig.2. Time course of the Dex-induced increase in the concentration of opioids in hybrid cells. At the indicated times before extraction of the cells (P 18) 1.2 ml of a 50 μM solution of Dex was added to a number (10–14) of Petri dishes filled with 60 ml growth medium (final conc. 1 μM). At the time of extraction the cellular protein content/Petri dish was 5–6 mg. The extracts were partially purified. Their content of opioids was determined by radioimmunoassay.

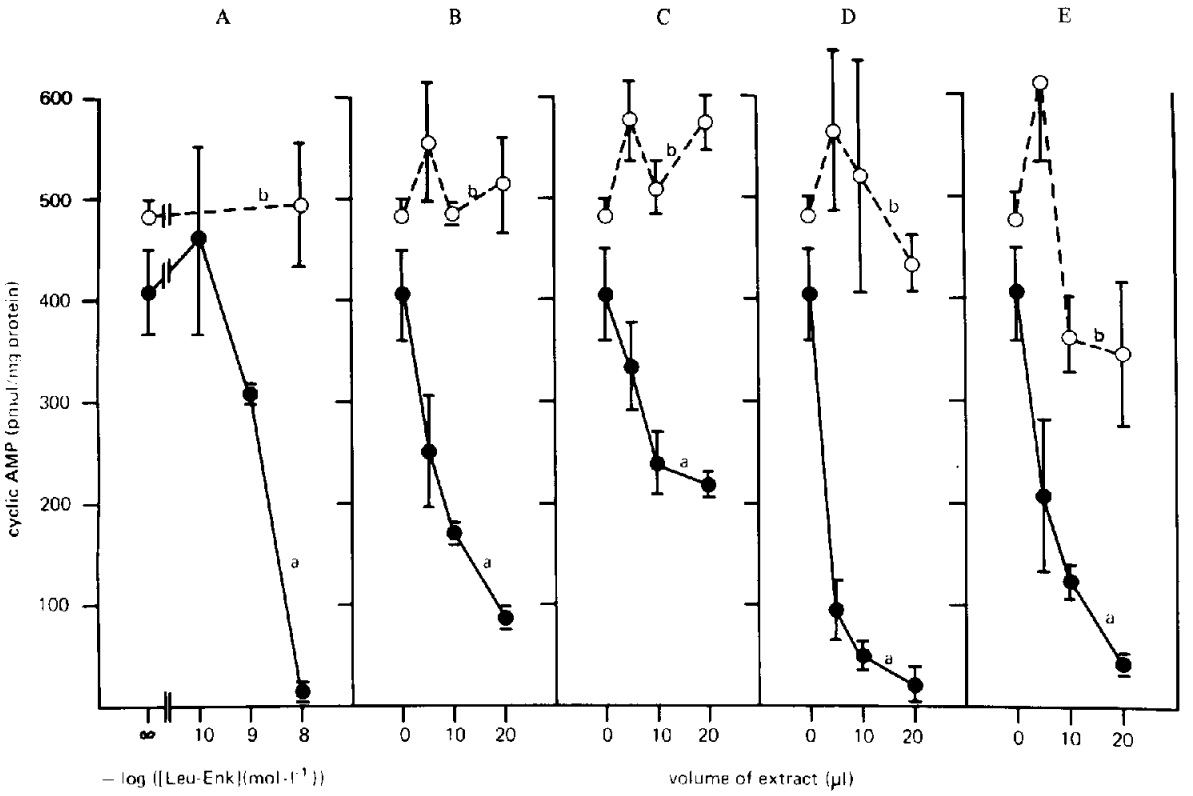

Fig.3. Leu-Enk (A) and HPLC-fractions of extracts of hybrid cells that had been cultured in the absence (B,C) or presence (D,E) of Dex, inhibit the PGE_1-elicited formation of cyclic AMP in hybrid cells. The assays were carried out in the absence (curves a) or presence (curves b) of 10 µM naloxone: 2.2×10^5 viable cells/well, 16 mm diam., 96% viability, passage no. 17. PGE_1 was 0.3 µM. Each value is the mean ± SD of data obtained from two parallel incubations. Each extract was prepared from cells of 30 Petri dishes, each containing 6.0–7.0 mg cellular protein. Cells were cultured for 4 days in the presence of 1 µM Dex. Final volume of the fractions was 200 µl: Leu-Enk (A); Met-Enk-like fraction (B,D); Leu-Enk-like fraction (C,E). The recoveries after the purification of the extracts were: for [^{3}H]Met-Enk 25% (B) and 20% (D); for [^{3}H]Leu-Enk 34% (C) and 28% (E).

sary to cause the same inhibition as in the controls (fig.3B,C, curves a). This indicates, that after treatment with Dex both fractions contain an elevated amount of opioids. The effects of all fractions and of synthetic Leu-Enk are prevented by the opioid antagonist naloxone (fig.3, curves b). The ratio in the hybrid cell extracts of Met- to Leu-Enk-like peptide of 3–4:1 is unaltered by treatment with Dex.

