Coordinate, Equimolar Secretion of Smaller Peptide Products Derived from Pro-ACTH/Endorphin by Mouse Pituitary Tumor Cells

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ABSTRACT The secretion of peptide products derived from pro-ACTH/endorphin was examined with several radioimmunoassays and with polyacrylamide gel analyses of immunoprecipitates of radioactively labeled peptides. In studies using a mouse pituitary tumor cell line the accumulation of each of the four molecular forms of adrenocorticotropic hormone (ACTH) in tissue culture medium was shown to be a linear function of time. No evidence for self-inhibition of secretion by accumulated, secreted peptides (i.e., ultra-short feedback) was found. Furthermore, synthetic human ACTH and synthetic camel β-endorphin did not alter secretion of peptides when added to the culture medium at levels up to 10,000 times physiological. Stimulation of the release of ACTH-, endorphin-, lipotropin-, and 16k fragment immunoreactive material by norepinephrine was fully blocked by cobalt; by this criterion, stimulated release was calcium dependent. All the smaller molecules derived from the pro-ACTH/endorphin common precursor were secreted in equimolar amounts under all circumstances tested, within the precision of these studies (±11%). Norepinephrine and cobalt did not significantly alter the secretion of pro-ACTH/endorphin and ACTH biosynthetic intermediate. The stimulation of secretion by norephinephrine and inhibition of secretion by cobalt was restricted to the lower molecular weight products derived from pro-ACTH/endorphin: glycosylated and nonglycosylated ACTH(1-39); β-lipotropin, β-endorphin, and γ-lipotropin; and 16k fragment.

The AtT-20/D-16v mouse pituitary tumor cell line, which was derived from anterior pituitary tissue, provided the first system in which the common precursor to adrenocorticotropic hormone (ACTH)[1] and β-lipotropin (βLPH)/β-endorphin was demonstrated (38). There is now considerable support for the existence of a similar common precursor to ACTH-related and endorphin-related peptides (pro-ACTH/endorphin) in many species and in several different tissues (reviewed in references 5, 16, and 22). The ACTH/endorphin biosynthetic pathway that operates in AtT20/D-16v cells is summarized in Fig. 1.

[1] *Abbreviations used in this paper*: ACTH, adrenocorticotropic hormone or corticotropin; βLPH, β-lipotropin; γLPH, γ-lipotropin or roughly the NH_2-terminal two-thirds of βLPH; αMSH; α-melanotropin or *N*-acetyl-ACTH(1-13)NH_2; CLIP, corticotropin-like intermediate lobe peptide or ACTH(18-39); 16k fragment, the NH_2-terminal non-ACTH, non-βLPH region of pro-ACTH/endorphin, containing a melanotropin-like region called γMSH; 31k, 16k, etc., molecules with apparent molecular weights of 31,000, 16,000, etc., when determined by SDS polyacrylamide gel electrophoresis using a borate/acetate buffer system.

Under a variety of circumstances, coordinate secretion of immunoreactive ACTH-related and βLPH-related peptides has been observed in humans and in experimental animals; however, the amounts of ACTH- and βLPH-related material found in plasma have not always been equimolar (1, 3, 20, 23, 24, 31, 61). Similarly, studies with pituitary tissue in vitro have usually demonstrated coordinate, although not always equimolar, secretion of ACTH- and βLPH-related material (2, 13, 15, 32, 42, 47, 51, 52, 65). Given the common biosynthetic precursor to ACTH and LPH, nonequimolar secretion of ACTH- and LPH-related material would require selective release of portions of the common precursor molecule (recall Fig. 1). Although many investigators report that ACTH- and LPH-related material can invariably be located in the same cells by immunohistochemical staining, there are reports that some cells in the pituitary and brain contain only ACTH- or LPH-related molecules. Whether these observations reflect independent synthesis of ACTH- and βLPH-related material, selective intracellular degradation of portions of a common precursor molecule, or an artifact of immunostaining is not yet clear (4, 27,

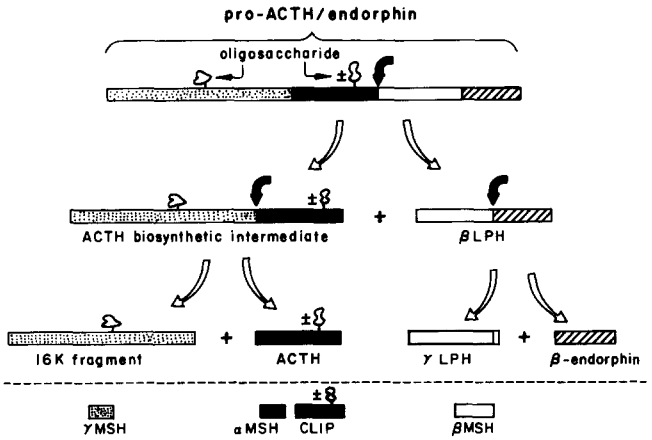

FIGURE 1 Structure and biosynthetic processing of pro-ACTH/endorphin in mouse pituitary tumor cells. The structure and biosynthetic processing of mouse pituitary tumor cell pro-ACTH/endorphin are summarized in reference 16; structures below the dashed line are shown for comparative purpose.

39, 41, 44, 48, 49, 68, 69).

Another important question concerns the suggestion that a number of peptide hormones directly inhibit their own secretion (called an "ultra-short feedback loop"); data on the existence of an ultra-short feedback loop for ACTH release are conflicting (17, 22, 26, 28, 47, 53).

