Immunocytochemical Demonstration of Tissue-type Plasminogen Activator in Endocrine Cells of the Rat Pituitary Gland

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ABSTRACT We immunocytochemically stained rat pituitary glands using antibodies against plasminogen activators of the tissue type (t-PA) and the urokinase type (u-PA). A large population of endocrine cells in the anterior lobe of the gland displayed intense cytoplasmic immunoreactivity with anti-t-PA. In some areas of the intermediate lobe we found a weak staining, and we observed weakly staining granular structures in the posterior lobe. Controls included absorption of the antibodies with highly purified t-PA. In addition, SDS PAGE followed by immunoblotting of pituitary gland extracts revealed only one band with an electrophoretic mobility similar to that of t-PA when stained with anti-t-PA IgG. No u-PA immunoreactivity was detected in the rat pituitary gland. Sequential staining experiments using antibodies against growth hormone and t-PA demonstrated that the t-PA–immunoreactive cells constitute a large subpopulation of the growth hormone–containing cells. These findings represent the first direct evidence for the presence of t-PA in cell types other than endothelial cells in the intact normal organism. In this article we discuss the implications of the results for a possible role of t-PA in the posttranslational processing of prohormones.

Plasminogen activators are serine proteases, able to convert the proenzyme plasminogen into the active protease plasmin. It is well documented that there are at least two types of mammalian plasminogen activators. The two types that can be distinguished by differences in molecular weight (M_r) and immunological reactivity (for reviews see references 1–4) are products of different genes (5–8). Both the urokinase-type plasminogen activator (u-PA),[1] $M_r \sim 50{,}000$, and the tissue-type plasminogen activator (t-PA), $M_r \sim 70{,}000$, are secreted from cells in culture as partly or completely inactive proenzymes (9–12), and it is generally believed that they exert their biological functions by activation of extracellular plasminogen.

Production and release of plasminogen activators have been implicated in thrombolysis (for reviews see references 2 and 3) and a variety of biological processes involving tissue degradation (for reviews see references 4 and 13), for example the postlactational involution of the mammary gland (14), ovulation (15), implantation of the fertilized ovum (16, 17), inflammation (18), and cancer (4, 13, 19–21). A role of plasminogen activators in the processing of prohormones has also been proposed (22–24). u-PA seems to be the type of plasminogen activator involved in tissue degradation (for review see reference 4), whereas t-PA until now has been linked primarily with thrombolysis (3).

Recent immunocytochemical studies have shown that u-PA immunoreactivity is widely distributed in the normal mouse. Most notably, u-PA was found in widely disseminated connective tissue cells with a fibroblast-like morphology that occurred in high numbers in the lamina propria of the gastrointestinal tract and to a lesser extent in a number of other organs. u-PA immunoreactivity was also found in the epithelial cells of the proximal and distal kidney tubules and the ductus deferens, pulmonary pneumocytes, decidual cells of the placenta, and epithelial cells of involuting mammary glands, whereas no immunoreactivity was found in endothelial cells (25). Strong u-PA immunoreactivity has also been

[1] *Abbreviations used in this paper:* TBS, 0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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demonstrated in invasively growing areas of the murine Lewis lung carcinoma (21).

t-PA immunoreactivity has been demonstrated in endothelial cells of veins and other blood vessels of several human tissues (26), but no systematic immunocytochemical studies on the occurrence of t-PA in the intact organism have been reported. We now report that t-PA immunoreactivity is present in endocrine cells of intact rat pituitary glands and that these cells constitute a large subpopulation of the cells that contain growth hormone immunoreactivity.

MATERIALS AND METHODS

Materials: We obtained the following materials from the indicated sources: protein-A Sepharose and cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden); swine IgG anti-rabbit immunoglobulins, rabbit peroxidase-anti-peroxidase complexes and peroxidase-coupled swine IgG anti-rabbit immunoglobulins (Dakopatts, Copenhagen); peroxidase-coupled protein A (Amersham International, Amersham, U.K.); *o*-phenylenediamine and Tween 20 (Merck A.G. Darmstadt); 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO); Immunoplate I (NUNC, Roskilde, Denmark); bovine serum albumin (BSA; Behring, Warburg, Federal Republic of Germany); and Millipore nitrocellulose sheets GSWP 00010 (Millipore, Bedford, MA). All other materials were those described previously (9, 10, 12, 21, 25–31) or of the best commercially available grade.

