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Steroidogenic Activity of High Molecular Weight Forms of Corticotropin†

Judith C. Gasson*

ABSTRACT: The high molecular weight forms of adrenocorticotropic hormone (ACTH) produced by mouse pituitary tumor cells (AtT-20/D-16v) were separated from each other by gel filtration; their ability to stimulate steroidogenesis by isolated rat adrenal cortical cells was studied. Pools of pro-ACTH/endorphin, ACTH biosynthetic intermediate, and glycosylated ACTH(1–39) were obtained; on the basis of $NaDodSO_4$–polyacrylamide gel electrophoresis, over 97% of the immunoactive ACTH was found to have the expected molecular weight. Suspensions of isolated rat adrenal cortical cells were incubated overnight in tissue culture medium and used in a 2-h steroid production assay. Synthetic human ACTH(1–39) [hACTH(1–39)] was used as a bioassay and immunoassay standard; 60 pM hACTH(1–39) stimulated half-maximal production of fluorogenic steroid. The amount of pro-ACTH/endorphin, ACTH biosynthetic intermediate, or glycosylated ACTH(1–39) added was estimated with an ACTH(17–24) immunoassay. All three high molecular weight forms of ACTH are capable of stimulating the same maximal level of steroidogenesis as hACTH(1–39). Glycosylated ACTH(1–39) is equipotent with hACTH(1–39); ACTH biosynthetic intermediate and pro-ACTH/endorphin are, respectively, 100- and 300-fold less potent than hACTH(1–39). Steroid production in response to all four forms of ACTH is linear in time. All of the different forms of ACTH stimulate the synthesis of corticosterone and related steroids; no significant production of cortisol or aldosterone was observed. β-Lipotropin (βLPH) and 16K fragment, which comprise the non-ACTH regions of pro-ACTH/endorphin and are secreted by the pituitary tumor cells, did not stimulate or interfere with steroidogenesis. Brief incubations of pro-ACTH/endorphin and ACTH biosynthetic intermediate with trypsin generated lower molecular weight forms of ACTH and increased biological activity 50-fold; thus, the decreased steroidogenic potency of these forms of ACTH is thought to be due to structural constraints on the ACTH(1–39)-like sequence in these larger precursor molecules.

Pulse-labeling studies using a mouse pituitary tumor cell line (AtT-20/D-16v), as well as isolated rat anterior and intermediate pituitary cells, have shown that glycosylated and nonglycosylated forms of adrenocorticotropic hormone [ACTH(1-39)] are synthesized as part of a larger glycoprotein molecule (Mains & Eipper, 1976; Mains et al., 1977; Eipper & Mains, 1978a). Since this glycoprotein is the biosynthetic precursor for both ACTH- and endorphin-containing molecules, it is referred to as pro-ACTH/endorphin. Quantitative pulse–chase experiments have shown that the major pathway for ACTH biosynthesis in mouse pituitary tumor cells is as shown in Scheme I. The ACTH peptide backbone makes up roughly the middle third of the pro-ACTH/endorphin molecule. The peptide extension to the carboxyl terminal side of the ACTH(1–39)-like region is similar to known βLPH molecules; a β-endorphin-like sequence [βLPH(61–91)] lies at the carboxyl-terminal end of the precursor. The amino-terminal third of the precursor molecule is referred to as 16K fragment, and at present very little is known about its structure or possible biological activity (Eipper & Mains, 1978b).

Pro-ACTH/endorphin, ACTH biosynthetic intermediate, and glycosylated ACTH(1–39) are secreted by pituitary tumor cells (Eipper & Mains, 1975; Eipper et al., 1976; Allen et al., 1978) and by isolated rat anterior and intermediate pituitary cells (Eipper & Mains, 1978a; Vale et al., 1978); in addition, high molecular weight forms of ACTH have been demonstrated in normal human plasma and the plasma of patients with pituitary or ectopic ACTH-secreting tumors (Yalow & Berson, 1973; Gewirtz & Yalow, 1974; Orth & Nicholson,

† From the Department of Physiology, University of Colorado Medical Center, Denver, Colorado 80262. *Received March 22, 1979; revised manuscript received May 29, 1979.* This research was supported by National Institutes of Health Grants AM 18929 and AM 19859.

* Present address: The Salk Institute, Regulatory Biology Laboratory, P.O. Box 85800, San Diego, CA 92138.

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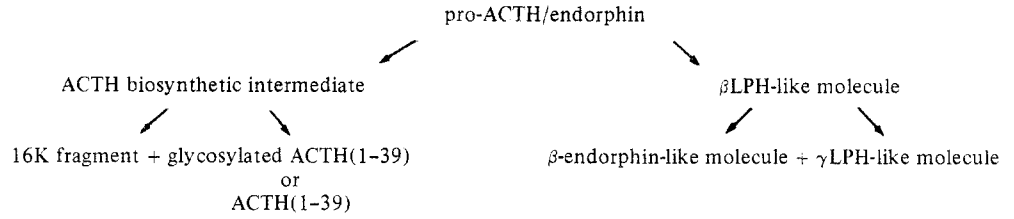
Scheme I



1977a,b). For determination of whether these high molecular weight forms of ACTH possess steroidogenic activity, bioassays were performed by using isolated rat adrenal cortical cells.

Experimental Procedures

Preparation of Separated Forms of ACTH. Tissue culture medium (without serum) was collected from confluent plates of AtT-20/D-16v mouse pituitary tumor cells every 24 h for a week and stored frozen with phenylmethanesulfonyl fluoride and iodoacetamide (each at 0.3 mg/mL) until use (Eipper & Mains, 1975). Immunoreactive ACTH, endorphin, and 16K fragment reach concentrations of about 0.2 μM in serum-free medium. Two different methods were used to prepare high molecular weight forms of ACTH. In method A the protein present in 500 mL of tissue culture medium was concentrated by mixing the medium with an equal volume of 48 mM HCl and 7.5 mL of CG-50 resin (added as a 1:1 slurry of resin in 0.025 M acetic acid) (Island et al., 1965). The resin plus acidified medium was stirred for 4 h at 4 °C, and the resin was collected in a 50-mL plastic syringe plugged with siliconized glass wool. The resin was washed with 3 volumes of 5% (v/v) acetic acid, and the protein was eluted with 60% acetic acid. The eluate was diluted with 2 volumes of ice-cold distilled water and lyophilized. The lyophilisate was dissolved in 1.5 mL of 1% acetic acid containing 2% β-mercaptoethanol and clarified by centrifugation at 1200*g* for 10 min at 4 °C. The pellet was reextracted with 0.5 mL of 1% acetic acid and 2% β-mercaptoethanol and centrifuged. The two supernatants were pooled and loaded onto a Sephadex G-75 superfine column (1.5 × 55 cm) equilibrated and eluted with 1% acetic acid at 4 °C. The void volume and total volume were determined by measuring absorbance at 280 nm. The concentrations of ACTH, endorphin, and 16K fragment (the non-ACTH, non-lipotropin region of the ACTH/endorphin common precursor; Eipper & Mains, 1978b) in the column fractions were estimated by radioimmunoassays. An antiserum with specificity for ACTH(17–24) (antiserum Bertha) was used to locate ACTH-containing material (Mains & Eipper, 1976; unpublished experiments). An antiserum with specificity for β-endorphin(14–27) (antiserum RB-100) was used to locate endorphin-containing material (Guillemin et al., 1977). An antiserum with specificity for the 16K fragment region (antiserum Georgie) was used to locate the 16K fragment containing material (Eipper & Mains, 1978a).