Control of secretion of immunoreactive ACTH from AtT-20/D-16v cells has been shown to be responsive to stimulation by hypothalamic factors, arginine-vasopressin, norepinephrine or cyclic nucleotide analogues, and to feedback inhibition by glucocorticoids but not sex steroids or mineralocorticoids (2, 22, 53–55, 64, 67). Thus, the tumor cells provide a model system for studying hormone release from corticotropes of the anterior pituitary. The AtT-20/D-16v cells were used to address the question of whether coordinate, equimolar secretion of the various products derived from pro-ACTH/endorphin occurs during both basal and stimulated secretion, or whether selective secretion of certain regions of the common precursor could be observed. In addition, the molecular forms of the pro-ACTH/endorphin-derived molecules secreted under basal and stimulated conditions were compared. The possibility that ACTH or endorphin inhibits its own release from AtT-20/D-16v cells was also investigated.

MATERIALS AND METHODS

Monolayer cultures of AtT-20/D-16v mouse pituitary tumor cells were maintained in Dulbecco-Vogt modified Eagle's medium with 2.5% horse serum, glucose, glutamine, and antibiotics (10). Release of immunoreactive molecules from identical dishes of tumor cells was determined after incubation in fresh complete culture medium containing test agents that might alter secretion; medium was removed from the cells, treated with protease inhibitors (phenylmethylsulfonyl fluoride and iodoacetamide; 0.3 mg/ml each) (34), and secreted hormones were measured by the appropriate radioimmunoassays and bioassays, as described previously (10, 13, 14, 18, 34, 35). No degradation or interconversion of hormones occurs after secretion under these incubation conditions (35; see also Results).

The release of radiolabeled proteins was studied by incubation of identical microwells of tumor cells (usually 5×10^4 cells/well) in 100 μl of complete culture medium containing [^{3}H]phenylalanine (150 μM; 55 Ci/mmol; New England Nuclear, Boston, Mass.), [^{3}H]tyrosine (150 μM; 53 or 13.4 Ci/mmol [see appropriate legends] New England Nuclear), or [^{3}H]histidine (150 μM; 12 Ci/mmol; New England Nuclear) (34, 35). After various periods of labeling, radioactive medium was removed and treated with protease inhibitors as above. In some experiments, several microwells of cells were incubated for 7–15 h in radioactive medium; one microwell of cells was extracted with 5 N acetic acid plus protease inhibitors (34), and the remaining microwells were rinsed with complete nonradioactive medium and then incubated for 60 min in fresh, nonradioactive medium containing test substances. This procedure diluted the radiolabel at least 1,000-fold and halted further measurable incorporation of the radiolabel into protein (34, 35). Medium containing secreted protein was removed and combined with protease inhibitors; cells were extracted with acid plus protease inhibitors. In all experiments, the total amount of radioactive pro-ACTH/endorphin-related material present in the cell extracts at the end of the prelabeling period was quantitatively accounted for by the sum of the labeled hormone in the cell extracts plus the culture medium after the 1-h chase incubation in nonradioactive medium (±15%) (35).

Radiolabeled hormones were isolated from cell extracts and culture media by sequential double antibody immunoprecipitation by use of affinity-purified rabbit antibodies and goat anti-rabbit immunoglobulin (34–36). The immunoprecipitation methods have previously been shown to be both specific and quantitative. Antibody specificity was determined by blocking immunoprecipitations with an excess of the appropriate synthetic or purified mouse tumor peptide. Immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis (11.25% acrylamide, 0.59% *N,N'*-methylenebisacrylamide, borate/acetate buffer, pH 8.5) or by gel filtration in 6 M guanidine HCl, 0.2 mg/ml bovine serum albumin (Sephadex G-50 superfine) (12).

The number of tyrosine residues in the various molecules biosynthetically derived from mouse tumor pro-ACTH/endorphin was determined from the amino acid compositions of the purified proteins and from analyses of radiolabeled tryptic and chymotryptic peptides (11, 12, 14, 29). The number of tyrosine residues in each of the major mouse tumor cell pro-ACTH/endorphin-related molecules is: β-endorphin, 1; γLPH, 1; βLPH, 2; ACTH(1–39), 2(one in α-melanotropin [αMSH] and the other in corticotropin-like intermediate lobe peptide [CLIP]); 16k fragment, 1 (in the region known as γMSH); ACTH biosynthetic intermediate, 3; pro-ACTH/endorphin, 5.

RESULTS

Linear Accumulation of ACTH in Culture Medium as a Function of Time

In agreement with the results of Herbert et al. (22) and Watanabe et al. (67), it was found in five experiments that the daily accumulation of immunoactive and bioactive ACTH [μg ACTH(1-39) equivalents/milligram cell protein/day] in several culture media (F-10, L-15, Dulbecco's modified Eagle's medium) was constant (±20%) for a wide range of cell densities, both in the presence and absence of horse serum. Accumulation of immunoactive ACTH in the medium was linear in time for up to 24 h in each of 13 experiments; one such experiment is shown in Fig. 2. Similar results were obtained with five different ACTH antisera with specificities that span the structure of ACTH(1-39). Accumulation of bioactive ACTH in the medium was also linear in time, using two different adrenal cell bioassay systems. The amount of bioactive ACTH was roughly two-thirds of the amount of immunoactive ACTH in the medium; the relatively low steroidogenic potency of pro-ACTH/endorphin and ACTH biosynthetic intermediate (18) would account for this result.

