Synthesis and Secretion of Corticotropins, Melanotropins, and Endorphins by Rat Intermediate Pituitary Cells*

Richard E. Mains and Betty A. Eipper
From the Department of Physiology, University of Colorado Medical Center, Denver, Colorado 80262

(Received for publication, February 23, 1979)

The synthesis and secretion of various intermediate pituitary proteins was studied by using dispersed intermediate pituitary cell suspensions. Control studies indicated that the isolated cells were obtained in good yield and that after more than 24 h in culture the isolated cells continued to synthesize a collection of proteins similar to those found in freshly extracted intermediate pituitary tissue.

Rat intermediate pituitary cells synthesized a molecule (M₀, 30,000; called 30K) that contained antigenic determinants for β-endorphin, γ-lipotropin, corticotropin (ACTH), and 16K fragment (the NH₂-terminal region of mouse tumor cell pro-ACTH/endorphin). This 30K molecule, two high molecular weight forms of ACTH(10K and 20K), and 10K fragment were all shown to be glycoproteins.

Continuous labeling and pulse-chase incubations were used to define the intracellular biosynthetic processing of the 30K molecule. After a 15-min pulse incubation the 30K molecule was the only labeled protein containing antigenic determinants for β-endorphin, γ-lipotropin, ACTH, or 16K fragment. A β-lipotropin-like molecule served as a biosynthetic intermediate in the production of proteins similar to β-endorphin and γ-lipotropin. Methionine-enkephalin and α-endorphin were not major products in the intermediate lobe cells. Molecules similar to α-melanocyte-stimulating hormone and corticotropin-like intermediate lobe peptide (ACTH(18-39)) were also derived from the same 30K molecule; 20K ACTH served as a biosynthetic intermediate in this conversion. In rat intermediate pituitary cells ACTH(1-39) was not a major final product of the intracellular biosynthetic processing of the 30K molecule. The 30K molecule also served as a precursor to a protein similar to mouse tumor cell 16K fragment and related smaller proteins. With rat intermediate pituitary cells, pulse-chase experiments utilizing [35S]methionine demonstrated almost quantitative conversion of the 30K precursor into labeled proteins similar to β-endorphin and α-melanocyte-stimulating hormone.

In the absence of added secretagogues, small amounts of all of the smaller proteins derived from the 30K precursor were secreted coordinately into the culture medium.

Previous work defined the biosynthetic pathways for adrenocorticotropic hormone (ACTH, corticotropin) and β-endorphin in the mouse pituitary tumor cell line, AtT-20/D-16v. A diagram of the structure of the common precursor molecule and the proteolytic cleavages involved in creating ACTH and β-endorphin is shown in Fig. 1 (1-6). Cell free protein synthesis has confirmed the existence of a common precursor to ACTH and β-endorphin in the AtT-20 tumor and in rat and bovine pituitaries and has shown that the ACTH- and β-endorphin-containing primary gene product is very similar in the tumor cells and in the anterior and intermediate lobes of the pituitary (7-13).

The AtT-20 tumor line was derived from an anterior pituitary tumor and the pattern of small immunoreactive peptides found in extracts of AtT-20/D-16v cells and in extracts of mouse or rat anterior pituitary are very similar (14-21). In contrast, the pattern of smaller immunoreactive peptides in the intermediate lobe of the pituitary of the mouse and rat differs strikingly from the pattern in tumor cells and anterior pituitary: the intermediate lobe contains α-melanocyte-stimulating hormone (αMSH; N-acetyl-ACTH(1-13)NH₂) and corticotropin-like intermediate lobe peptide (CLIP; ACTH(18-39)), which do not occur in substantial amounts in the anterior lobe; the intermediate lobe contains primarily β-endorphin and relatively little β-lipotropin (βLPH), while the anterior lobe contains more βLPH than β-endorphin (18-26). Since the two lobes of the pituitary synthesize very similar primary gene products but contain very different collections of final products derived from the larger molecule, it seems likely that tissue-specific differences in proteolytic enzymes and other biosynthetic processing enzymes are crucial in generating the differences observed between the lobes.

Quantitative analyses of pulse-chase experiments with the AtT-20/D-16v tumor cells have shown that each molecule of pro-ACTH/endorphin that undergoes proteolytic processing generates a smaller ACTH-related molecule and a smaller β-endorphin-related molecule (5). As expected, synthesis and release of ACTH-related and β-endorphin-related proteins are closely coupled in the basal state, during glucocorticoid feedback inhibition, and during stimulation by hypothalamic corticotropin-releasing factor or by cyclic nucleotides (18, 19, 27-29). ACTH and β-endorphin release from normal anterior pituitary tissue is regulated by the same factors that affect hormone release from the tumor cells (18, 27-32). However, the physiological mechanisms controlling release of ACTH- and β-endorphin-related proteins from the intermediate lobe are different: cells in the intermediate lobe are not sensitive to or corticotropin; βLPH, β-lipotropin; αMSH, α-melanotropin (N-acetyl-ACTH(1-13)NH₂); CLIP, corticotropin-like intermediate lobe peptide (ACTH(18-39)); βMSH, β-melanotropin (corresponds to βLPH(41-58) in several species); SDS, sodium dodecyl sulfate; Dns, danyl, 5-dimethylaminonaphthalene-1-sulfonyl; 31K, 16K, etc., apparent molecular weights of 31,000, 16,000, etc., based on SDS-gel electrophoresis.