Animals: Male (unless otherwise stated) Wistar rats, 300–350 g, were anesthetized with diethylether and fixed by intracardiac perfusion with ~60 ml cold (4°C) 0.01 M sodium phosphate, pH 7.4, with 0.14 M NaCl (PBS) followed by ~60 ml cold (4°C) 1% (wt/vol) paraformaldehyde solution in 10 mM sodium phosphate buffer, pH 7.4. Alternatively, tissue was removed from diethylether-anesthetized animals without any preceding perfusion and immediately frozen in isopentane on dry ice. Tissue used for zymographic or immunoblotting analysis was removed from animals perfused with ~60 ml of PBS after diethylether anesthesia.

Tissue Treatment: For immunocytochemistry, pituitary glands were dissected from animals perfused with paraformaldehyde and postfixed for 14–16 h at 4°C, then rinsed for 4–6 h in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% (wt/vol) sucrose. Pituitary glands were then frozen in melting Freon 13 and cryostat sections (6–8 μm) were cut at −20°C and collected on chrome alum-gelatine-coated slides.

For zymographic and immunoblotting analysis freshly PBS-perfused pituitary glands were washed with PBS, blotted dry on filter paper, weighed, minced, and homogenized with 75 mM potassium acetate buffer, pH 4.2, containing 0.3 M sodium chloride, 0.1 M L-arginine, 10 mM EDTA, and 0.25% (wt/vol) Triton X-100 (32), 10 μl/mg wet weight. The extracts were centrifuged (16,000 *g*) at 4°C for 10 min and the supernatants were stored frozen (−20°C) until analysis.

Enzyme-linked Immunosorbent Assay: Immunoplates were coated overnight at 37°C with 2 μg/ml of antigen or BSA in 0.1 M Na_2CO_3, pH 9.8, 100 μl/well. The plates were washed and the remaining protein-binding sites were blocked by incubation with 200 μl 0.25% (wt/vol) BSA in PBS. The primary antibody under investigation was added (100 μl/well). Usually a fivefold serial dilution, made with PBS containing 0.1% (wt/vol) Tween 20 (PBS-Tween) starting from 10 μg/ml, was used. The plate was washed again and incubated with peroxidase-linked swine IgG anti-rabbit immunoglobulins, diluted 1:800 in PBS-Tween, 100 μl/well. After washing was done with PBS-Tween and once with distilled H_2O, the peroxidase reaction was performed for 5 min with 0.1% *o*-phenylenediamine, 0.01% H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.0, 100 μl/well. The reaction was stopped by adding 100 μl 1 M H_2SO_4, and the absorbance was read at 492 nm with a Dynatech Microelisa Minireader (Dynatech, Alexandria, VA). Incubation with first and second antibody layer was performed at 37°C for 1 h while shaking. All other procedures were done at room temperature. All washings were performed four times with PBS-Tween unless otherwise stated. Controls included wells coated with BSA to exclude nonspecific binding of the first antibody layer, and the absorbance readings of wells without the first antibody layer were subtracted.

Antibodies: Rabbit antibodies against human t-PA were produced by immunization of rabbits with t-PA purified by affinity chromatography using a monoclonal antibody as described (26, 31). Preimmune and immune IgG were purified by affinity chromatography on protein A-Sepharose (31), and the IgG concentration was determined by absorbance measurement at 280 nm using an extinction coefficient $E^{1\%,1cm}_{280nm}$ of 13.7. The enzyme-linked immunosorbent assay showed that the rabbit antibodies raised against human t-PA reacted with the mouse monoclonal anti-t-PA IgG that had been used for purification of the human t-PA. We found no reaction when we used preimmune IgG from the same rabbit. The presence of these contaminating antibodies was therefore probably due to trace amounts of monoclonal antibody leaking from the affinity chromatography column during the elution of human t-PA. The rabbit anti-t-PA antibodies used in this study were therefore absorbed by passing 2.2 mg anti-t-PA IgG through a PBS-equilibrated column (2 ml) with ~2 mg monoclonal antibody coupled to cyanogen bromide-activated Sepharose. The rabbit anti-human t-PA IgG did not cross-react with human u-PA (26), but various findings (see Results) indicated that they cross-reacted with rat t-PA. Rabbit IgG antibodies against highly purified mouse u-PA were produced, purified, and absorbed with a glutaraldehyde polymer of mouse proteins depleted of u-PA as described (25, 28). We previously reported that the rabbit anti-mouse u-PA did not cross-react with mouse t-PA, whereas it cross-reacted with rat u-PA (25). Swine IgG anti-rabbit immunoglobulin was absorbed by incubating 1 ml of the IgG preparation with 50 μl rat serum for 20 h at 4°C, which was then centrifuged (100,000 *g*) at 4°C for 60 min.

For the sequential staining experiments rabbit antibodies directed against human growth hormone (NIADDK-anti-hGH-IC-2) and ACTH (NIADDK-anti-hACTH-IC-1) were kindly donated by the National Hormone and Pituitary Program of the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Bethesda, MD.