For a study of the secretion of each of the four major molecular forms of ACTH, identical microwells of AtT-20/D-16v cells were incubated for increasing periods of time in medium containing a radioactive amino acid and the release of each molecular form of ACTH was examined by immunoprecipitation and SDS polyacrylamide gel electrophoresis. In five experiments, the accumulation of each form of radioactive ACTH was found to be linear in time after an initial lag period (during which the intracellular hormone pools were labeling); one such experiment is shown in Fig. 3. At the end of 30 min of incubation, the only form of radioactive ACTH in the medium was pro-ACTH/endorphin. Later in the incubation, labeled ACTH biosynthetic intermediate began appearing in the medium at a rate that remained constant for the duration of the study. After a lag of 1–1.5 h, the two forms of ACTH(1-

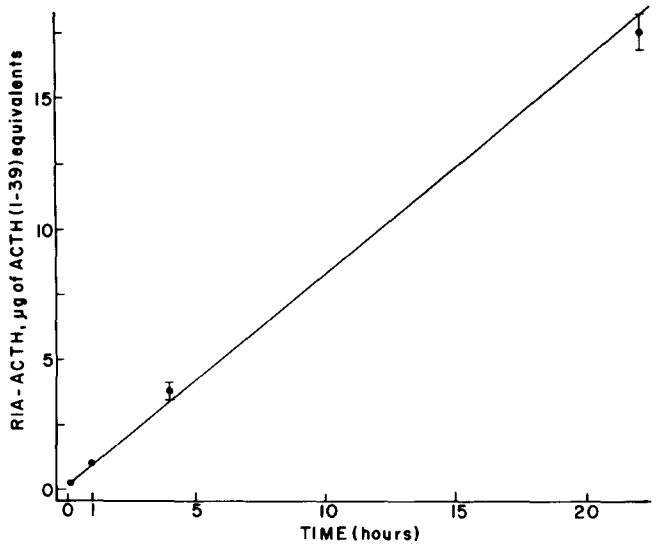

FIGURE 2 Accumulation of immunoactive ACTH in AtT-20/D-16v culture medium as a function of time. The accumulation of immunoactive ACTH in the medium of a dense dish of AtT-20/D-16v cells was measured; cells (2.2 mg cellular protein) in a 60-mm culture dish were fed with 5 ml of Dulbecco's modified Eagle's medium containing 2.5% horse serum; 3 h later (time zero in the figure) this medium was removed and 2.60 ml of fresh medium was placed on the cells. Samples of medium (0.20 ml) were removed at 10 min, 60 min, 4 h, and 22 h. ACTH was measured by radioimmunoassay with an NH_2-terminal ACTH antiserum and with a bioassay using Y-1 mouse adrenal tumor cells (10); only the immunoassay data are shown. The vertical bars represent the standard deviations of the data from two independent radioimmunoassays of the same samples (a total of 10–12 determinations per data point).

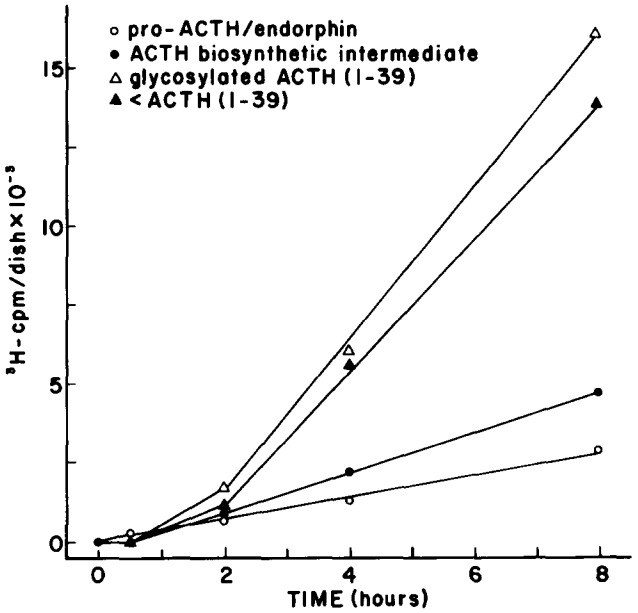

FIGURE 3 Accumulation of multiple radiolabeled forms of ACTH in AtT-20/D-16v cell culture medium. Four identical microwells of mouse pituitary tumor cells were incubated in complete medium containing [^{3}H]tyrosine (2,3-side chain labeled; 13.4 Ci/mmol; 150 μM) for the times indicated. Samples of culture medium were analyzed by immunoprecipitation with the middle ACTH antiserum (Bertha; reference 34) and SDS polyacrylamide gel electrophoresis. The amount of label appearing in each form of ACTH at each time was calculated by summing the radioactivity in each peak of the SDS polyacrylamide gel profile and these totals are plotted. The incorporation of radiolabel into 12.5% trichloroacetic acid-precipitable cellular material was a linear function of time for the entire incubation; at 8 h, the cell extracts contained 7.5×10^6 cpm (25×10^6 dpm) acid-precipitable material.

39) (with and without the oligosaccharide chain attached in the COOH-terminal half of the molecule) began appearing in the medium at a constant rate.

Coordinate, Equimolar Secretion of Immunoactive Molecules Related to Pro-ACTH/Endorphin

The secretion of ACTH-related, endorphin-related, LPH-related, and 16k fragment-related molecules by the mouse pituitary tumor cells was first studied using radioimmunoassays. In 17 experiments, the basal release of immunoactive ACTH-, endorphin-, and 16k fragment-related molecules was nearly equimolar (within ±25%). Because basal release of ACTH-related molecules was linear in time for many hours (Figs. 2 and 3), the effects of agents that alter secretory rates were studied by examining the accumulation of hormones in the medium after a convenient fixed period of time, generally 1 or 2 h.

