Abstract. The intermediate lobe of the rat pituitary gland produces a series of peptides related to ACTH and LPH. The spontaneous and isoproterenol-stimulated release of such peptides was studied during in vitro superfusion of rat neurointermediate lobes with Krebs-Ringer medium. Products released into the superfusion medium were quantified by direct measurement or after chromatography on Sephadex G-50. ACTH bioactivity was determined by use of adrenal cortical cell suspension assay. In addition, NH2-terminal ACTH, CO2H-terminal ACTH, α-MSH and β-endorphin radioimmunoassays were used. The results show that
1. neurointermediate lobes of rats secrete spontaneously various ACTH- and LPH-related peptides in amounts proportional to the amounts in which these peptides are found in extracts of the neurointermediate lobe;
2. the β-adrenergic agonist, isoproterenol, stimulated the spontaneous release of various peptides, including α-MSH, ACTH, CLIP, glycosylated CLIP, and β-endorphin-like peptides;
3. isoproterenol induced a dose-dependent (10^-9 to 10^-7 M), parallel increase in the release of α-MSH and ACTH following similar time courses and showing identical EC50 values (about 10^-8 M).
Although the spontaneous release of α-MSH and ACTH from rat neurointermediate lobes is not strictly coupled under the conditions used in this study, isoproterenol seems to affect the spontaneous release of these peptides to the same relative extent.

A series of biologically active peptides related to ACTH and LPH are formed in the corticotrophs of the anterior lobe and the melanotrophs of the intermediate lobe of the pituitary gland by proteolytic cleavage of a common precursor molecule (Mains et al. 1977; Roberts & Herbert 1977; Rubinstein et al. 1978; Nakanishi et al. 1979). Although the precursor produced in the corticotrophs is likely to be identical to that formed in the melanotrophs (Mains & Eipper 1975, 1979; Eipper & Mains 1978), both cell types produce different species of ACTH- and LPH-related peptides. For example, the anterior lobe of the rat contains microgram amounts of ACTH1-39, but very little of its fragments. In contrast, the intermediate lobe contains only small amounts of ACTH1-39, but microgram quantities of α-MSH and CLIP, peptides with a primary sequence identical to ACTH1-13 and ACTH18-39, respectively. The occurrence of equimolar amounts of CLIP and αMSH (Scott et al. 1974), and recent observations from pulse label experiments (Mains & Eipper 1979), are supportive of the hypothesis that in the intermediate lobe, ACTH1-39 (or larger molecular forms of ACTH) serves as a prohormone for α-MSH and CLIP. Likewise, β-LPH might serve as a prohormone for γ-LPH and β-endorphin, which are the dominant LPH-related peptides in the intermediate lobe (Crine et al. 1978; Mains & Eipper 1979; Jackson & Lowry 1980).

In addition to a possible precursor role, results of in vitro studies have shown that ACTH and β-LPH can also be secreted from the intermediate lobe (Kraicer 1977; Mains & Eipper 1979; Tilders 1980).
1979). In contrast to the close coupling observed between the release of ACTH- and LPH-related peptides from corticotrophs (Guillemin et al. 1977; Gillies & Lowry 1978; Vale et al. 1978), the results of various experiments are in support of the view that the release of various peptides from the melanotrophs is not necessarily coupled (Kraicer et al. 1978; Vaudry et al. 1978; Tilders 1979; Smelik & Tilders 1980). This leads to the intriguing question of whether the relative quantities in which the various peptides are secreted by the intermediate lobe can be influenced by specific stimuli.

We have studied this question by measuring the spontaneous and stimulated release of various ACTH- and LPH-related peptides from neuro-intermediate lobes of rats superfused in vitro. In the studies reported here, the β-adrenergic agonist isoproterenol was used as a stimulus since this agent is known to increase the release of α-MSH in vitro and in vivo (Bower et al. 1974; Smelik & Tilders 1980).

Materials and Methods

Superfusion

Adult female Wistar rats were decapitated, and the pituitary glands were removed immediately. The neuro-intermediate lobes (NIL) of the pituitary glands were carefully separated from the anterior lobes and transferred (5–10 lobes per superfusion chamber unless otherwise stated) to a superfusion apparatus described earlier (Mulder & Smelik 1977). The lobes were superfused continuously at a rate of 0.25 ml/min with Krebs-Ringer bicarbonate buffer (KRB) equilibrated in an atmosphere of 95% oxygen and 5% carbon dioxide at 37°C. KRB was supplemented with glucose (2 mg/ml) and bovine serum albumin (5 mg/ml, Sigma, Fraction V).