Immunocytochemistry: Cryostat sections of fixed tissue were washed with 0.05 M Tris-HCl, pH 7.4, with 0.15 M NaCl (TBS) containing 1% (wt/vol) Triton X-100 (TBS-Triton) for 15 min, incubated with 1% (wt/vol) human serum albumin in TBS for 30 min, briefly rinsed in TBS-Triton, and incubated with different concentrations of antibodies. We found optimal staining with concentrations of 4–10 μg IgG/ml when an 18 h incubation at 4°C was used followed by a 1 h re-equilibration at room temperature. The site of the antigen-antibody reaction was demonstrated by sequential incubation with swine IgG anti-rabbit immunoglobulin (diluted 1:30), horseradish peroxidase-coupled protein A (diluted 1:200), and rabbit peroxidase-antiperoxidase complexes (diluted 1:70), each for 30 min at room temperature, with intense washing with TBS-Triton between each incubation period. All antibody dilutions were performed with TBS containing 0.25% BSA. Peroxidase activity was demonstrated with diaminobenzidine-H_2O_2 (29) and sections were lightly counterstained with haematoxylin.

Controls were as recommended by Sternberger (33) and included (*a*) omission of first or second layer of antibodies or omission of peroxidase-coupled protein A and peroxidase-antiperoxidase complexes, (*b*) substitution of primary antibodies by preimmune IgG from the same rabbit, and (*c*) absorption of the antibodies with the corresponding antigen. Absorption with t-PA and u-PA was performed by passing the IgG preparations twice through Sepharose columns (1 ml) coupled with highly purified preparations of human t-PA (see reference 10) and mouse u-PA (see reference 27), respectively. As a control, aliquots of the respective IgG preparations were passed twice through similar columns coupled with the same amount of BSA. All columns were equilibrated with TBS and developed by gravity. Enzyme-linked immunosorbent analysis with human t-PA as antigen demonstrated that the concentration of t-PA-absorbed anti-t-PA needed to obtain identical absorbance readings was ~3,000-fold higher than that needed of nonabsorbed anti-t-PA IgG.

For the sequential staining experiments, cryostat sections were initially stained for t-PA as described above, except that the peroxidase activity was demonstrated with 3-amino-9-ethylcarbazole (29). After photography the peroxidase reaction product was eluted with ethanol, and antibodies bound to the sections were removed by oxidation as described (30, 34). Thereafter incubation of each section with swine IgG anti-rabbit immunoglobulin and rabbit peroxidase-antiperoxidase complexes was followed by development with 3-amino-9-ethylcarbazole to ensure the efficiency of the antibody elution. Sections completely negative after this development were then processed for staining with rabbit anti-human growth hormone or rabbit anti-human ACTH, diluted 1/100–1/250 using a 30-min incubation at room temperature. This was followed by incubation with swine IgG anti-rabbit immunoglobulins, and rabbit peroxidase-antiperoxidase complexes and the sections were developed with diaminobenzidine-H_2O_2 as described above.

Electophoresis and Immunoblotting: SDS PAGE and electrophoretic transfer of the proteins to nitrocellulose paper were performed as described (25). The nitrocellulose paper was air dried and fixed in 1% (wt/vol) paraformaldehyde in 10 mM sodium phosphate buffer, pH 7.4, for 1 h at 4°C. Immunocytochemical staining of the nitrocellulose replicas was performed identically to the staining of tissue sections, except that the washing with TBS-Triton was done at 37°C. We performed zymographic analysis for plasminogen activators of tissue extracts separated by SDS PAGE as described (27, 35), by layering the polyacrylamide gel on a fibrin-agarose gel with or without plasminogen. Marker proteins were as described (9, 10).

RESULTS

Immunocytochemical Findings

Immunocytochemical staining of rat pituitary glands with anti-t-PA revealed immunoreactivity in a number of endocrine cells in the anterior lobe of the rat pituitary gland (Fig. 1). Some of the cells were intensively stained, and others stained more weakly. Some endocrine cells of the anterior lobe contained no detectable immunoreactive material. The t-PA-immunoreactive material was located solely in the cytoplasm of the cells, often with a distinct granular appearance (Fig. 2). In some areas of the pars intermedia of rat pituitary gland we found a weak staining (result not shown), and in other areas we observed no immunoreactivity (Fig. 1). The posterior lobe contained many small areas, with apparently granular weak immunoreactivity (Fig. 1). To evaluate the possibility that the histological location of t-PA was somewhat altered during the perfusion fixation, rat pituitary glands were excised and frozen in isopentane on dry ice. Sections were thawed in 1% (wt/vol) paraformaldehyde in 10 mM sodium