Norepinephrine was previously reported to stimulate release of ACTH from rat anterior pituitary tissue in vitro (51, 65, 66); we confirmed that secretion of pro-ACTH/endorphin-related molecules from AtT-20 cells was stimulated in a graded fashion by norepinephrine in the concentration range of 0.01–1.0 μM. The stimulation by norepinephrine was a mixed α- and β-agonist action: stimulated release was partially blocked by addition of propranolol (a β-blocker; 0.05-1 μM) and partially mimicked by phenylephrine (an α-agonist; 1–10 μM) and by isoproterenol (a β-agonist; 1–10 μM). In six experiments, secretion of immunoactive ACTH, endorphin, and 16k fragment remained nearly equimolar in the presence of 1.5- to 3.0-fold stimulations of hormone release above the basal rate; one such experiment is reported in Table I. Hormone release was stimulated from 2.2- to 2.9-fold, depending on the immunoassay used.

In studying the actions of test substances on hormone release by pituitary cells, it is crucial to monitor the general state of health of the cells. The ability of the cells to incorporate a radioactive amino acid into material precipitable with 12.5% trichloroacetic acid was used as a measure of cellular health. By use of this criterion, satisfactory conditions for studying directly the role of extracellular calcium in hormone secretion could not be established; only when cell health was compromised (by addition of EDTA or EGTA, 1–2.5 mM) could hormone secretion be inhibited. An alternate approach for investigating the role of calcium in secretion involved the use of calcium antagonists; cobalt is known to be a potent competitive antagonist of extracellular calcium actions in many secretory systems (9, 30, 59, 62). Addition of $CoCl_2$ at concentrations up to 1 mM did not inhibit the ability of the tumor cells to incorporate radioactive amino acids into acid-precipitable material (see legend to Table I). As shown in Table I, $CoCl_2$ had no significant inhibitory effect on the basal secretion of immunoactive ACTH, endorphin, 16k fragment, or γLPH. The stimulation of release caused by 10 μM norepinephrine was abolished in the presence of 1 mM $CoCl_2$. By the criterion of cobalt inhibition, basal secretion from AtT-20/D-16v cells is not calcium-dependent, whereas the norepinephrine-stimulated increment in secretion is calcium-dependent.

TABLE I

Stimulation and Inhibition of Release of Pro-ACTH/ Endorphin-related Products

Additions to medium	Immunoactivity			
	ACTH	Endorphin	LPH	16k fragment
None	1.0	1.0	1.0	1.0
$CoCl_2$	0.8	0.9	0.8	0.8
Norepinephrine	2.2	2.4	2.2	2.9
Norepinephrine + $CoCl_2$	0.7	0.8	0.9	1.0

Identical 35-mm dishes of AtT-20/D-16v cells were incubated for 60 min in 1.00 ml of fresh, complete culture medium containing [^{3}H]leucine (5 μCi/ml, final sp act 6.26 mCi/mmol); medium contained no additives ("none"), 1 mM $CoCl_2$, and/or 10 μM norepinephrine bitartrate (made fresh within 2 min of the start of the 60-min incubation). Media were harvested with protease inhibitors (34). Cell health was monitored by determining the ability of the cells to synthesize new, radioactive proteins during the test treatments. The cells were extracted with 0.8 ml of 100 mM NaOH and the extracts were precipitated with 0.8 ml of 25% trichloroacetic acid; the four sets of duplicate cell extracts were identical, and contained 10,800 ± 1,100 cpm (mean ± SD) of acid-precipitable material. Release is reported as a fraction of basal release, which was set at 1.0. Data for release are highly dependent on the antibodies and immunoassay standards used. For these experiments, the basal concentrations were (in nM): ACTH, 5.6; endorphin, 5.4; 16k fragment, 7.3; LPH, 6.0. All immunoassays were performed at least in duplicate, with at least 10 points averaged for each number reported; the standard deviation of each average value in the table was ≤12%.

TABLE II

Test of Ultra-short Feedback

	Immunoactive Peptide			
	ACTH	β-endorphin	16k fragment	γLPH
	nM			
Medium + Cells				
Control	21	27	20	24
+ hACTH	214	26	23	26
+ $\beta_c$endo	24	299	22	28
Medium − Cells				
Control	<0.2	<0.2	<0.02	<0.02
+ hACTH	188	<0.2	<0.02	<0.02
+ $\beta_c$endo	<0.2	271	<0.02	<0.02

Identical dishes of AtT-20/D-16v cells were incubated in fresh medium with 2.5% horse serum for 6 h, and medium was harvested with protease inhibitors. To some dishes, synthetic human ACTH(1-39) (nominally 195 nM) or camel β-endorphin(1-31) (nominally 259 nM) were added. Control samples of media with added peptides (but without cells) were also incubated for 6 h and harvested. Three or four dishes were used with each of the three media tested; all radioimmunoassays yielded ≥5 data points per dish. The standard deviations of all values shown were ≤10%.

Absence of Self-inhibition of Release ("Ultra-short Loop Feedback")

Based on accumulation of immunoactive, bioactive, or radiolabeled ACTH in the culture medium (Figs. 2 and 3), the AtT-20/D-16v cells used in this work do not seem to exhibit "ultra-short loop feedback." Other reports of AtT-20 secretion are in disagreement on this point (22, 53, 60, 67). Because basal and stimulated secretion of all the peptide products derived from pro-ACTH/endorphin is roughly equimolar (Table 1), a further test of whether ACTH or β-endorphin can inhibit secretion by AtT-20 cells is possible. Synthetic ACTH or β-endorphin was added to culture media at the start of the 6-h incubation.