After superfusion for 60 min with KRB, superfusion was continued for 30 min with KRB containing isoproterenol at concentrations of 10⁻⁶ - 10⁻⁸ M, followed by another 15 min with isoproterenol-free KRB. The superfusion medium was collected in 5- or 10-min fractions. In two experiments, 30 NIL were superfused, and 5-min fractions were collected alternately for chromatography and for the bioassay of ACTH.

Gel filtration

For chromatography, superfusion medium samples were collected on ice in tubes containing 100 μl of 1.6% glycine in 1 M HCl and frozen immediately. Two 5-min superfusion medium samples collected 10 min apart before, during, and after superfusion with isoproterenol were pooled to form the 'pre-', 'during,' and 'post-stimulation' samples. Each of these pooled samples was subjected to chromatography on Sephadex G-50 superfine under acid dissociating conditions using 1% formic acid as an eluate (Jackson & Lowry 1980). The column was equilibrated with 5 μg of ACTH₁₋₃₉, CLIP and β-endorphin.

Determination of peptides released

Bioassay of ACTH. The amount of biologically active corticotrophin (ACTH₃₉) was quantified by using the dispersed adrenocortical cell assay of Sayers as described in detail (Mulder & Smelik 1977). Medium samples were adjusted to pH 3.5 with HCl and stored at -20°C or assayed immediately. Superfusion medium samples induced a dose-dependent increase in corticosterone production. The slope of this relationship was identical to that of the dose-response relationships obtained from NIL or anterior lobe extracts of rat pituitary glands and from ACTH₁₋₃₉ and ACTH₁₋₂₄. ACTH₁₋₂₄ was used as a standard (biological activity 100 mU/μg).

Radioimmunoassays of α-MSH. For the direct quantification of immunoreactive α-MSH (α-MSHᵢ) in superfusion medium samples, EDTA was added to a final concentration of 10 mM, and the samples were stored at -20°C until assayed. The concentration of α-MSHᵢ was measured by using a specific radioimmunoassay as described in detail (Penny & Thody 1978), which showed low cross-reactivity with ACTH₁₋₃₉ (0.15%) or CLIP (< 10%). Synthetic α-MSH (CIBA-GEIGY) was used for labelling purposes and as a standard. Medium samples, NIL extracts, and synthetic α-MSH induced parallel, concentration-dependent displacement of [¹²⁵I]-labelled α-MSH.

For the quantification of α-MSHᵢ, in fractions obtained after column chromatography, we used the α-MSH assay described by Scott et al. (1976). Both α-MSH assays show similar characteristics.

Radioimmunoassays of ACTH. ACTH₁ was quantified by using the NH₂-terminal and the CO₂H-terminal ACTH assays described elsewhere in detail (Ratcliffe et al. 1972). In both assays, ACTH₁₋₃₉ was used as a standard and for labelling purposes. In the CO₂H-terminal assay, α-MSH did not crossreact, but CLIP showed full crossreaction with ACTH₁₋₃₉. In the NH₂-terminal assay, CLIP did not crossreact, and α-MSH showed only a slight crossreaction with h-ACTH₁₋₃₉.

Radioimmunoassay of β-endorphin. β-Endorphin was quantified by using an antiserum that was raised in rabbits against human β-LPH as described by Jackson & Lowry (1980).

β-Endorphin was used for labelling purposes as a standard. Crossreaction between β-LPH and β-endorphin was 100%.
**Pituitary extracts**

Anterior lobes or NIL were homogenized in 0.1 M HCl (1 lobe/ml) and incubated at 4°C for 1 h. Homogenates were then centrifuged at 4°C (10 min, 5000 × g), and the supernate was removed and stored at −20°C until assayed.

**Peptides and drugs**

The following peptides were used: human ACTH$_{1-39}$ and ovine CLIP (both purified in the Department of Chemical Pathology), synthetic human ACTH$_{1-24}$ (Organon, Oss) synthetic α-MSH (CIBA-GEIGY) and purified procine β-endorphin (a gift from Dr. D. G. Smyth).

Isoproterenol (DL isoprenaline, O.P.G., Amsterdam) was dissolved in ice-cold 0.01 M HCl and diluted in warm KRB just prior to use. Propranolol (DL-propranolol HCl, Sigma) was dissolved in distilled water and diluted in KRB.

**Results**

**Spontaneous release of ACTH$_b$ and α-MSH$_i$**

During superfusion of rat NIL with KRB, the release of α-MSH$_i$ was initially high but fell rapidly, which is in agreement with a previous report of MSH bioactivity (Tilders et al. 1979). After 30–40 min, the release rate of α-MSH$_i$ showed a very slow linear decline with time or no change at all, for at least 2 h. In contrast, the release of ACTH$_b$ did not stabilize but showed a progressive decline throughout the superfusion run, as illustrated in Fig. 1. After about 40 min, the decline in the release rate of ACTH became linear with time.

