Plasminogen Activators of the Pituitary Gland: Enzyme Characterization and Hormonal Modulation

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ABSTRACT We studied plasminogen activator (PA) of the rat pituitary gland in organ and cell monolayer culture. Both anterior and intermediate lobes contain, synthesize and secrete a mixture consisting of the two known types of PA: urokinase and so-called tissue PA. Both enzymes were formed essentially by all PA secreting cells, and PA was identified specifically in mammotrophs, corticotrophs, and luteinizing hormone containing gonadotrophs.

Pituitary PA production was modulated on exposure to a variety of biological effectors: anterior lobe PA secretion was stimulated by agents that raised intracellular cAMP concentration; this process depended on de novo enzyme synthesis. Enzyme production was repressed by androgens and glucocorticoids. When anterior lobe cultures were maintained in plasminogen-free media, the extracellular, secreted forms of ACTH consisted almost exclusively of the high molecular weight forms (31,000 and 23,000); the smaller forms (13,000 and 4,500) were also found in the extracellular medium of cultures supplemented with plasminogen. In contrast, the size distribution of intracellular ACTH species was unaffected by the presence of plasminogen. These results resemble those previously obtained with pancreatic islets and are consistent with the possibility that plasmin, generated by PA secretion, participates in prohormone processing.

PA synthesis in intermediate lobe explants was stimulated by exposure to dibutyryl cAMP, and repressed by hydrocortisone. In accordance with the dopaminergic control of intermediate lobe function in some vertebrates, apomorphine strongly repressed PA synthesis in intermediate, but not anterior lobe cultures.

A growing body of evidence indicates that plasminogen activator (PA)[1] production is a pervasive cellular mechanism for initiating localized proteolysis (33). This process is common to a broad spectrum of cell types, is correlated with a variety of physiological phenomena and, in most cases, is modulated by specific hormones. PA is particularly conspicuous in endocrine tissue including the thyroid (J.-D. Vassalli, unpublished observations), parathyroid (5), and pancreatic islets; in the latter, the rate of PA synthesis is determined by extracellular glucose concentration and enzyme production may provide a mechanism for prohormone processing (46).

Recent studies of adrenocorticotropin (ACTH) structure and synthesis in amphibian (8, 22) and mammalian pituitary (9, 26, 35) have shown that the predominant 4,500-mol-wt form of corticotropin is derived from a larger (31,000 mol wt) precursor that carries the antigenic determinants for both ACTH and β-lipotropin: corticotropin is apparently generated by proteolytic processing at sites consisting of two adjacent positively charged amino acids. Such sites are common to many proteins, including the prohormones for insulin (4, 38), ACTH (27), and parathyroid hormone (15) among others (18, 34), and indicate that processing is probably catalyzed by an enzyme with trypsin-like specificity. This inference is strengthened by the finding that brief trypsinization can convert both proinsulin (38) and "big" ACTH to the respective hormonal derivatives (12); however, trypsin itself cannot normally mediate prohormone conversion since it is produced only in the exocrine pancreas, and another enzyme must therefore be responsible for hormone processing.

A number of considerations prompted us to examine PA production in the pituitary gland: firstly, as already noted, PA is synthesized abundantly in other endocrines that secrete peptide hormones, but not in the adrenal cortex where only steroid hormones are produced (M. McConnell and E. Reich,

[1] *Abbreviations used in this paper*: ACTH, adrenocorticotropin; dbcAMP, dibutyryl cyclic AMP; LH, luteinizing hormone; α-MSH, α-melanotropic-stimulating hormone; PA, plasminogen activator.

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unpublished observations). Secondly, the anterior pituitary produces several peptide hormones, and it appeared of interest to determine which, if any, of the corresponding specific cell types produced PA. Thirdly, hormone formation in the pituitary can be modulated by a variety of effector molecules that also influence PA production in other tissues, and it seemed worthwhile to establish whether pituitary PA and hormone syntheses were coordinated in any way. Finally plasmin, a trypsin-like enzyme that is generated by plasminogen activator, has been shown to convert proinsulin to a molecule electrophoretically indistinguishable from insulin (46); thus the possibility that plasmin might participate, by analogy, in the processing of ACTH precursors invited exploration.

MATERIALS AND METHODS

Female Sprague Dawley rats weighing 150–200 g were purchased from Charles River Breeding Laboratories, Inc., (Wilmington, MA). [^{35}S]Methionine (specific activity 1,000 Ci/mM) was obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY). Dulbecco's minimal essential medium and fetal bovine serum were from Grand Island Biological Co. (Grand Island, NY); hydrocortisone, cholera toxin, 2-dibutyryl cAMP (dbcAMP), apomorphine, Trasylol, phenylmethylsulphonyl fluoride, and iodoacetamide were from Sigma Chemical Co. (St. Louis, MO); urokinase reference standard from Leo Pharmaceutical Products (Ballerup, Denmark); casein (as milk powder) was from Carnation; tissue culture plasticware was obtained from Falcon Labware (Oxnard, CA); α-melanotropic-stimulating hormone (α-MSH), luteinizing-hormone-releasing hormone and tyrotropic-releasing hormone were from Bachem Co. (Torrance, CA). All the other reagents were of the highest grade commercially available.