An alternate method (method B) was also used to prepare high molecular weight forms of ACTH from spent serum-free tissue culture medium. Method B does not expose the ACTH molecules to acid pH. The spent medium was concentrated 10-fold by rotary evaporation in a siliconized round-bottomed flask. The concentrate was dialyzed against two changes of 500 mL of 15 mM NaCl, 5 mM Hepes, and 5 mM EDTA at pH 7.4 at 4 °C for a total of 4 h. The dialyzed concentrate was further concentrated by rotary evaporation to a final volume equal to 1% of the starting volume. This final concentrate was fractionated on a Sephadex G-100 superfine column (1 × 60 cm) equilibrated and eluted at 4 °C with 15 mM NaCl, 5 mM Hepes, and 0.05 mg/mL bovine serum albumin, at pH 7.4. The void volume and total volume were determined by measuring the absorbance of the fractions at 280 nm. Bovine serum albumin (treated with phenylmethanesulfonyl fluoride) was added to each fraction to a final concentration of 5 mg/mL in order to prevent loss of immunoactive ACTH during storage of the column fractions at −10 °C.

The apparent molecular weight and purity of the ACTH pools obtained by both methods A and B were determined by borate–acetate-buffered $NaDodSO_4$–polyacrylamide gel electrophoresis (Davies & Stark, 1970); gels were analyzed by radioimmunoassay or were stained with Coomassie brilliant blue and scanned at 580 nm.

Highly purified preparations of mouse tumor cell glycosylated ACTH, βLPH, and 16K fragment were provided by B. A. Eipper. These samples were obtained by method A, followed by ion-exchange chromatography using carboxymethylcellulose and gel filtration.

Preparation of Isolated Rat Adrenal Cortical Cells. Male Sprague–Dawley rats (Charles River Breeding Co.; 150–450 g) were killed by decapitation between 1:00 and 3:00 p.m. The adrenal glands were rapidly removed, decapsulated, and minced at room temperature in sterile saline. During the course of this work modifications of several methods of preparing isolated adrenal cortical cells have been used (Sayers et al., 1971; Lowry et al., 1973; Albano et al., 1974; Hornsby et al., 1974; Ramachandran & Suyama, 1975). The most consistent results (cell yield and steroidogenesis) were obtained by using an adaptation of the methods developed by Vale and co-workers (Vale et al., 1972; Vale & Rivier, 1977) for anterior pituitary cultures. The tissue was dispersed by using a mixture of collagenase, hyaluronidase, and DNase in Hepes–Tricine-buffered Dulbecco–Vogt modified Eagle's medium (DMEM) for 20 min; dissociations were performed in a sterile 15-mL conical plastic tube containing a 1.0-cm Teflon stirring bar suspended in a 37 °C water bath over a magnetic stirrer set at the lowest speed. The cells were then incubated in Hepes–Tricine-buffered DMEM containing 3 mg/mL trypsin (Trypsin I-300; ICN Pharmaceuticals) at 37 °C for an additional 20 min. Following trypsinization, the cells were dispersed gently with a Pasteur pipet and washed in phosphate-buffered saline containing 0.5 mg/mL lima bean trypsin inhibitor. The cell pellet was resuspended in DMEM containing 5 mg/mL bovine serum albumin, 0.5 mg/mL lima bean trypsin inhibitor, kanamycin sulfate, glucose, and glutamine (Eipper & Mains, 1975); the cell suspension was pipetted into sterile plastic tubes. Each experiment utilized the adrenals from two rats and consisted of 30–50 tubes with 10000–25000 adrenal cells per tube. The isolated rat adrenal cortical cells were incubated overnight at 36 °C, 10% CO_2 and 90% air, in a humid atmosphere before use in the ACTH bioassay.

Bioassay of ACTH. The isolated rat adrenal cortical cells were collected by centrifugation (100*g*; 3 min) at room temperature; fresh DMEM with bovine serum albumin and

lima bean trypsin inhibitor (490 μL) was added to each tube. Synthetic hACTH(1–39) standard (provided by Drs. W. Rittel and P. A. Desaulles, CIBA-GEIGY) or a test sample of ACTH was added to each tube in a volume of 10 μL. All samples and the standard were serially diluted in DMEM containing bovine serum albumin and lima bean trypsin inhibitor; all samples were bioassayed in duplicate. The concentration of ACTH in the test samples [pro-ACTH/endorphin, ACTH biosynthetic intermediate, and glycosylated ACTH(1–39)] was estimated by radioimmunoassay with the ACTH(17–24) antibody (Mains & Eipper, 1976). Analyses of the tryptic peptides of [^{3}H]tyrosine- and [^{3}H]tryptophan-labeled pro-ACTH/endorphin and ACTH biosynthetic intermediate make it possible to calculate approximate molar extinction coefficients for these molecules at 280 nm (B. A. Eipper, personal communication). On the basis of the measured concentration of immunoactive ACTH and these calculated molar extinction coefficients, 30–50% of the observed absorbance at 280 nm is due to the high molecular weight ACTH.

Isolated rat adrenal cortical cells were incubated with test samples or standard for 2 h at 37 °C, in a humid 10% CO_2 atmosphere. The potency of the test molecules relative to the synthetic hACTH(1–39) standard was calculated by determining the ratio of the concentration (estimated by radioimmunoassay) of the test molecule to the concentration of the standard required to give the same percent of maximal steroid production in each experiment. When the data from several experiments are averaged, the result is presented as mean ± 1 standard deviation.

Steroid concentration in each tube (medium plus cells) was measured by the fluorescence method of Peterson (1957) as modified by Sayers et al. (1971). Concentrations of ACTH which produce maximal steroidogenesis evoke a mean 50-fold increase over background steroid production (tubes containing cells and no ACTH). A corticosterone standard was included in each assay.

Product Identification. The steroid products synthesized by the isolated rat adrenal cells were identified by studying the conversion of [^{3}H]pregnenolone to radioactive glucocorticoids and mineralocorticoids. [^{3}H]Pregnenolone (labeled in the 7 position; 18 Ci/mmol; Amersham/Searle) was evaporated to dryness in a glass tube under a stream of nitrogen. Medium containing bovine serum albumin and lima bean trypsin inhibitor was added to the tube and shaken; this medium containing [^{3}H]pregnenolone at a final concentration of 0.1–0.5 μM was used for the incubation of isolated rat adrenal cortical cells with synthetic hACTH(1–39) or a form of high molecular weight ACTH as in previous experiments. Short-term experiments were performed as described under Bioassay of ACTH using medium containing labeled pregnenolone. For long-term experiments the isolated rat adrenal cortical cells were preincubated overnight with the standard or test ACTH molecules. The medium was replaced, as in other experiments, with medium containing [^{3}H]pregnenolone, and the same standard or test molecule was added. After 2 h of incubation, the culture tubes containing isolated adrenal cortical cells, [^{3}H]pregnenolone, and ACTH were centrifuged gently. The medium was removed, an aliquot was saved for steroid assay, and the remainder was frozen on dry ice and stored at −70 °C. The steroid products formed from [^{3}H]-pregnenolone were identified by a modification of the method of O'Hare & Neville (1973). The medium was thawed, and 10–20 μL was added to a tube containing 1.0 mL of methylene chloride and 80–90 μL of distilled water. The following standards were added (10 μL of each at a concentration of 0.5 mg/mL in methanol): corticosterone, cortisol, aldosterone, progesterone, 18-hydroxydeoxycorticosterone, pregnenolone, and deoxycorticosterone. The sample was mixed; aqueous and organic phases were separated by centrifugation. The aqueous layer was discarded, and the methylene chloride layer was evaporated under a stream of nitrogen to a final volume of 15–40 μL but not to dryness. An aliquot was removed to determine the initial input of radioactivity applied to the chromatogram. The remaining sample was spotted in one corner of a 20 × 20 cm thin-layer chromatography plate (ITLC-SAF; Gelman). The chromatogram was developed in the first dimension in benzene–acetone (115:35). The chromatogram was allowed to dry, and the standards were visualized with ultraviolet light and traced. The thin-layer plate was cut at $M_{solvent}$ = 0.55, and the lower half (containing the origin and aldosterone, cortisol, 18-hydroxydeoxycorticosterone, and corticosterone standards) was chromatographed in the second dimension in chloroform–ethanol (142.5:7.5) to separate the cortisol and 18-hydroxydeoxycorticosterone standards fully. The upper half (containing the deoxycorticosterone, progesterone, and pregnenolone standards) was chromatographed in the second dimension in ethyl acetate–cyclohexane (50:50). The standards were visualized and traced; pregnenolone was visualized with vanillin (Levin et al., 1969). Strips of the thin-layer chromatography plate were cut into 0.5 × 2 cm sections and counted in a liquid scintillation counter. The recovery of total radioactivity was determined as well as the percent of the total radioactivity associated with the internal standards.

