ADRENOCORTICOTROPIN AND β-LIPOTROPIN IN THE HYPOTHALAMUS

Localization in the Same Arcuate Neurons by Sequential Immunocytochemical Procedures

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ABSTRACT

Adrenocorticotropic hormone (ACTH) and β-lipotrophic hormone (β-LPH) have been localized by immunoperoxidase methods in nerve cells and fibers of the hypothalamus and brain stem of the ewe. 6-μm sections were immunostained first for either ACTH or β-LPH. The reaction products and the antibody complexes were then eluted completely from the tissue, and the same section was immunostained for the second peptide. Absorption of the primary antisera with a variety of peptide fragments of ACTH and β-LPH demonstrated, immunocytochemically as well as by radioimmunoassay, that the ACTH and β-LPH antisera were directed to the COOH- and NH₂-termini of the peptides, respectively. Neither antiserum recognized any portion of the heterologous peptide. In the sequential staining procedure on the same tissue section, preincubation of the antisera with the homologous peptide abolished the staining, whereas preincubation with the heterologous peptide did not affect it, regardless of the order followed.

Every nerve cell in the arcuate nucleus that contained ACTH also contained β-LPH, but β-LPH cells appeared, probably falsely, to be twice as numerous as ACTH cells. β-LPH-positive fibers in and beyond the hypothalamus were also more numerous and stained more intensively than ACTH fibers. The salient exception was fibers in the infundibular zona externa, where the opposite was true.

Our observations establish that ACTH and β-LPH are contained in the same nerve cells. They strongly favor biosynthesis in brain, probably from a common precursor molecule, as has been demonstrated in the pituitary gland. The complexity of the cytologic distribution pattern described suggests that the two peptides are not processed in the same manner by the nerve cell.
Recent data suggest that adrenocorticotropin (ACTH) and β-lipotropin (β-LPH) may be synthesized from a common precursor in the brain as well as the adrenohypophysis. Their highest concentration in brain has been found in the hypothalamus and pituitary (3). Their synthesis in brain. The pattern of distribution also suggests that, once synthesized, probably as portions of a single precursor molecule, the two peptides are not processed in the same manner by the cell.

**MATERIALS AND METHODS**

**Tissue Preparation**

The brains of nine ewes, five of them pregnant at term, were removed under phenobarbital anesthesia. The hypothalamus and pituitary were quickly dissected en bloc and immersed in Bouin’s solution for 36 h. The fixed blocks were washed overnight in running tap water, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. 6-μm serial sections were cut in the coronal plane. Every tenth section was stained with cresyl violet for histoanatomic orientation.

**Primary Antisera**

Antiserum to ACTH was generated in a rabbit using synthetic human ACTH (Ciba-Geigy Corp., Pharmaceuticals Div., Summit, N. J.) conjugated with bovine thyroglobulin by the carboximide method. 500 μg of the immunogen (determined by the Lowry protein estimation method) in 1 ml of 0.9% saline was emulsified with 1 ml of complete Freund’s adjuvant and injected into multiple intradermal sites along the animal’s back. 50 and 25 μg of the immunogen in incomplete Freund’s adjuvant were administered by the same route one and three months after the initial injection. 2 wk later, a 1:40,000 titer of 44% binding for a trace of radiolabeled (125I-iodo-) human ACTH was obtained.

Cross-reactivities of the antiserum to different ACTH fragments and related peptides were determined. These were performed at a final titer of 1:10,000 incubated at 10°C for 8 h before separation (see Table I).

Cross-reactivity studies were also performed at a final dilution of 1:500 (the titer used in immunocytochemistry) to ascertain whether any high-affinity, low-capacity binding sites for ACTH fragments were present which would become “unmasked” at this higher concentration (at a titer of 1:10,000 they could have presumably been diluted beyond detection). Cross-reactivity data at 1:500 were the same as at a dilution of 1:10,000, except that α-melanocyte-stimulating hormone (α-MSH) cross-reactivity increased from 1.3 to 2.6, cross-reactivity with 1-24 ACTH increased from 28 to 35, and cross-reactivity with 25-39 ACTH from 38 to 57 (see Table I).