Preparation of Pituitary Cultures

ANTERIOR LOBE: Cells were prepared from the pituitaries of Sprague-Dawley female rats, weighing 150–200 g, by a trypsin digestion technique (17). After decapitation, the pituitary glands were removed from the skull and the anterior lobes were trimmed free of other tissue and minced with scalpels. The minced tissue was incubated under agitation at 37°C for 2 h in Dulbecco's medium containing 1 mg/ml bovine serum albumin and 1 mg/ml trypsin (Worthington Biomedical Co., Freehold, NJ) At the end of this treatment, the cells and small pieces of tissue were collected by centrifugation, washed twice with medium containing antibiotics and 10% plasminogen depleted fetal bovine serum (30) and resuspended in fresh medium. The suspension was then passed twice through a 20 gauge hypodermic needle to facilitate dispersion of the cells. After a final wash, the cells were plated in 0.5 ml of the above medium at the desired densities (usually 1×10^5 cells/well) in Linbro wells (FB 16–24 TC; Linbro Scientific, Hamden, CT). The recovery of viable cells was usually 5×10^5 per pituitary. Following incubation for 48 h, the medium was removed, the cultures were washed twice with phosphate buffer and then incubated with serum free medium plus or minus the effectors to be tested; 8 h later the medium was again changed and fresh serum free medium (250 μl) supplemented with the effectors was added. Conditioned medium was then harvested at the desired time intervals, usually 16–18 h, and assayed. Cells were also collected, washed in phosphate buffer, and lysed in 0.1% Triton X-100.

INTERMEDIATE LOBE: The pars nervosa and pars intermedia were carefully separated from the pars distalis, sectioned longitudinally, and both halves were placed onto lens paper floating on culture medium. The lens papers were incubated at 37°C in individual wells of multiwell plates with 0.3 ml of serum free medium, supplemented with effectors and exposed to an atmosphere of 95% O_2/5% CO_2 saturated with water vapor. After 24–48 h, the organ cultures were transferred to a ^{125}I-fibrin plate containing 300 μl of medium, 10 μg/ml of purified human plasminogen was added and the fibrinolytic activity was determined. To determine the protein content, the explants were washed three times with cold phosphate buffer and dissolved in 0.1 N NaOH for 30 h.

ASSAY OF PLASMINOGEN ACTIVATOR: All assays were performed in triplicate and all values are given as the mean of the three determinations. Multiwell plates coated with ^{125}I-fibrin were prepared as previously described (40); each well contained 10 μg of fibrinogen (1×10^5 cpm/well). Aliquots of incubation media and cell lysates were assayed in 250 μl of 0.1 M Tris HCl, pH 8.1, containing 2 μg purified human plasminogen (6). All experiments included appropriate controls for reagents and media. Plasminogen independent proteolysis was monitored by performing parallel assays in the absence of plasminogen; such plasminogen independent proteolysis never exceeded 1% of the total activity. The PA content of incubation media and cell lysates is expressed in Ploug units, as determined with reference to a standard preparation of urokinase (urokinase reference standard; Leo Pharmaceutical Products, Ballerup, Denmark).

PA production by individual cells was assayed by a casein/agar overlay procedure (44): cells plated in Falcon 60 mm tissue-culture dishes (Falcon Labware) were overlaid with a casein/agar mixture containing plasminogen (40 μg/ml). Development of caseinolytic plaques was observed by dark field illumination.

Detection of plasminogen activator following SDS PAGE was performed according to Granelli-Piperno and Reich (13). To immunoprecipitate the enzyme conditioned medium (containing the equivalent of 40–80 mU urokinase) was first incubated for 16 h at 4°C with either antiurokinase Ig (60 μg) or anti-tissue activator Ig (50 μg); protein A sepharose was then added for 30 min at 22°C to precipitate the enzyme-Ig complexes from which enzyme was eluted in 1% SDS solution.

ANTIBODY PRODUCTION: Antibodies to ACTH were raised in rabbits injected weekly with 1 mg/ml of human ACTH (1–39) (generously provided by Organon) for 3 wk. Antibodies to prolactin and luteinizing hormone (LH) were raised in mice injected with 50 μg of highly purified antigens (generous gift of Dr. D. Burleigh [ICN Pharmaceuticals, Inc.]). The animals were boosted after 3 wk with 10 μg of antigen and antisera were collected after 4 d. Anti-urokinase antibodies were supplied by Dr. D. Belin and anti-tissue activator antibodies were from Dr. W.-D. Schleuning of this laboratory.

IMMUNOFLUORESCENCE: Cells were fixed for 10 min in absolute methanol, washed twice with phosphate buffer, pH 7.4, and incubated with diluted antisera (usually 1:10) at 37°C for 30 min. After three 10-min washes, the first at 37°C and the last two at room temperature, fluorescein conjugated goat anti-rabbit and anti-mouse IgG (diluted 1:100) were added, and a second sequence of incubation and washing was repeated as above.

INCUBATION OF ANTERIOR LOBE CELLS WITH RADIOACTIVE AMINO ACIDS: 1×10^6 anterior lobe cells were plated in 35-mm Falcon tissue culture dishes for 48 h in Dulbecco's medium supplemented with 10% fetal calf serum or plasminogen-depleted fetal calf serum. The cells were then labeled for 10 h with 200 μCi/ml of [^{35}S]methionine (Amersham-Searle, Arlington Heights, IL; 1,000 ci/mmol), in methionine-deficient medium supplemented with appropriate serum. The medium was harvested, the protease inhibitors phenylmethylsulphonyl fluoride, iodoacetamide, pepstatin (1 mM each), Trasylol (100 U/ml) were added, the medium was centrifuged to remove cell debris, and ACTH was immunoprecipitated. To examine the intracellular compartment, we extracted cells in 5 N acetic acid containing 5 mg/ml BSA and the above protease inhibitors. The extracts were frozen and thawed three times, incubated at 4°C overnight, centrifuged to remove insoluble material, and lyophilized.

ACTH IMMUNOPRECIPITATION: ACTH antiserum was purified by affinity chromatography using a sepharose-ACTH (1–24) column as described by Mains and Eipper (24). Immunoprecipitation was carried out as follows: briefly, culture medium and lyophilized samples (resuspended in 50 mM Tris-HCl, pH 7.4) were made 1% in SDS, boiled for 5 min, after which Triton X-100 was added to a final concentration of 5%. The samples were immunoprecipitated for 16 h at 4°C, protein A Sepharose was added, the mixture incubated for 30 min at room temperature and then centrifuged. The pellets were washed three times with NETTS buffer (0.5 M NaCl, 0.2% SDS, 1% Triton X-100, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4) and once with 0.5 M NaCl, 0.1% SDS, 50 mM Tris-HCl, H 7.4. Proteins were eluted by boiling the pellets in electrophoresis sample buffer. Samples were analyzed by electrophoresis in 12.5% SDS polyacrylamide gels according to Laemmli (19), using molecular weight markers from Pharmacia Fine Chemicals (Piscataway, NJ). After electrophoresis the individual lanes of the gel were cut into 2-mm slices, dissolved in 20% H_2O_2 for 16 h at 45°C, mixed with Aquasol, and radioactivity determined in a scintillation counter. Proteins were determined using a Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) developed by Bradford (3).