Trypsin Treatment. Samples of hACTH(1–39) and glycosylated ACTH(1–39), ACTH biosynthetic intermediate, or pro-ACTH/endorphin (all prepared by gel filtration in mild acetic acid) were lyophilized and dissolved in 0.05 M sodium phosphate buffer (pH 8.0) containing 1 mg/mL bovine serum albumin (treated with phenylmethanesulfonyl fluoride and dialyzed extensively into 0.9% NaCl); at least 95% of the protein (based on weight) in each sample was bovine serum albumin. Trypsin (TPCK treated; Worthington) was added in 0.05 M sodium phosphate buffer (pH 8.0) to a final concentration of 1.0 μg/mL, and the samples were incubated at room temperature for the specified times. The reactions were terminated by the addition of lima bean trypsin inhibitor (900-fold molar excess over trypsin) and boiling for 3 min. The control samples (0 time) were prepared by adding the lima bean trypsin inhibitor before the trypsin and boiling the sample immediately after the addition of trypsin. An aliquot of each sample was serially diluted, and steroidogenic potency was measured. The remaining sample was lyophilized and fractionated by $NaDodSO_4$–polyacrylamide gel electrophoresis; the gels were immunoassayed with the ACTH(17–24) antibody, the 16K fragment antibody, and β-endorphin(14–27) antibody.

Results

Isolated Rat Adrenal Cortical Cells. Standard methods for preparation of isolated rat adrenal cortical cells for use in ACTH bioassays have employed enzymatic digestion with either trypsin (Sayers et al., 1971; Lowry et al., 1973) or collagenase (Albano et al., 1974; Hornsby et al., 1974; Ramachandran & Suyama, 1975). A modification of the method developed by Vale and co-workers (Vale et al., 1972; Vale & Rivier, 1977) for anterior pituitary tissue, which utilizes collagenase digestion followed by trypsinization, was found to produce more consistent results in these experiments. An overnight incubation of the isolated adrenal cortical cell

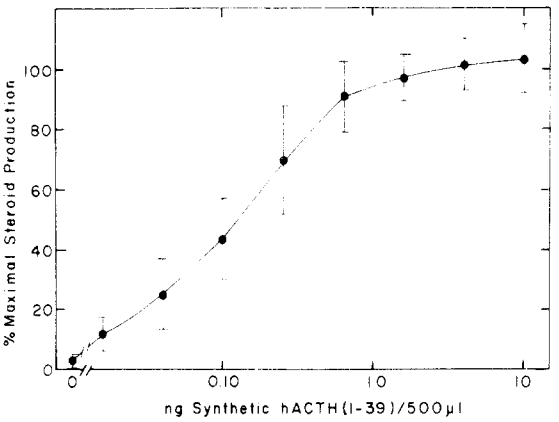


FIGURE 1: Dose–response curve for hACTH(1–39). Isolated rat adrenal cortical cells were prepared as described under Experimental Procedures. Following an overnight incubation, the tissue culture medium was replaced with fresh medium containing a serial dilution of synthetic hACTH(1–39). Maximal steroid production was determined by averaging the data points in the plateau phase of the curve; all data points were graphed as percent maximal steroid production. Data presented are the mean ± 1 SD of 16 separate experiments. The average calculated amount of glucocorticoid [(10 pg/stimulated adrenal cell)/2 h] is consistent with previously reported values (Haning et al., 1970; Sayers et al., 1974; Lowry et al., 1973).

suspensions in sterile culture tubes containing complete tissue culture medium was included for two reasons: steroids produced by cells damaged in the dissociation process are not measured since this medium is discarded; it might be possible for the cells, which have been exposed to trypsin during dissociation, to replace membrane receptor proteins which may have been destroyed.

Steroid production by isolated rat adrenal cortical cells in response to synthetic hACTH(1–39) was found to be linear in time; in most experiments steroid production during a 2-h incubation was measured. The log dose–response curve for synthetic hACTH(1–39) is shown in Figure 1; the average midpoint of the bioassay is 60 pM hACTH(1–39) [range 33–107 pM hACTH(1–39)]. The isolated rat adrenal cortical cells are sensitive to reasonable levels of ACTH; in the rat, basal levels of ACTH are 5–20 pM, and ACTH levels after stress are as high as 500 pM (Rees et al., 1971). A full dose–response curve for hACTH(1–39) is included in each experiment, and the steroidogenic potency of the different forms of ACTH is always determined relative to the hACTH(1–39) standard.

Preparation of Separated Forms of ACTH. High molecular weight forms of ACTH were prepared from the tissue culture medium of AtT-20/D-16v mouse pituitary tumor cells. For preparation of samples containing only pro-ACTH/endorphin, ACTH biosynthetic intermediate, or glycosylated ACTH-(1–39), the protein present in a large volume of medium was concentrated and fractionated by gel filtration, as shown in Figure 2A. Molecules related to ACTH, β-endorphin, and 16K fragment are all secreted into the tissue culture medium; the concentration of each of these molecules was estimated by radioimmunoassay. Only molecules with ACTH immunoreactivity affect the steroidogenic response of the isolated adrenal cells. In Figure 2B, aliquots of various column fractions were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and ACTH(17–24) radioimmunoassay to determine which fractions contained a single molecular weight form of ACTH. In the samples of pro-ACTH/endorphin, ACTH biosynthetic intermediate, or glycosylated ACTH-(1–39) selected, greater than 97% of the ACTH immunoactivity is present in the appropriate single peak of high