7885
These results do not, however, yield any information about the molecular forms of the immunoactive proteins or about their rates of synthesis. Fig. 3 shows an analysis of two aliquots of an intermediate pituitary cell suspension after incubation in medium containing \[^{[H]}\text{phenylalanine}\] for 6 h; one sample (short term cells) was incubated in radioactive medium immediately after dissociation, and one sample (long term cells) was preincubated for 24 h before labeling. The molecular forms of NH\(_2\)-terminal ACTH-related, 16K fragment-related, and NH\(_2\)-terminal \(\beta\)-endorphin-related material synthesized by short term cells and long term cells were similar. More detailed analyses of the peptide products would be required to determine whether subtle changes in processing occurred during the time in culture (such as alterations in the degree of \(N\)-acyetylation of the \(\alpha\)MSH-like material in Fig. 3A).

The major products one would expect to be produced from a pro-ACTH/endorphin-like molecule, based on the mouse pituitary tumor cell work (Fig. 1), were being produced by the intermediate lobe cell suspensions. However, the post-translational processing of the apparent precursor molecule by the intermediate lobe cells appeared to differ from the processing observed in the tumor cells: an \(\alpha\)MSH-like molecule was produced in the intermediate lobe cells but not in the AtT-20 cells; the intermediate lobe cells contained primarily \(\beta\)-endorphin-like material with very little \(\beta\)LPH-like material while the AtT-20 cells contained similar amounts of \(\beta\)-endorphin and \(\beta\)LPH; the 16K fragment-like molecule appeared to undergo a greater amount of proteolytic processing in the intermediate lobe cells than it did in the AtT-20 cells. This further proteolytic processing of 16K fragment cannot yet be interpreted, since the specificity of the 16K fragment antibody is not known.

In various incubations of intermediate pituitary cells with labeled precursors, the ratio of total immunoprecipitable radioactivity (sum of all pro-ACTH/endorphin antibodies) to total acid-precipitable radioactivity (12.5% trichloroacetic acid) varied from 5 to 20% depending on the labeled amino acid used. In a number of studies by other workers, the amount of \(\beta\)-endorphin, \(\beta\)MSH, CLIP, or \(\alpha\)MSH in the intermediate lobe was found to be 2 to 5 pmol of peptide/\(\mu\)g of protein (20, 22–24, 36, 52–60). Using the fact (see below) that \(\beta\)-endorphin and \(\alpha\)MSH are produced from a molecule similar in size to tumor cell pro-ACTH/endorphin (with a molecular weight during gel filtration in 6 M guanidine HCl of about 26,500), it can be calculated that roughly 10% of the protein in the intermediate lobe should be immunoprecipitable with the appropriate pro-ACTH/endorphin antibodies.\(^2\)

When an aliquot of short term cells identical with the cells used in Fig. 3 was incubated in Krebs-Ringer bicarbonate with glucose, albumin, and \[^{[H]}\text{phenylalanine}\] at 3 to 4 \(\mu\)M (conditions commonly used in other studies of intermediate lobe cell suspensions), only about 1% as much acid-precipitable material was produced; less than 1% as much immunoprecipitable material, compared to the results in Fig. 3, was produced.

Identification of Proteins Related to pro-ACTH/endorphin in Intermediate Pituitary—Antibodies to different regions of mouse tumor cell pro-ACTH/endorphin (Fig. 2) were used sequentially to show that rat and mouse intermediate pituitary cells synthesized a collection of pro-ACTH/endorphin-related molecules similar to those produced by the tumor cells (Fig. 1). An example of this type of analysis of rat intermediate pituitary cells is shown in Fig. 4; cells were incubated for 12 h in medium containing \[^{[H]}\text{phenylalanine}\]

\(^2\)"Methods," some "Results" (including Table I and Figs. 2, 5, 7, 11, and 13), and all of the references are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-335, cite author(s), and include a check or money order for $2.25 per set of photocopies.
the supernatant remaining after immunoprecipitation with P-endorphin was analyzed by using the 16K fragment antibody as the P-endorphin. When the 16K fragment antibody was added to weights of 22,000, 14,000, and 4,500. When a second aliquot of detected only three pools of ACTH with apparent molecular weights of 31,000, 13,000, and 3,500; these peaks correspond to this cell extract was analyzed by using the middle ACTH antibody, the NH2-terminal ACTH antibody immu-
mnoprecipitated a peak at 15K and a smaller cross-reactive peak at 5K; peaks at the expected positions for CLIP and glycosylated CLIP were seen using the middle ACTH antibody after the NH2-terminal ACTH antibody (5K and 13K, respectively).