The ACTH$_b$ content of NIL superfused for 2 h with KRB appeared to be identical (3.68 ± 0.26 mU per NIL, mean ± SEM, N = 10) to that of their non-superfused controls extracted directly after preparation (4.12 ± 0.49 mU per NIL, N = 10). The total amount of ACTH released into the medium during 2 h of superfusion with KRB was less than 4% of the ACTH content of the NIL after superfusion. Also, the α-MSH$_i$ content of superfused and non-superfused NIL was identical (1.27 ± 0.13 µg per lobe, N = 10, and 1.14 ± 0.08 µg per lobe, N = 10, respectively), and the total amount of α-MSH$_i$ released during 2 h of superfusion with KRB was less than 5% of the α-MSH content of the NIL after superfusion.

![Fig. 1.](image)

Spontaneous release of α-MSH$_i$ and ACTH$_b$ from rat neurointermediate lobes superfused with KRB. On the ordinates: the average amount of α-MSH$_i$ and ACTH$_b$ released into the medium during 10-min collection periods per NIL.
Effect of isoproterenol on the release of α-MSH and ACTH from rat NIL superfused in vitro. For comparison, release of α-MSH\textsubscript{i} and ACTH\textsubscript{b} is expressed as percentage of the calculated spontaneous release (see text). 'ISO' indicates the period of superfusion with KRB containing \(10^{-7}\) M of isoproterenol. Data represent mean and SEM of 4 independent experiments.

**Fig. 2.**

Effects of isoproterenol on release of ACTH\textsubscript{b} and α-MSH\textsubscript{i}

When, after 60 min of superfusion with KRB, NIL were superfused for 30 min with isoproterenol-containing medium, the release rates of ACTH and α-MSH increased, reaching maximal values within 10 min after the beginning of exposure to isoproterenol. In pilot studies we found that within 10

**Fig. 3.**

Effect of various concentrations of isoproterenol on the release of ACTH\textsubscript{b} and α-MSH\textsubscript{i} from rat NIL superfused with KRB (panel A) or KRB containing propranolol, \(10^{-7}\) M (panel B). Release of α-MSH\textsubscript{i} (hatched bars) and of ACTH\textsubscript{b} (solid bars) is expressed as percentage of the calculated spontaneous release (Fig. 2). Data represent mean and SEM of 3–6 experiments.
min after termination of the exposure to isoproterenol (10^{-7} M), the release rates of α-MSH_i and ACTH_b were identical to those of NIL superfused with KRB only. This allowed us to compute the spontaneous release of ACTH_b and α-MSH_i from each group of NIL by linear extrapolation of the release rate between 55 and 100 min (cf. Fig. 1). For direct comparison, the release rates of α-MSH_i and ACTH_b were expressed as percentage of their calculated spontaneous release rates.

The time course of the isoproterenol-induced increase of α-MSH_i was similar to the time course of the increase in ACTH_b release at all doses tested (10^{-9} - 10^{-6} M). As illustrated in Fig. 2, the isoproterenol-induced increase in α-MSH_i and ACTH_b release was not maintained during the entire period of exposure to isoproterenol. By using the same regimen as shown in Fig. 2, different concentrations of isoproterenol were tested for their effects on α-MSH_i and ACTH_b release, and the maximal peak heights were calculated. As summarized in Fig. 3, isoproterenol at concentrations of 10^{-9} - 10^{-7} M induced a dose-dependent stimulation of ACTH_b release which was parallel to its effect on α-MSH_i release. The secretion of α-MSH_i and ACTH_b did not further increase at higher isoproterenol concentrations (10^{-6} M). The ED_{50} of isoproterenol for stimulating the release of α-MSH_i and ACTH_b was about 10^{-8} M.

When propranolol (10^{-7} M) was present throughout the superfusion experiment, the α-MSH_i and ACTH_b releasing effects of 10^{-8} M isoproterenol were completely blocked and those of 10^{-7} M isoproterenol were partially prevented.

Chromatography of ACTH- and LPH-related peptides released spontaneously.

