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Altered Ratios of Beta-Endorphin:Beta-Lipotropin Released from Anterior Lobe Corticotropes with Increased Secretory Drive. II. Repeated Stress

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Abstract

A number of stimuli including acute footshock and electrically-induced seizures lead to release of β-endorphin immunoreactivity from the anterior pituitary corticotropes. Gel filtration of this β-endorphin immunoreactivity indicates that approximately 3-fold more β-endorphin than β-lipotropin is released into plasma following these acute stressors. A similar preponderance of β-endorphin over β-lipotropin is seen in the media of short-term anterior lobe cell suspensions stimulated with ovine corticotropin-releasing hormone. Previous studies indicated that footshock stress, when administered repeatedly, can increase the biosynthesis of anterior lobe proopiomelanocortin (POMC) as indicated by increased steady state adrenocorticotropin/β-endorphin content as well as increased POMC mRNA levels and increased POMC biosynthesis and rate of processing as measured by pulse-labeling and pulse-chase studies. The goal of the present studies was to determine whether this increased biosynthetic drive results in an alteration in the end products secreted with repeated stress. Acute footshock in a rat which has received 14 days of chronic footshock releases proportionately more β-lipotropin than is released in a naive rat. Chronic electrically-induced seizures, which also increase anterior lobe POMC derived peptide stores, lead to a similar shift in the ratio of β-lipotropin:β-endorphin released following stress. These data suggest that chronic drive and the subsequent changes in POMC peptide stores may lead to a decrease in the proportion of β-endorphin size immunoreactivity in the releasable pool of the anterior lobe corticotrope, thus altering the hormonal signal from the anterior lobe corticotrope.

The previous paper focused on removal of glucocorticoids by either surgical adrenalectomy or pharmacological blockade as a means of driving corticotrope secretion *in vivo*. However, both of these stimuli produce increased drive for corticotrope secretion by opening up the feedback loop system. Do the same changes occur in situations of increased demand in a closed loop system? To explore this question, the current studies examine the effects of two stimuli to the hypothalamic-pituitary-adrenal (HPA) axis: footshock stress and electrically-induced seizures (ECS). Unlike the studies with adrenalectomy and metyrapone, where glucocorticoid exposure is decreased, chronic stress and repeated seizures drive glucocorticoid secretion as well as corticotrope secretion, leading to a different hormonal and cellular environment for the corticotrope than adrenalectomy. However, common to all these paradigms is increased corticotrope secretion.

The effects of acute and chronic footshock stress on anterior lobe release and biosynthesis have been characterized in a series of studies. We have previously reported that both acute footshock stress or ovine corticotropin-releasing hormone (oCRH) administered to a short-term anterior lobe suspension preferentially releases β-endorphin over β-lipotropin (β-LPH) (1). Pulse-labeling and pulse-chase experiments from this laboratory have demonstrated that this same acute stress, which produces a significant depletion of β-endorphin stores in the anterior lobe, is followed by increases both in the rate of biosynthesis of the precursor, proopiomelanocortin (POMC), and in the rate of conversion of POMC to its end products, β-LPH and β-endorphin (2, 3). These same studies demonstrated that footshock stress, when administered one-half hour daily in a repetitive phasic design for 14 days, produced an increase in POMC mRNA steady state levels and increased stores of POMC (the protein precursor) and its cleavage products, β-endorphin and β-LPH; pulse-chase studies have demonstrated a normal rate of POMC post-translational processing as measured by the conversion of the precursor to its final products (2, 3). Thus, while acute stress increases the rate of proteolytic cleavage of POMC in a naive rat, the same stressor does not lead to this acceleration in processing in a rat which was

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previously exposed to chronic stress, i.e. a rat with increased POMC mRNA levels and increased peptide stores.