RESULTS

Anterior Lobe

PRODUCTION AND SECRETION OF PA; CHARACTERIZATION OF PITUITARY PA: In view of the structural and functional complexity of the pituitary, we used separated lobes rather than intact glands as starting material for assessing PA production either in organ culture or, after suitable dispersion to single cell suspensions, in monolayer culture. As seen in Fig. 1, secretion is maintained in primary monolayer cultures for at least 7 d, during which enzyme activity in the condi-

tioned medium continues to increase. This accumulation represents de novo synthesis and not merely release of preformed intracellular stores, since the appearance of PA is blocked by inhibitors of genetic transcription (actinomycin 3 μg/ml) or translation (cycloheximide 5 μg/ml). Similar results have been obtained using anterior lobe explants in organ culture using the method of Ossowski et al. (29).

Two discrete and catalytically efficient plasminogen activators are known to be produced by mammalian tissues, namely, urokinase and the so-called tissue PA, and both of these were identified in gland extracts and in conditioned media from monolayer cultures. The results of zymographic assays (Fig. 2*A*) show the presence of two major PA activities whose electrophoretic mobilities correspond to apparent molecular weights of 80,000 and 48,000, respectively. The activity of the slower-migrating component was selectively and completely blocked by rabbit antisera raised against highly purified, homogeneous human tissue PA, and was thereby identified as the homologous rat enzyme (Fig. 2*B*). The more rapidly migrating 48,000-mol-wt enzyme was identified as rat urokinase by two criteria: its mobility was identical with that of the predominant PA species in rat urine, and catalytic activity was completely blocked by purified immunoglobulins from antisera to pure mouse urokinase (Fig. 2*B*). As in all other known mammalian preparations, both enzymes were irreversibly inactivated by exposure to diisopropylfluorophosphate (1 mM, 40°C, 24 h). The synthesis of both forms of PA was also modulated in response to hormonal effectors.

IDENTIFICATION OF PA-PRODUCING PITUITARY CELLS: The cellular composition of the anterior lobe is complex, reflecting the large number of different hormones produced in this portion of the gland. PA production in the other normal tissues so far analyzed has always been correlated with specific physiological processes or responses, and it was therefore of interest to determine whether pituitary PA activity could be assigned to one or more of the known cell types. To this end, monolayer cultures containing widely dispersed cells

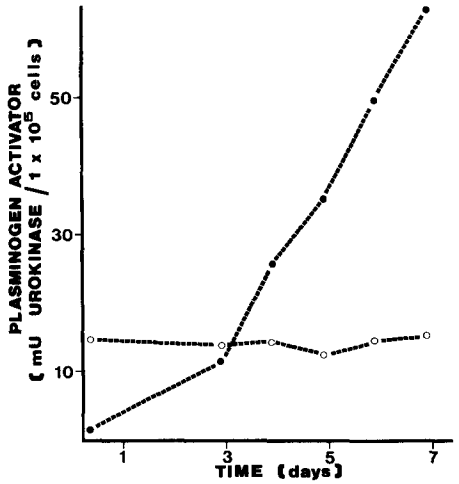

FIGURE 1 Plasminogen activator production by anterior pituitary cells as a function of time in culture. Pituitary cells were plated at a density of 1 × 10^5 cells/well. After 48 h the cultures were washed and 250 μl of medium containing 10% plasminogen depleted acid-treated fetal bovine serum were added to each well. The medium was collected at 24-h intervals and cells from three wells were lysed in 250 μl of 0.1% Triton X-100. Aliquots of culture medium (20 μl) and cell lysate (5 μl) were assayed for plasminogen activator. The results for both intracellular and extracellular enzyme are expressed as mU urokinase per 1 × 10^5 cells (three cultures per group). ●, conditioned medium (accumulated activity at the indicated time); O, cell lysate.

