JCI The Journal of Clinical Investigation

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J Clin Invest. 1985;75(3):832-835. https://doi.org/10.1172/JCI111779.

Research Article

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N-Acetyl Endorphin in Rat Spermatogonia and Primary Spermatocytes

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Abstract

In previous reports modest levels of β-endorphin have been found by radioimmunoassay in rat testis, and localized by immunofluorescence to the interstitial cells. We have confirmed these previous reports and extended them by showing that the majority of testicular endorphins are acetylated forms, *N*-acetyl γ-endorphin, *N*-acetyl α-endorphin, and *N*-acetyl β-endorphin$_{1-27}$. In addition, *N*-acetylated endorphins are not found in interstitial cells, but are confined to spermatogonia and primary spermatocytes.

Introduction

Pro-opiomelanocortin-derived peptides, including ACTH and β-endorphin (EP)[1], have been demonstrated in hypothalamus, anterior pituitary, and intermediate lobe by techniques such as hybridization of specific complementary DNA probes (1, 2). By immunoassay and immunofluorescence techniques, β-EP has been detected in various nonneuronal tissues such as the gastrointestinal tract (3), pancreas (4), placenta (5), ovary (6), and testis (7). In both intracranial and other tissues, the extent of proteolytic cleavage and posttranslational processing (glycosylation, amidation, and acetylation) varies widely. Although α-*N*-acetylated EPs have been demonstrated primarily in the neurointermediate lobe of the pituitary (8), a recent report noted that acetylated EPs were present as a minor component in the testis (9). In the present study we have used specific antisera to demonstrate that *N*-acetyl (Ac) EPs are a major immunoreactive form of the peptide in the testis, and that, in contrast with β-EP which appears confined to interstitial cells, *N*-Ac-EPs are found in spermatogonia and primary spermatocytes.

Methods

Mature male Sprague-Dawley rats (>200 g body wt) were obtained from the Central Animal House, Monash University, Clayton, Australia and killed by decapitation. Two complementary antisera were used for indirect immunofluorescence studies and radioimmunoassay (RIA): R56, which recognizes antigenic determinants in the mid portion (18–26) of β-EP and its *N*-acetyl derivatives, and R92, which recognizes *N*-Ac-EPs but not their unmodified precursors.

Synthetic ovine α-*N*-Ac-β-EP$_{1-27}$ (Peninsula Laboratories, Belmont, CA) was used as an antigen for R92, and in the RIA studies, both for radioiodination and standard. R92 was raised in a rabbit immunized with ovine α-*N*-Ac-β-EP$_{1-27}$ conjugated to bovine thyroglobulin with 1-ethyl-3(3-dimethylamino-propyl) carbodiimide-HCl. Protein-antigen conjugates were dialyzed, lyophilized, emulsified with Freund's complete adjuvant, and injected at 2–4-wk intervals into rabbits at multiple intradermal sites until a satisfactory antibody titre was obtained. The R92 antiserum does not recognize ACTH$_{1-39}$, Synacthen, α-melanocyte stimulating hormone, corticotropin-like intermediate lobe peptide (CLIP), Met-enkephalin, and Leu-enkephalin, and has a cross-reactivity of <0.15% with β-EP$_{1-31}$, β-EP$_{1-16}$ (α-EP), β-EP$_{1-17}$ (γ-EP). The antibody, however, cross-reacts 100% with α-*N*-Ac-α-EP and α-*N*-Ac-γ-EP on a molar basis. The R56 has been previously described in detail (6); it recognizes β-EP$_{1-31}$, β-EP$_{1-27}$, and their *N*-acetylated derivatives equivalently on a molar basis, but fails to recognize α-EP, γ-EP, or *N*-acetylated forms of these shorter peptides.