Do these processing rate differences lead to differences in the end product of POMC processing that is preferentially secreted? To address this question, we undertook a comparison of β-endorphin forms released *in vivo* into plasma following acute and chronic/acute footshock stress. Repeated ECS also results in changes in the HPA axis including increased CRH mRNA accompanied by a decrease in CRH content in the CRH neurons of the paraventricular nucleus, increased adrenocorticotropin (ACTH) and β-endorphin peptide stores in anterior lobe corticotropes, and increased plasma corticosterone levels (4). Again these changes indicate increased secretory drive to the corticotrope following repeated ECS. Similar to the effects of adrenalectomy, we found differences in the proportion of β-endorphin to β-LPH released with acute versus chronic stress, despite similar ratios of these peptides in the anterior pituitary stores. These data provide further evidence for our previous observation that increased drive to secrete can affect the molar ratios of β-endorphin:β-LPH in the releasable pools and that the overall molar ratio of these peptides stored in the anterior lobe does not predict the actual forms released (1).

Results

Footshock stress

Forms released in vivo. To assess forms released *in vivo*, we combined column chromatography with RIAs for both total β-endorphin/β-LPH immunoreactivity (IR) and a specific RIA for N-acetyl-β-endorphin-IR. Since both N-acetyl-β-endorphin-IR and α-melanocyte-stimulating hormone (α-MSH)-IR are derived exclusively from the intermediate lobe, these serve as markers of intermediate lobe release. The mean values of β-endorphin/β-LPH-IR, N-acetyl-β-endorphin-IR and α-MSH-IR across the three experiments for control, acute footshock stress, chronic stress/rest and chronic stress/acute stress are shown in Table 1. Each experiment combined the plasma from 8 to 16 rats for column chromatography. The errors shown are the error of the mean of the three experiments. Acute footshock stress is accompanied by a large increase in β-endorphin/β-LPH-IR in plasma (329 fmoles/ml). These data are in agreement with our previous observation of a 5-fold increase in both β-endorphin/β-LPH-IR and ACTH-IR following acute footshock stress (2). Both N-acetyl-β-endorphin and α-MSH-IR demonstrate a much smaller release (14 to 28 fmoles/ml) from the intermediate lobe with footshock stress. This is also in agreement with our previously reported data (5). These data confirm the anterior lobe corticotrope origin of the β-endorphin/β-LPH-IR released by footshock stress. The proportions of β-LPH and β-endorphin following acute footshock as determined by gel filtration are shown in Table 2. The β-endorphin/β-LPH-IR in plasma released by acute stress can be shown to consist of both β-LPH and β-endorphin with β-endorphin as the predominant form (ratio of β-endorphin:β-LPH = 2.7 : 1). As previously reported (1) these data demonstrate that acute stress is accompanied by predominantly β-endorphin release. The data from the N-acetyl-β-endorphin RIA demonstrate that less than 10% of the total β-endorphin size material in the plasma of rats following acute stress is N-acetylated. A comparison of the ratio of β-LPH and β-endorphin released into plasma by acute stress in naive versus

TABLE 1. Mean Plasma β-Endorphin/β-LPH-IR, N-Acetyl-β-Endorphin-IR and α-MSH-IR Concentrations (pM) Across Three Experiments.

	Control	Acute stress	Chronic stress/rest	Chronic stress/acute stress
β-Endorphin/β-LPH-IR	63 ± 43	392 ± 83	142 ± 61	432 ± 155
β-Endorphin/β-LPH-IR released		329		290
N-Acetyl-β-endorphin-IR	42 ± 16	61 ± 15	38 ± 12	66 ± 8
N-Acetyl-β-endorphin-IR released		19		28
α-MSH-IR	37 ± 6	51 ± 8	105 ± 5	151 ± 40
α-MSH released		14		46

TABLE 2. Plasma Forms of β-Endorphin-IR Following Footshock Stress.