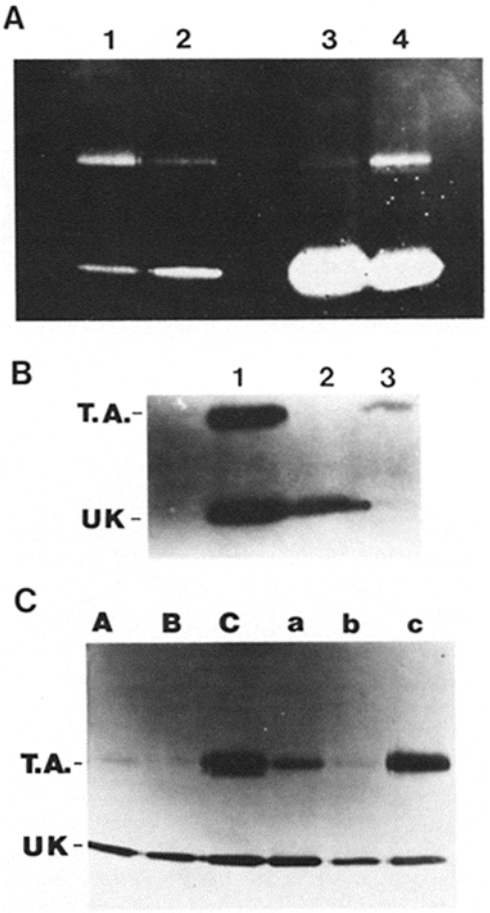

FIGURE 2 (*A*) Plasminogen activators of anterior and intermediate lobes of rat pituitary. Zymographic analysis of conditioned media and cell lysates was performed exactly as described in reference 9. The samples were applied to an 11% SDS polyacrylamide slab gel. After electrophoresis, SDS was extracted, the slab was applied to fibrin-agar indicator gel containing plasminogen, incubating for 12 h at 37°C, and the indicator gel fixed and stained. No lytic zones were observed in the absence of plasminogen. Lane *1*: Cell lysate from 5 × 10^4 anterior lobe cells. Lane *2*: Conditioned medium from 5 × 10^4 anterior lobe cells. Lane *3*: Cell lysate from three intermediate lobes. Lane *4*: Conditioned medium from three intermediate lobes. (*B*) Identification of plasminogen activators of anterior lobe cultures after immunoprecipitation with anti-urokinase or anti-tissue activator antibodies. Aliquots of conditioned medium were immunoprecipitated as described in Materials and Methods. The immunoprecipitates were applied to an 11% SDS polyacrylamide slab gel. After electrophoresis the gel was analyzed for plasminogen activators by layering onto a plasminogen containing fibrin-agar indicator gel. Lane *1*: Control conditioned medium before immunoprecipitation. Lane *2*: Immunoprecipitate with anti-urokinase Ig. Lane *3*: Immunoprecipitate with anti-tissue activator Ig. (C) Identification of plasminogen activators of anterior lobe cultures after treatment with cAMP or hydrocortisone. Aliquots of conditioned media and cell extracts were analyzed on an 11% SDS polyacrylamide slab gel. After electrophoresis the gel was analyzed for plasminogen activators by layering in onto a plasminogen containing fibrinagar indicator gel. *A, a:* Conditioned medium and lysate, respectively, from control cultures. *B, b:* the same from hydrocortisone treated (10^{-7} M) cultures. *C, c:* the same from cultures exposed to cAMP (10^{-3} M).

were prepared and the individual cells assayed for PA production using an agar overlay procedure in which enzyme secretion is easily detected by the appearance of surrounding clear lytic zones (44). After incubation for a period sufficient to generate distinct lytic zones, the culture was fixed, the PA producing zones were marked, the agar overlay was removed, and the cells in separate cultures were then characterized by immunofluorescence using monospecific antisera directed, respectively, against prolactin, LH, and corticotropin (see Fig. 3). From the results in Table I, it can be seen that (*a*) in all cultures, ~35–40% of anterior lobe cells were PA producing; (*b*) PA production was detected in each of the specific populations that were examined: approximately one third of the mammotrophs and gonadotrophs, and about one fifth of the corticotrophs were PA secreting cells. Thus enzyme production was not limited either to a single, or to a minor subpopulation of anterior lobe cells. Because both kinds of PA were observed in zymographic assays of conditioned media, an experiment identical with that in Table I was performed to determine the distribution of cells producing the urokinase and tissue activator forms of PA. In this case, lytic zone production was monitored as usual, but with the agar overlay containing Ig directed against urokinase, tissue activator, both enzymes, or neither. The results were that each IgG alone reduced the diameter, but not the number of lytic zones, whereas the presence of both antibodies eliminated all lytic plaques. It follows that most, and probably all of the PA producing anterior lobe cells are secreting a mixture of the two PA enzymes.

MODULATION OF ANTERIOR LOBE PA PRODUCTION: There are several reasons why it was of interest to study the modulation of pituitary PA synthesis: firstly, PA production in virtually all nonneoplastic cells is modulated by a variety of hormones and hormone-like agents, and it was therefore desirable to compare the previously documented patterns of PA modulation with that of the pituitary, itself a distinctly hormone-responsive tissue. Secondly, the anterior pituitary, like the endocrine pancreas, is specialized for the production of polypeptide hormones, and PA production in the islets of Langerhans is closely correlated with β-cell responses to glucose and other effectors that are known to modulate insulin production (46). Hence, it seemed worth exploring the extent to which the regulatory patterns of pituitary PA synthesis resembled that of the pancreatic islets.

It is well established from the work of several laboratories that some pituitary hormone production is modulated by changes in intracellular cAMP concentrations (23, 31), and the data in Fig. 4 show that pituitary PA secretion is strongly stimulated by the addition of dbcAMP to the culture medium. The cAMP phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine produced a similar effect, as did cholera toxin, an agent that stimulates adenyl cyclase activity; its action was maximal at the low concentration of 10^{-11} M. The stimulation of PA secretion both by dbcAMP and cholera toxin was completely blocked in the presence of actinomycin (3 μg/ml)

TABLE I

Single Cell Assay for Plasminogen Activator and Hormone Production

Antiserum	PA^+, H^-	PA^+, H^+	PA^-, H^+	PA^-, H^-
Prolactin	23.7	15.0	28.4	32.9
LH	29.9	10.7	19.2	40.0
ACTH	33.3	1.8	8.4	56.7

Anterior pituitary cells were plated at a density of 1×10^4 cells in 60-mm tissue culture dishes. After 24–48 h the cultures were washed and overlaid with a casein-agar mixture containing plasminogen, incubated at 37°C for 5 h and then fixed (Materials and Methods). Lytic zones were identified by dark-field microscopy, marked, the casein-agar overlay was removed, and the plates washed twice with phosphate buffer and processed for immunofluorescence. Separate, parallel cultures were analyzed using, respectively antisera to prolactin, LH, and ACTH antisera. The results are expressed as the percent of cells that were positive for plasminogen activator and negative (not fluorescent) for the hormone production (PA^+, H^-); positive for both plasminogen activator and the hormone (PA^+, H^+); negative for plasminogen activator and positive for the hormone (PA^-, H^+); or negative for both plasminogen activator and the hormone (PA^-, H^-). The values given are the means of four separate experiments.