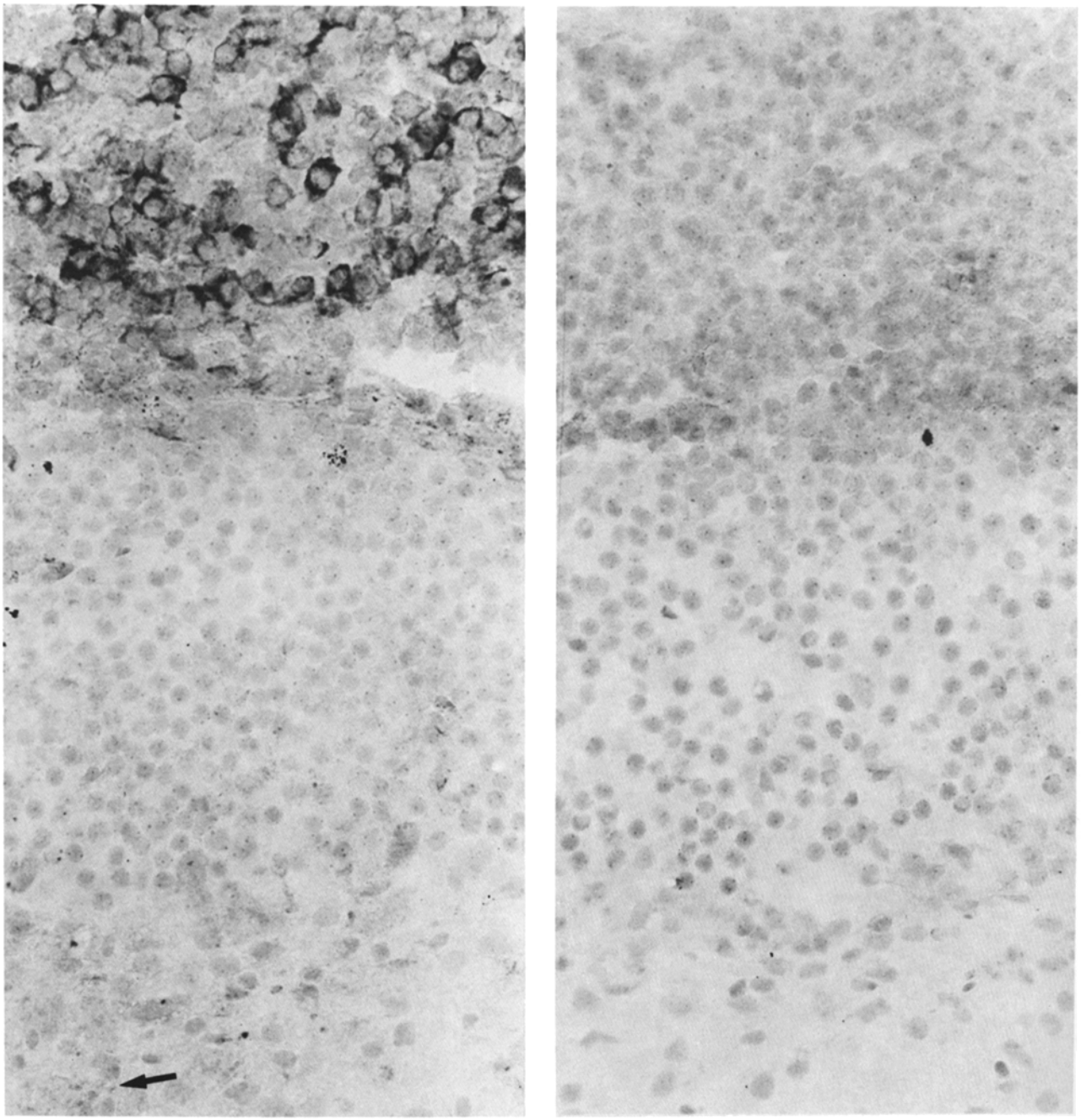

FIGURE 1 Rat pituitary gland stained with antiserum to t-PA. Sections were incubated with rabbit anti-human t-PA IgG absorbed with Sepharose-coupled BSA (*left*) or purified human t-PA (*right*). Note the presence of a number of strongly stained endocrine cells in the anterior lobe (*left, top*) and the lack of cells stained in this section of the intermediate lobe (*left, center*). In all sections analyzed no or only a few weakly stained cells were observed in the intermediate lobe (results not shown). Notice also the weak apparently granular staining in the posterior lobe (arrow, *left, bottom*), and the lack of staining when the anti-t-PA was preabsorbed with purified human t-PA (*right*).

