NH$_2$-terminal specificity and axonal localization of adrenocorticotropin binding sites in rat median eminence*

(Mark Van Houten, Masood N. Khan, Raymond J. Walsh, Barry A. Baquiran, Leo P. Renaud, Charles Bourque, Salvatore Soro, Serge Gauthier, Michel Chretien, and Barry I. Posner)

Division of Medicine, McGill University, Montreal, Quebec, Canada; Department of Anatomy, George Washington Medical Center, Washington, DC; Department of Neurology, Neurosurgery and Psychiatry, McGill University, Montreal, Quebec, Canada; Institut de Recherches Clinique de Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada

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Abstract

Adrenocorticotropin binding sites in the rat median eminence have been localized in vivo. These binding sites occur in the basalar zone, which is rich in axonal endings. Using competitive binding and quantitative light-microscope radioautography, we found that the median-eminence binding site, in contradistinction to the adrenal receptor, binds specifically the residue 4–10 region of the adrenocorticotropin molecule. Using quantitative electron-microscope radioautography and median-eminence deafferentation, we localized the binding sites to axon terminals in this region. In time-delayed uptake studies using light-microscope radioautography, we failed to observe concentration of radiolabel in neurons of the basal hypothalamus after the direct injection of radiiodinated adrenocorticotropin(1–24) into the median eminence.

Binding sites for blood-borne adrenocorticotropin (ACTH) have been localized to the rat median eminence by quantitative radioautography (1). The median eminence is one of the four circumventricular organs of the brain, which are targets for direct interaction with a variety of circulating peptide hormones (2). Of these four regions, the median eminence is the only brain region in which specific binding sites for blood-borne adrenocorticotropin have been demonstrated (1). In view of the various purportedly direct effects of blood-borne adrenocorticotropin and related peptides on brain function (3, 4), we have attempted to determine the cellular localization and binding specificity of adrenocorticotropin binding sites in the rat median eminence by using quantitative light- and electron-microscope radioautography. In addition, we report the results of pilot radioautographic investigations of brain uptake of circulating adrenocorticotropin (5–8).

Methods

Synthetic ACTH$_1$–24 (Cortrosyn), a gift from Organon, was iodinated using lactoperoxidase under mild conditions for 30 sec, as described by McIlhinney and Schulster (8). Monoiodinated hormone prepared by this method has been shown to retain its steroidogenic properties and capacity to bind receptors isolated from rat adrenocortical cells (8). Iodinated peptide was purified by the silicic acid adsorption method of Rees et al. (9) with minor modifications, as follows. Ten milligrams of silicic acid (Bio-Sil A, mesh 200–325, Bio-Rad), which had been suspended in 2 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 1.5% (wt/vol) bovine serum albumin, was added to the iodination mixture to terminate the iodination reaction. After 30 min at room temperature, the pellet was washed three times with 2 ml of cold distilled water and once with 1 M HCl. The adsorbed hormone was eluted in 2 ml of acetone/water/acetic acid (40:59:1, vol/vol) and concentrated by evaporation under a stream of N$_2$. The purified $^{125}$I-labeled ACTH$_1$–24 ($^{125}$I-ACTH$_1$–24) had a specific activity of 124 µCi/µg (1 Ci = 37 GBq). After purification, >98% of the radioactivity could be adsorbed by silicic acid, and >95% could be precipitated by 10% (wt/vol) trichloroacetic acid. The labeled hormone retained its immunologic activity, as judged by its specific binding to a high-affinity antiserum (A-7) against the steroidogenic region of ACTH$_1$–24 (10).

To visualize binding sites for blood-borne adrenocorticotropin by light-microscope radioautography, $^{125}$I-ACTH$_1$–24 (1.1 x 10$^8$ cpm) in 0.3 ml of 25 mM Tris Cl, pH 7.4/1.5% bovine serum albumin was injected into the left cardiac ventricle of anesthetized 200-g male Sprague–Dawley rats (Charles River Breeding Laboratories, St. Constant, Quebec, Canada), as described previously (1). The peptide specificity of the binding sites was evaluated in companion rats that were injected with $^{125}$I-ACTH$_1$–24 mixed with various amounts (1-, 3-, 30-, or 1000-fold molar excess) of unlabeled ACTH$_1$–24, α-melanotropin (α-MSH), β-melanotropin (β-MSH), or β-lipotropin (β-LPH). The gravimetric amount of coinjected unlabeled ACTH$_1$–24, α-MSH, β-MSH, or β-LPH perfold excess was calculated uniformly on a molar basis with respect to the amount of $^{125}$I-ACTH$_1$–24. A total of 30 rats were used in binding specificity studies. The number of rats injected with a particular mixture of labeled and unlabeled peptide is reported in the appropriate figure legend. The cellular location of binding sites in the median eminence was studied using electron-microscope radioautography of two additional pairs of rats, which were injected intracardially with $^{125}$I-ACTH$_1$–24 (1.1 x 10$^8$ cpm) in the absence or presence of a 1000-fold (1 mg) excess of coinjected unlabeled ACTH$_1$–24.