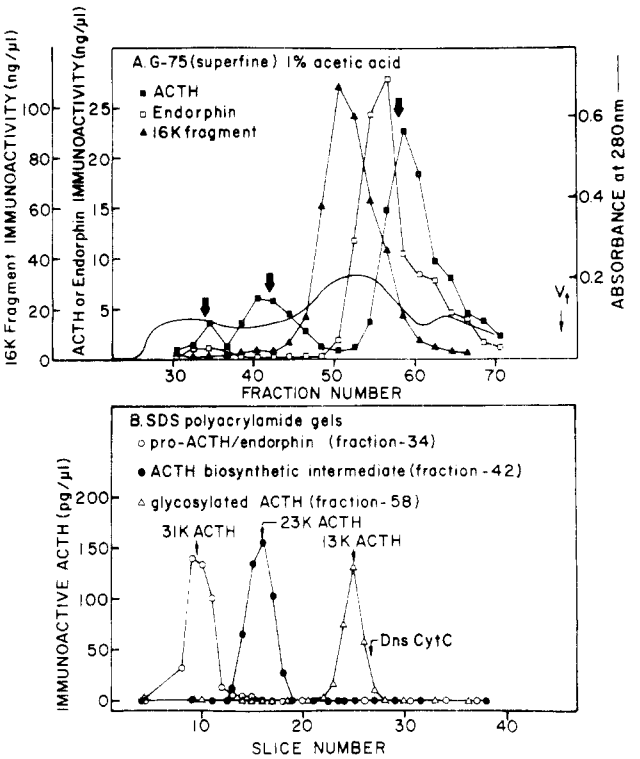


FIGURE 2: Preparation of separated forms of ACTH. (A) The protein present in 500 mL of spent zero serum tissue culture medium from AtT-20/D-16v mouse pituitary tumor cells was concentrated as described under Experimental Procedures and fractionated on a Sephadex G-75(SF) column in 1% acetic acid at 4 °C. On the basis of the results of radioimmunoassays for ACTH(17–24), β-endorphin(14–27), and 16K fragment, the fractions indicated by the heavy arrow (↓) were selected for analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. (B) For determination of the molecular weight of the ACTH-related material in the fractions analyzed, the gels were sliced, eluted, and immunoassayed with the ACTH(17–24) antibody. Recoveries of immunoactive ACTH were >95%.

molecular weight ACTH. Until each of the high molecular weight forms of ACTH has been purified to homogeneity, an ACTH radioimmunoassay can only give an estimate of their concentration. By use of the absorbance of the fractions at 280 nm and the molar extinction coefficient for each form of ACTH, it can be calculated that approximately 40% of the protein present in the samples of pro-ACTH/endorphin and ACTH biosynthetic intermediate shown in Figure 2 is actually the desired molecule. In addition, aliquots from these pools of high molecular weight ACTH were fractionated by $NaDodSO_4$–polyacrylamide gel electrophoresis; scans of the stained gels indicated that 30–50% of the protein was in the appropriate molecular weight range. These pools of separated forms of ACTH were used to determine the steroidogenic potency of the molecules.

Dose–Response Curves for High Molecular Weight Forms of ACTH. The dose–response curves obtained for each of the three high molecular weight forms of ACTH are shown in Figure 3. All three high molecular weight forms of ACTH are capable of stimulating the same maximal level of steroidogenesis as the synthetic hACTH(1–39) standard. Glycosylated ACTH(1–39) is equipotent to the hACTH(1–39) standard in this system as reported in Table I. The mean potency of ACTH biosynthetic intermediate is 0.9% times that of the hACTH(1–39) standard. Pro-ACTH/endorphin is 0.33% as potent as the hACTH(1–39) standard.

Given the greatly reduced potency of pro-ACTH/endorphin and ACTH biosynthetic intermediate, one must consider the possibility that these molecules exhibit bioactivity only after

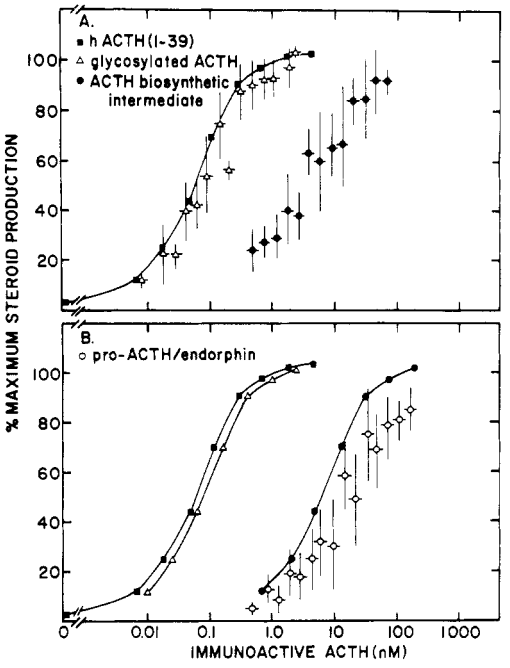


FIGURE 3: Relative steroidogenic potency of high molecular weight forms of ACTH. Isolated rat adrenal cortical cells were incubated with a single form of ACTH for 2 h, and the steroid concentration in each sample was measured by using the acid–ethanol fluorescence assay. (A) The dose–response curve for hACTH(1–39) is reproduced from Figure 1. Data points for glycosylated ACTH(1–39), ACTH biosynthetic intermediate, and pro-ACTH/endorphin are the mean ± 1 standard deviation for at least eight experiments. The ACTH concentration in the samples was estimated by radioimmunoassay; all samples were serially diluted for bioassay and immunoassay, and the curves generated were parallel to the hACTH(1–39) standard in both cases. (B) Calculated average dose–response curves for glycosylated ACTH(1–39) and ACTH biosynthetic intermediate were obtained by using the average potency (determined by using only points between 20 and 80% maximal steroid production; the arithmetic mean of the logarithms of the individually calculated potencies was computed, and the antilogarithm was taken as the average potency).

Table I: Relative Steroidogenic Potency of Pro-ACTH/Endorphin and Related Peptides[a]

form of ACTH	method of preparation	mean potency rel to hACTH-(1-39)	potency range (±1 SD)
glycosylated ACTH(1-39)	neutral pH	0.56	0.31-1.00
	acid pH	0.75	0.45-1.25
	CM-cellulose	1.1	0.90-1.40
ACTH biosynthetic intermediate	neutral pH	0.010	0.004-0.028
	acid pH	0.0091	0.004-0.020
pro-ACTH/endorphin	acid pH	0.0033	0.0017-0.0062

[a] The relative potencies of separated or highly purified pro-ACTH/endorphin and related peptides were determined as described in Figure 3. In addition to the usual CG-50 concentration and mild acetic acid gel filtration method of preparing partially purified fractions for the adrenal cell bioassay, ion-exchange chromatography was used to prepare more highly purified samples for bioassay; the pool of glycosylated ACTH(1-39) was greater than 90% pure (the samples were provided by B. A. Eipper). In order to avoid exposure of the molecules to acidic conditions, a neutral pH method of preparing separated forms of ACTH for bioassay was also employed (method B under Experimental Procedures).

proteolysis into some smaller forms of ACTH. If this were the case, steroid production in response to pro-ACTH/endorphin and ACTH biosynthetic intermediate might not be linear in time. Figure 4 shows that, at the shortest times

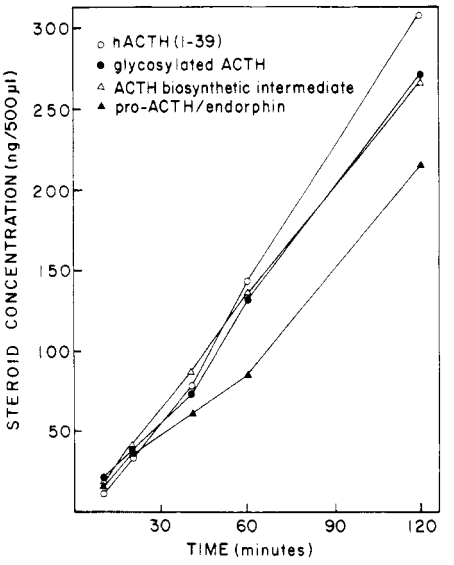


FIGURE 4: Time course of steroid production. Isolated rat adrenal cortical cells were incubated with maximally or nearly maximally active concentrations of hACTH(1–39), glycosylated ACTH(1–39), ACTH biosynthetic intermediate, or pro-ACTH/endorphin for the times indicated; production of fluorogenic steroid was measured.

assayed, no detectable difference can be seen among any of the four forms of ACTH. In all cases the rate of steroid accumulation is approximately linear for up to 2 h.