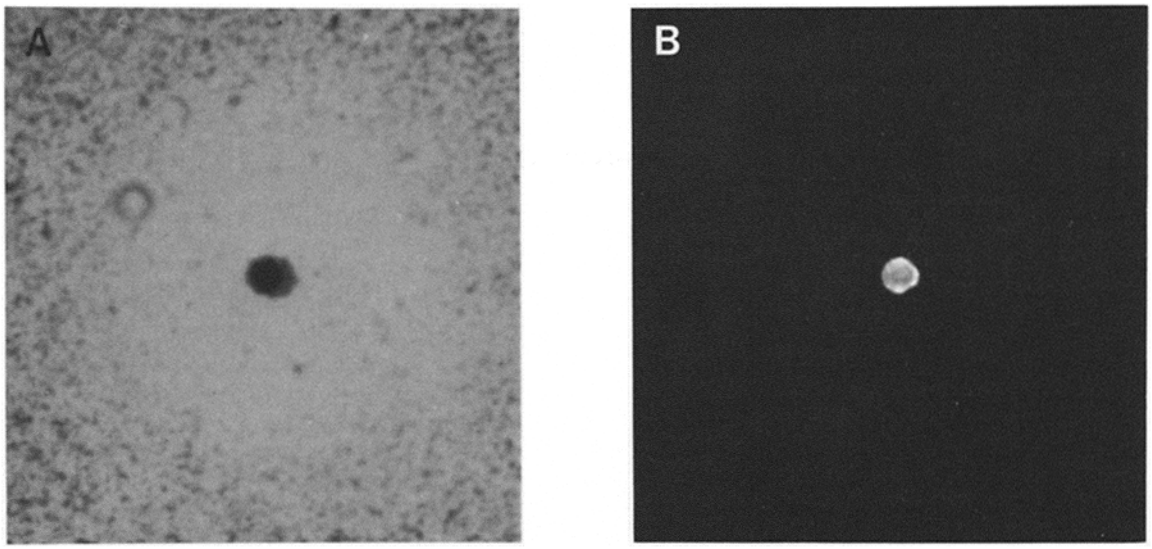

FIGURE 3 Single cell assay for plasminogen activator and hormone production. Anterior pituitary cells were plated at a density of 1×10^4 cells in 60mm tissue culture dishes. After 24–28 h, the cultures were washed and overlaid with a casein-agar mixture containing plasminogen, incubated at 37°C for 5 h and then fixed. Lytic zones were identified by dark-field microscopy, marked, the casein-agar overlay was removed, and the plates were processed for immunofluorescence using anti-prolactin antiserum. (*A*) Cell surrounded by lytic area. (*B*) The same cell stained with anti-prolactin. (Indirect immunofluorescence was performed as described in Materials and Methods.) Approximately × 50.

or cycloheximide (5 μg/ml), indicating that this process required genetic transcription and translation. The cyclic nucleotide mediated induction of PA secretion therefore represents do novo synthesis, and not simply the discharge of intracellular enzyme.

The anterior lobe responds to a variety of hormonal stimuli by changing polypeptide hormone production; hence, if PA synthesis was somehow coordinated with hormone formation, changes in enzyme production might also be induced by physiological regulators of pituitary function. As seen in Fig. 5, hydrocortisone, which suppresses pituitary ACTH production, also progressively reduced PA synthesis throughout a physiological range of concentrations; the mineralocorticoid aldosterone was somewhat less effective. Likewise, the androgen testosterone repressed PA synthesis, but only at pharmacological levels, whereas β-estradiol and progesterone at physiological concentrations showed no effect. The inhibitory actions of testosterone and hydrocortisone were additive, the two steroids together blocking enzyme production almost completely, an effect that presumably results from the two hormones acting on a mixture of different cell types. There are two further points of interest concerning the modulation of anterior lobe PA: the first is that the inhibitory effect of hydrocortisone was dominant over that of cAMP, since cultures pretreated with glucocorticoid showed no stimulation of PA synthesis in response to dbcAMP. The second concerns the differential response of the two PA species to modulation: cAMP predominantly stimulated the production of the tissue activator form, whereas hydrocortisone repressed the synthesis of both (Fig. 2*C*).

We also tested the effect of a variety of agents on PA production—some of these are significant for pituitary physiology, whereas others had previously been shown to modulate enzyme synthesis by other cell types. In the concentration range 10 ng–10 μg/ml, the following compounds had no effect on anterior lobe PA production: vasopressin, epinephrine, apomorphine, highly purified rat-follicle-stimulating hormone, human ACTH, ovine prolactin, bovine insulin, synthetic α-MSH, tyrosine, and the hypothalamic releasing factors luteinizing-hormone-releasing hormone and thyrotropic-releasing hormone. Also without effect were the same concentrations of retinoids, concanavalin A, and phorbol ester, all

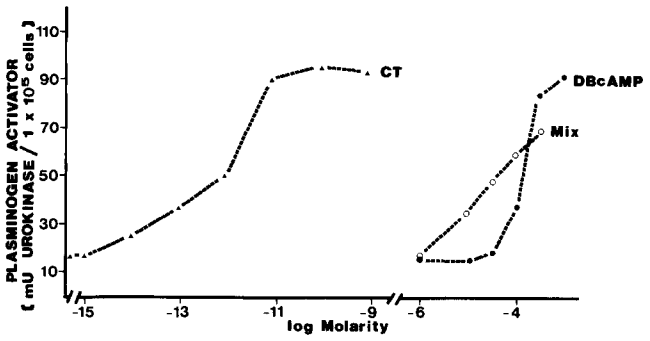

FIGURE 4 Effect of cyclic nucleotide and related compounds on plasminogen activator production in anterior pituitary cultures: dose response curves. Anterior cells were plated at a density of 1 × 10^5 cells per well and exposed to the various agents as described in Materials and Methods. Aliquots (20 μl) of culture media were assayed for PA and the results are expressed as mU urokinase in the medium per 1 × 10^5 cells. Each point represents the average of the values obtained from two parallel cultures. ▲, cholera toxin; ●, dbcAMP; O, 1-methyl-3-isobutylxanthine.