In all radioautographic studies, 5 min after the injection of the various solutions the rats were perfused via the left cardiac ventricle, first with Ringer’s solution/0.28 M lactate until the jugular veins were free of blood, and then with either Bouin’s fixative solution (for light-microscope radioautography) or a buffered solution of mixed aldehydes (for electron-microscope radioautography) (11). Shortly after perfusion/fixedation, the adrenals and coronal slabs of medial basal hypothalamus were removed. For light-microscope radioautography the tissue was dehydrated in graded alcohol solutions and paraffin-embedded, so that 5-μm-thick tissue sections could be mounted on precleaned glass microscope slides, stained with modified Harris’ hematoxylin, and coated with

Abbreviations: ACTH, adrenocorticotropin; $^{125}$I-ACTH$_1$–24, $^{125}$I-labeled adrenocorticotropin(1–24); $^{125}$I-WGA, $^{125}$I-labeled wheat germ agglutinin; MSH, melanotropin; LPH, lipotropin.

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Kodak NTB2 emulsion according to standardized procedures (12). Radioautographs were exposed in the dark at 4°C for 4 days before development for 6 min in fresh Kodak D-170 solution.

For electron-microscope radioautography, trimmed coronal blocks of median eminence were post-fixed in 2% (wt/vol) OsO₄ in 0.1 M cacodylate buffer for 4 hr, dehydrated, and embedded in Epon. Thin sections (pale gold interference color) of median eminence were covered with a monolayer of Ilford L-4 emulsion (Ilford, Toronto) and exposed for 4 months before development in fresh Kodak D-19 (13). Radioautographs were immersed in isoamyl acetate (Tousimis, Montreal) for 10 min, post-stained in uranyl acetate and lead citrate, and examined in a Hitachi HS-7S electron microscope.

The quantitation of light-microscope radioautographic reactions was accomplished with the aid of a Diavert photomicroscope (Reichert, Vienna) fitted with an 11 × 11 mm ocular grid frame (Whipple, Natick, MA). The average reaction intensity (silver grains per unit area) over the median eminence in each rat was quantitated by counting the number of grains observed within a total of 14 unit areas at a total magnification of ×1600. Unstained sections served to confirm the absence of spurious chemographic artifacts, and stained sections from a nonradioactive brain showed an insignificant level of background fog.

All grain views in the electron microscope were photographed and enlarged to a final magnification of ×25,000. The relative contents of label in neuronal and glial structures were analyzed by allocating and tabulating grains according to the underlying structure (direct scoring) and by application of a 100-nm resolution circle (14) to evaluate the degree of ambiguity in grain localization. Labeled nerve terminals were further categorized according to the ratio of clear and dense-core vesicles that they contained.

Confirmation of the localization obtained by electron microscopic analysis was sought in animals with introduced brain lesions. Four adult male Sprague-Dawley rats were placed in a stereotaxic apparatus, and the median eminence of each rat was deafferented unilaterally from the hypothalamus by hemicircular rotation of an extrudable Halazs-type knife, as previously described (15). Two weeks after placement of the knife cuts, the rats were injected intracardially with 125I-ACTH₁⁻₂₄ (1.0 × 10⁶ cpm), either alone (2 rats) or with 50 μg of unlabeled ACTH₁⁻₂₄ (2 rats). Light-microscope radioautographs of coronal sections through the center of the median eminence were prepared as above. The average intensity of radioautographic reactions over that aspect of the median eminence ipsilateral to the deafferentation was compared to the intensity of the reactions over the contralateral aspect with intact axonal connections with the hypothalamus, by methods described previously (15). In each rat median eminence, 32 unit areas were scored from the deafferented and the intact side, respectively.