Experiment 1	β-LPH	β-Endorphin	β-Endorphin : β-LPH
Control	5	145	29 : 1
Acute stress	144	375	2.6 : 1
Chronic stress/rest	17	162	9.5 : 1
Chronic/acute stress	303	438	1.45 : 1
Experiment 2	β-LPH	β-Endorphin	β-LPH : β-Endorphin
Control	3	20	6.6 : 1
Acute stress	136	384	2.7 : 1
Chronic/acute stress	130	124	0.95 : 1
		Acute stress	Mean 2.65 ± 0.07 : 1
		Chronic/acute stress	Mean 1.2 ± 0.3 : 1

Values are expressed as fmoles/ml plasma.

chronically stressed rats is shown in Table 2. In these two experiments, a larger proportion of the β-endorphin-IR released by chronic/acute stress is β-LPH than that released following acute stress rats (ratio of β-endorphin:β-LPH = 2.7 : 1 following acute footshock; ratio of β-endorphin:β-LPH = 1.2 : 1 following chronic/acute footshock). The differences in absolute amounts of β-endorphin-IR between experiments are not due to differences in recovery from the sieving column but were present in the unsieved plasma and represent real differences between groups of animals in their response to this same stressor.

Forms released in vitro. These *in vivo* data were also confirmed in an *in vitro* system using short-term anterior lobe suspensions from control and chronic stress/rest rats exposed to this same footshock stressor. The anterior pituitaries from 10 rats were pooled for each group. Both baseline and oCRH stimulated release were examined. The data are shown in Fig. 1. In control and chronic stress/rest suspensions, the basal unstimulated release shows approximately equal amounts of β-LPH and β-endorphin released into the medium (data not shown). Following treatment with 1 nM oCRH, there is a 300% increase in β-endorphin-IR (β-LPH plus β-endorphin) secreted into the media from the control and a 375% increase in β-endorphin-IR secreted into the media from the chronic stress/rest suspensions. The media from control anterior lobe suspension shows approximately 2-fold more β-endorphin than β-LPH (ratio β-endorphin : β-LPH = 1.8 : 1) following oCRH stimulation, while the media from chronic stress/rest suspensions shows a ratio of β-endorphin : β-LPH of 1.1 : 1, again suggesting substantially less β-endorphin is released from these anterior lobes from chronically stressed rats. Also included in this figure are data from media from anterior lobe suspensions from acute stress (β-endorphin : β-LPH = 2.5 : 1) and chronic/acute stress (β-endorphin : β-LPH = 1.2 : 1), suggesting this acute challenge and release *in vivo* did not change the response to oCRH *in vitro*. Thus, the mean ratio of β-endorphin : β-LPH for the stimulated release from the pituitaries from rats not exposed to chronic footshock is 2.2 : 1, while the ratio of β-endorphin : β-LPH for the stimulated release from pituitaries from chronic footshock rats is 1.2 : 1. These ratios are similar to that observed in the adrenalectomy/metyrapone studies (control ratio of β-endorphin : β-LPH = 2.0 : 1; adrenalectomy + metyrapone ratio of β-endorphin : β-LPH = 1.2 : 1) presented in the previous paper. If the *in vitro* data from both metyrapone- and adrenalectomy-treated rats are combined with the *in vitro* data on rats receiving chronic footshock, there is a significant decrease in the ratio of β-endorphin : β-LPH released by oCRH in these chronic treatment groups ($n = 4$, $P = 0.0014$, unpaired, two-tailed t-test).