Superfusion medium samples were subjected to chromatography on Sephadex G-50, and the eluted fractions were assayed by using four different radioimmunoassay systems as shown in Fig. 4. The elution profile of α-MSH_i showed one predominant peak with an elution pattern identical to that of synthetic α-MSH. Both the NH_2- and CO_2H-terminal ACTH assays showed a small amount of ACTH_i near the void volume, probably reflecting large molecular forms of ACTH. A small peak of ACTH_i with the elution volume of ACTH_{1-39} was detected in the NH_2-terminal ACTH assay which contained more than 50% of the NH_2-ACTH_i recovered from the column. Considerably more ACTH_i was found in the CO_2H-terminal ACTH assay, which showed two major peaks. Peak I eluted slightly earlier than did ACTH_{1-39}, and this material is probably identical to big CLIP found in NIL extracts (Jackson & Lowry 1980). Peak II had the same elution volume as CLIP.

Most of the β-endorphin_i was present in two major peaks, reflecting the situation in NIL extracts (Jackson & Lowry 1980). The first peak, β-endorphin_i I, had the same elution volume as β-endorphin, whereas the second peak, β-endorphin_i...
phrin II,' eluted slightly later. No activity was found near the void volume, but some samples showed detectable activity at the elution volume of rat β-LPH (kav 0.24–0.28).

**Effect of isoproterenol on the release of ACTH- and LPH-related peptides**

The effect of isoproterenol on the release of these peptides was studied by collecting superfusion medium samples of rat NIL before, during, and after exposure to isoproterenol (10⁻⁷ M). α-MSH; and NH₂-terminal ACTH₁ and ACTH₂ were measured directly in the medium. The concentrations of β-endorphin; I and II and of CO₂H-terminal ACTH₁ and ACTH₂ were calculated from the chromatograms. The results, summarized in Table 1, show that with the exception of β-endorphin; I, each of the activities was found in higher concentrations during than before or after exposure to isoproterenol. Since pooled medium fractions were analyzed in this experiment rather than fractions containing the peak effects, the amplitude of the effect of isoproterenol on the release of α-MSH; and ACTH₂ seemed smaller than that shown in Figs. 2 and 3.

**Table 1.**

<table>
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<tr>
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<th>ng peptide per ml superfusion medium</th>
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<tr>
<td></td>
<td>Pre</td>
<td>Iso</td>
</tr>
<tr>
<td>β-Endorphin; I</td>
<td>24.00</td>
<td>26.60</td>
</tr>
<tr>
<td>β-Endorphin; II</td>
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<td>45.20</td>
</tr>
<tr>
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</tr>
<tr>
<td>NH₂-ACTH₁(d)</td>
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<td>1.84</td>
</tr>
<tr>
<td>ACTH₂(d)</td>
<td>1.10</td>
<td>1.52</td>
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* A group of 30 NIL was superfused with KRB for 60 min and then for 30 min with 10⁻⁷ M isoproterenol containing KRB followed by another 30 min with KRB. Medium fractions were collected before (Pre), during (Iso), and after (Post) superfusion with isoproterenol. The concentration of various peptides in the pooled medium samples was determined after chromatography or by direct measurement (d). Data represent mean biological activity (b) or immunoreactivity (i) in ng of standard (β-endorphin, ACTH₁₋₃₉, α-MSH).

**Discussion**

In the present study, we investigated the spontaneous and stimulated release of various ACTH- and LPH-related peptides from the intermediate lobe of rats in vitro. In order to obtain accurate information on the secretion rate of these various peptides, without interference of possible direct feedback effects (Kastin et al. 1971) or extracellular breakdown of the large molecular forms which are very susceptible to proteolytic cleavage, we decided to use a superfusion method rather than an incubation procedure employed by others (Bower et al. 1974; Kraicer & Morris 1976; Mains & Eipper 1979). Intermediate lobe cell suspensions have been used in studies on peptide release (Kraicer 1977; Kraicer et al. 1978), but their use is endangered by possible artifacts caused by the dispersion procedure. In order to avoid this, the present studies were carried out by using freshly prepared NIL.

During superfusion, rat NIL spontaneously secrete a family of ACTH- and LPH-related peptides in amounts proportional to the amounts in which these peptides are found in NIL extracts. Most of the β-endorphin; found in the medium was present in two moieties with elution profiles identical to those of the two forms of β-endorphin; found in NIL extracts (Jackson & Lowry 1980). The substance(s) appearing as β-endorphin; I (cf. Fig. 4) had an elution volume identical to that of pβ-endorphin and therefore might represent rat β-endorphin. β-Endorphin; II, which was not found in anterior lobe extracts, appears to be a smaller peptide. Furthermore, only a small amount of β-endorphin; with an elution coefficient of about 0.25 was secreted, and this material is likely to represent rat β-LPH, which was also found in only small quantities in NIL extracts (Jackson & Lowry 1980).