Direct injections of peptides into the median eminence were done in the following manner (16). Male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of urethane (25% wt/vol solution; 1.25 g/kg) and secured in a stereotaxic apparatus. Body temperature was maintained at 38.5°C, and the heart rate was monitored continuously during the experiment. The hypothalamus was exposed via a transphenygal approach. Bleeding during surgery was controlled by insertion of a mixture of nontoxic Plasticon in Vaseline into the diploic spaces occupied by venous sinuses. A glass micropipette with a tip diameter of ~10 μm and bent at a 15–20° angle within 2 mm of the tip was glued onto the needle of a 10-μl Hamilton syringe containing 125I-ACTH₁⁻₂₄ (3.84 × 10⁶ cpm/5 μl) of 1.5% bovine serum albumin/25 mM Tris Cl, pH 7.4) or 125I-labeled wheat germ agglutinin (125I-WGA; 4.00 × 10⁶ cpm/5 μl of 50 mM sodium phosphate, pH 7.8; iodinated by the chloramine-T method (17)). Under visual guidance, the tip of the micropipette was inserted under the ventral-most surface of the median eminence. Into the median eminence of each of two rats, ~5 μl of the 125I-ACTH₁⁻₂₄ solution was slowly infused over 20 min, using a manually driven micromanipulator. Two other rats were each injected in the same manner with 5 μl of the 125I-WGA solution, so that the molar amounts of injected tracer were equal to those injected in the 125I-ACTH₁⁻₂₄ study. After a survival period of 8 hrs (18), during which the rats remained anesthetized and were periodically monitored in the stereotaxic apparatus, the rats were fixed by perfusion through the heart with Bouin’s solution. Coronal blocks of medial basal hypothalamus were sectioned at 50-μm intervals at the level of the arcuate nucleus. Mounted and stained 5-μm-thick sections were radioautographed as described above, using a 2-month exposure.

RESULTS

The peptide specificity of adrenocorticotropic binding sites was compared in the median eminence and adrenal zona fasciculata (Fig. 1 A and B). Whereas a 1000-fold molar excess of coinjected unlabeled ACTH₁⁻₂₄ markedly blocked the binding of 125I-ACTH₁⁻₂₄ in both median eminence and adrenal, a 1000-fold molar excess of α- or β-MSH blocked binding to the median eminence but not to the adrenal. The inhibition of 125I-ACTH₁⁻₂₄ binding to median eminence was compared at several concentrations of unlabeled ACTH₁⁻₂₄, α-MSH, and β-LPH (Fig. 2). On a molar basis, ACTH₁⁻₂₄ was consistently more effective than α-MSH, which in turn was more effective than β-LPH.

In electron-microscope radioautographs of the median eminence of rats injected intracardially with 125I-ACTH₁⁻₂₄ 5 min prior to brain fixation, three-fourths of the bound radioactivity was localized to nerve terminals and preterminal axons (Table 1 and Fig. 3). Classification of labeled terminals according to vesicle content showed a nonrandom association: 65% of the labeled terminals contained a mixed population of clear and dense-core vesicles in a ratio of 1:1 to 10:1 (Fig. 4).

![Fig. 1. The average reaction intensity, expressed as grains/unit area (mean ± SD), over the median eminence (A) and the adrenal zona fasciculata (B) was compared among rats injected systemically with 125I-ACTH₁⁻₂₄ in the absence or presence of a 1000-fold molar excess of coinjected unlabeled ACTH₁⁻₂₄, α-MSH, or β-LPH. Each bar represents an average of 24 unit areas from a single rat.](image-url)
In electron-microscope radioautographs of rats coinjected with a 1000-fold excess of unlabeled ACTH$^{1-24}$, the reverse situation was obtained, i.e., three-fourths of the residual bound radiolabel was localized to glia (Table 1). Classification of the few nerve terminals that bound label in the presence of receptor blockade revealed a more randomized association, in that they contained pure vesicle populations or various mixtures of vesicle types without a dominant vesicle population ratio (Fig. 4).

Hemicircular axonal deafferentiation of the median eminence produced a striking unilateral loss of total binding (Fig. 5), but no significant change in nonspecific binding (Fig. 6). In both rats, unilateral deafferentiation resulted in 50%–60% loss of specific binding capacity.

Discrete cellular uptake of radiolabel was observed in light-microscope radioautographs of the medial basal hypothalamus of rats 8 hr after the median eminence was injected with $^{125}$I-WGA. In agreement with previous reports of the localization of tuberoinfundibular neurons by mapping with retrogradely transported morphologic markers (18, 19), we observed labeled neurons restricted to the supraoptic, paraventricular, arcuate, and ventromedial nuclei, where the few retrogradely labeled neurons were concentrated in the posterior ventrolateral subdivision (20) (Fig. 7). In our rats, an average of 1–3 labeled cells per tissue section was observed throughout the rostrocaudal extent of the arcuate nucleus. In

![Graph showing radioautographic reaction over the median eminence](image1)

**Fig. 2.** The average radioautographic reaction over the median eminence following the coinjection of $^{125}$I-ACTH$^{1-24}$ and various amounts of unlabeled ACTH$^{1-24}$ ( ), $\alpha$-MSH ( ), or $\beta$-LPH ( ) is expressed as a percent of the average reaction obtained from rats injected with $^{125}$I-ACTH$^{1-24}$ alone (% of max). Each symbol represents an average of 24 unit areas from a single rat.