For immunohistochemistry, tissues were sliced ~2 mm thick and fixed in Bouin's solution for 4 h at room temperature. Following dehydration with 70 and 80% ethanol, tissues were cleared and paraffin infiltrated by an Autotechnicon Ultrapressor (Swords, Dublin, Ireland); the specimens were embedded in paraffin wax at 60°C. 3–4-μm sections were prepared, followed by routine delipidation. For immunofluorescence studies, the rehydrated sections were incubated with R92 or R56 antiserum (1:80 dilution) in antiserum dilution buffer consisting of 0.1 M lysine, 1 mg/ml bovine serum albumin (BSA), 0.1% sodium azide, and 0.3% Triton X-100 in PBS at 4°C for 24 h. After 3 × 10-min phosphate-buffered saline (PBS) washes, fluorescein-ated sheep anti-rabbit Ig was added at a 1:20 dilution for 30 min at room temperature. The sections were rinsed a further 3 × 10-min before being mounted in PBS/glycerol-containing *p*-phenylenediamine to reduce fluorescence leaching and viewed under water immersion objectives on a Leitz Dialux 20 fluorescence microscope (Leitz, Inc., Rockleigh, NJ). Specificity of staining reactions was demonstrated by the absence of specific staining with antiserum dilution buffer, with normal rabbit serum, or with antiserum diluted 1:32 preabsorbed with an equal volume of 5 μg/ml synthetic human β-EP (R56) or synthetic human N-Ac-β-EP$_{1-27}$ (R92). Staining for β-EP and N-Ac-EP was also unaffected by prior absorption with ACTH, Met-enkephalin, or arginine vasopressin at concentrations > 50 μg/ml.

For RIA of β-EP and *N*-Ac-EP activity, testes were minced in 4 ml of 0.1 M HCl, boiled for 15 min, chilled on ice, and homogenized with a Polytron (Brinkman Instruments, Westbury, NY; speed setting, 2.5, 1 × 5-s burst). Homogenates were centrifuged (15,000 *g*, 30 min at 4°C) and the supernatant passed through Sep-Pak columns (Waters Associates, Milford, MA). Peptides were eluted with 2 ml of 70% CH_3CN in 11 mM trifluoracetic acid (TFA); the eluates were evaporated to dryness at 37°C and either processed immediately or stored at −20°C. The dried eluate was dissolved in 11 mM TFA and subjected to reverse-phase high performance liquid chromatography (HPLC) on a μBondapak column (Waters Associates) as previously described (10). The column was eluted with a linear gradient of 30–80% B over 30 min, where A is 11 mM TFA and B is 11 mM TFA containing 70% acetonitrile; these conditions were chosen to separate various molecular forms of *N*-Ac-EP. The eluant was monitored at 214 nm and the flow rate was 1 ml/min. Fractions (0.5 ml) were evaporated and redissolved in the appropriate buffer prior to RIA. Extracts or reconstituted column fractions (1 ml, 0.5% BSA in PBS, 0.02 M EDTA, pH 7.6) were assayed and duplicate values determined at two dilutions. In assays

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Received for publication 16 July 1984 and in revised form 11 October 1984.

1. *Abbreviations used in this paper:* Ac, acetyl; EP, endorphin; α-EP, β-EP$_{1-16}$; γ-EP, β-EP$_{1-17}$; HPLC, high performance liquid chromatography; ir, immunoreactive; TFA, trifluoroacetic acid.

J. Clin. Invest.

0021-9738/85/03/0832/04 $1.00
Volume 75, March 1985, 832–835

using both R56 (β-EP) and R92 (N-Ac-EP), testicular extracts diluted parallel to the standard curve (data not shown).

Recoveries, both hot and cold, were 70–80% through extraction of tissue to initial concentration on Sep-Paks, and ≥90% over the Sep-Pak and HPLC procedures. Minor oxidation of *N*-Ac-EP appears to occur over the extraction and processing steps, as judged both by tracer studies and by studies in which samples were prepared in buffers containing either dilute H_2O_2 or reducing agents. In contrast, there does not appear to be substantial nonspecific cleavage of longer to shorter forms of EP due to acid protease activity during extraction. Under the same conditions as used above, controls studies on rat pituitary neuro-intermediate lobe, show that the predominant forms of *N*-Ac-EP immunoreactivity are *N*-Ac-β-EP_{1-27}, *N*-Ac-β-EP_{1-31}, and *N*-Ac_{1-26}; a small peak co-eluting with *N*-Ac-γ-EP was seen, and no immunoreactive (ir)-*N*-Ac-α-EP (Cheng, M. C., A. I. Smith, J. A. Clements, and J. W. Funder, manuscript in preparation).

Results

The distinct and contrasting pattern of immunofluorescent staining obtained with antisera R56 and R92 is shown in Fig. 1. As previously reported (5), immunoreactive β-EP appears confined to the interstitial cells with no suggestion of any positively staining cells within the tubules (left). In contrast, *N*-Ac-EP immunofluorescence-positive cells are abundant within the seminiferous tubules (right), but conspicuously absent from the interstitium.