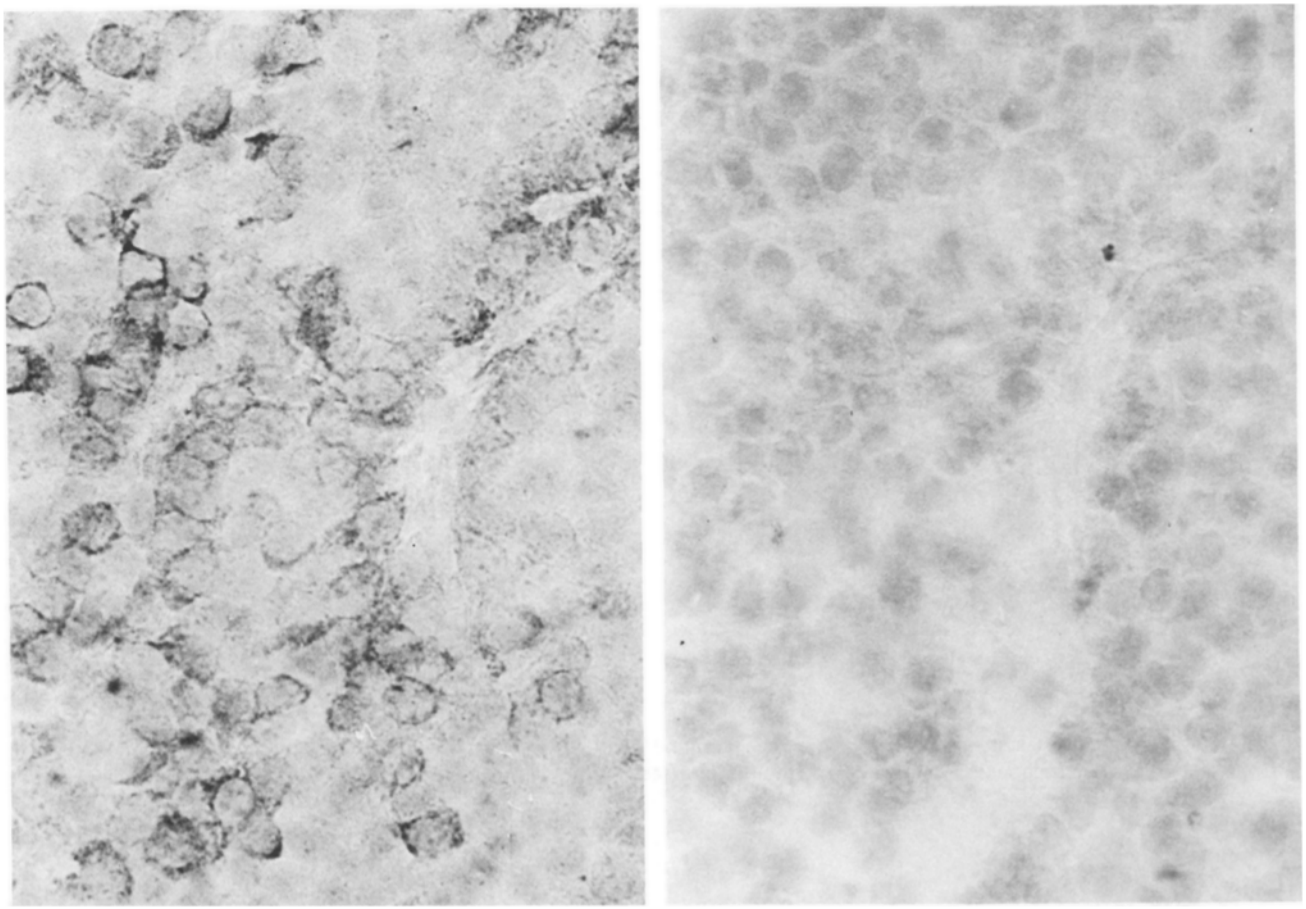

FIGURE 2 High magnification of rat pituitary anterior lobe stained with antiserum to t-PA absorbed with Sepharose-coupled BSA (*left*) or purified human t-PA (*right*). Note that the immunoreactive material is located in the cytoplasm of endocrine cells, often with a granular appearance.

phosphate, pH 7.4, and stained immunocytochemically. The cellular localization of the immunoreactivity was similar to that found with perfused tissue, although the staining intensity was lower and the tissue morphology much less satisfactory (results not shown). No staining was found when the primary antibody was replaced either by preimmune IgG, TBS-BSA, or anti-t-PA previously absorbed with highly purified t-PA. No endogeneous peroxidase was observed in the perfusion-fixed pituitaries.