It is conventional to use strong acetic acid during isolation of ACTH(1–39), and Figure 3 shows that glycosylated ACTH(1–39) exhibits full bioactivity even after exposure to strong acetic acid. With the two larger forms of ACTH (pro-ACTH/endorphin and ACTH biosynthetic intermediate), it was necessary to consider that their decreased steroidogenic potency was due to exposure to acid pH. Therefore, separated forms of ACTH were prepared by techniques which did not expose the molecules to acid pH. This alternate technique (see Experimental Procedures) involves concentration of the culture medium from the AtT-20 cells by rotary evaporation and fractionation on a Sephadex G-100 superfine column at neutral pH. The homogeneity of the various pools of ACTH was again verified by $NaDodSO_4$–polyacrylamide gel electrophoresis and radioimmunoassay for ACTH; sufficient amounts of separated glycosylated ACTH and ACTH biosynthetic intermediate were obtained for bioassay. Table I shows that use of the neutral pH technique did not result in a significant increase in the biological activity of ACTH biosynthetic intermediate or glycosylated ACTH(1–39) relative to the hACTH(1–39) standard.

Figure 2A shows that relatively high concentrations of 16K fragment and β-lipotropin-like material cofractionate with glycosylated ACTH(1–39). For investigation of the effect of the presence of these other molecules on the steroidogenic activity of glycosylated ACTH, samples of highly purified mouse tumor βLPH, 16K fragment, and glycosylated ACTH(1–39) (all prepared as described under Experimental Procedures) were bioassayed. As shown in Table I, highly purified glycosylated ACTH(1–39) retains full biological activity. Mouse tumor 16K fragment and βLPH possess no detectable steroidogenic activity in this bioassay system; on a molar basis, 16K fragment and βLPH are at least 10 000- and 250-fold less potent, respectively, than the hACTH(1–39) standard. Furthermore, the addition of a 100-fold molar excess of βLPH or 16K fragment to glycosylated ACTH(1–39) does not affect the steroidogenic response of the isolated rat adrenal cortical cells to glycosylated ACTH(1–39) (not shown).

If pro-ACTH/endorphin and ACTH biosynthetic intermediate were weak, full agonists in this bioassay system, one could predict that a large molar excess of one of these molecules could decrease the steroidogenic response of the adrenal cells to hACTH(1–39). To test this possibility, isolated rat adrenal cortical cells were incubated with hACTH(1–39) plus concentrations of pro-ACTH/endorphin or ACTH biosynthetic intermediate at least 150-fold greater on a molar basis. Great excesses of pro-ACTH/endorphin or ACTH biosynthetic intermediate did not inhibit the steroidogenic response to hACTH(1–39); the responses were additive. The fact that the response could be accurately predicted, based on the relative potencies of these two high molecular weight forms of ACTH, indicates that pro-ACTH/endorphin and ACTH biosynthetic intermediate are not antagonists in this system (i.e., binding to a receptor but not evoking a full biological response).

Identification of Steroid Products. Another possible explanation for the relative inactivity of ACTH biosynthetic intermediate and pro-ACTH/endorphin is that these two forms of ACTH are stimulating the production of a steroid for which the fluorescence assay is less sensitive. In addition, it has been suggested that the type of glucocorticoids produced by adrenal cortical cells is determined by the form of ACTH present (Coslovsky & Yalow, 1974). For identification of the glucocorticoid products secreted, isolated rat adrenal cortical cells were incubated in complete culture medium containing [^{3}H]pregnenolone, and the labeled steroid products were identified by two-dimensional thin-layer chromatography. In the short-term incubations, adrenal cortical cells were incubated with hACTH(1–39), glycosylated ACTH, ACTH biosynthetic intermediate or pro-ACTH/endorphin, and [^{3}H]pregnenolone for 2 h. In the long-term experiments the adrenal cortical cells were preincubated with a single form of ACTH for 20 h and then incubated in fresh culture medium containing [^{3}H]pregnenolone and the same form of ACTH for 2 h. Table II shows that, in both the short- and long-term incubations, all four forms of ACTH stimulated production of the same major steroid products: corticosterone, 18-hydroxydeoxycorticosterone, and deoxycorticosterone. There were no significant differences in the products formed during the short- and long-term incubations. After the 20-h preincubation with ACTH in the long-term experiment, maximum steroid production per cell decreased about twofold. This observation suggests that target cell desensitization may have occurred as a result of the preincubation step (Hornsby & Gill, 1977; Morera et al., 1978).

Trypsinization of High Molecular Weight Forms of ACTH. Studies using a variety of ACTH analogues have shown that alteration of the amino terminus of ACTH(1–39) diminishes its steroidogenic potency (Waller & Dixon, 1960); in ACTH biosynthetic intermediate and pro-ACTH/endorphin, 16K fragment is extended from the amino-terminal end of the ACTH(1–39)-like segment (Eipper & Mains, 1978b). The reduced biological activity of these two forms of ACTH might be due to the presence of this 16K fragment peptide extension. Tryptic digests of ACTH biosynthetic intermediate or pro-ACTH/endorphin radiolabeled with tyrosine, methionine, phenylalanine, or arginine contain a labeled peptide that comigrates with ACTH(1–8) in several different separation systems; therefore, a trypsin-sensitive site immediately precedes the ACTH(1–39)-like segment in these larger molecules (Eipper & Mains, 1978b). Trypsin-like cleavages are involved in the proteolytic processing of several prohormone molecules (Kemmler et al., 1971; Goltzman et al., 1976). Therefore,

Table II: Identification of Steroids Produced by Isolated Adrenal Cells

	short-term incubation[a]	
products created from [^{3}H]-pregnenolone (%)	hACTH(1-39), 118-fold stimulation[c]	glycosylated ACTH, ACTH biosynthetic intermediate, and pro-ACTH/endorphin, 58- to 121-fold stimulation[c]
aldosterone	0.001	0.002
cortisol	0.003	0.002
18-hydroxydeoxycorticosterone	27	26–32
corticosterone	45	43–51
deoxycorticosterone	10	10–12
progesterone	19	10–11
	long-term incubation[b]	
products created from [^{3}H]-pregnenolone (%)	hACTH(1-39), 110-fold stimulation[c]	glycosylated ACTH, ACTH biosynthetic intermediate, and pro-ACTH/endorphin, 68- to 110-fold stimulation[c]
aldosterone	0.9	0.12–0.79
cortisol	0.09	0–0.1
18-hydroxydeoxycorticosterone	32	34–36
corticosterone	55	48–52
deoxycorticosterone	4	5–8
progesterone	6	4–8

[a] Short-term incubations. Isolated rat adrenal cortical cells were incubated with a single form of ACTH in complete culture medium containing [^{3}H]pregnenolone for 2 h. Steroid products were identified by two-dimensional thin-layer chromatography as described under Experimental Procedures. [b] Long-term incubations. Isolated rat adrenal cortical cells were preincubated with a single form of ACTH for 20 h at a dose barely adequate to give maximal steroid production. After the preincubation, the medium was replaced with fresh medium containing the same form of ACTH, at the same concentration as the preincubation step, and [^{3}H]pregnenolone for 2 h. Samples were chromatographed as above. Recovery of radioactivity in all experiments was greater than 80%; at least 30 000 cpm was used in each chromatogram. [c] Stimulation of steroidogenesis over unstimulated control.

preparations of ACTH biosynthetic intermediate and pro-ACTH/endorphin were subjected to mild trypsin treatment to determine if trypsin were capable of cleaving off the 16K fragment extension while leaving the ACTH(1–39)-like region intact. Trypsin-treated samples were analyzed for steroidogenic potency and for apparent molecular weight.