For the incubations shown in Figs. 4 and 5 it is possible to estimate how much of the ACTH- and P-endorphin-related protein in the cells became labeled during the incubation period. The P-endorphin content of the sample analyzed in Fig. 4 was estimated from Table I. The labeling achieved in this 12-h incubation represents 13.3 Ci/mmol of P-endorphin, or 6.7 Ci/mmol of phenylalanine (since rat P-endorphin contains 2 phenylalanine residues); this level is 12% of the theoretical maximum labeling (55 Ci/mmol). Similarly, if we take the aMSH content of mouse intermediate pituitary to be 28 pmol (20, 56), the labeling of aMSH-like material achieved during the 16-h incubation shown in Fig. 5 represents 21% of its theoretical maximum.

The high molecular weight forms of mouse tumor cell ACTH are glycoproteins: an oligosaccharide chain is sometimes linked to an asparagine residue in the COOH-terminal region of the ACTH(1-39)-like sequence and the 16K fragment region is also glycosylated (2, 4, 6; see Fig. 1). To determine whether the rat intermediate pituitary cells also produced glycoprotein forms of ACTH and 16K fragment, cells were incubated with [3H]glucosamine and the proteins synthesized were analyzed by the sequential immunoprecipitation technique (Fig. 6). The three high molecular weight forms of ACTH and 16K fragment were found to be glycoproteins. As expected, if labeled glucosamine was incorporated into 45K ACTH, the aMSH-like molecule (2K), the BLPH-like molecule, or the P-endorphin-like molecule. Thus the pattern of glycosylation of the rat intermediate pituitary 30K molecule appeared to be qualitatively similar to the pattern of glycosylation of mouse tumor cell pro-ACTH/endorphin (Fig. 1).

The Initial Intracellular Product Related to ACTH, P-endorphin, and 16K fragment—Rat intermediate pituitary cells were incubated for short times in medium containing [3H]leucine in order to examine the initial pattern of labeling of ACTH- and P-endorphin-related proteins (Fig. 7). Cell extracts were immunoprecipitated with middle ACTH antibody; there was a progressive build-up of a labeled 30K molecule similar in size to mouse tumor cell pro-ACTH/endorphin. The time course of accumulation of this labeled

and an extract was immunoprecipitated with a sequence of four antibodies. The NH2-terminal P-endorphin immunoprecipitated contained labeled peaks with apparent molecular weights of 31,000, 13,000, and 3,500; these peaks correspond to mouse tumor cell pro-ACTH/endorphin, BLPH, and P-endorphin, respectively. The middle ACTH antibody was added to the supernatant from this first immunoprecipitation and detected only three pools of ACTH with apparent molecular weights of 22,000, 14,000, and 4,500. When a second aliquot of this cell extract was analyzed by using the middle ACTH antibody before the P-endorphin antibody, the 31K peak was found in the middle ACTH immunoprecipitate and was absent from the P-endorphin immunoprecipitate; the results of this crossed immunoprecipitation experiment show that one 31K molecule contains antigenic determinants for both ACTH and P-endorphin. When the 16K fragment antibody was added to the supernatant remaining after immunoprecipitation with the NH2-terminal P-endorphin and middle ACTH antibodies, it detected a broad peak of labeled material with an apparent molecular weight of 16,000; thus antibody to mouse tumor cell 16K fragment detected a similar cross-reactive protein in rat pituitary. When a separate aliquot of the same cell extract was analyzed by using the 16K fragment antibody as the initial antibody, the 31K and 22K peaks appeared in the 16K fragment immunoprecipitate. These results are consistent with a model for the structure of the rat 31K molecule that is similar to the model for the structure of mouse tumor cell pro-

FIG. 3. Comparison of freshly isolated and aged rat inter-
mediate pituitary cells. Rat intermediate pituitary cells were prepared from two 200-g male rats and incubated for 6 h in medium containing [3H]phenylalanine (22 Ci/mmol); equal aliquots of cells were either incubated with labeled medium immediately for 6 h (short term cells) or incubated for 24 h in XMEMM-CO2 containing rat serum and bovine serum albumin and then incubated for 6 h in labeled medium (long term cells). Immunoprecipitations of cell extracts (equivalent to 0.0067 of an intermediate lobe) were performed using three antisera: immunoprecipitates prepared with NH2-terminal ACTH (Part A) and 16K fragment (Part B) antibodies were analyzed on 12.5% acrylamide borate-acetate-buffered SDS-polyacrylamide gels; immunoprecipitates prepared with NH2-terminal P-endorphin antibody (Part C) were analyzed on 12% acrylamide Tris-HCl buffered SDS-gels. When the endorphin sample (Part C) was analyzed on the borate-acetate-buffered gel system, the 36K peak had an apparent molecular weight of 31K.

thus appeared to contain 13 to 14 amino acids. When analyzed in a similar way, AtT-20/D-16v tumor cells did not produce significant amounts of aMSH-like material. When the NH2-terminal ACTH antibody was used as the initial antibody, a pattern similar to that shown in Fig. 3A was obtained. When mouse intermediate pituitary cells were prepared and incubated with [3H]phenylalanine, the results of sequential analyses with a series of antibodies were very similar to the results obtained with rat intermediate pituitary cells (Fig. 5): an aMSH-like molecule was detected; a P-endorphin-like molecule accounted for most of the beta-endorphin-containing material; the 16K fragment antisera precipitated a peak at 15K and a smaller cross-reactive peak at 5K; peaks at the expected positions for CLIP and glycosylated CLIP were seen using the middle ACTH antibody after the NH2-terminal ACTH antibody (5K and 13K, respectively).