Experiments with sequential staining of the same sections for t-PA followed by staining for growth hormone demonstrated that the t-PA–immunoreactive cells also contained growth hormone immunoreactivity (Fig. 3). However, some cells that contained growth hormone immunoreactivity were negative when stained for t-PA (Fig. 3). Apparently, all t-PA–staining cells also contained growth hormone.

When the staining pattern for t-PA and ACTH was compared using the sequential staining method, we observed no correlation (results not shown).

Staining with anti-u-PA did not lead to detection of immunoreactivity in any part of the rat pituitary gland (results not shown).

Zymographic and Immunoblotting Analysis

Zymographic analysis of extracts of rat pituitary glands showed the presence of only one plasminogen activator with an apparent M_r of ~67,000 (Fig. 4). We previously reported that the high molecular weight form of u-PA from rat urine under identical conditions migrates with an M_r of ~48,000. No plasminogen activator with this electrophoretic mobility was detected in the rat pituitary gland extracts (Fig. 4). The plasminogen activator of M_r ~67,000 was removed from the extracts by passage through a Sepharose-column coupled with anti-human t-PA IgG (Fig. 4). Furthermore, analysis of the same pituitary gland extracts with SDS PAGE followed by immunoblotting with the use of anti-human t-PA showed one stainable band, with an M_r ~ 67,000, similar to that of human t-PA (Fig. 5).

DISCUSSION

The findings that (*a*) the apparent M_r of the plasminogen activator of rat pituitary gland extracts was similar to that previously reported for human t-PA (~67,000, reference 31), (*b*) it binds to anti-human t-PA IgG, and (*c*) a protein band with a similar electrophoretic mobility is stained with anti-human t-PA IgG in immunoblotting indicate that this plasminogen activator is t-PA and that the anti-human t-PA IgG cross-reacts with rat t-PA. The latter conclusion is further supported by the fact that staining of other rat tissues with anti-human t-PA showed immunoreactivity in endothelial cells of veins and other blood vessels (results not shown) in a manner comparable to that reported for human tissues with the same antibodies (26).

Staining and absorption controls clearly demonstrated that

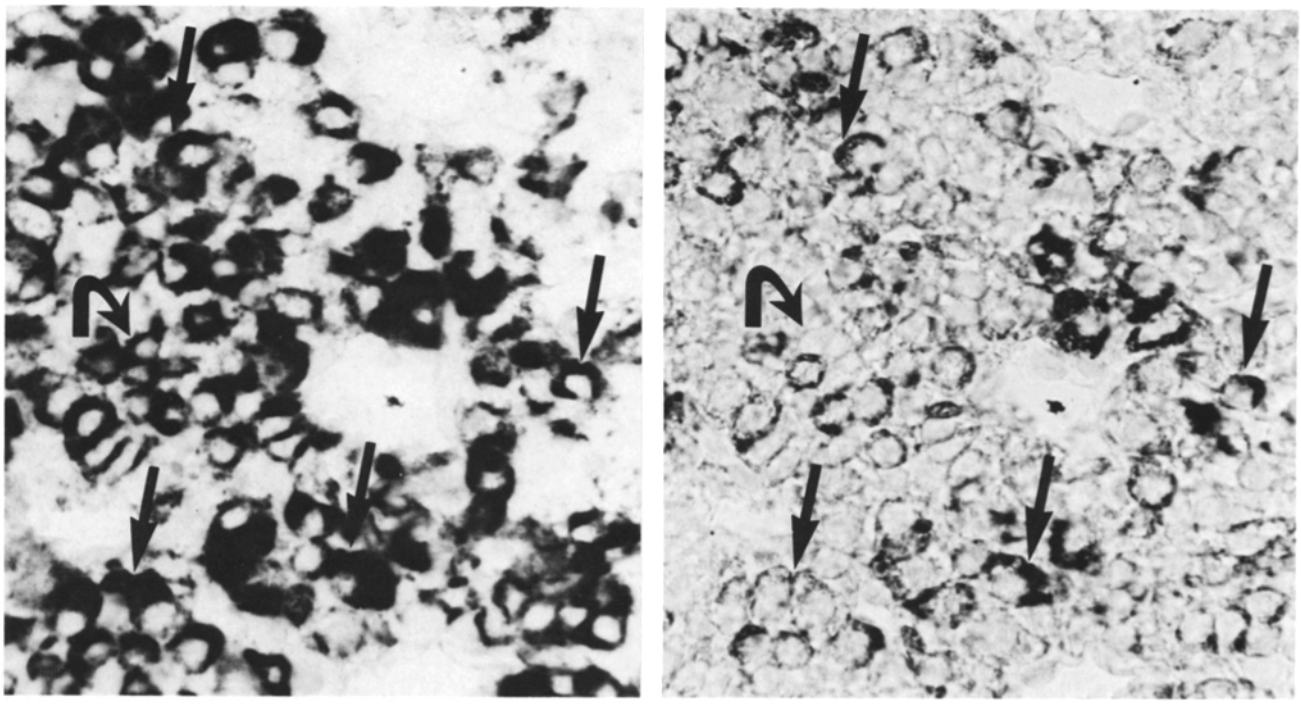

FIGURE 3 Sequential staining for t-PA (*left*) and growth hormone (*right*). After staining for t-PA the reaction product and antibodies were eluted and the section was stained for growth hormone (see Materials and Methods). Note that all the t-PA-containing cells contain growth hormone (straight arrows) but that a few apparently t-PA-negative cells contain growth hormone immunoreactivity (curved arrow).