![Representative electron-microscope radioautograph illustrating the radiolabeling of nerve terminals and axons (A) following the systemic injection of $^{125}$I-ACTH$^{1-24}$. (×14,500.)](image2)

**Fig. 3.** Contrast, no such localized accumulations of radiolabel was observed in any tissue sections of medial basal hypothalami of rats injected via the median eminence with $^{125}$I-ACTH$^{1-24}$.

**DISCUSSION**

The median eminence and adrenals of the rat bind different regions of the ACTH$^{1-24}$ polypeptide. Both $\alpha$- and $\beta$-MSH, which contain the peptide sequence representing the NH$_2$-terminal ACTH$^{1-10}$ segment (21), block the binding of $^{125}$I-ACTH$^{1-24}$ to the median eminence. Blockade by $\beta$-LPH, which also contains this peptide sequence (21), further supports the NH$_2$-terminal specificity of the median eminence site. The 4–10 region of ACTH$^{1-24}$ appears to be active in influencing memory and learning functions (3, 4) and in modulating tuberoinfundibular dopaminergic activity (22, 23). Median eminence binding sites could be involved in directly mediating the central feedback effects of hormones with this

![Graph showing cellular sites of $^{125}$I-ACTH$^{1-24}$ binding in rat median eminence](image3)

**Table 1.** Cellular sites of $^{125}$I-ACTH$^{1-24}$ binding in rat median eminence

<table>
<thead>
<tr>
<th>Site</th>
<th>% total grains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuronal</td>
<td>75.5</td>
</tr>
<tr>
<td>Axonal</td>
<td>23.8</td>
</tr>
<tr>
<td>Terminal</td>
<td>51.7</td>
</tr>
<tr>
<td>Glial</td>
<td>20.5</td>
</tr>
<tr>
<td>Unidentified</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Animals were injected intracardially with $^{125}$I-ACTH$^{1-24}$ alone (Control) or coinjected with a 1000-fold molar excess of unlabeled ACTH$^{1-24}$ (Blocked). Grains were scored in electron-microscope radioautographs of median eminence: Control, 530 total grains, 8.5% shared neuronal/glia. Blocked, 81 total grains, 11% shared neuronal/glia. In both situations, grains shared between neurons and glia represented an insignificant percentage of all grains scored by the direct-hit method (14).

![Graph showing ratio of vesicle types](image4)

**Fig. 4.** Labeled nerve terminals were classified according to their vesicle content, and the various categories are represented as a percent of all labeled terminals scored in electron-microscope radioautographs of median eminence of rats either injected with $^{125}$I-ACTH$^{1-24}$ alone (Top) or coinjected with $^{125}$I- and unlabeled ACTH$^{1-24}$ (Bottom).
peptide sequence. Presumably the adrenal binding sites recognize a longer portion of ACTH1-24, which is the core for full steroidogenic activity (24).

Nerve terminals and preterminal axons in the median eminence appear to possess sites of specific binding, because the heavy labeling of these structures by 125I-ACTH1-24 is blocked by coinfected unlabeled ACTH1-24, and axonal deafferentation of the median eminence greatly reduced specific binding. Labeled nerve terminals are characterized by a low ratio of clear to dense-core vesicles. Although the functional significance of this morphological profile is unknown, this observation indicates that specific binding sites of ACTH1-24 are not randomly associated with nerve terminals in the median eminence.

Direct injection of 125I-WGA into the median eminence resulted in retrograde uptake and concentration of the radiolabel by hypothalamic neurons. This testifies to the general reliability and reproducibility of this method for detection of tuberoinfundibular neurons (18, 19). Indeed, tuberoinfundibular neurons appear to have the capacity to take up and concentrate other circulating proteins and polypeptides (18, 19). This capacity has been cited in support of a theory that would account for the presence of adrenocorticotropic and related peptides in arcuate hypothalamic neurons on the basis of direct uptake from the portal blood (1, 5-7). Under our experimental conditions, however, we could not provide radioautographic evidence to support the uptake hypothesis with regard to adrenocorticotropic. This failure may reflect unexpected complications, such as selection of an inappropriate interval for uptake, enzymatic deiodination resulting in the loss of the radiolabel, and damage to axon terminals during injection. More likely, adrenocorticotropic is not handled by the hypothalamus in the same manner in vivo as are certain morphologic markers.

Previously, Van Houten et al. (11) reported the localiz-