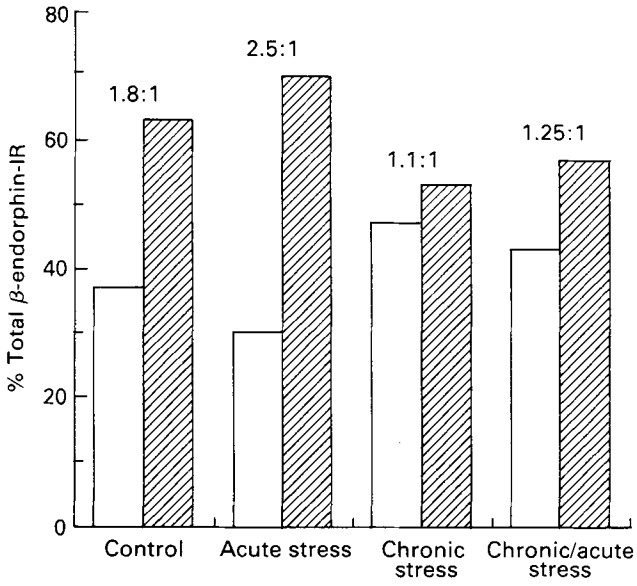

FIG. 1. Release of β-endorphin (▨) and β-LPH (□) in response to oCRH in short-term anterior lobe suspensions from control rats, acute footshock rats, chronic stress rest and chronic/acute footshock stress rats. These data were calculated by adding the values of fractions in each peak following G-50 sieving, then subtracting baseline readings from oCRH stimulated peaks. The basal unstimulated release demonstrated equimolar release of β-endorphin and β-LPH in the control and chronic stress/rest suspension (data not shown). The data are presented as percent of total β-endorphin-IR, with percent β-LPH plus percent β-endorphin totaling 100%. Anterior lobe suspensions from both control and acute footshock rats demonstrate a 2-fold greater release of β-endorphin than β-LPH following oCRH stimulation. In contrast, anterior lobe suspensions from chronic stress/rest and chronic/acute footshock stress show a 1 : 1 ratio of β-endorphin to β-LPH released with oCRH stimulation.

Corticotrope content and forms. One possible explanation for the changes in forms released *in vivo* and *in vitro* following chronic footshock stress would be a change in molar ratio of β-LPH to β-endorphin in the corticotrope stores. Table 3 shows the sieving data for β-endorphin and β-LPH from anterior pituitary cell extracts from control, acute stress, chronic stress/rest and chronic/acute stress animals. The cell extracts from chronic stress/rest group demonstrated a 2-fold increase in β-endorphin/β-LPH-IR in comparison to the control group. The molar ratios show a slight predominance of β-LPH over β-endorphin in control animals, which does not appear to change with chronic stress. If anything, the chronic stress/rest groups show more β-endorphin than β-LPH. Likewise, the cell extracts from the anterior lobe suspensions show similar ratios of β-LPH : β-endorphin in control and chronic stress/rest groups (data not shown).

Electroconvulsive seizures

Since footshock is a 30 min stressor, it is possible that there are differentially releasable pools over the time of acute stress. To explore this possibility, ECS was selected as a stimulus since it

TABLE 3. Anterior Pituitary : Immunoreactive Forms Present (pmoles/sieving peak).

	POMC (31K)	β-LPH	β-Endorphin	β-LPH : β-Endorphin
Control	57	327	271	1.2 : 1
Acute stress	24.9	202	144	1.4 : 1
Chronic stress	45	616	640	0.96 : 1
Chronic/acute stress	32	456	356	1.3 : 1

is a brief stimulus that leads to release of β-endorphin/β-LPH into plasma of a similar magnitude to that seen with chronic footshock. In this experiment, acute ECS led to a 30% decrease in anterior pituitary stores of ACTH, with an increase in plasma β-endorphin/β-LPH concentrations from 38±4 fmoles/ml to 136±25 fmoles/ml. Chronic ECS led to a 26% increase in anterior pituitary stores of ACTH. Chronic/acute ECS increased plasma β-endorphin/β-LPH from 45±13 fmoles/ml to 183± 18 fmoles/ml. There was no significant change in N-acetyl β-endorphin following acute or chronic/acute ECS (acute ECS 27±10 to 16±5 pM; chronic ECS 19±4 to 33±8 pM). The plasma from baseline and 5 min post-ECS from both naive and chronic ECS rats were then pooled and applied to a molecular sieving column to determine ratio of β-LPH to β-endorphin released by acute ECS. Five minutes following acute ECS, the ratio of β-endorphin : β-LPH released was 3.4 : 1. The ratio of β-endorphin : β-LPH 5 min following acute ECS in the chronic ECS rats was 0.9 : 1, in agreement with the footshock data demonstrating that chronic stimulation of corticotrope secretion by stress can shift the proportion of β-LPH released with re-challenge. If we compare the ratio of β-endorphin : β-LPH release by acute stress versus chronic stress across the three footshock and one ECS experiments, there is a significant decrease in the proportion of β-endorphin released with chronic stress (n = 4, P = 0.0021, two-tailed, unpaired *t*-test).