Table II shows that the peptides added to the media were stable in the medium in the absence of cells, and that only the homologous assay detected its peptide. Again, basal or control secretion of immunoactive ACTH, endorphin, 16k fragment, and LPH was equimolar. Synthetic ACTH was not detectably degraded in the presence of the AtT-20/D-16v cells, and did not alter the secretion of endorphin, 16k fragment, and LPH. ^{125}I-labeled ACTH was also stable (>98.6% remained intact, as determined by gel filtration) after 13 h in the presence of the tumor cells (not shown). Likewise, synthetic endorphin was not measurably degraded by the cells and did not alter secretion of ACTH, 16k fragment, and LPH. The concentrations of synthetic ACTH and endorphin added to the media were about 10,000-fold greater than physiological levels (1, 3, 23, 24, 31, 61).

Coordinate Secretion of Radiolabeled Molecules Related to Pro-ACTH/ Endorphin

To investigate which molecular forms of ACTH, LPH, endorphin, and 16k fragment-related material were secreted under basal and stimulated conditions, AtT-20/D-16v cells were incubated in medium containing [^{3}H]tyrosine for 7 h (a period of time sufficient to label >90% of the intracellular hormone stores [15]). Secretion of radiolabeled hormones was examined during an additional hour of incubation in fresh, nonradioactive medium containing agents capable of altering the secretory rate. It has been shown that, under these incubation conditions, no significant intra- or extracellular degradation of pro-ACTH/endorphin-related material occurred (15, 35, 36). The present experiments confirmed the previous results.

The release of [^{3}H]tyrosine-labeled molecules immunoprecipitable with a middle ACTH antibody during basal, norepinephrine (1 μM), and cobalt (1 mM) treatments is shown in Fig. 4*A*. As in Fig. 3, all four major size classes of ACTH-related material were released into the medium under basal conditions: pro-ACTH/endorphin [31k], ACTH biosynthetic intermediate [21k], glycosylated ACTH(1-39) [13.5k] and ACTH(1-39) or <ACTH(1-39) [≤4.5k]. Norepinephrine stimulated the secretion of each of the two smaller peaks of ACTH about fivefold, while the release of pro-ACTH/endorphin and ACTH biosynthetic intermediate was not significantly altered. Cobalt had a negligible effect on basal secretion of any of the four forms of ACTH.

In addition to the ACTH-related material recognized by the middle ACTH antibody, a small amount of NH_2-terminal ACTH-immunoprecipitable material (approximately the size of αMSH) was also secreted (Fig. 4*B*); this material was obtained from the labeled molecules not immunoprecipitated by the middle ACTH antiserum. On a molar basis, the secreted NH_2-terminal ACTH-related material amounted to only a tenth to a twentieth of the amount of secreted ACTH(1-39) plus glycosylated ACTH(1-39). The middle ACTH antibody will immunoprecipitate molecules similar to CLIP [ACTH(18-39); reference 37], so the small amount of CLIP-like material expected to correspond to the NH_2-terminal ACTH-reactive material in Fig. 4*B* would be present in the 13.5k and 4.5k peaks in Fig. 4*A*. Norepinephrine stimulated secretion of NH_2-terminal ACTH-related material eightfold. Analyses of tryptic peptides showed that none of NH_2-terminal ACTH-reactive material in Fig. 4*B* had an acetylated amino terminus and thus is not genuine αMSH.

The accumulation of labeled endorphin-related molecules in the medium was examined after ACTH-related molecules had

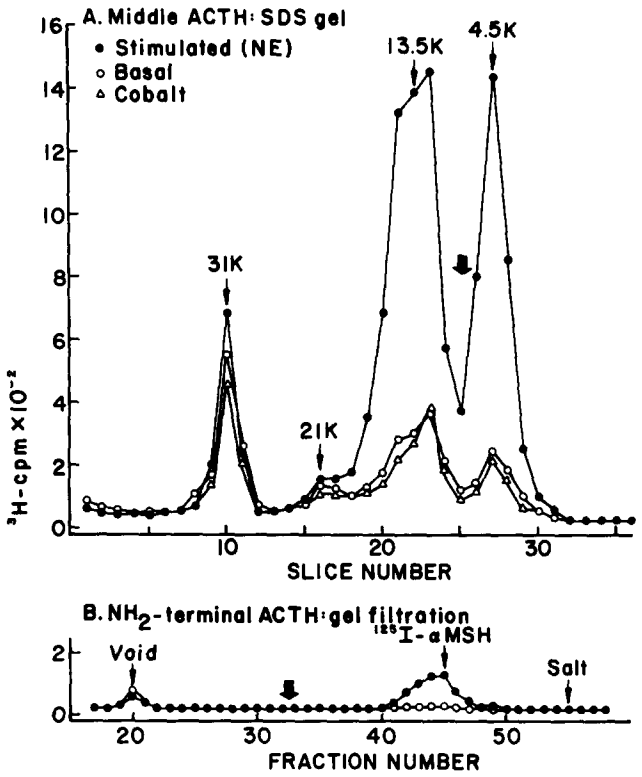

FIGURE 4 Release of radiolabeled ACTH-related molecules. Three identical microwells of AtT-20/D-16v cells were incubated for 7 h in basal, complete culture medium containing [^{3}H]tyrosine (150 μM; 3,5-ring labeled; 53 Ci/mmol) and then rinsed and chased in 500 μl complete nonradioactive medium for 1 h containing no additives ("basal"), 1 μM norepinephrine bitartrate ("NE"), or 1 mM $CoCl_2$ ("cobalt"). Samples of medium (150 μl) were immunoprecipitated with middle ACTH antiserum (*A*) and analyzed on SDS polyacrylamide gels. The supernate from the middle ACTH immunoprecipitate was immunoprecipitated with the NH_2-terminal ACTH antibody (which reacts with αMSH as well as ACTH) and analyzed by gel filtration in 6 M guanidine HCl (*B*). In *A*, the heavy arrow marks the position of the internal dansylated cytochrome *c* marker; in *B*, the arrow marks the position of the internal glucagon marker.