As shown in Figure 5, brief trypsin treatment of ACTH biosynthetic intermediate greatly increased its steroidogenic potency. The intact ACTH biosynthetic intermediate was about 1% as potent as hACTH(1–39); 2 min of mild trypsin treatment increased its steroidogenic potency 50-fold. After 10 min of mild trypsin digestion the steroidogenic potency declined 5-fold. In order to understand how trypsin treatment increased the steroidogenic potency of ACTH biosynthetic intermediate, aliquots of the samples bioassayed in Figure 5 were fractionated by $NaDodSO_4$–polyacrylamide gel electrophoresis and assayed with radioimmunoassays for ACTH(17–24) and 16K fragment as shown in Figure 6. After 2 min of mild trypsin treatment the ACTH(17–24) assay detects three peaks of material; the peak at 21 000 daltons represents undigested ACTH biosynthetic intermediate; the peak of ACTH immunoactivity at 14 000 daltons is the size of glycosylated ACTH(1–39); the third peak comigrates with ^{125}I-labeled pACTH(1–39). The pool of ACTH biosynthetic

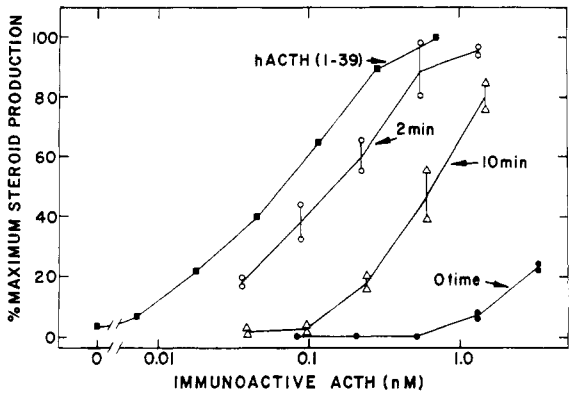


FIGURE 5: Effect of trypsin treatment on the steroidogenic activity of ACTH biosynthetic intermediate. Identical samples of ACTH biosynthetic intermediate (containing bovine serum albumin) were incubated with trypsin (see Experimental Procedures) at room temperature for the times indicated. After 10 min of trypsin treatment, the ACTH(17–24) immunoactivity decreased about 40%. All samples were bioassayed in duplicate; the mean values obtained for hACTH(1–39) are shown for reference. The potency of ACTH biosynthetic intermediate relative to the hACTH(1–39) standard is increased from 1% at 0 time to 51% at 2 min; the 10-min sample is 9% as potent as hACTH(1–39).

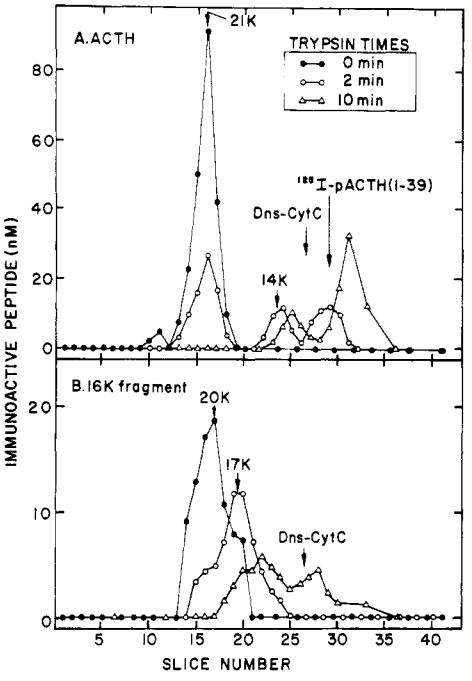


FIGURE 6: Effect of trypsin treatment on ACTH biosynthetic intermediate. Aliquots of trypsin-treated ACTH biosynthetic intermediate (Figure 5) were fractionated by $NaDodSO_4$–polyacrylamide gel electrophoresis and immunoassayed with the ACTH(17–24) antibody (part A) and the 16K fragment antibody (part B). The three different gels (0 time, 2 min, and 10 min) have been aligned so that the internal dansylated cytochrome *c* markers are coincident. The apparent molecular weights are indicated.

intermediate used in these experiments includes molecules having an oligosaccharide chain attached to their ACTH-(1–39)-like segment [the precursor to glycosylated ACTH-(1–39)] and molecules lacking this oligosaccharide chain [the precursor to ACTH(1–39)]. After 10 min of mild trypsin treatment, no undigested ACTH biosynthetic intermediate remains, and the lower molecular weight peaks of immunoactive ACTH have shifted to positions slightly smaller than those of glycosylated ACTH(1–39) and ACTH(1–39). This result is consistent with the suggestion that the decrease in bioactivity seen at 10 min is due to tryptic cleavages within the ACTH(1–39) sequence. With the 16K fragment ra-

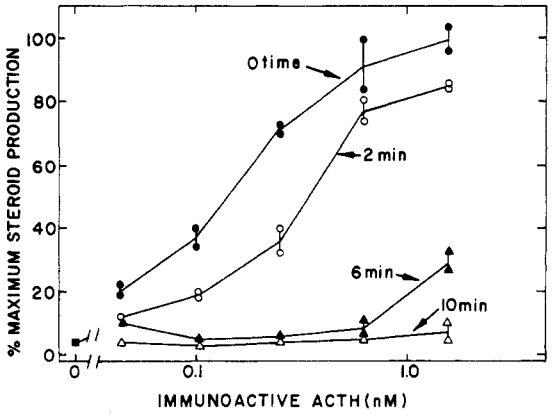


FIGURE 7: Effect of trypsin treatment on the steroidogenic activity of glycosylated ACTH(1–39). Identical samples of glycosylated ACTH(1–39) (containing bovine serum albumin) were exposed to trypsin for the times indicated (see Experimental Procedures) and bioassayed. No loss of ACTH(17–24) immunoactivity was seen after the exposure of glycosylated ACTH(1–39) to trypsin for 10 min. After 2 min glycosylated ACTH(1–39) was 40% as bioactive as the control; after 6 min bioactivity fell to 4.5% of the control value.

dioimmunoassay, the control sample (0 time) shows a peak of intact ACTH biosynthetic intermediate at 20 000 daltons; the shoulder on this peak represents the 16K fragment activity present in the pool of ACTH biosynthetic intermediate. After 2 min of mild trypsin digestion, most of the 16K fragment immunoactivity is associated with a single broad peak at 17 000 daltons; a small amount of intact ACTH biosynthetic intermediate is still present. After 10 min of mild trypsinization a large amount of the 16K fragment immunoactivity is cleaved into smaller cross-reactive peptides.

These results suggest that trypsin is capable of partially mimicking the normal intracellular proteolytic cleavages of ACTH biosynthetic intermediate. The 16K fragment extension is removed from the amino terminus of the ACTH-(1–39)-like region; removal of the 16K fragment extension exposes the amino terminus of the ACTH(1–39)-like region and accounts for the increased steroidogenic potency following trypsin treatment. The region between the 16K fragment and the ACTH(1–39)-like sequence in ACTH biosynthetic intermediate is apparently more sensitive to tryptic cleavage than sites within the ACTH(1–39)-like segment; longer trypsin treatment results in cleavage within the ACTH(1–39)-like segment and a decrease in steroidogenic potency. Figure 7 shows that a similar trypsin treatment of glycosylated ACTH(1–39) or hACTH(1–39) (data not shown) simply causes a progressive decrease in steroidogenic potency.