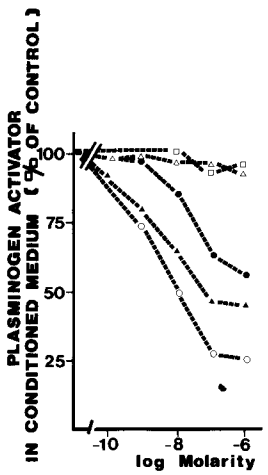

FIGURE 5 Effect of steroids on plasminogen activator production in anterior pituitary cultures: dose response curves. Anterior pituitary cells were plated (1 × 10^5 cells per well) and exposed to the indicated steroids as described in Materials and Methods. Aliquots (20 μl) of conditioned media were assayed for PA and the results expressed as the percentage of the enzyme level present in the medium of control, hormone-free cultures. The level of enzyme in conditioned medium from control cultures (100%) was 55 mU urokinase/10^5 cells. ●, aldosterone; ▲, hydrocortisone; O, dihydrotestosterone; △, estradiol; □, progesterone.

of which strongly induce PA production either in chick fibroblasts and mouse teratoma cells (41, 47, 48), or macrophages and leukocytes, respectively (14, 45).

FORMS OF ACTH PRODUCED BY THE ANTERIOR LOBE: It is known that several forms of ACTH are normally present in the mammalian circulation (49, 50)—these include the predominant, small form of M_r 4,500, in addition to larger species of M_r 13,000 (a glycosylated version of the M_r 4,500 species), M_r 23,000 and 31,000, the latter being considered as precursor molecules subject to proteolytic processing (9, 35). All of these molecular weight species are present within pituitary cells, but it has not been proven that the fully processed intracellular forms (i.e., 4,500 and 13,000) are released in response to physiological stimuli and are the precursors to the circulating extracellular forms. It is, however, also clear from studies of cultured pituitary tumor cell lines that intracellular corticotropin (4,500) is derived from a larger molecule (31,000 mol wt) that carries antigenic determinants both for β-lipotropin and ACTH (9, 35). This same large precursor is secreted in substantial amounts by cultured pituitary cells (9), a finding that is in accord with similar observations on gastrin (49), LH (25), and insulin (37), where, in each case, the prohormone form accounts for a substantial fraction of the total secretion product. The fact that large precursors are secreted in all of these systems suggests that some prohormone conversion may occur extracellularly, a possibility previously envisaged for insulin production in the islets of Langerhans (46). These considerations prompted us to examine the spectrum of ACTH molecules synthesized in the presence and absence of plasminogen.

Pituitary cultures were maintained in media supplemented either with regular serum or with serum depleted of plasminogen. After suitable labeling with [^{35}S]methionine, the cells and medium were analysed separately and the immunoreactive ACTH molecules were harvested by precipitation and analysed by SDS PAGE. As seen in Fig. 6, cell extracts from cultures maintained in plasminogen depleted media contained all four species of ACTH molecules (31,000, 23,000, 13,000, and 4,500 mol wt), whereas only the two largest—31,000 and 23,000 mol wt—were present in the extracellular phase. Cultures maintained in plasminogen containing serum gave an identical pattern for the intracellular hormone. However, in the culture medium there was a substantial reduction of the large ACTH species—31,000 and 23,000 mol wt—

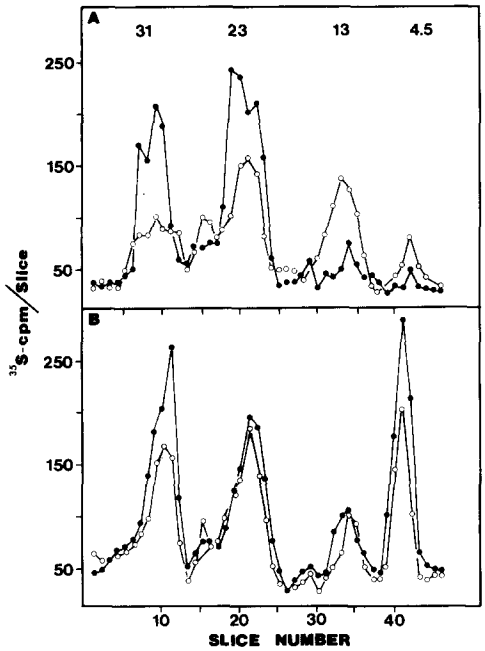

FIGURE 6 Conditioned medium and cell extracts from anterior lobe cultures: immunoprecipitation of corticotropin. Anterior lobe cells were plated at a density of 1×10^6 cells per well and incubated for 48 h in medium supplemented with 5% of either native or plasminogen depleted fetal bovine serum. The cultures were then labeled for 10 h in medium containing [^{35}S]methionine (200 μCi/ml). The conditioned media and cell extracts were incubated with antiserum to ACTH and the immunoprecipitates analyzed on SDS polyacrylamide gels. After the completion of electrophoresis the individual gel lanes were sliced and the slices assayed for radioactivity. (*A*) Immunoreactive corticotropin molecules precipitated from conditioned medium of cultures incubated with native (○) or plasminogen depleted (●) fetal bovine serum. (*B*) Immunoreactive corticotropin molecules precipitated from cell extracts of cultures incubated with native (○) or plasminogen depleted (●) fetal bovine serum. Molecular weights ($\times 10^{-3}$) in top of *A*.

accompanied by an increase in the fraction of smaller molecules at 13,000 and 4,500 mol wt.

Comparable experiments performed with a mouse pituitary tumor cell line gave identical results for extracellular hormone (not shown)—namely, that in the absence of plasminogen, only the 31,000- and 23,000-mol-wt forms were routinely detectable.