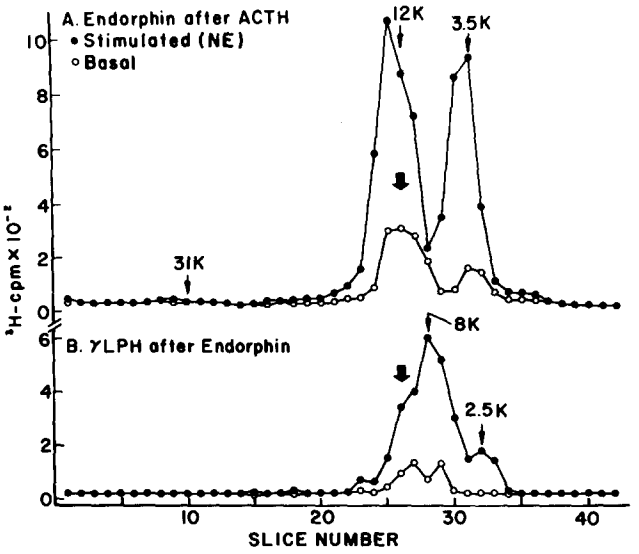

FIGURE 5 Release of radiolabeled LPH-related molecules. Samples of medium analyzed with ACTH antisera in Fig. 4 were next immunoprecipitated with antisera to endorphin (*A*) and then with antisera to mouse tumor cell γLPH (*B*) and analyzed on SDS polyacrylamide gels. The heavy arrow marks the position of the internal dansylated cytochrome *c* marker on each gel.

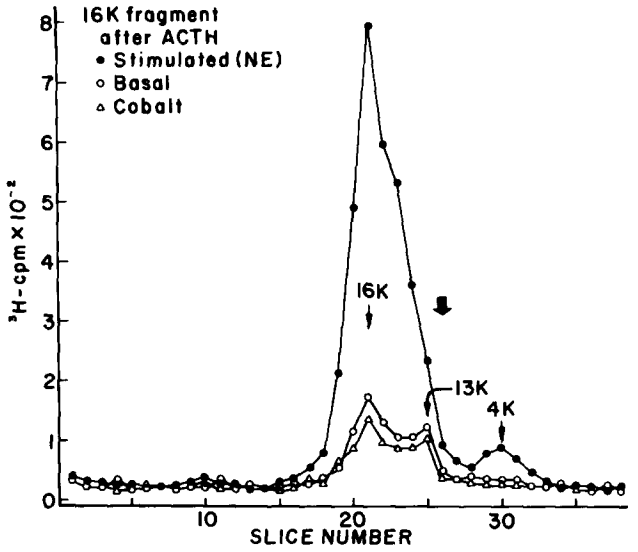

FIGURE 6 Release of radiolabeled 16k fragment-related molecules. Samples identical to those analyzed in Fig. 4 were immunoprecipitated with ACTH antisera and next immunoprecipitated with antisera to mouse tumor cell 16k fragment; the 16k fragment immunoprecipitates were analyzed on SDS polyacrylamide gels. The heavy arrow marks the position of the internal dansylated cytochrome *c* marker on each gel.

been removed from the medium by immunoprecipitation. Addition of norepinephrine stimulated the secretion of βLPH(12k) and β-endorphin (3.5k) three- and sixfold, respectively, over basal levels (Fig. 5*A*). $CoCl_2$ (1 mM) did not significantly alter basal secretion (not shown), in agreement with the lack of effect of cobalt on basal ACTH secretion (Fig. 4*A*).

After ACTH- and endorphin-related molecules were removed from the medium, antiserum to mouse tumor cell γLPH was used to examine secretion of γLPH-related material (8k; Fig. 5*B*). Norepinephrine stimulated secretion of γLPH-reactive radioactive molecules sixfold; cobalt again had no significant effect on basal secretion. A small amount of 2.5k γLPH-related material appeared in the norepinephrine-stimulated sample but not in the basal or cobalt samples.

Finally, the same samples of medium were used to investigate release of 16k fragment-related material (Fig. 6). After removal of ACTH-related material (as in Fig. 4), the 16k fragment antibody immunoprecipitated a heterogeneous collection of 16k fragment-related material with apparent molecular weights of 18k to 13k. Norepinephrine stimulated the secretion of the heterogeneous 16k fragment-related material fivefold; in addition, a peak of lower molecular weight 16k fragment-related material was detected (4k). As before, cobalt did not significantly alter basal secretion of 16k fragment.

Results completely in agreement with the data in Figs. 4–6 were obtained in two other experiments in which [^{3}H]phenylalanine was used to label the molecules before secretion, and in one experiment in which [^{3}H]histidine was used.