The rabbit antiserum to ovine β-LPH used in this study was previously characterized by radioimmunoassay (9) and by immunoperoxidase technique on sheep brain (31). Immunostaining of brain sections was abolished completely by preincubation of 1 ml of the antiserum at a 1:1,000 dilution with 1 μg of synthetic ovine β-LPH (C. H. Li), whereas 5 μg of porcine 1-39 ACTH (Third International Standard), 0.3 μg of met- or leu-enkephalin (61-65 β-LPH), 1.5 μg of β-endorphin (61-91 β-LPH), and 1 μg of β-MSH (37-58 β-LPH) had no effect.

**Immunocytochemical Methods**

Deparaffinized sections on glass slides were rehy-
TABLE I

| Peptide tested | Source           | Cross-reactivity* |
|----------------|------------------|-------------------|
| Human 1-39 ACTH | Ciba-Geigy       | 100               |
| Porcine 1-39 ACTH | Organon         | 100               |
| Human 4-10 ACTH  | Organon          | 1.3              |
| Human 1-10 ACTH  | Organon          | 1.1              |
| Human 1-16 NH₂   | Organon          | 1.7              |
| ACTH            |                  |                   |
| Human 11-24 ACTH| Organon          | 16                |
| Human 17-39 ACTH| Organon          | 100               |
| Porcine 25-39 ACTH | Organon       | 38                |
| Human 1-24 ACTH  | Organon          | 28                |
| α-MSH           | Ciba-Geigy       | 1.3              |
| β-ovine LPH     | C. H. Li         | 0                 |
| β-porcine endorphin | R. Guillemin | 0                 |
| α-porcine endorphin | R. Guillemin | 0                 |
| γ-porcine LPH   | L. Graf           | 0                 |
| γ-porcine endorphin | R. Guillemin | 0                 |
| Human β-MSH     | National Pituitary | 0              |

Performed at a final titer of 1:10,000 with an 8-h incubation at 10°C before separation.

* Cross-reactivities are expressed as the dose of peptide necessary to give 50% replacement of initial binding divided by the dose of synthetic human 1-39 ACTH giving similar displacement × 100.

Elution of Bound Antibody Complexes from the Tissue Sections

To perform sequential staining for ACTH and β-LPH by indirect immunoperoxidase technique, the antibody used to identify ACTH in the first part of the experiment had to be removed from the tissue before identification of β-LPH could be accomplished on the same section. Since we postulated that ACTH and β-LPH would identify any antibodies to ACTH remaining from the first staining sequence, and in the same sites. This likelihood was demonstrable when sections stained for ACTH, after removal of the chloronaphthol reaction product, were successfully restained on sequential addition of sheep anti-rabbit gamma globulin, PAP, and 4-Cl-1-naphthol (or 3,3' dianimobenzidine) again. This restaining procedure, in fact, provided an essential test for the efficacy of the primary antibody elution methods we tried. Such standard antibody elution methods as high ionic strength (14), low pH (14), and electrophoresis (26), either by themselves or in the presence of excess ACTH, failed to dissociate all of the tissue-bound antibody. Complete immunohistologic elution was obtained by the oxidation method of Tramu et al. (24), as described below.

Elution Technique

After specific staining for one of the hormones and photography, the blue reaction products to 4-Cl-1-naphthol were removed and the sections were rehydrated by the methods outlined above. The tissue-bound antibodies were then eluted by immersion for 1 min with agitation in an oxidizing solution of 1 vol of 2.5% KMnO₄, 1 vol of 5% H₂SO₄, and 30 vol of distilled water. At this concentration, disruption of tissue morphology was not excessive. The sections were directly decolorized in 0.5% sodium metabisulfite (Na₂S₂O₅) in distilled water for 3-5 min, washed in running tap water for 2 h, and subsequently washed in PBS before repeating the immunocytochemical sequence using the antiserum to the second hormone as the first reactant. Immunoreactive cells and fibers were identified by light microscopy, and the sections were rephotographed for comparison with the photomicrographs from the first immunocytochemical sequence.