4 By extrapolation from Table I, a 150-g rat intermediate pituitary cell suspension would contain about 305 pmol of beta-endorphin; the sample analyzed (0.0017 of a lobe) would contain 0.52 pmol of beta-endorphin. The sample contained 4960 cpm of immunoprecipitable radioactivity; at 30% counting efficiency for tritium, this is 15,200 dpm or 6.91 nCi of label. Dividing 6.91 nCi by 0.52 pmol of beta-endorphin, we get 13.3 Ci/mmol of beta-endorphin; since rat beta-endorphin contains 2 phenylalanine residues, this represents 6.7 Ci/mmol of phenylalanine. The theoretical maximum level of labeling is determined by the input [3H]phenylalanine and is 56 Ci/mmol; the labeling achieved represents 12% of the maximum possible.

5 B. A. Eipper and R. E. Mains, manuscript in preparation.
30K pool of molecules in the intermediate pituitary cells was very similar to the time course of accumulation of labeled proparathyroid hormone in parathyroid tissue (62): there was a brief lag period before labeled immunoprecipitable molecules were detected; after this lag period the pool of labeled precursor built up to a steady level in less than an hour. The molecular events underlying the relatively simple pattern shown in Fig. 7 could be fairly complex: based on analyses in other systems (62-66), post-translational cleavage of a signal peptide, if there were one, might be occurring; the oligosaccharide chains, after being transferred to the nascent chains from their lipid carrier, would be undergoing further processing during this time period. The simple separation system used in Fig. 7 was not capable of resolving the several different forms of the initial product which were probably present in the cells at these early times; the analysis did indicate that no higher or lower molecular weight forms of labeled immunoreactive material were detectable even at these short times of incubation.

To establish that the approximately 30K molecule observed in Fig. 7 contained antigenic determinants for β-endorphin and 16K fragment as well as for ACTH (and thus could serve as a common precursor for βLPH/β-endorphin, 16K fragment, and ACTH/αMSH), sequential immunoprecipitation studies were performed. Aliquots of cell extracts from a 15-min incubation of rat intermediate pituitary cells in medium containing [3H]methionine were immunoprecipitated with NH2-terminal ACTH antibody, NH2-terminal β-endorphin antibody, and 16K fragment antiserum (Fig. 8). Each immunoprecipitate contained the same amount of radioactivity (±11%) with the same apparent molecular weight (29 to 30K). In each case, incubation of the supernatant from the first immunoprecipitation with an antisemir with different specificity did not result in precipitation of additional labeled 29 to 30K material.

Thus the initial intracellular product in rat intermediate pituitary cells contained antigenic determinants for several of the peptide regions predicted based on the mouse tumor cell pro-ACTH/endorphin model (Fig. 1).

Final Products from the Common Precursor—The initial intracellular product observed above could serve as a precursor to a wide variety of smaller protein products; before analyzing the details of the intermediate steps in the biosynthetic pathways, the end points of the processing pathway were determined. In an experiment similar to the one in Fig. 8, rat intermediate pituitary cells were incubated in [3S]methionine for 15 min; one aliquot of cells was incubated immediately (pulse) and an equal aliquot was incubated for 6 h in complete nonradioactive culture medium before extraction (chase). Samples were immunoprecipitated with various antibodies and analyzed on SDS-polyacrylamide gels (Fig. 9). After the 15-min pulse, the endorphin antisemir immunoprecipitated only the expected peak of 30K material. Following the 6-h chase incubation, antibodies to ACTH, β-endorphin, and 16K fragment did not detect any radioactivity remaining at the 30K position; molecules similar to β-endorphin, αMSH, and 16K fragment were detected as final intracellular products. There are 3 methionine residues in mouse tumor cell pro-ACTH/endorphin: one at position 5 of β-endorphin, one at position 4 of ACTH, and one in the middle region of 16K fragment (6); based on analyses of trypsin and chymotryptic peptides, the methionine content of the rat pituitary 30K molecule appears to be similar.

After the 6-h chase period (Fig. 9B), the β-endorphin immunoprecipitate contained a single [3S]methionine-labeled peak with an apparent molecular weight of 3.5K; this 3.5K peak contained 31% of the radioactivity present in the immunoprecipitable 30K pool after the 15-min pulse. No peak of
radioactivity associated with a protein as large as βLPH (12K in this gel system) was found. Based on gel filtration in 6 M guanidine HCl, less than 10% of the labeled 3.5K endorphin-related material was as small as α-endorphin. After a 6-h continuous labeling period in [35S]methionine-containing medium, we detected synthesis of <0.4% as much immunoprecipitable [35S]methionine-enkephalin compared to [35S]methionine-β-endorphin (using methionine-enkephalin antiserum K-26, kindly provided by Doctors E. Weber and K. H. Voigt, University of Ulm, West Germany).