The neurohormones acetylcholine and opioid peptides released by the hybrids might regulate via the muscarinic [18] and opioid receptors, respectively, the content of opioids in these cells. However, the level of opioid peptides in the hybrids is neither affected by the agonists carbachol and morphine nor by the antagonists atropine and naloxone. Also diazepam is without effect (not shown).

4. Discussion

Adrenalectomy leads to an increase in the concentration of β-endorphin in the anterior lobe of the pituitary gland [10]. In congruence herewith is the decrease caused by dexamethasone in the intracellular concentration of β-endorphin in vitro in cultured pituitary tumor cells AtT-20 [19] and in vivo in the anterior lobe [10]. In contrast, after large doses of Dex levels of enkephalin were slightly increased in rat striatum [10]. In accord with this report is our finding with the aid of two different assays that exposure of neuroblastoma × glioma hybrid cells to Dex raises the intracellular level of enkephalin-like peptides. With 3–5-fold this stimulation is much more pronounced than in the in vivo experiments [10]. This

difference may be due to different concentrations of glucocorticoids in brain and in the culture medium. The latter contains fetal calf serum necessary for the growth of the hybrids. On the other hand, adrenalectomy does not influence enkephalin levels in brain [10]. In view of these results, the hybrid cells appear to be a useful model for studying the regulation of enkephalin-like peptides by glucocorticoids. This study and the fact that the hybrid cells do not contain endorphins indicates that the enkephalin and the endorphin systems are different.

Of the steroid hormones tested, only the corticoids alter the level of opioids in the hybrids, the maximal effect being observed at 1 μM Dex and after an incubation of at least 1 day. Similar specificity and optimal conditions have been reported for the action of glucocorticoids in other systems [20–23], e.g., the induction of glutamine synthetase in primary brain cell cultures [20]. The equal increase by glucocorticoids in the concentrations of the Met- and Leu-Enk-like peptides may be explained by the induction of a common precursor protein, such as has been isolated recently from bovine adrenal medulla [24]. It is not known whether the increase in the level of enkephalin-like material is due to enhanced synthesis or to decreased degradation or release. However, the latter is unlikely, since hydrocortisone inhibits the release of prostaglandins from fat cells already within minutes [25]. On the other hand, it is conceivable that the enzymes presumably involved in the processing of the precursor molecule are regulated by glucocorticoids.

The decrease at >1 μM Dex in the dose–response curve (fig.1) may be explained in at least two ways:

(i) Dex may perturbe membranes and thus cause an enhanced release of opioids;

(ii) Dex may exert a cytotoxic effect, as has been observed for the induction of tyrosine hydroxylase in rat pheochromocytoma cells [17]. In accord herewith would be the reduction by 35% in protein content per dish, noticed at 50 μM Dex (fig.1).

As to the cell type, the elevation by glucocorticoids of opioid levels in hybrid cells appears to be somewhat specific. We observed no such regulation of opioid levels in glioma cells, although it is demonstrated by the induction of glutamine synthetase [20,21] that these cells are indeed susceptible to glucocorticoids.

The level of opioids is not altered via the muscarinic cholinergic or the opioid receptors present on the hybrid cells. Agonists or antagonists for both receptors are without clearcut reproducible effects. This is in contrast to the adrenal medulla where acetylcholine causes via a nicotinic cholinergic receptor [26] a significant release of opioid peptides from the chromaffin cells [4]. This difference may be due to differences in the state of differentiation or the cholinergic receptors involved. Hybrid cells become differentiated and electrically excitable after treatment with dibutyryl adenosine 3′:5′-cyclic AMP. It remains to be elucidated whether or not under such conditions acetylcholine causes an increased release of opioids from hybrid cells. In contrast to the rat brain [9], in hybrid cells diazepam causes no alteration in the level of opioid peptides. It is not known whether the lack of effect of diazepam on the level of cyclic AMP in the hybrids [27] is of any significance in this context.

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