Controls

Method controls included replacement of the primary antiserum with PBS or with nonimmune rabbit serum at the same dilution. Absorption controls for each primary antiserum were carried out by preabsorption for 24 h at 4°C with synthetic ACTH, β-LPH, and several synthetic fragments of them before application of the primary antiserum in the immunoperoxidase sequence on adjacent sections of the hypothalamus and pituitary.

Preabsorption of 1 ml of 1:1,000 dilution of the
antiserum to ovine \( \beta \)-LPH with 1 \( \mu \)g of synthetic ovine \( \beta \)-LPH abolished all reactivity in hypothalamus and pituitary; 1 \( \mu \)g of porcine 1-39 ACTH had no effect. Preabsorption of 1 ml of 1:500 dilution of the antiserum to ACTH with 1.0, 0.1, and 0.01 \( \mu \)g of porcine 1-39 ACTH had a graded effect: complete abolition of staining in the anterior pituitary and hypothalamus by 1 \( \mu \)g, reduction by approximately one-half with 0.1 \( \mu \)g in the hypothalamus only, and no appreciable effect with 0.01 \( \mu \)g. Preabsorption with 3 \( \mu \)g of ovine \( \beta \)-LPH, 1 \( \mu \)g of 1-13 ACTH (Organon Inc., West Orange, N. J.), or 1 \( \mu \)g of 11-24 ACTH had no effect on ACTH reactivity, whereas 1 \( \mu \)g of 25-39 ACTH abolished ACTH staining in the brain and reduced it significantly in the anterior pituitary (see Table I for sources of supply of peptides).

Cross-absorption experiments were carried out in sequential staining procedures using antisera to both hormones. Sections were reacted first with antiserum to ACTH preincubated with synthetic ovine \( \beta \)-LPH, and the reaction products to 4-Chl-naphthol were photographed. The same sections, after removal of the bound precipitates to 4-Chl-naphthol and elution of the tissue bound antibodies to ACTH, were then reacted with antiserum to \( \beta \)-LPH preincubated with porcine 1-39 ACTH. The procedure was repeated, inverting the order of the primary antisera. In both instances, parallel experiments were performed in which the second primary antibody was preincubated with the homologous rather than the heterologous antigen.

RESULTS

In the pituitary, both ACTH and \( \beta \)-LPH were found in the cytoplasm of all the cells of the intermediate lobe and also in the same cells in scattered groups in the anterior lobe (Fig. 1a and b), as reported by Pelletier et al. (19). The intensity and the intracytoplasmic distribution of the reactive products to ACTH and \( \beta \)-LPH were identical. There was no appreciable difference between the pregnant and nonpregnant ewes, in either the pituitary or the hypothalamus.

In the hypothalamus, many of the cells of the arcuate nucleus, as well as scattered ones somewhat lateral to it, were immunoreactive for \( \beta \)-LPH. Only half as many cells in the same regions were reactive for ACTH. In adjacent sections stained alternately for the two hormones, it was rarely possible to be certain that the same cells contained both peptides, partly because these are relatively small nerve cells whose profile usually changes materially from one 6-\( \mu \)m thick section to the next, partly because of the relative paucity

\[ \text{FIGURE 1} \quad (a \text{ and} \ b) \text{ Photomicrographs of the same 6-\( \mu \)m section of sheep anterior pituitary immuno-stained by the peroxidase-antiperoxidase indirect method for ACTH (a) and, after complete elution of the ACTH immune complex from the section, similarly stained for \( \beta \)-LPH (b). The distribution of reactive products is identical despite mild loss of general histologic detail in b after the oxidizing elution procedure. Glycerol wet mount. \times 750.} \]
of reliable reference landmarks like blood vessels, and partly because of the considerable discrepancy in the number of ACTH- and β-LPH-positive cells. The series of sequential staining for the two hormones on the same section (with intervening elution of the first primary antibody), however, demonstrated consistently that every cell that contained ACTH also contained β-LPH (Fig. 2a and b).