Direct evidence that plasmin, generated in the extracellular medium, was capable of converting the precursor molecules to mature, small ACTH-like species was obtained from the experiment whose results are summarized in Table II. Here, anterior pituitary cultures were first labeled by incubation with [^{35}S]methionine in the absence of plasminogen. The isolated conditioned media were supplemented with 50 μg/ml plasminogen and then incubated further and the ratio of radioactivity in the residual ACTH precursor molecules to that in small ACTH products was determined after different times of incubation: the precursor compartment decreased and radioactivity in ACTH accumulated progressively as a function of time, the ratio changing by a factor of 4.7. The high recovery of the total radioactivity in the two compartments—>90% in every case—is noteworthy. It is clear from the time course of the reaction that the conversion to ACTH slows considerably after 40 min of incubation. It is known that plasmin is autolytic, that plasmin attacks plasminogen without activating it, and, in certain systems, also destroys PA by proteolysis (32, 43). Any of the preceding reactions could explain the apparent slowing of the reaction, as could the reduction in precursor concentration resulting from the observed conversion, but we have so far not performed any experiments to explore which of these or other alternative mechanisms might be operating.

PA Production by Intermediate Lobe

It is known that the intermediate lobe synthesizes large ACTH precursors and their partial degradation products—4,500-mol-wt ACTH, and β-lipotropin and α-MSH. Hence, if anterior lobe PA synthesis bore some physiological relationship to ACTH production, the intermediate lobe might also be expected to form PA. To explore PA production by the intermediate lobe, explants of the portion of the gland were cultured in wells coated with ^{125}I-fibrin and the rate of plasminogen-dependent solubilization of radioactivity was measured. The results in Fig. 7*A* show that the intermediate lobes produced significant amounts of PA. As in the anterior lobe, both forms of PA were produced (Fig. 2*A*), and enzyme synthesis was both strongly repressed by hydrocortisone and stimulated by dbcAMP. Because the intermediate lobe of lower vertebrates is subject to dopaminergic neural control (21), we also tested the effect of the dopamine antagonist apomorphine; this agent also strongly inhibited intermediate lobe enzyme production (Fig. 7*B*) and it is of interest that apomorphine did not affect PA synthesis in anterior lobe cultures. This differential response to apomorphine provides good incidental evidence for the absence of cross contamination of anterior and intermediate lobe cultures, respectively.

DISCUSSION

The presence of PA in pituitary tissue was previously reported by Astrup (2), who assigned the enzyme exclusively to the vascular endothelium. Without excluding some, presumably minor, contributions from the endothelium, our results leave no doubt that PA is produced by the glandular cells of both the anterior and intermediate lobes. Further, PA synthesis in pituitary tissue is modulated in response to a variety of physiological stimuli just as it is in most normal tissues, including other endocrines.

Anterior lobe PA activity reflects the secretion of the two plasminogen activating enzymes, namely, urokinase and the

TABLE II

Effect of Plasminogen on the Processing of ACTH Precursor

Time	0	10′	20′	40′	60′
% Precursor	81	73	69	61	58
% ACTH	10	19	21	31	34
Ratio:Precursor/Product	8.1	3.8	3.3	1.96	1.70

Anterior lobe cells were plated at a density of 1×10^6 cells/ml and incubated for 48 h in medium supplemented with 5% plasminogen-depleted fetal bovine serum. The cultures were then washed and labeled for 10 h in medium containing [^{35}S]methionine (200 μCi/ml). The conditioned media were collected, freed of cells, and aliquots were then incubated at 37°C for different times with 50 μg/ml plasminogen. Samples were immunoprecipitated with an ACTH antiserum and the immunoprecipitates analysed on SDS PAGE. The individual gel lanes were sliced and the slices assayed for radioactivity. Results are expressed as percentage of the total radioactivity recovered in the respective ACTH components in each gel lane (~9,000 cpm were applied to each lane). The ACTH precursor is here defined as the total of the 30,000- and 23,000-mol-wt components, while ACTH represents the total in the 13,000- and 4,500-mol-wt species.

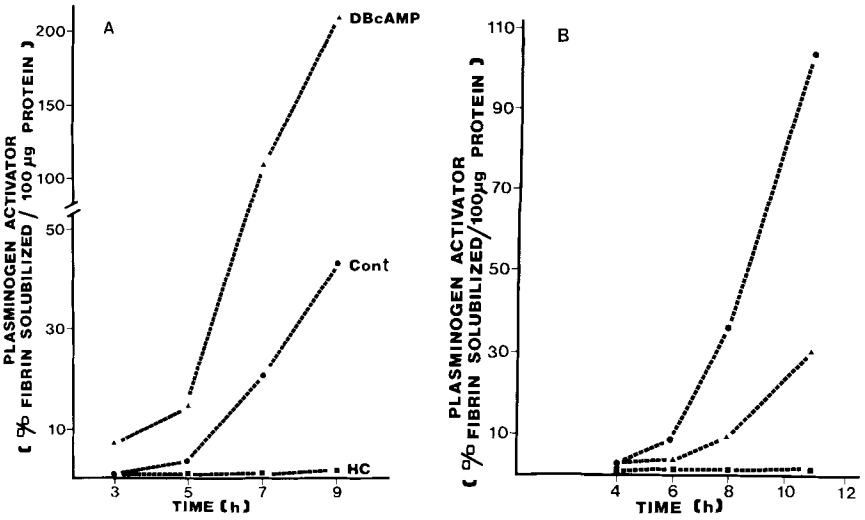

FIGURE 7 Plasminogen activator production in organ cultures of pituitary intermediate lobes. Intermediate lobes, carefully freed of anterior and posterior lobe tissue, were sectioned longitudinally and applied to a circle of lens paper that was floated on the surface of 300 µl of medium in a well of a Linbro multiwell plate. After 24 h of incubation the explants were transferred to a second well that was coated with ^{125}I-fibrin and contained 300 µl of medium supplemented with 10 µg/ml highly purified human plasminogen in addition to the effectors to be tested. Aliquots of medium were removed at the indicated times and assayed for solubilized radioactivity. Four separate explants were exposed to each treatment in parallel and the results averaged. The protein content of each group of four explants was determined and the amount of fibrin degraded at each time point is expressed as a percentage of the total fibrin originally present in the well. Similar experiments were performed on four separate occasions, and the results in all cases were qualitatively identical with those presented in the figure. (*A*) Effects of hydrocortisone and dbcAMP. ●, control, no addition; ▲, dbcAMP, 10^{-3} M; ■, hydrocortisone 10^{-7} M. (*B*) ●, control, no addition; ▲, apomorphine 10^{-6} M; ■, apomorphine 10^{-5} M.

so-called tissue activator, and the synthesis of both forms is modulated in response to stimuli that can be considered physiological. Since most PA-producing cells are in fact secreting both enzymes concurrently, it is noteworthy that their secretion rates can be modulated differentially in response to at least one effector, hydrocortisone.