The predominant ACTH-related peptides that were secreted spontaneously were the NH₂-terminal fragment α-MSH (acetyl-ACTH₁₋₃₉-NH₂), the CO₂H-terminal fragment CLIP (ACTH₁₈₋₃₉) and a CO₂H-terminal fragment also found in NIL extracts, which has recently been identified as a glycosylated form of CLIP (Mains & Eipper 1979; Jackson & Lowry 1980). In comparison to the CO₂H-terminal ACTH₁, only small amounts of ACTH-like material were detected in the NH₂-terminal ACTH assay, which recognizes that part of the ACTH molecule which also contains the
The dissociation between the release of α-MSH and ACTH, which has been found even more pronounced under membrane-depolarizing conditions (Vaudry et al. 1978; Tilders 1979; Smelik & Tilders 1980), may be a consequence of differences in cellular compartmentalization of these two entities, since a small population of corticotroph-like cells has been reported to exist in the intermediate lobe of some mammals (Stoeckel et al. 1973). Alternatively, it may be a consequence of differences in subcellular compartmentalization related to the fact that ACTH is an intermediate in the biosynthesis of α-MSH and CLIP, rather than a major end product in the intermediate lobe.

In view of the latter hypothesis, it was of interest to establish whether catecholamines that are known to affect the release of α-MSH in vitro and in vivo would have similar effects on the release of other ACTH- and LPH-related peptides.

In addition to the MSH-release-inhibiting effects of dopamine and of high concentrations of epinephrine and norepinephrine (Hadley et al. 1977) which are probably mediated by interaction of these catecholamines with dopamine receptors (Tilders et al. 1980b), low dose of epinephrine have been found to enhance MSH release from rat NIL (Bower et al. 1974; Tilders et al. 1980b) and from dispersed intermediate lobe cells (Tilders et al., to be published). Since the effect of low dose of epinephrine could be mimicked by the β-adrenergic agonist, isoproterenol, and blocked by the β-adrenergic antagonist, propranolol, it is likely that the melanotrophs exhibit β-adrenergic receptor sites.

Unlike the situation during spontaneous release, stimulation of β-adrenergic receptors by superfusing rat NIL with isoproterenol-containing medium resulted in a coupled increase in the release of ACTHβ and α-MSHα. The release of both entities followed a similar time course (Fig. 2) and showed identical dose-response relationships with an apparent EC50 of about 10−8 M isoproterenol (Fig. 3). Thus, although the absolute amount of α-MSHα released under influence of isoproterenol is much larger than the absolute amount of ACTHβ, we do not interpret this in terms of a selective effect of isoproterenol on the release of α-MSH since both entities are stimulated to the same relative extent. Moreover, isoproterenol also stimulated the release of other ACTH- and LPH-related peptides including CLIP, glycosylated CLIP, and β-endorphin I. Although the isoproterenol-induced release of β-endorphin I was only small in the present study, isoproterenol was found to stimulate the release of β-endorphin I and β-endorphin II to a similar extent in experiments with dispersed intermediate lobe cells (Jackson, unpublished results), and induced a strong stimulation of the release of rat β-endorphin from NIL in vitro (Vermes et al. 1980).

The ability of the melanotrophs to respond to β-adrenergic stimulation with an increase in the release of ACTH- and LPH-related peptides is specific for this part of the pituitary gland and is not found in the corticotrophs of the anterior lobe (Vale et al. 1978). Although the physiological role of such a β-adrenergic mechanism in the control of the secretory activity of the intermediate lobe remains to be established, β-adrenergic-stimulating agents may be valuable tools to stimulate selectively the secretion of ACTH- and LPH-related peptides from the intermediate lobe as shown in recent in vivo studies (Tilders et al. 1980a).
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Note added in proof
Recently, Kebabian and co-workers (Cote et al. 1980; Munemura et al. 1980) have characterized the \( \beta \)-adrenergic receptors that are present on the intermediate lobe cells of rats. According to biochemical criteria, these \( \beta \)-adrenergic receptors belong to the \( \beta_2 \) subcategory. Furthermore, they presented evidence to support the view that \( \beta \)-adrenergic stimulation of \( \alpha \) MSH release involves an increase in adenylate cyclase activity.
Cote T, Munemura M, Eskay R L & Kebabian J W (1980): Biochemical identification of the \( \beta \)-adrenoceptor and evidence for the involvement of an adenosine 3',5'-monophosphate system in the \( \beta \)-adrenergically induced release of \( \alpha \)-melanocyte-stimulating hormone in the intermediate lobe of the rat pituitary gland. Endocrinology 107: 108.

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