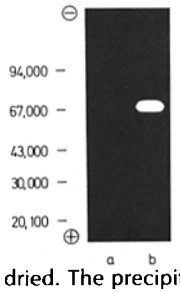

FIGURE 4 Zymographic analysis of extracts of rat pituitary glands. Pituitary glands of six rats were extracted and 25 μl of the extract was applied to each of two Sepharose columns coupled with either 2 mg anti-t-PA IgG (lane *a*) or 2 mg preimmune IgG (lane *b*) and equilibrated with TBS. The columns were developed with TBS and the run-through and wash fractions (2.4 ml) were collected, dialyzed against 0.1% (wt/vol) SDS, and freeze-dried. The precipitate was dissolved in sample buffer without SDS and subjected to SDS PAGE followed by zymography for plasminogen activators for 4 h at 37°C. A zymogram of the original pituitary extract was identical to that shown in lane *b*. No lysis was seen when plasminogen was omitted from the agarose gel (results not shown). The localization of marker proteins stained by Coomassie Blue are indicated.

the staining of the pituitary gland with anti-t-PA was due to immunological binding of the purified IgG preparation to tissue components. The results of the absorption experiments with the highly pure t-PA (detection limit for contaminating proteins was ~5% as evaluated by SDS PAGE followed by Coomassie Blue staining), obtained by affinity chromatography with a monoclonal antibody, make it unlikely that the staining is due to contaminating antibodies. Furthermore, the finding that the immunoblotting analysis of the pituitary gland extracts revealed that only one band was stained with anti-t-PA, and the fact that this band had an electrophoretic mobility similar to that of human t-PA, makes it unlikely that the staining or part of the staining is due to cross-reaction of the anti-t-PA IgG with molecules different from rat t-PA. We thus find it very likely that the staining demonstrated in this study was due to the presence of authentic t-PA. These findings represent the first direct evidence for the presence of t-PA in (and probably production by) cell types other than endothelial cells (26, 36) in the intact normal organism and therefore point to t-PA's having functions other than participating in thrombolysis (see below and reference 4 for a further discussion).

Our sequential staining experiments showed that the cells containing t-PA immunoreactivity also contained growth hormone immunoreactivity and that the t-PA-containing cells constitute a large subpopulation of the somatotrophs. After elution of the t-PA staining, each section was stained again with swine anti-rabbit immunoglobulin and rabbit peroxidase-antiperoxidase complexes, and only sections completely devoid of reaction were stained for the second antigen. Furthermore, the finding that some t-PA-negative cells display growth hormone immunoreactivity, the finding of a more intensive staining with anti-human growth hormone antiserum, and the lack of correspondence when anti-ACTH antiserum was employed all make it improbable that the antibody elution was insufficient.

The lack of detectable u-PA immunoreactivity was not due to a lack of cross-reaction of the anti-mouse u-PA IgG with rat u-PA, because such a cross-reaction has been demonstrated (28). It is, however, possible that the anti-mouse u-PA IgG could not stain rat u-PA under the conditions used, and we therefore stained rat kidney with the anti-u-PA IgG. These experiments revealed a distribution of u-PA immunoreactivity in epithelial cells of proximal and distal kidney tubules (results not shown) similar to that previously reported in mouse kidney (25), indicating that these antibodies can stain rat u-PA. The lack of detectable u-PA immunoreactivity in the rat pituitary gland agrees with similar results previously reported for the mouse pituitary gland (25).