The distribution of *N*-Ac-EP-positive cells within the tubules (right, Fig. 1) suggests that the peptide is present in spermatogonia and primary spermatocytes. This cellular localization is supported by the data shown in Fig. 2, where, in different tubules, the immunofluorescence-positive cells appear to be concentrated on the basement membrane (left), clearly separate from the basement membrane (center), or almost in continuity with the tubular lumen (right). Such differences between tubules are consistent with the wave pattern of sperm maturation in the rat (11), with different tubules showing germ cells at different stages of maturity. Testicular sections from immature rats showed no *N*-Ac-EP-positive cells by immunofluorescence; pituitary sections showed intense and uniform staining of the pars intermedia, but no staining in the pars anterior (data not shown).

In mature rat testes, we found ir-β-EP levels of 0.09±0.01 pmol/g tissue, mean±SEM, n = 8, comparable with previously reported levels (7). In the same extracts, levels of *N*-Ac-EP immunoreactivity were 0.13±0.01 pmol/g tissue. On HPLC, the predominant form of *N*-Ac-EP immunoreactivity is *N*-Ac-γ-EP, with smaller but distinct peaks of *N*-Ac-α-EP and *N*-Ac-β-EP_{1-27} (Fig. 3). The minor peaks on each side of *N*-Ac-