The perikaryal staining for ACTH was considerably less intense in the hypothalamus than in the pituitary, where the amount of preabsorption antigen required to block staining was at least one order of magnitude greater than in the nerve cells. Also, in contrast to the pituitary, where the staining intensities of β-LPH and ACTH in the same cell were indistinguishable, the staining in the hypothalamic perikarya was uniformly more pronounced for β-LPH than for ACTH. For this reason, but also because the oxidative antibody elution procedure diminished further homologous or heterologous tissue immunoreactivity to some extent, it was preferable to stain for ACTH before β-LPH, although results were substantially the same regardless of the order followed. Most noticeably in the cells that contained small amounts of immunoreactive products, whether to ACTH or β-LPH, these products were concentrated close to the cell nucleus. Immunoreactive products similar to those in the perikaryon were sometimes seen in stubby processes extending from the cell body that were interpreted as dendrites.

The beaded appearance characteristic of axons was evident in the moderate number of immunoreactive fibers encountered in and about the arcuate nucleus, the dorsomedial and medial paraventricular hypothalamic nuclei, and the mesencephalic central grey. As in the arcuate perikarya, β-LPH fibers were more numerous and stained more intensely than ACTH fibers in these sites, where occasional fibers could be identified with certainty as containing both hormones. In the median eminence, however, ACTH fibers were much more numerous than β-LPH fibers and stained more intensely. They were present in the zona interna and were also clearly outlined projecting to the zona externa. ACTH fibers were distributed evenly along the length and breadth of the zona externa, whereas β-LPH fibers were concentrated in the lateral extents of the portal bed in the rostral and caudal, but not the mid, portion of the infundibulum.

Figure 2 (a and b) Photomicrographs of the same 6-μm section of sheep arcuate nucleus immunostained by the peroxidase-antiperoxidase indirect method, a has been stained with ACTH antiserum preabsorbed with β-LPH. After complete elution of the ACTH antibody complex from the tissue, the section was restained with antiserum to β-LPH preincubated with ACTH, and rephotographed (b). Reaction products to both antisera are present in the same nerve cell. They are slightly less intense for β-LPH (b) than ACTH (a) because the elution procedure diminishes the intensity of the products from a subsequent immunostaining procedure; under comparable technical circumstances, β-LPH staining was always more intense than ACTH in the arcuate perikary. Glycerol wet mount, x 750.
The specificity of the immunohistochemical reaction for each of the two peptides in the sequential staining approach was established by two complementary experiments (Figs. 2a, and b, 3a, and b, 4a, and b). Elution of the primary antibody from the tissue in the first part of the sequence was shown to be complete because reincubation of the section with only the secondary reactants failed to produce any staining, whereas reincubation with the complete sequence of reactants restored staining. Secondly, the series of cross-absorption experiments, in which the antiserum against ACTH and β-LPH were preabsorbed with the heterologous antigens, had no effect on the sequential staining results reported (Fig. 2a and b). These results were confirmed by the combined preabsorption and sequential staining experiments, in which preabsorption of the first primary antiserum with the heterologous antigen produced undiminished staining and, after elution, preabsorption of the second primary antiserum with the homologous and heterologous antigen, respectively, abolished and did not abolish staining regardless of the order in which the primary antisera were reacted.

DISCUSSION
The absorption controls in these experiments demonstrate that the antisera to ACTH and β-LPH employed do not render visible any portion of the heterologous antigen molecule. On tissue sections, our antiserum to ACTH recognizes the COOH-terminus of the molecule only, since only the 25–39 fragment blocked the staining, but the radioimmunoassay data indicate that the antiserum also contains lower-affinity antibodies against more proximal fragments of the peptide (Table I). The antigenic determinant of the β-LPH antiserum probably resides, by histochemical criteria, somewhere in the 1–36 sequence, since preincubation with β-MSH, met5- and leu3-enkephalin, or β-endorphin did not alter reactivity, whereas complete β-LPH abolished it. The same specificity of the antiserum was previously shown by radioimmunoassay (8).