The presence of plasminogen activators of nondetermined type in endocrine tissue was first noted in extracts of human

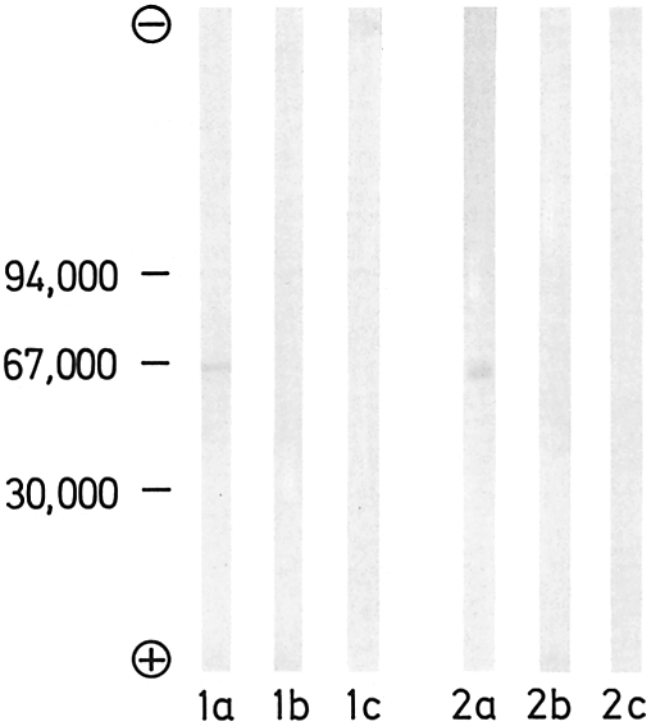

FIGURE 5 SDS PAGE followed by immunoblotting analysis of extracts of rat pituitary glands. 25 μl of the extract (see legend to Fig. 4) was applied to each of three lanes (*1a–1c*). Purified human t-PA (2.5 ng) was applied to each of three other lanes (*2a–2c*). After SDS PAGE the proteins were transferred by electrophoresis to nitrocellulose sheets. The paper was cut longitudinally and stained immunocytochemically using anti-t-PA IgG (10 μg/ml) previously passed through Sepharose columns coupled with BSA (lanes *a*) or purified human t-PA (lanes *b*), or using preimmune IgG (10 μg/ml) (lanes *c*). The localization of marker proteins stained by Amido black (21) are indicated.

pituitary, thyroid, and adrenal glands (37). By overlaying of tissue sections with a fibrin layer containing plasminogen, plasminogen activator of a nondetermined type was demonstrated in mouse hypothalamus and amygdala (22). Isolated islets of Langerhans, maintained in culture, were found to secrete plasminogen activator, and this secretion could be modulated by a number of substances known to modulate the secretion of insulin (23).

It was recently reported that rat pituitary gland cells in culture secreted both u-PA and t-PA, and it was demonstrated that the total plasminogen activator activity in the cell cultures could be modulated by a number of biological effector molecules, such as different hormones (24). That report points to subpopulations of the prolactin-, LH-, and ACTH-containing cells as producers of plasminogen activators, at least in cell culture. The present results apparently disagree, as we found virtually all t-PA–containing cells to contain growth hormone immunoreactivity, and because we did not observe any correlation between the presence of t-PA and ACTH immunoreactivity. Furthermore, our results apparently disagree as to the presence of u-PA in the pituitary gland cells. This latter apparent discrepancy was not due to the fact that female rats were used in the previous study and male rats in the present study, because immunocytochemical staining of pituitary glands from female rats did not reveal any detectable u-PA immunoreactivity (results not shown). The differences in the findings concerning u-PA in the two studies might however be explained by cultured cells' not necessarily being representative of the corresponding cell types in the intact organism with respect to production and release of plasminogen activators. This could be due to a selection of cells taking place when cultures are established and to differences in the presence of regulatory factors between the microenvironment of cells in the intact organism and in culture (see references 4 and 25 for further discussion). Note also that u-PA may have been present in the intact pituitary gland in the rat (and in the mouse; see reference 25) in amounts below the detection limit, and that our results determine the presence of a certain amount of the respective plasminogen activators in the cells at the time of fixation and do not necessarily reflect the site or amount of activator production.

Many data now indicate that processing of prohormones to polypeptide hormones takes place in the secretion granules of endocrine cells and neurons, and both serine and thiol proteases, among other enzymes, have been implicated in the posttranslational proteolysis (38–42). It has previously been proposed that plasminogen activators play a role in these processes (22–24). Our results support this notion with respect to the somatotrophs of the pituitary gland and point to t-PA's and not u-PA's being the activator possibly involved in posttranslational processing in these cells.

At present the only known natural substrate for t-PA is plasminogen, which is converted to plasmin by a cleavage of an arginine–valine bond (43). t-PA could be involved in the processing of prohormones either by a direct catalytic activity on the respective prohormones or by activation of plasminogen to plasmin, which in turn could catalyze the processing. Alternative possibilities are that t-PA or plasmin could participate in prohormone conversion by activation of processing enzymes present in a proenzyme form. To evaluate these possibilities further studies are needed of the localization of t-PA on the electron microscopic level and of the possible presence of plasminogen in the t-PA immunoreactive cells.

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