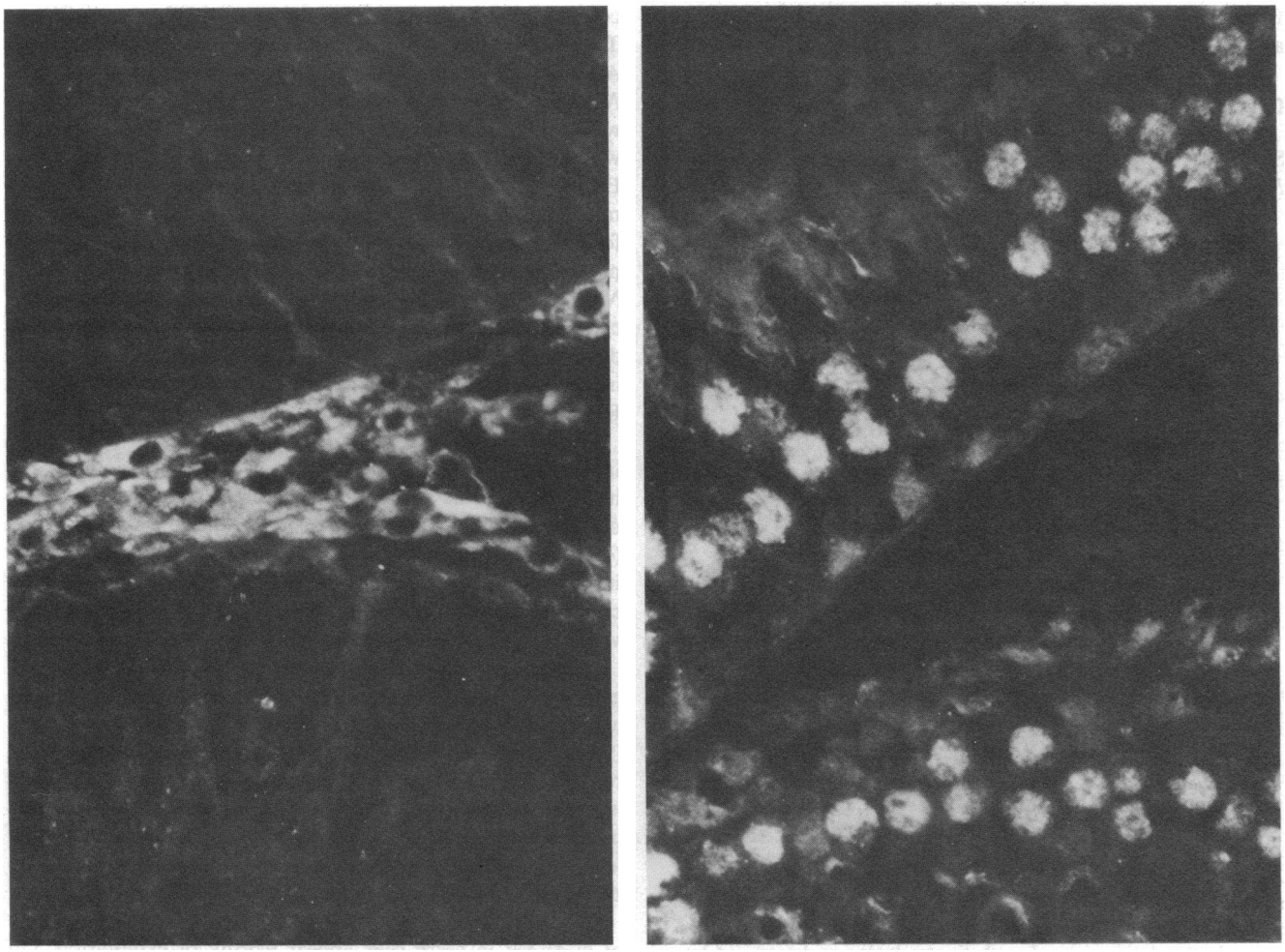

Figure 1. Immunofluorescence staining of sections of rat testis for β-EP (*left*) and *N*-Ac-EP (*right*).

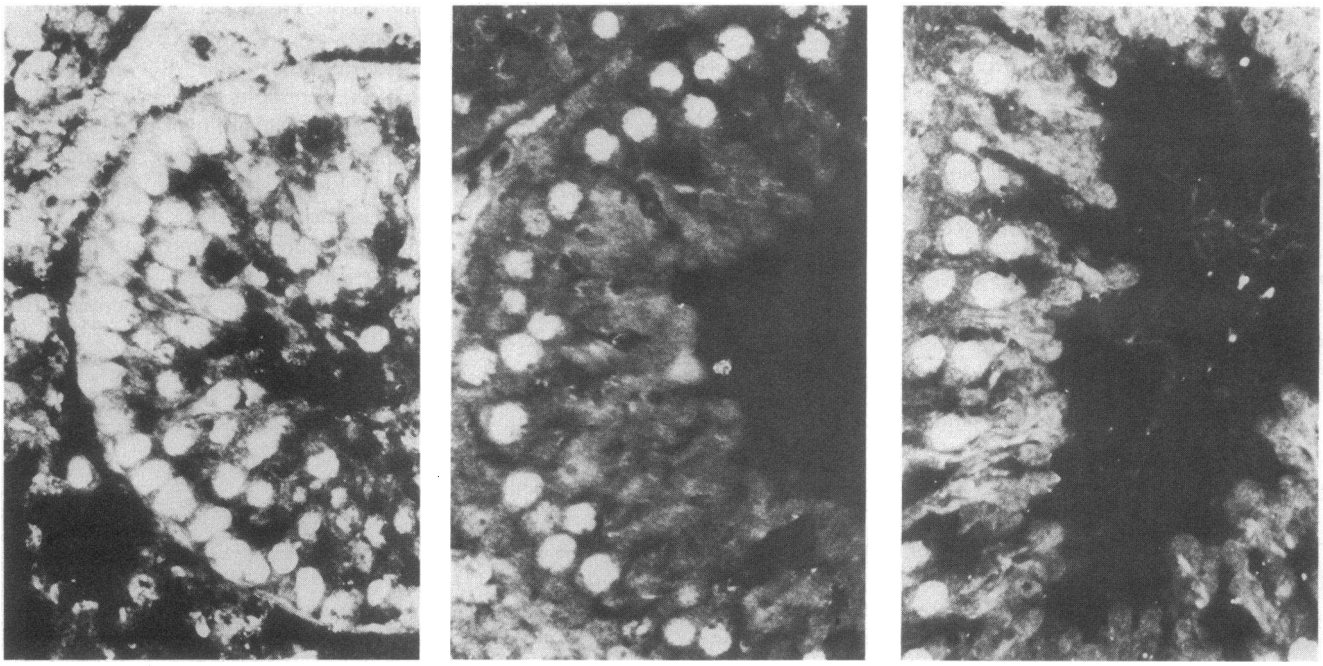

Figure 2. Indirect immunofluorescence microscopy of sections of rat testis, showing progression of ir-*N*-Ac-EP-positive staining cells from basement membrane (*left*) to juxta-luminal (*right*).

α-EP coelute with the sulphoxides of *N*-Ac-α-EP and *N*-Ac-γ-EP; the shoulder eluting before *N*-Ac-β-EP_{1-27} may represent, at least in part, *N*-Ac-β-EP_{1-31}.