The NH2-terminal ACTH antibody immunoprecipitated a single peak of [35S]methionine-labeled material that co-migrated with 131I-labeled αMSH (Fig. 9B); this αMSH-like molecule contained 38% of the radioactivity initially present in the immunoprecipitable 30K pool. The αMSH-like molecule was 13 to 14 amino acids in length, as judged by gel filtration in 6 M guanidine HCl. Paper electrophoretic analyses (pH 6.35 and pH 3.5) of the tryptic peptides of the αMSH-like molecule labeled with [3H]phenylalanine or with [35S]methionine were consistent with the suggestion that most (but not all) of the molecules had an acetylated NH2 terminus (αMSH-N-acetyl-ACTH(1-13)NH2); substantial amounts of αMSH-related material was as small as βLPH (12K and 4.5K, contained only 18% of the radioactivity immunoprecipitable after the 15-min pulse. The relatively low yield of labeled 16K fragment-related material present after the 6-h chase may have been due to conversion of 16K fragment into smaller [35S]methionine-containing peptides that were not recognized by the antibody. Until the location of the methionine residue in 16K fragment and the specificity of the antisera are known, this question will remain unresolved.

About 87% of the radioactivity present as immunoprecipitable 30K material after a 15-min pulse was found in the cells as immunoprecipitable smaller proteins after a 6-h chase; only about 4% of the initial immunoprecipitable radioactivity appeared in the culture medium during the 6-h chase. Thus nearly all of the newly synthesized precursor was converted into a distinct set of smaller molecules.

Samples of the whole cell extract (without prior immunoprecipitation or trichloroacetic acid precipitation) were also analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 9); as expected, a heterogeneous collection of newly synthesized proteins was observed. After the 15-min pulse, 12% of the [35S]methionine-labeled material on the gel was immunoprecipitated by the β-endorphin antibody. Although a peak at 30K could be discerned in the analysis of the whole cell extract, only half of the 30K material was immunoprecipitated by any of the pro-ACTH/endorphin antibodies. After the 6-h chase, there was still labeled material in the whole cell extract at the 31K position, but none of the pro-ACTH/endorphin antibodies detected any immunoprecipitable labeled molecules of that size. After the 6-h chase, the whole cell extract contained a peak at 3.5K; the αMSH-like molecule, β-endorphin-like molecule, and some of the 16K fragment-related material found after the 6-h chase incubation all contributed to formation of this peak of labeled low molecular weight material observed in the whole cell extract (Fig. 9B).

Further Analysis of the Biosynthetic Pathway of ACTH and αMSH—The results in Figs. 7 to 9 showed that a single 30K molecule was the common precursor for several smaller proteins in the intermediate lobe of the rat pituitary. In order to understand the steps involved in converting the approximately 30,000-dalton precursor molecule into 1500- to 4500-dalton products, additional experiments were performed. Several aliquots of rat intermediate pituitary cells were incubated in medium containing [3H]phenylalanine for progressively increasing periods of time and two different ACTH antibodies were used to determine the kinetics of appearance of labeled ACTH-related proteins (Fig. 10). The peak of immunoprecipitable material at 31K built up in time and reached a plateau level; after labeled 31K material appeared in the cells, radioactivity appeared at the 22K position and built up to a plateau level. The 31K molecule corresponds to mouse pituitary tumor cell pro-ACTH/endorphin and the 22K peak corresponds to ACTH biosynthetic intermediate (Fig. 1). When the middle ACTH antibody was used, peaks of radioactivity at 14K and 4.5K continued to accumulate for the entire 12-h incubation. The NH2-terminal ACTH antibody precipitated an αMSH-like molecule from the supernatant left after precipitation with the middle ACTH antibody.

The intact 14K and 4.5K molecules had masses of 4600 and 2800 daltons, respectively, during gel filtration in 6 M guanidine HCl and thus were significantly smaller than the glycosylated ACTH(1-39) and ACTH(1-39) found in the mouse pituitary tumor cells. Rat ACTH(1-39) would be expected to contain phenylalanine residues at positions 7, 35, and 39 (4, 24, 68). Tryptic digests of the [3H]phenylalanine-labeled 14K...
In order to determine whether the βLPH-like molecule served as a biosynthetic intermediate on the pathway from the 31K molecule to 3.5K endorphin, pulse-chase experiments were carried out (Fig. 12). Rat intermediate pituitary cells were incubated in medium containing [35S]methionine for 15 min and harvested immediately or incubated in nonradioactive medium before harvest; cell extracts were analyzed by immunoprecipitation with NH2-terminal β-endorphin antibody. Analysis of the chymotryptic peptides of [35S]methionine-labeled rat βLPH-like and β-endorphin-like molecules has shown that both of these molecules contain only 1 methionine residue. After the pulse, the only labeled immunoprecipitable molecule present was the common precursor (29K). After a 1-h chase in nonradioactive medium a substantial amount of labeled precursor was converted into a βLPH-like molecule (12.5K), but no labeled β-endorphin (3.5K) had appeared. After a 2-h incubation in unlabeled medium, the 3.5K endorphin became labeled. Finally, after a 6-h chase period, 3.5K endorphin was the only labeled β-endorphin-related molecule remaining in the cells (the same result as in Fig. 9). This result shows that a βLPH-like molecule is an obligatory biosynthetic intermediate in the production of a β-endorphin-like molecule from the 30K common precursor in rat intermediate pituitary cells. In the pulse-chase experiment shown in Fig. 12, 34% of the radioactivity present in the 30K common precursor after a 15-min pulse was detected in the 3.5K endorphin product after a 6-h chase. In three separate pulse-chase experiments (one with [3H]phenylalanine and two with [35S]methionine), the half-life of the 30K common precursor during the chase periods was about 30 min in the rat intermediate pituitary cells.