Discussion

In summary, with acute footshock stress substantial amounts of β-endorphin size material are released from the anterior lobe, while with chronic footshock stress, more β-LPH than β-endorphin is released *in vivo*. Short-term anterior lobe suspensions confirm the *in vivo* data; oCRH stimulates release of 1.8-fold more β-endorphin than β-LPH in control suspensions, while it stimulates release of approximately equal amounts of β-endorphin and β-LPH in suspensions from chronically stressed rats. Chronic ECS, which also increases peptide stores of POMC derived peptides in the anterior lobe, leads to a shift in the ratio of β-endorphin to β-LPH to favor more β-LPH release.

Although it is generally agreed that under normal conditions POMC, the prohormone, is not secreted from either the anterior lobe or intermediate lobe, it is less clear whether β-endorphin or β-LPH is the preferred end product of the anterior lobe POMC system. The molar ratios of β-LPH to β-endorphin in the anterior lobe suggest that β-LPH is the predominant stored product (6–9). Our previous data examining acute footshock stress-induced release of β-endorphin-IR demonstrated that what is released from the anterior lobe does not match the molar ratios of the stored materials, presumably because the total content does not accurately reflect the releasable pools (1). Similarly, these experiments demonstrate that there are shifts in the proportions of β-LPH and β-endorphin released under situations of chronic drive but this same manipulation does not necessarily produce changes in the ratio of stored materials. Our previous studies using short-term anterior lobe suspensions from acutely footshock-stressed rats demonstrated: 1) increased biosynthesis of POMC in pulse-chase experiments, 2) an increased rate of processing of POMC to β-LPH/β-endorphin in these same experiments, and 3) continued preferential secretion of β-endorphin size material with oCRH stimulation despite depletion of the β-endorphin/β-LPH stores of the anterior pituitary to 80% of control values; this suggested that the last step of the processing of β-LPH to β-endorphin can occur rapidly in situations of high demand, (1, 3) presumably due to alterations in post-translational events. However, in the case of the chronically footshock-stressed animals, there is a 2-fold increase in the β-endorphin/β-LPH and ACTH-IR content of the corticotropes, and a slight decrease in the rate of processing of the precursor POMC as measured by pulse-chase, even when the animals are re-challenged with acute stress (2, 3). This suggests that rather than replenishing releasable pools by acutely increasing POMC processing, the anterior lobe maintains its ability to respond to this chronic demand by increased stores. In these same animals, equimolar amounts of β-LPH and β-endorphin are released with stress or oCRH stimulation of anterior lobe suspensions, possibly because this mechanism for rapidly replenishing β-endorphin stores is no longer operating.