Although the available data are insufficient to permit any precise interpretations, the patterns of pituitary PA modulation by hormones raise many intriguing questions that provide targets for future work. For example, the large increase in PA production induced by increased cAMP levels is unlikely to be mediated by the response of a single cell type. If that is so, it implies that at least several pituitary cell types are probably targets for polypeptide or other hormones that activate adenyl cyclase. The results obtained to date already exclude posterior pituitary hormones, epinephrine and at least two releasing factors as candidates for this role, and the identification of the putative hormones thus presents a challenge for the future. Further, the inhibition of PA production by hydrocortisone, both alone and in combination with testosterone, must involve many, and perhaps all cell types of the anterior lobe. This would suggest that the spectrum of anterior lobe functions which can be modulated by these steroids may be broader than could be deduced using physiological evidence obtained from intact animals. Pituitary cell cultures exposed to various hormone treatments and analysed by the combination of immunofluorescence and lytic zone techniques as described above can be used as a convenient assay-system for testing these and other hypotheses, because they permit each subpopulation to be assayed for changes in PA production, if a suitably specific antiserum is available for the hormone in question.

One result that deserves comment is the finding (Table I) that only a fraction of each hormone-specific cell population also produced PA; for the three hormones studied the proportion of PA-positive cells ranging from 35% for prolactin and LH to 18% for ACTH. We suggest that, at least under conditions of submaximal stimulation, the cells of each hormone-specific population may be physiologically heterogeneous with respect to the rate of hormone synthesis and secretion. This is analogous with the thyroid where it is well established from pulse-labeling data that only a minor fraction of the follicular cells in euthyroid glands is active in hormone synthesis at any instant (20). To explain our observations on pituitary cultures, we propose a working hypothesis that embodies some features of hormone synthesis found in the thyroid and in pancreatic islets: we suggest that the three pituitary cell types we studied are functioning under conditions of submaximal stimulation and that each can be imagined to consist of two subpopulations, a "active" fraction in which peptide products are directed along a pathway that leads to secretion, and an "idling" fraction in which newly formed peptides are being channelled to intracellular sites for storage and degradation. Since the effects of cAMP and hydrocortisone on pituitary PA and hormone synthesis (1, 10, 31) are qualitatively identical, we suggest that PA and hormone secretion occur in parallel, just as they do in pancreatic islets exposed to glucose (46); we therefore propose that PA production is a property of the "active" cell population, and that it can serve as an indirect measure on a marker for hormone secretion.

If the endocrine cells do indeed exist as "active" and "idling" subpopulations, both of which are synthesizing hormone precursors, there would seem to be two possible mechanisms for managing the intracellular traffic of hormone-related products: in the first, the peptides would be directed to an organelle, such as a secretion granule, where they could be held in reserve until mobilized in response to a secretory

stimulus. The existence of this "classical" pathway is well documented, having been demonstrated in the exocrine pancreas (39). If this type of pathway predominated in the processing of pituitary peptides, the profile of secreted, extracellular hormonal peptides would necessarily be identical with, or closely resemble, those in the main intracellular pool(s). Some of our results are not consistent with the pancreatic exocrine model and suggest a possible alternative. Specifically, the profiles of ACTH-related molecules in the intra- and extracellular compartments were different when pituitary cultures were labeled in the absence of plasminogen (Fig. 6): the smaller, mature species—4,500 and 13,000—mol wt were present within the cells (presumably the "idling" fraction), but, in plasminogen-free media, only the large forms were extracellular. This result indicates that the extracellular molecules could not have been drawn from the same pool as the bulk of intracellular species; they must therefore have followed a pathway to secretion that by-passed the intracellular sites where the small ACTH peptides are produced and stored. With these data in mind, we suggest that newly formed hormone precursors can be directed along one of two intracellular pathways, the first leading directly to secretion, and the second leading to intracellular sites of storage and degradation. Our results do not exclude the possibility that some of the latter pool could be secreted in response to hyperstimulation. A similar model has been suggested by the data of Track et al. (42) to account for the hormone labeling patterns he observed in pancreatic islets. Our hypotheses, while consistent with all of our data, should be regarded as tentative; they also suggest a variety of experimental tests that will be the subject of future work.

What is the role of PA secretion in the pituitary? Our data are still insufficient to support any firm conclusions. Despite these reservations, it is probably significant, firstly, that PA and ACTH are modulated in parallel in response to increased cyclic nucleotide levels and to hydrocortisone, and, secondly, that plasmin, generated by the cellular secretion of PA, can convert some secreted "big" ACTH to the smaller 13,000- and 4,500-mol-wt forms. These findings are consistent with the suggestion that some prohormone conversion may be catalysed, at least in part, by plasmin, a possibility previously envisioned also for the processing of proinsulin by the islets of Langerhans (46). In the latter tissue granuli-associated thiol proteinases have also been reported to be capable of catalysing the proinsulin-insulin conversion (7, 11). In view of their acidic pH optima it is difficult to exclude the possibility that these enzymes may be of lysosomal origin, and perhaps involved in the metabolic turnover of intracellular (pro)hormone.

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