If the processing of the rat pituitary βLPH-like molecule into a β-endorphin-like molecule follows the scheme outlined in Fig. 1, a γLPH-like molecule should be created as βLPH is cleaved to β-endorphin; a direct demonstration of this expected result is shown in Fig. 13. The γLPH-like molecule (6 to 6.5K) was further processed into smaller immunoprecipitable [3H]phenylalanine-containing material (2.3K).

**Appearance of Labeled Hormones in the Culture Medium**—Cells in the intermediate lobe of the pituitary are thought to be under direct neural regulation (33–38). Thus suspensions of isolated rat intermediate pituitary cells are clearly different from cells in situ since the isolated cells lack
neural innervation. Using the rat intermediate pituitary cell suspensions, the basal rate of appearance and molecular form of radiolabeled hormones in the medium were determined. The $^{[35S]}$methionine-labeled ACTH-, β-endorphin-, and 16K fragment-related proteins which appeared in the culture medium during a pulse-chase incubation are shown in Fig. 14. Under basal conditions, only a small percentage of the intracellular labeled hormone stores were released into the medium (approximately 4%/6 h); the sample of medium analyzed in Fig. 14 corresponded to 10 times the amount of cell extract analyzed in Fig. 9. As expected for healthy cells which were not simply leaking their hormone content into the medium nonspecifically, no secretion of newly synthesized labeled hormone occurred during the first 15 min of the chase period. After 45 min, a substantial amount of the 29 to 31K precursor was secreted and only small amounts of other ACTH-, β-endorphin-, or 16K fragment-related molecules appeared in the medium. After 6 h in the nonradioactive chase medium, a little more of the precursor appeared in the medium along with substantial amounts of each of the smaller ACTH-, αMSH, β-endorphin-, and 16K fragment-related molecules. In a basal state, the isolated rat intermediate pituitary cells secreted the various smaller peptide products derived from the common precursor in a coordinated manner; secretion of with substantial amounts of each of the smaller ACTH-, αMSH, CLIP, and 16K fragment. As was found previously in the intermediate pituitary tumor cells, the higher molecular weight forms of ACTH and 16K fragment were glycoprotein; a molecule with the properties expected of glycosylated CLIP was also identified.

At the present time we are hesitant to assign a name to the 30K common precursor in the intermediate pituitary. In the mouse anterior pituitary tumor cells (AtT-20/D-16v), a similar 30K molecule has been called pro-ACTH/endorphin by analogy to proinsulin and proparathyroid hormone; this molecule serves as a precursor to ACTH, which has a clear role in physiology, and β-endorphin, which may also have effects as a circulating hormone (69–72). Since the intermediate pituitary contains and secretes very little intact ACTH(1–39), a more appropriate name for the intermediate pituitary 30K molecule might be pro-αMSH, pro-CLIP, pro-γLPH, or pro-β-endorphin. However, as noted by Scott et al. (68), there is still no agreement about which intermediate lobe products are biologically important. In an effort to avoid confusion we will refer to the precursor for the various smaller intermediate pituitary products as 30K precursor.

The dispersed cell preparations used in these studies are thought to serve as an accurate model for studying several important aspects of intermediate pituitary metabolism. Most previous studies on hormone synthesis by intermediate pituitary tissue failed to report cell or hormone yields and the stability of in vitro preparations was often not examined; there have been many reports of failure to demonstrate ACTH, αMSH, or βMSH biosynthesis in vitro by intermediate pituitary tissue. Among the previous successful studies, Scott et al. (68) used rat intermediate posterior lobes in organ culture to demonstrate the synthesis of αMSH, CLIP, and several other unidentified peptides; a complete culture medium was utilized and the level of labeling achieved for αMSH and CLIP using $^{[14C]}$proline for 6 h was roughly 2% of the theoretical maximum (68). The studies reported here, using dispersed cells, were also carried out in complete culture medium, and the level of labeling of the various hormonal products reached at least 10% of the theoretical maximum after 12 to 16 h of incubation. Much lower levels of labeling (less than 0.005% of the theoretical maximum) were obtained in other studies when intermediate pituitary slices or cells were incubated in a simple saline solution containing glucose, albumin, and a single radioactive amino acid (43–45, 73–75). These quantitative differences may reflect the dramatic and rapid changes that occur in mRNA and tRNA pools, amino acid transport, protein synthesis and breakdown, and lysosomal activity in tissues deprived of adequate amino acid supplies (76–80).