Discussion

Since the β-EP antiserum (R56) recognizes essentially 100% *N*-Ac-β-EP_{1-27} on a molar basis with β-EP_{1-31}, it is not possible that the predominant *N*-Ac-EP immunoreactivity can be accounted for by acetylated derivatives of β-EP_{1-27} or β-EP_{1-31}. This is consistent with a recent report (9) of the absence of *N*-Ac-β-EP in testicular extracts. Three minor peaks of testicular *N*-Ac-EP-like immunoreactivity were found on HPLC, of which one co-eluted with *N*-Ac-α-EP. In this study, however, we find that more than half of the testicular immunoreactive EP is *N*-acetylated, as has been shown to be the case for the rat intermediate lobe of the pituitary gland (12–14), but not for the rat brain (15, 16). In the intermediate lobe the predominant forms are the *N*-Ac-β-EP_{1-31}, *N*-Ac-β-EP_{1-27} and *N*-Ac-β-EP_{1-26}; in the spermatogonia and primary spermatocytes, shorter acetylated β-EP derivatives predominate, as demonstrated directly by RIA of HPLC fractions, and indirectly in that they are not seen on immunofluorescence with antiserum R56, which recognizes the longer acetylated derivatives.

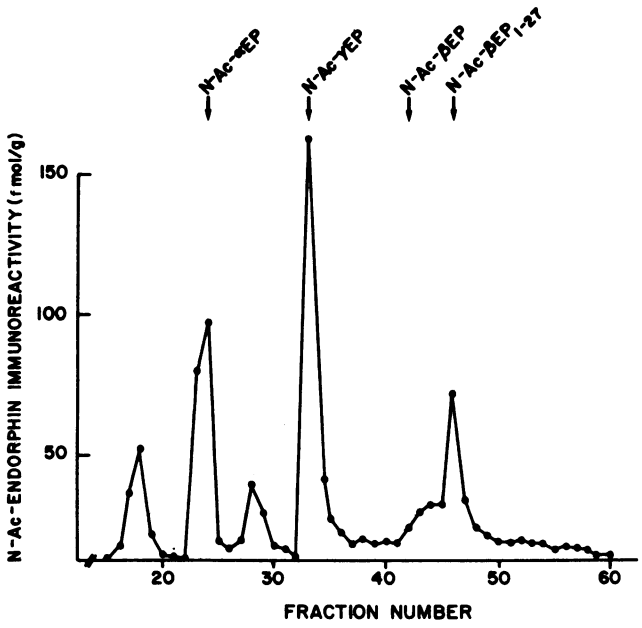

Figure 3. Profile of *N*-Ac-EP immunoreactivity (antiserum R92) after reverse-phase HPLC of Sep-Pak-purified extract of testes pooled from six mature Sprague-Dawley rats. Fractions were collected at 0.5 min intervals; the elution time of synthetic standards was determined by uv absorbance at 214 nm in separate runs.

The findings of ir-N-Ac-EP in germ cells is of potential importance in at least two ways. The phenomenon may in itself be useful as a temporal marker of spermatocyte development; in other organs, for instance, the pineal, acetylation (of serotonin) has recently been shown (17) to be temporally important. Similarly, in the retina, a complementary role for cholinesterase has been proposed in the processing and activation of pro-enkephalin (18).

Secondly, the relatively high levels of ir-N-Ac-EP in the germ cells suggest the possibility of a physiological role. The lower levels of testicular ir-β-EP have previously been suggested (9) as consistent with a paracrine rather than an endocrine role; though the testicular content of ir-N-Ac-EP is higher, it is still orders of magnitude lower than that of the pars intermedia. Accordingly, it seems probable that potential physiological roles for N-Ac-EP in germ cells may similarly be paracrine (or autocrine), rather than endocrine.

Two possible areas of potential physiological importance of N-Ac-EP in germ cells appear worthy of exploration. On the one hand, though *N*-acetylated EPs do not bind to opiate

receptors, the possibility that there are N-Ac-EP-specific receptors mediating specific physiological processes is one currently unexplored. There is ample precedent, e.g., choline acetyl transferase, for the process of esterification to result in activation rather than inactivation.

Alternatively, since *N*-acetylation renders EPs opiate receptor inactive, the process in the germ cell may represent a disposal mechanism, to block what might otherwise be physiologically unwanted effects of opiate-active peptides from germ cells, locally in the reproductive tract. It is, for example, well established that the vas deferens contains opiate receptors; one classical opiate bioassay utilizes the mouse vas deferens. Whether the physiological role of *N*-acetylation by germ cells is one of activation or disposal, interventions which reduce or abolish the process may interfere with sperm production or capacitation, and thus conceivably be of eventual importance as antifertility agents.

Acknowledgments

We thank Dr. H. W. G. Baker for his comments and help in preparing the manuscript.

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