Pro-ACTH/endorphin consists of ACTH biosynthetic intermediate with a βLPH-like segment extended from the carboxyl terminus of the ACTH-like region. Figure 8 shows the results of mild trypsin treatment on the steroidogenic potency of pro-ACTH/endorphin. The intact pro-ACTH/endorphin was 300-fold less potent than hACTH(1–39); 2 min of exposure to trypsin increased its steroidogenic potency about 50-fold. After 10 min of trypsinization the steroidogenic potency fell from the 2-min level. Aliquots of these samples were fractionated by $NaDodSO_4$–polyacrylamide gel electrophoresis and analyzed by radioimmunoassay for ACTH-(17–24), 16K fragment, and β-endorphin(14–27) as shown in Figure 9. In the control sample all three immunoassays show the expected single peak of immunoactivity at 30 000 daltons. After 2 min of mild trypsinization a small amount of undigested pro-ACTH/endorphin remains; no intact pro-ACTH/endorphin remains in the 10-min sample. When immunoassayed with the 16K fragment antibody in Figure 9B,

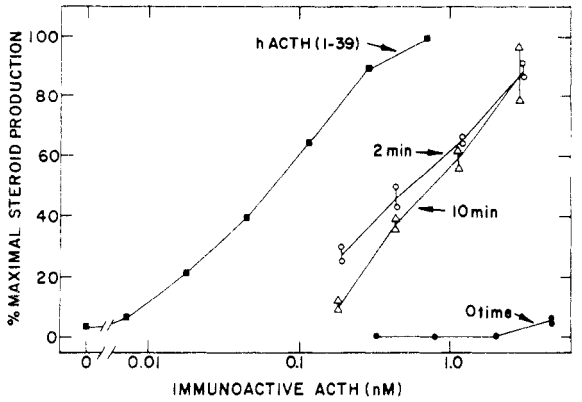


FIGURE 8: Effect of trypsin treatment on the steroidogenic activity of proACTH/endorphin. Identical samples of pro-ACTH/endorphin (containing bovine serum albumin) were incubated with trypsin (see Experimental Procedures) at room temperature for the times indicated. Trypsin treatment for 10 min decreased the ACTH(17–24) immunoactivity about 40%. All samples were bioassayed in duplicate; mean values obtained for hACTH(1–39) are shown for reference. The relative biological potency of pro-ACTH/endorphin to the standard is increased from 0.3% at time 0 to 17% at 2 min; the 10-min sample is 8% as potent as hACTH(1–39).

the products generated from pro-ACTH/endorphin appear to be very similar to the 16K fragment related material generated from ACTH biosynthetic intermediate (recall Figure 6B). After 2 min of trypsin treatment there is a prominent peak at 16 500 daltons, and after 10 min most of the activity is seen in two peaks smaller than 16K fragment. Trypsin appears to attack the junction between 16K fragment and the ACTH-(1–39)-like segment in a similar way in both pro-ACTH/endorphin and ACTH biosynthetic intermediate. Trypsin treatment of pro-ACTH/endorphin does not generate a molecule similar to the naturally occurring ACTH biosynthetic intermediate. As seen in Figure 9A, at 2 min there is a heterogeneous collection of ACTH-containing fragments which are smaller than the ACTH biosynthetic intermediate. Some of these ACTH-containing fragments must have the amino terminus exposed in order to account for the increased steroidogenic potency seen in Figure 8 and the release of 16K fragment seen in Figure 9B. No peaks of glycosylated ACTH(1–39) and ACTH(1–39) (as in Figure 6A) are seen; this observation suggests that trypsin is not carrying out the cleavage between the ACTH(1–39)-like and βLPH-like segments as efficiently as the intracellular enzymes. After 10 min, the ACTH-containing material is still very heterogeneous, although most of the material is smaller than 16 500 daltons. It seems likely that this sample contains a population of ACTH-containing molecules with and without segments of the βLPH-like molecule attached to the carboxyl terminus as well as biologically inactive cleavage products of ACTH which are smaller than the ^{125}I-labeled pACTH(1–39) marker.

The fate of the carboxyl-terminal portion of pro-ACTH/endorphin was investigated by using the β-endorphin radioimmunoassay as shown in Figure 9C. After exposure to trypsin for 2 min, three smaller peaks of endorphin immunoactivity are seen. The peaks at 16 500 and 13 000 daltons are the size peptides that are produced when the 16K fragment is removed from the pro-ACTH/endorphin molecule and no further cleavages are made; the two peaks could simply represent molecules differing by the presence or absence of an oligosaccharide chain in their ACTH(1–39)-like segment. As seen in Figure 9A, ACTH immunoactivity is seen in the same regions of the gel. The peak of endorphin immunoactivity at 13 000 daltons would also include any βLPH-like molecules created. A peak of endorphin-containing material

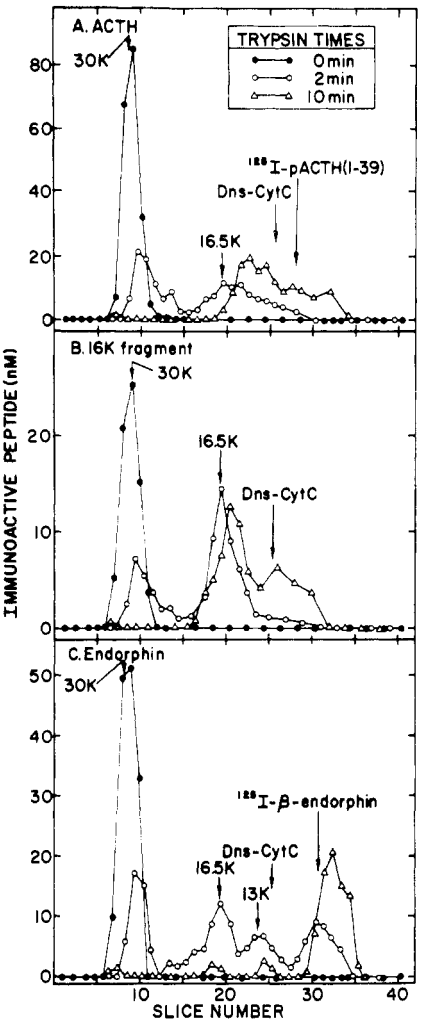


FIGURE 9: Effect of trypsin treatment on pro-ACTH/endorphin. Aliquots of trypsin-treated pro-ACTH/endorphin (Figure 8) were fractionated by NaDodSO$_4$–polyacrylamide gel electrophoresis and immunoassayed with the ACTH(17–24) antibody (part A), the 16K fragment antibody (part B), and the β-endorphin(14–27) antibody (part C). The three different gels have been aligned so that the internal dansylated cytochrome *c* markers are coincident. The apparent molecular weights are indicated.

that comigrates with ^{125}I-labeled β-endorphin is also observed. After 10 min of trypsinization, small amounts of immunoactive β-endorphin-containing material remain at 16 500 and 13 000 daltons, and material that is smaller than the ^{125}I-labeled β-endorphin marker has appeared. The presence of the βLPH-like sequence in pro-ACTH/endorphin seems to be responsible for the production of a heterogeneous population of ACTH-containing molecules after mild trypsin treatment. Trypsin, under the conditions used in these experiments, is not capable of mimicking accurately the intracellular cleavage of pro-ACTH/endorphin; however, trypsin is capable of increasing the steroidogenic potency 50-fold.