The early steps in the intracellular processing of the 30K common precursor molecule in intermediate pituitary tissue are similar to the processing pathway defined in the AtT-20 mouse pituitary tumor cells (Fig. 1). However, intermediate pituitary cells carry out several important additional steps of processing. As predicted by Scott et al. (22), in the intermediate lobe cells the ACTH-like segment of the 30K precursor is cleaved to yield molecules similar to αMSH and CLIP. These biosynthetic results are consistent with the fact that the amount of intact, biologically active ACTH(1–39) found in intermediate pituitary extracts is only a few percent of the amount of αMSH or CLIP found in this tissue (20, 22–24, 36, 56, 60, 68, 81, 82). In the AtT-20 cells and in anterior pituitary tissue there is very little production of αMSH or CLIP and most of the ACTH(1–39) segment is kept intact.

In both the rat intermediate pituitary cells and the mouse pituitary tumor cells a βLPH-like molecule serves as a biosynthetic intermediate in the production of a β-endorphin-like molecule. In the intermediate pituitary this conversion goes essentially to completion and β-endorphin is the major product; however, in the tumor cells and in anterior pituitary, both βLPH and β-endorphin appear to be final products. In this
work, no detectable labeled methionine-enkephalin was produced by the intermediate pituitary cells. Studies utilizing radioimmunoassay and bioassay techniques have also found much less methionine-enkephalin than β-endorphin in the pituitary (53–55, 58). Although the sequence of methionine-enkephalin is contained within the structure of β-endorphin (1–31), there is no evidence that methionine-enkephalin is derived biosynthetically from β-endorphin, in both the brain and the pituitary, methionine-enkephalin and β-endorphin always occur in separate and distinct cells (58, 83–85). Along with β-endorphin, it was possible to identify a γLPH-like molecule in the intermediate pituitary cells; γLPH was generated during the cleavage of βLPH to produce β-endorphin, and in the intermediate pituitary cells the γLPH also underwent additional intracellular proteolytic processing.

A glycoprotein similar to the 16K fragment region of the mouse tumor cell pro-ACTH/endorphin molecule was also found in the intermediate pituitary cell extracts; in addition, smaller material related to 16K fragment was detected. The interpretation of these results must await further information about the structure of 16K fragment and about the specificity of the 16K fragment antiserum used.

In the intermediate pituitary cells, there is no evidence for significant degradation of any segment of the 30K precursor molecule. Instead, discrete cleavages are made at a number of well-defined sites and all of the resultant pieces are conserved. Similarly, in the proteolytic processing of proinsulin and parathyroid hormone into final product hormone, the peptide segments removed are not degraded (62, 86, 87). It is not clear why, in another study on rat intermediate pituitary cell suspensions, there appeared to be a tremendous loss of 30K precursor during intracellular biosynthetic processing (45).

Hormone secretion from the intermediate pituitary of amphibians and fishes, and several other lower vertebrates is under tonic inhibitory neural control; when the dopaminergic neural inhibition is removed, the intermediate pituitary secretes at a high rate (33–38, 88, 89). In contrast, control of hormone secretion from the mammalian intermediate pituitary is not yet well understood (34, 37, 60, 81, 90). Previous studies of rat intermediate pituitary tissue in vitro have found ACTH (1–39) and CLIP secretion rates very similar to those found in this work (a few per cent of the cellular hormone content in 6 h) (37, 60, 68, 81, 90); in contrast, the AtT-20 tumor cells have been shown to secrete an amount of hormone equal to their hormone content every 6 to 8 h. Like the AtT-20 cells, the rat intermediate pituitary cells were found to secrete all of the products created intracellularly from the 30K precursor in a coordinate manner. In addition, some intact 30K precursor was secreted by the intermediate pituitary cells, as was found earlier for the AtT-20/D-16v cells (1–6, 18, 21). On a molar basis, only about 0.6% of the newly synthesized 30K precursor was secreted by the intermediate pituitary cells; in the AtT-20 tumor cells, 8% of the newly synthesized pro-ACTH/endorphin was secreted (5). The basal secretion of 30K precursor may represent secretion from a distinct intracellular pool, as has recently been suggested for basal secretion from the exocrine pancreas by Dagorn (91).

There are a large number of small biologically active peptides with modified amino acids at their NH2 or COOH termini, or both; the biosynthesis of most of these small peptides is poorly understood. Since the precursors and intermediates in the biosynthesis of αMSH are now identified, intermediate pituitary cells should prove to be a useful model system for studying acetylation and amidation. The methylation of certain amino acid residues in myosin is controlled by the type of nerve which innervates a fast or slow skeletal muscle (92, 93); perhaps the innervation of intermediate pituitary cells conveys a signal promoting acetylation and amidation of αMSH.

REFERENCES

See p. 7894.
Synthesis and Secretion by Rat Intermediate Pituitary Cells

ICHIKO T. MISHI and Betty A. Tupper

METHODS

Preparation of Cell Suspensions. Intermediate pituitary tissue and dissociated cells were always kept in a COMPLETE tissue culture medium, at the time of the medium changes. All experiments were performed on tissue cultures grown in a tissue culture incubator at 37°C, 95% air, 5% CO2. Cultures were washed several times with tissue culture medium, at least twice, and used in the presence of charcoal-stripped serum.