Coordinate Secretion is Equimolar Secretion

The radioactivity in each peak in Figs. 4–6 was summed and normalized in Table III; molecules have been grouped according to major branch points in the biosynthetic pathway (Fig. 1). The number of tyrosine residues in each molecule was used

TABLE III

Equimolar, Coordinate Secretion of Smaller Peptide Products Derived from Pro-ACTH/ Endorphin

Form of molecule	No. of tyrosine residues	cpm (Normalized)/molecule		Ratio, increment: basal
		Basal	NE-increment	
Pro-ACTH/endorphin	5	200	25	0.1
ACTH biosynthetic intermediate	3	140	−9	−0.1
Glycosylated ACTH(1-39)*	2	666	2,218	3.3
ACTH(1-39)*	2	359	1,507	4.2
NH_2-terminal ACTH-reactive material	1	55	393	7.1
Total ACTH(1-39)-related‡	2	1,052	3,922	3.7
βLPH	2	552	1,247	2.3
β-endorphin	1	437	2,139	4.9
γLPH	1	405	1,953	4.8
Total βLPH-related§	2	973	3,293	3.4
Total 16k fragment-related	1	981	3,798	3.9

Data from Figs. 4-6 are summarized in tabular form. Normalized cpm/molecule equals cpm/molecule divided by the number of tyrosine residues in that molecule. Data are for 150 μl of medium; total cpm released per microwell (500 μl) can be obtained by multiplying data by 3.33 and by the number of tyrosine residues. Repetition of all the original immunoprecipitations, followed by SDS gel analyses, yielded values within 10% of those shown in this table; these data are for the particular set of immunoprecipitations shown in Figs. 4-6. The total secretion of ACTH-related molecules, including pro-ACTH/endorphin and ACTH biosynthetic intermediate, was 1,392 normalized cpm; for βLPH, including pro-ACTH/endorphin, 1,173 normalized cpm; for 16k fragment, including pro-ACTH/endorphin and biosynthetic intermediate, 1,321 normalized cpm.

* Presumably includes a small amount of material similar to CLIP to correspond to the NH_2-terminal material, in addition to intact ACTH(1-39).

‡ Calculated by adding total ACTH-related radioactivity (middle + NH_2-terminal) and then dividing by two tyrosine residues.

§ Calculated by adding βLPH + β-endorphin + γLPH radioactivity and then dividing by two tyrosine residues.

to normalize the amount of radioactivity in each protein. After dividing the amount of radioactivity in each peak by the appropriate number of tyrosine residues, the normalized radioactivities can be compared directly on a molar basis. This comparison does not depend on equilibration of the intracellular and extracellular radioactive amino acid pools. Rather, the calculation depends only on the validity of the pathway in Fig. 1 and on the correctness of the assignments of tyrosine residues.

Two important points should be noted about the data for pro-ACTH/endorphin and ACTH biosynthetic intermediate. First, the two biggest forms of ACTH comprise ~25% of the moles of ACTH-related material secreted by the tumor cells in the basal state, in agreement with our previous tumor cell data (10, 34, 38). Second, there was no significant change (±13%) in the secretion of pro-ACTH/endorphin and ACTH biosynthetic intermediate in the presence of norepinephrine. In the stimulated state, then, the two largest forms of ACTH accounted for only 7% of the total moles of ACTH secreted.

In the basal state, the accumulation of 16k fragment-, LPH-, and ACTH-related material in the medium was equal (981, 973, 1,052 normalized cpm/molecule, respectively). Within the LPH class, the basal secretion of β-endorphin (437 normalized cpm/molecule) matched the basal secretion of γLPH (405 normalized cpm/molecule). Each of these results is predicted from Fig. 1 if no degradation of the major pieces of pro-ACTH/endorphin occurs either before or after secretion.

After stimulation with norepinephrine, the equimolar secretion of products derived from pro-ACTH/endorphin was maintained. For example, the secretion of 16k fragment-related material (4,779 normalized cpm/molecule) was closely matched by the secretion of ACTH(1-39)-related material (4,974 normalized cpm/molecule) and only exceeded the secretion of LPH-related material (4,266 normalized cpm/molecule) by 11%. Again, the stimulated secretion of β-endorphin matched the level for γLPH (within 8%).

As a consequence of equimolar, coordinate release of the peptide products derived from pro-ACTH/endorphin, in both basal and stimulated states, the increments in secretion caused by norepinephrine were also equimolar: ACTH(1-39)-related, βLPH-related, and 16k fragment-related increases were the same within ±9%; β-endorphin and γLPH increases were equal within ±5%.

In both [^{3}H]phenylalanine experiments discussed above, the basal and incremental secretions of smaller peptide products derived from pro-ACTH/endorphin were also equimolar within ±11%. In the [^{3}H]histidine experiment, only endorphin-related molecules were examined; norepinephrine stimulated secretion of βLPH and β-endorphin but had no effect on pro-ACTH/endorphin secretion. Cobalt fully blocked the increased secretion of βLPH and β-endorphin caused by norepinephrine.

DISCUSSION

In this work, the AtT-20/D-16v mouse pituitary tumor cells were used to demonstrate coordinate, equimolar release of all the smaller peptides derived biosynthetically from pro-ACTH/endorphin. Coordinate equimolar release was observed during both basal and norepinephrine-stimulated secretion, within the limits of the methods employed (±11%). The use of quantitative immunoprecipitation of radiolabeled molecules allowed a direct molar comparison of secretion of different regions of pro-ACTH/endorphin, and avoided the difficulties inherent when exclusively using radioimmunoassays to estimate several different molecular forms of a hormone. It is important to note that most of the 16k fragment secreted was intact or nearly intact 16k fragment, and not cleaved to products such as the potential 12 amino acid product γMSH (43) (Fig. 1). Primary rat anterior pituitary cultures were also found to secrete almost exclusively intact 16k fragment under basal conditions (15).