Another possibility is that there are differentially releasable pools, i.e. an immediate release pool and a sustained release pool (10). A time course study with acute footshock (1) demonstrated no difference in the ratios of β-endorphin : β-LPH following 5 or 30 min of acute footshock. The shift in the ratio of forms released also occurs with the chronic application of the very brief stressor of ECS (chronic ECS group), suggesting that different releasable pools, i.e. an immediate release pool and a sustained release pool, would not explain the differences in forms released by repeated stress. Likewise, the *in vitro* cell suspensions demonstrate that following acute footshock stress, the anterior lobe continues to release β-endorphin preferentially over β-LPH, despite a 20% depletion of the β-endorphin/β-LPH-IR content of the anterior lobe by acute stress. The *in vitro* pituitary release data from the chronically stressed animals demonstrate the release of primarily β-LPH with oCRH stimulation even from animals which have not been stressed for 24 h (chronic stress/rest). These data support the hypothesis that there are changes in the composition of the releasable pool with chronic stressors that leads to increased stores of peptide, such that a larger proportion of the granules in the releasable pool contain β-LPH rather than β-endorphin. They are also compatible with the data of Allen *et al.* (11) suggesting that older ACTH-containing granules are selectively released over newly synthesized granules. In situations of high demand, there is presumably less time to fully process β-LPH to β-endorphin and both old and new granules contain primarily β-LPH. Similar *in vitro* data were reported by Ham and Smyth (12, 13) demonstrating that acute CRH in anterior lobe primary cultures released primarily β-endorphin while chronic CRH releases primarily β-LPH. These data are in partial agreement with the report by Wand *et al.* (14) examining the effects of chronic secretagog exposure on the release of β-LPH and β-endorphin in rat primary anterior lobe cultures. Using pulse-labeling for 12 h, then measuring the released radiolabeled β-LPH and β-endorphin, they concluded that chronic secretagog exposure causes proportionately more β-LPH release than in control cultures. However, in both control cultures and cultures exposed to chronic CRH, they report a predominance of β-LPH over β-endorphin. Their data are in agreement with work with AtT-20 cells showing more β-LPH than β-endorphin released with CRH (9). Such differences may be due to the use of long-term primary cultures rather than whole animals; *in vivo* additional factors such as glucocorticoids

may interact with endogenous secretogogs to regulate POMC processing and release.

At the current time, we do not know the target organs for peripheral β-endorphin release or all the physiological functions of β-LPH and β-endorphin. However, β-endorphin is a relatively stable, opioid active peptide and can interact at opioid receptors in a number of peripheral sites including the gut, immune systems, reproductive system, pituitary (not corticotropes) and even the median eminence of the hypothalamus. A number of these systems are modified by stress through both neural and hormonal mechanisms. A decrease in the amount of opioid active β-endorphin released with chronic stress could have profound effects on a number of different physiological systems. Additionally, we have only characterized one time point, 14 days. It is possible that these alterations may be of a greater magnitude at another time point, as seen in the adrenalectomy time course study in the previous paper.

In conclusion, these studies suggest that the secreted product from the anterior lobe POMC system is controlled by an interplay between mRNA levels, peptide stores and processing rates of the precursor which determine the nature of the releasable pool. The composition of this releasable pool is modifiable by repetitive stimulation and these modifications, in turn, appear to lead to distinctly different peripheral biological consequences. Although the peripheral targets of β-endorphin and β-LPH are unknown, changes in the release of opioid active β-endorphin may affect a number of peripheral systems in which opioids are known to be active. In the intermediate lobe POMC system, increased stores following repeated stress lead to processing changes in some products more than others, so that not all end products are increased to the same extent (5). Such changes in the end products increased with chronic stress may lead to a different ratio of products being secreted at different times. This suggests the possibility that peptidergic endocrine cells may deliver somewhat different messages to their targets, depending upon regulatory adaptations that can occur as a consequence of repeated demands. This would endow these cells with a level of flexibility controlled by their physiological context which is yet to be fully explored.

Materials and Methods

Stressors

Both initial and repeated exposure to stimuli were examined. 'Acute' designates the first exposure to the stimulus. 'Chronic' designates once daily administration of the stimulus for 7 to 14 days as discussed below. Rats were sacrificed either 24 h after the last exposure (chronic/rest) or were re-challenged immediately prior to sacrifice (chronic/acute). Intermittent footshock was administered as follows: acute stress consisted of one 30-min session (12 shocks/min; 2.5 mAmp) followed by decapitation. Chronic stress consisted of 30-min daily stress sessions for 14 days with rats either given 24 h to recover from last stress (chronic stress/rest) or re-stressed immediately before decapitation (chronic stress/acute stress). For acute ECS, an 80 mAmp electric shock lasting 0.2 s was administered to the brain via ear electrode clips. The animals were decapitated 3 to 5 min following the application of the shock. For chronic ECS studies, daily ECS was administered once daily for 7 days and animals were either decapitated 24 h later (chronic/rest) or following an eighth session (chronic/acute). In plasma experiments acute stress and chronic/acute stress animals were paired for comparison. These stressors were approved by the University of Michigan Committee on the Use and Care of Animals.