Immunological methods cannot unequivocally demonstrate the presence of complete hormonal sequences. Data from other sources, however, support the likelihood that the cellular localizations reported denote the presence of the complete hormone sequences. α-MSH has been visualized in perikarya of the arcuate nucleus (5, 18). With antisera to different fragments of ACTH, no difference has been found by radioimmunoassay between brain and pituitary ACTH in four mammalian species (13). Genetic considerations also

Figure 3 (a and b) Photomicrographs of the same 6-μm section of sheep arcuate nucleus immunostained by the peroxidase-antiperoxidase indirect method. a has been stained with ACTH antiserum preabsorbed with β-LPH. After complete elution of the ACTH antibody complex from the tissue, the section was restained with the same ACTH antiserum preincubated with ACTH (b). The immunoreactive cell in a (arrow) is not reactive in b (arrow), demonstrating that the ACTH antiserum staining reaction is not affected by preincubation with β-LPH, but is completely blocked in the same cell by preincubation with ACTH. (C) capillary. Glycerol wet mount. × 750.
lend support to the identity of hormone and transmitter peptides as they are demonstrated in different neural systems and in extraneural organs. What is much less likely is that the complete amino acid sequence is always or even usually present in a free form. Since the discovery of proinsulin (22, 23), the hypothesis that the final form of these peptides arises by enzymatic cleavage from precursor molecules has gained experimental support rapidly (1, 7, 12, 15, 20), thereby introducing another likely regulatory dimension in transmitter physiology. ACTH and β-LPH themselves are fragments of an ~31,000-dalton protein first isolated from a pituitary tumor cell line (12). Studies of biosynthesis in bovine brain indicate that a larger precursor is also formed in the arcuate nucleus (11).

Some of the data discussed, as well as immunocytochemical evidence that in the pituitary ACTH and β-LPH are located in the same cells (19), have led to the supposition that the two peptides may also be found in the same cells in the brain (1, 29). The sequential staining technique we have employed demonstrates conclusively that every ovine arcuate perikaryon that contains ACTH also contains β-LPH. The converse, however, is not apparent, since half of the β-LPH reactive cells do not stain with the ACTH antiserum. The generally weaker staining of ACTH than β-LPH might suggest a critical difference in the potency of the two antisera, or a differential loss of the antigen in the course of tissue preparation, but in the pituitary the staining intensities of ACTH and β-LPH in the same cell are equivalent. Furthermore, nerve fibers in the median eminence stain more strongly for ACTH than β-LPH—the opposite of arcuate perikarya and of axons elsewhere in the hypothalamus and in the midbrain. The differences in staining, therefore, probably reflect differences in the amount of peptide present.

The much smaller amounts of immunocytochemically reactive ACTH and β-LPH in the hypothalamus compared to the pituitary, intracellularly as well as regionally, are in accord with regional tissue assay data (9). The complex pattern of distribution of ACTH and β-LPH in brain
strongly suggests neuronal synthesis of these peptides. More important, the concentration of reactive products in the immediate vicinity of the nerve cell nucleus is characteristic of well-established secretory systems in the hypothalamus (4, 21), presumably because this is where the synthesizing and packaging apparatus of the cell is located (6).

The ACTH pattern of weak staining in the arcuate perikarya and strong staining in the terminal axons of the median eminence, compared to the opposite staining pattern for /3-LPH, is open to a number of interpretations: (a) mechanisms of degradation are different for the peptide fragments; (b) conformational differences in molecular structure alter immunoreactivity of the peptides in different ways; (c) binding of the peptides to other molecules masks or modifies antigenic sites; and (d) peptide cleavage, axonal transport, storage, and/or secretion proceed at different rates. Two possible explanations for the visualization of less ACTH than /3-LPH in the arcuate nucleus in this study are suggested by other experiments. Since the rat arcuate nucleus has been shown to contain four times more a-MSH than ACTH (8), the ACTH molecule may be rapidly converted mainly to an NH2-terminal MSH than ACTH, the ACTH molecule may be present as part of a larger molecule partially purified from bovine pituitary (D. T. Krieger, A. Liotta, and M. J. Brownstein, unpublished observations).

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