Coordinate, equimolar secretion would be expected from the biosynthetic pathway in Fig. 1, if little or no degradation occurred before or after secretion. Previous work (35) showed that there is coordinate, equimolar synthesis and secretion of ACTH- and endorphin-related molecules in AtT-20/D-16v cells under basal conditions; the present work extends the previous results to include all of the smaller peptides derived from pro-ACTH/endorphin, and to study both the basal and stimulated states. The possibility of intracellular degradation of hormone during acute or chronic inhibition of secretion was not investigated in this work; such degradation has been seen for both parathyroid hormone (33, 40) and insulin (21).

Equimolar, coordinate secretion of all the peptides derived from pro-ACTH/endorphin would also be expected using normal anterior pituitary tissue; the biosynthetic pathway in the rat anterior pituitary is the same as that in the AtT-20 cells (37). In our studies, basal secretion from isolated rat anterior pituitary cells has always been coordinate and equimolar (15). Coordinate but clearly not equimolar secretion has been re-

ported in work on primary anterior pituitary cultures (2, 32, 42, 47, 52). Because the recovery of exogenous peptides in the sampling medium and the stability of endogenous secreted molecules were not reported in most other studies, it is difficult to compare previous results to the data in this paper.

One of the clearest demonstrations of inhibition of secretion by the products of prior secretion involves collagen; fibroblasts are inhibited from further secretion of collagen by the globular (but not the helical) region of previously secreted or exogenously added collagen molecules (70). Another good example is somatostatin secretion by pancreatic islets (25). In many other studies reporting inhibition of secretion by previously secreted peptides, tissue viability and stability of peptides after secretion were incompletely documented. The data on ACTH secretion from AtT-20 cells and from primary rat anterior pituitary tissue are conflicting (17, 22, 26, 28, 47, 53). Two previous reports of secretion by AtT-20 cells found secretion to be continuous for many hours (22, 67), as in this study. Because exogenous and endogenous ACTH (53), endorphins (55, 60), and melanotropins (57) are sometimes degraded in cultures with half-lives of the order of 10 min to 2 h, a lack of stability after secretion can be confused with a decreased secretory rate. Under the incubation conditions used in these and previous studies (Figs. 2 and 3; Table II; reference 35), endogenous and exogenous hormonal peptides were stable after secretion into the culture medium. It has been suggested that autoregulation of secretion only occurs at high cell densities and high hormone concentrations (53); in Fig. 2 and Table II, no evidence for autoregulation of secretion was seen at hormone levels four orders of magnitude above the highest levels found in blood (1, 3, 23, 24, 31, 61). Plateau levels of endorphin and ACTH secretion found in some other studies of AtT-20 cells (53, 60) were <1% of the levels achieved in this work (Fig. 2) and in previous studies by other groups (22, 67). The hormone levels shown in Fig. 2 and Table I are not overestimates, as the amounts have been determined with five different ACTH and two different endorphin radioimmunoassays, two different ACTH bioassays, an opiate bioassay, and by quantitative amino acid analyses after purification of the secreted hormonal peptides (10, 12, 14, 18, 29, 34).

The AtT-20/D-16v mouse pituitary tumor cells have a very high basal rate of secretion; ~15% of cellular hormone content is secreted per hour (10, 22, 34; Fig. 2). By comparison, basal secretion from primary rat anterior pituitary cells is only ~0.4% of cellular hormone content/hour (15). Even with such a high basal secretion rate, the mouse tumor cells were stimulated by norepinephrine to release additional hormone (Tables I and III, Figs. 4–6). When considering the actions of pro-ACTH/endorphin-related molecules after secretion, the important result is that the increased release caused by norepinephrine is almost exclusively in the smaller, more bioactive molecules; ACTH(1-39) and glycosylated ACTH(1-39) are much more active than the larger molecules at stimulating steroidogenesis in the adrenals (18), and only β-endorphin has substantial opiate activity (14). In similar but less fully analyzed experiments, stimulated primary anterior pituitary cells also released mainly smaller peptides (13, 65). In many other tissues, the molecular composition of basal secretion is not the same as stimulated secretion (7, 8, 33, 40, 50, 56, 63).

The ionic basis of secretion of peptides from AtT-20 cells is not yet clear. Depolarization of anterior pituitary cells by exposure to elevated potassium causes release of ACTH (30, 51); ionized external calcium is required for potassium- and hypothalamic extract-stimulated secretion of ACTH (19, 30, 51). The AtT-20 cells have been reported to increase their secretion rate when exposed to elevated potassium (54, 60), but only for periods of a few minutes; when AtT-20 cells are exposed to elevated potassium for 1 h (a time commonly used in studies of secretion of ACTH by normal anterior pituitary), no detectable increase over basal secretion is found (54, 60; R. E. Mains and B. A. Eipper, unpublished observations). Similarly, GH_3 rat pituitary tumor cells exhibit increased growth hormone secretion in elevated potassium only for a few minutes (45). Ionized extracellular calcium is required for stimulated but not basal secretion from both normal and tumor cells (Table I; Figs. 4–6; 19, 30, 54, 60). Basal secretion from many other peptide-secreting tissues is also independent of ionized extracellular calcium (6, 58, 59, 63). Interestingly, AtT-20/D-16v cells are capable of exhibiting regenerative changes in membrane potential which have pharmacological properties similar to calcium action potentials.[2]

These studies extend work on several peptide-secreting tissues which shows that the molecular compositions and ionic dependence of basal and stimulated secretions are often different. Frequently, the molecular composition of only the stimulated component of secretion correlates well with analyses of the molecular composition of the soluble contents of secretory granules, which are generally presumed to release their contents by exocytosis (46). The cellular basis of basal secretion is unclear; it could reflect heterogeneity among the cells or among secretory granules inside the cells. Alternatively, basal secretion might originate in subcellular organelles other than conventional secretory granules.

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