Tissue processing

Trunk blood was collected immediately following decapitation in K^- EDTA containing tubes. Pituitaries were removed from these animals, and anterior lobe and intermediate lobe were separated. For content and sieving studies, both lobes were frozen on dry ice immediately for subsequent extraction. For pituitary release experiments, anterior lobes were placed in oxygenated Krebs-Ringer bicarbonate buffer, pH 7.2 (Krebs). The anterior lobes from 10 animals of each group were dissociated by incubation for 60 min with collagenase (3 mg/ml) in Krebs as previously described (2). After 90 min stabilization in Krebs buffer, 1 nM oCRH was added to aliquots of the cells. Following a 60-min incubation period, the cells were centrifuged, and the media saved for subsequent extraction and sieving; the cellular stores of POMC and its products were extracted with 5 N acetic acid, a standard β-endorphin extraction procedure, using repeated freezing and thawing to lyse the cells. Plasma and culture media were extracted with Sep-Pak C_{18} cartridges using the procedure previously described for β-endorphin extraction from human plasma (15). To ensure that the *in vitro* manipulations did not introduce changes in stored forms, anterior pituitaries which were not cultured from 10 control and 10 chronically stressed animals were homogenized in an acetone/0.2 N HCl (3 : 1) mixture, centrifuged and the supernatant lyophilized. Gel filtration was used to determine ratios of β-LPH to β-endorphin. Samples were pooled for sieving and applied to a 90 × 1.5 cm G-50 superfine column developed in 1% formic acid. Blue dextran and cobalt chloride were used to mark the inclusion and exclusion volume of the column, and cytochrome C and [^{3}H]β-endorphin were used as markers for β-LPH and β-endorphin, respectively.

Radioimmunoassay

Fractions from G-50 sieving of plasma, pituitaries, media and cell suspensions were assayed using the β-endorphin RIA previously described (15). In brief, all fractions including fractions from the void volume were lyophilized then resuspended in 0.1% human serum albumin, pH 3.0 with 1 N HCl, for these assays. Standards were dissolved in the same diluent. The β-endorphin RIA used an antibody directed primarily to the mid-portion of the β-endorphin molecule that recognized both β-endorphin and β-LPH equally well, and also recognized the precursor, POMC. This immunoreactivity was designated as β-endorphin/β-LPH-IR. The antibody was used at a final dilution of 1/40,000 with [^{125}I]β-endorphin$_h$ as the radiolabeled tracer. β-Endorphin$_c$ or β-endorphin$_h$ were used as the standards. Under the conditions used in this assay, the antibody generally showed 80% to 100% cross-reactivity with N-acetylated-β-endorphin$_{1-31}$ and 50% cross-reactivity with N-acetyl-β-endorphin$_{1-27}$. The N-acetyl-β-endorphin assay utilized an antibody that was N-acetyl specific; it showed no cross-reactivity with non-acetylated β-endorphin$_{1-31}$. It demonstrated 100% cross-reactivity with N-acetyl-β-endorphin$_{1-27}$, N-acetyl β-endorphin$_{1-16}$ and N-acetyl γ-endorphin (N-acetyl-β-endorphin$_{1-17}$). It was used at a final dilution of 1/8000. [^{125}I]N-acetyl-β-endorphin$_{1-27}$ (human sequence) was used as the radiolabeled tracer. N-acetyl-β-endorphin$_{1-31c}$ was used as the unlabeled standard. α-MSH was measured with an antibody that was a generous gift of Reiner Martin and assayed as previously described (6). The antibody was used at a titer of 1 : 20,000. [^{125}I]des-acetyl-α-MSH was used as the radiolabeled tracer and α-MSH was used as the standard. This antibody requires amidation of the C-terminal for antigenic recognition of α-MSH. The IC_{50} of this assay was 60 fmoles/tube. Sample and standard preparations and assay buffer were the same as for the β-endorphin and N-acetyl-β-endorphin RIAs.

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