Discussion

The data presented here demonstrate that mouse glycosylated ACTH(1–39), ACTH biosynthetic intermediate, and pro-ACTH/endorphin are all capable of stimulating production of glucocorticoids by isolated rat adrenal cortical cells; all three high molecular weight forms of mouse ACTH cause the same maximal steroid production as synthetic hACTH-(1–39), as monitored by a fluorescence assay for glucocorticoids. In addition, each of the four forms of ACTH stimulates formation of the same glucocorticoid products from

[^{3}H]pregnenolone. Glycosylated ACTH(1–39) is equipotent with hACTH(1–39); ACTH biosynthetic intermediate and pro-ACTH/endorphin are 2 orders of magnitude less potent than hACTH(1–39).

The observed steroidogenic potency of glycosylated ACTH(1–39) is consistent with the findings of other investigators. Forms of ACTH with molecular weights of 6000–9000 have been described in mouse and rat pituitary and in mouse pituitary tumor cells (Lang et al., 1973; Orth et al., 1973; Eipper & Mains, 1975); on the basis of comparisons of ACTH bioassays and ACTH immunoassays, these forms of ACTH were found to be fully bioactive. Glycosylated ACTH(1–39) consists of an ACTH(1–39) peptide backbone with a single oligosaccharide chain attached to an asparagine residue in the carboxyl-terminal region of the molecule (Eipper & Mains, 1977); since amino-terminal fragments of ACTH(1–39) are fully bioactive (Ney et al., 1964), an alteration in the carboxyl-terminal end of the peptide might not be expected to have a significant effect on steroidogenic activity. Glycosylated ACTH(1–39) is secreted by mouse pituitary tumor cells and by cultured rat and mouse pituitary cells (Mains & Eipper, 1978; Eipper & Mains, 1978a; Vale et al., 1978; Allen et al., 1978). Pulse–chase studies have shown that glycosylated ACTH(1–39) and ACTH(1–39) are alternate products of the ACTH biosynthetic pathway (Mains & Eipper, 1976); on the basis of the work presented here, the actions of ACTH(1–39) and glycosylated ACTH(1–39) on isolated adrenal cortical cells are qualitatively and quantitatively similar.

It is more difficult to compare the results obtained for pro-ACTH/endorphin and ACTH biosynthetic intermediate to earlier studies on the steroidogenic activity of high molecular weight forms of ACTH. All previous studies used pools of high molecular weight ACTH that would have contained a mixture of pro-ACTH/endorphin and ACTH biosynthetic intermediate. "Big" ACTH from human nonpituitary tumors was found to have steroidogenic activity that was less than 4% of its immunoactivity (Gewirtz et al., 1974). Pools of 20 000- to 30 000-dalton ACTH from mouse pituitary and mouse pituitary tumor cells and "big" ACTH from a human pituitary tumor were found to have steroidogenic activity that was about 30% of their immunoactivity (Eipper & Mains, 1975; Krieger et al., 1976). The preparations of ACTH biosynthetic intermediate and pro-ACTH/endorphin used here contained a single form of ACTH and contained much less contaminating protein than samples in previous work.

Until the various different forms of ACTH have been completely purified, one can only estimate their concentration with ACTH radioimmunoassays. Therefore, steroidogenic potency of a given form of ACTH must be expressed as a ratio of bioactive ACTH to immunoactive ACTH. It is well documented that many ACTH immunoassays vastly underestimate the concentrations of these two high molecular weight forms of ACTH (Orth & Nicholson, 1977a; Eipper & Mains, 1978a; Allen et al., 1978); by using an ACTH antiserum that is relatively insensitive to pro-ACTH/endorphin and ACTH biosynthetic intermediate, it is possible to obtain a falsely high ratio of biological to immunological activity. The ACTH-(17–24) antibody used in the experiments described here cannot be underestimating the concentrations of pro-ACTH/endorphin or ACTH biosynthetic intermediate by more than a factor of 2 or 3 because the samples are about 30–50% pure. Finally, in the studies cited above, trypsin inhibitors were not included during the incubation of adrenal cells with high molecular weight forms of ACTH; in the incubations described here, lima bean trypsin inhibitor was included. Data presented here and studies by Gewirtz et al. (1974) show that tryptic cleavages can greatly increase the steroidogenic activity of pro-ACTH/endorphin and ACTH biosynthetic intermediate.

Several experiments have been presented to investigate further the low steroidogenic potency of pro-ACTH/endorphin and ACTH biosynthetic intermediate. ACTH biosynthetic intermediate and glycosylated ACTH(1–39) were separated without exposure to strong acid; no changes in the relative steroidogenic potency were observed. Samples of highly purified βLPH and 16K fragment, which are present in some of the pools of separated forms of ACTH, did not stimulate or inhibit the steroidogenic response of isolated rat adrenal cortical cells. Highly purified glycosylated ACTH(1–39) was not significantly different in relative steroidogenic potency from pools separated simply by size. Time-course studies showed no lag in the production of steroids with any of the four forms of ACTH; in addition, ACTH biosynthetic intermediate and pro-ACTH/endorphin do not act as partial agonists to hACTH(1–39). Since the same steroid products were synthesized in response to all of the forms of ACTH, the low steroidogenic potency is not due to the production of a steroid measured poorly in the fluorescence assay.

It has been proposed that the forms of ACTH secreted by the pituitary determine the type of glucocorticoid produced by the adrenal glands (Coslovsky & Yalow, 1974). According to this hypothesis, in species where the pituitary secretes predominantly "intermediate" ACTH [glycosylated ACTH(1–39)], the adrenal cortex produces corticosterone; in species where the pituitary secretes predominantly "little" ACTH [ACTH(1–39)], the adrenal cortex produces cortisol. The data presented here, as well as data in the literature, do not support this hypothesis. Isolated rat adrenal cortical cells secrete corticosterone (along with deoxycorticosterone and 18-hydroxydeoxycorticosterone), but no cortisol is secreted. These results are consistent with the work of O'Hare & Neville (1973), who have shown that cultured rat adrenal cortical cells produce corticosterone for up to 12 weeks in response to porcine ACTH(1–39). The absence of secretion of cortisol in response to ACTH(1–39) cannot be explained by a lack of ACTH(1–39) in the rat pituitary (Lang et al., 1973; Scott et al., 1974; Vale et al., 1978; Eipper & Mains, 1978a).

The low steroidogenic potency of ACTH biosynthetic intermediate and pro-ACTH/endorphin is thought to be due to the addition of the 16K fragment extension on the amino-terminal side of the ACTH(1–39) sequence. The addition of an acetyl group to the amino-terminal serine residue of ACTH(1–39) reduces its biological activity to less than 10% of control values (Waller & Dixon, 1960). Experiments in which pro-ACTH/endorphin and ACTH biosynthetic intermediate were incubated briefly with trypsin showed a 50-fold increase in steroidogenic potency relative to hACTH(1–39). The observed 50-fold increase demonstrates two points: first, the low steroidogenic potency of these two forms cannot be due to an artifact of the preparation of the molecules such as oxidation of the methionine residue at position four of ACTH(1–39); second, the ACTH(17–24) immunoassay cannot be greatly overestimating the concentrations of these molecules.

Analysis of the trypsin-treated pro-ACTH/endorphin and ACTH biosynthetic intermediate by $NaDodSO_4$–polyacrylamide gel electrophoresis showed formation of molecules similar to the 16K fragment and lower molecular weight forms of ACTH. Trypsin seems to be able to mimic the intracellular

enzymes that convert ACTH biosynthetic intermediate into 16K fragment and glycosylated or nonglycosylated ACTH-(1–39). However, trypsin does not mimic the intracellular enzymes that convert pro-ACTH/endorphin into a βLPH-like molecule plus ACTH biosynthetic intermediate.

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