The tissue was filtered through a 150 micron pop filter, to remove remaining denatured proteins. Tissue was then homogenized by sonication in 0.6 N HCl, and the extracts were neutralized to pH 7.2 with 0.6 N NaOH. The extracts were then centrifuged at 100,000g for 1 hour. The supernatant was lyophilized and stored at -20°C until further use. The pellets were resuspended in 0.6 N HCl and subjected to the same treatment as above.

Table 1. Recovery and Stability of Intermediate Pituitary Proteins in Isolated Cell Suspensions

| Fraction      | Protein Content | Recovery (%) |
|---------------|-----------------|--------------|
| t-1           | 140,000         | 85           |
| t-2           | 140,000         | 80           |
| t-3           | 140,000         | 75           |
| t-4           | 140,000         | 70           |
| t-5           | 140,000         | 65           |

Figure 1. Schematic diagram of the purification of mouse anti-ACTH from serum. (A) Affinity chromatography on IgG (xenogeneic) agarose beads and IgG (mouse) absorbate, followed by a second pass through xenogeneic agarose beads. (B) Chromatography on protein A (mouse) agarose beads. (C) Chromatography on protein A (immunogenic) agarose beads. (D) Chromatography on protein G (xenogeneic) agarose beads. (E) Chromatography on protein G (mouse) agarose beads.

Figure 2. Purification of mouse anti-ACTH from serum. (A) Affinity chromatography on IgG (xenogeneic) agarose beads and IgG (mouse) absorbate, followed by a second pass through xenogeneic agarose beads. (B) Chromatography on protein A (mouse) agarose beads. (C) Chromatography on protein A (immunogenic) agarose beads. (D) Chromatography on protein G (xenogeneic) agarose beads. (E) Chromatography on protein G (mouse) agarose beads.

Figure 3. Purification of mouse anti-ACTH from serum. (A) Affinity chromatography on IgG (xenogeneic) agarose beads and IgG (mouse) absorbate, followed by a second pass through xenogeneic agarose beads. (B) Chromatography on protein A (mouse) agarose beads. (C) Chromatography on protein A (immunogenic) agarose beads. (D) Chromatography on protein G (xenogeneic) agarose beads. (E) Chromatography on protein G (mouse) agarose beads.

Figure 4. Purification of mouse anti-ACTH from serum. (A) Affinity chromatography on IgG (xenogeneic) agarose beads and IgG (mouse) absorbate, followed by a second pass through xenogeneic agarose beads. (B) Chromatography on protein A (mouse) agarose beads. (C) Chromatography on protein A (immunogenic) agarose beads. (D) Chromatography on protein G (xenogeneic) agarose beads. (E) Chromatography on protein G (mouse) agarose beads.
Synthesis and Secretion by Rat Intermediate Pituitary Cells

Figure 7. Scrape labeling experiment analyzed with N-terminally-cleaved antibody. N-terminally-cleaved antibody was used to analyze additional effects of the same cell extracts and culture media described in Figure 10.

Figure 11. Pulse-chase experiment analyzed with antibody to mouse tumor cell P19. Rat intermediate pituitary cells were prepared as in Figure 4 and labeled with 3H-[35S]methionine for 60 min. After labeling, cells were washed and incubated in serum-free medium for 4 h. The medium was collected and assayed for immunoreactive material. The immunoreactive material was then harvested and analyzed by SDS-PAGE and autoradiography.

REFERENCES

1. Motes, S., R. B. G. Miller (1973) J. Biol. Chem. 248:3418-3426.
2. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
3. Pappas, S. A. & B. L. Martin (1975) J. Biol. Chem. 250:5001-5007.
4. Smith, J. T. & S. A. Pappas (1975) J. Biol. Chem. 250:5008-5014.
5. Gomori, G. & P. B. Martin (1975) J. Biol. Chem. 250:5015-5021.
6. Guy, R. A. & E. E. Engel (1975) J. Biol. Chem. 250:5022-5028.
7. Meeks, E. A. & J. L. Hensley (1975) J. Biol. Chem. 250:5029-5036.
8. Pappas, S. A. & B. L. Martin (1975) J. Biol. Chem. 250:5037-5042.
9. Smith, J. T. & S. A. Pappas (1975) J. Biol. Chem. 250:5043-5049.
10. Motes, S., R. B. G. Miller (1973) J. Biol. Chem. 248:3418-3426.
11. Motes, S., R. B. G. Miller (1973) J. Biol. Chem. 248:3418-3426.
12. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
13. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
14. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
15. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
16. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
17. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
18. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
19. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
20. Scriver, C. R., G. S. Schrier (1975) Endocrinology 97:1395-1400.
Synthesis and secretion of corticotropins, melanotropins, and endorphins by rat intermediate pituitary cells.
R E Mains and B A Eipper

J. Biol. Chem. 1979, 254:7885-7894.

Access the most updated version of this article at http://www.jbc.org/content/254/16/7885

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/16/7885.full.html#ref-list-1