Tissue Plasminogen Activator (t-PA) Is Targeted to the Regulated Secretory Pathway

CATECHOLAMINE STORAGE VESICLES AS A RESERVOIR FOR THE RAPID RELEASE OF t-PA*

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Robert J. Parmer‡, Manjula Mahata, Sushil Mahata, Matthew T. Sebald§, Daniel T. O’Connor, and Lindsey A. Miles¶

From the Department of Medicine and Center for Molecular Genetics, University of California, and Veterans Affairs Medical Center, San Diego, California 92161 and §Department of Vascular Biology (VB-1), The Scripps Research Institute, La Jolla, California 92037

Tissue-type plasminogen activator (t-PA) is a serine protease that plays a central role in the regulation of intravascular thrombolysis. The acute release of t-PA in vivo is induced by a variety of stimuli including exercise, trauma, and neural stimulation. These types of stimuli also result in sympathoadrenal activation and ectosytic release of amines and proteins from catecholamine storage vesicles of the adrenal medulla and sympathetic neurons. Therefore, we tested the hypothesis that t-PA is packaged in and released directly from catecholamine storage vesicles, using several chromaffin cell sources including the rat pheochromocytoma PC-12 chromaffin cell line, primary cultures of bovine adrenal chromaffin cells, and human pheochromocytoma. t-PA was expressed in chromaffin cells as detected by Northern blotting, immunoprecipitation of [35S]Met-labeled t-PA, and specific t-PA enzyme-linked immunosorbent assay of cell homogenates. In addition, chromaffin cell t-PA was enzymatically active by fibrin zymography. To explore the subcellular localization of the expressed t-PA, PC-12 cells were labeled with [3H]norepinephrine, homogenized, and subjected to sucrose density fractionation. [3H]Norepinephrine and t-PA antigen were colocalized to the same subcellular fraction with a major peak at 1.4  r sucrose, consistent with the buoyant density of catecholamine storage vesicles. In addition, catecholamine storage vesicles from isolated human pheochromocytoma tumors were enriched approximately 30-fold in t-PA antigen, compared with tumor homogenate. Furthermore, exposure of PC-12 cells or primary bovine adrenal chromaffin cells to chromaffin cell secretagogues (60  m nicotine, 55  mM KCl, or 2  mM BaCl2) resulted in co-release of t-PA in parallel with catecholamines. These data demonstrate that t-PA is expressed in chromaffin cells, is sorted into the regulated pathway of secretion, and is co-released with catecholamines by chromaffin cell stimulation. Catecholamine storage vesicles may be an important reservoir and sympathoadrenal activation an important physiologic mechanism for the rapid release of t-PA. In addition, expression of t-PA by chromaffin cells suggests a role for this protease in the proteolytic processing of chromaffin cell proteins.

Tissue plasminogen activator (t-PA)† is a serine protease playing the dominant role in elimination of fibrin from the vasculature by activating the circulating zymogen, plasminogen, to the primary fibrinolytic enzyme, plasmin (1, 2). The major source of circulating t-PA under basal conditions is thought to be the endothelial cell (3, 4). It is generally believed that t-PA follows the constitutive secretory pathway (5). However, t-PA can be released into the circulation within minutes in response to distinct types of stimulation (6–8). The rapidity of this response suggests secretion from stored pools rather than de novo synthesis (9). However, the mechanisms by which this response occurs in vivo and the cellular sources are not known.

Specific stimuli have been identified that induce the acute release of t-PA. Included among these stimuli are exercise (10), mental stress (11, 12), electroconvulsive therapy (13), and surgery (11). Of note, these types of stimuli also activate the sympathoadrenal system causing exocytotic release of amines and proteins from catecholamine storage vesicles of the adrenal medulla and sympathetic neurons (14). In addition, t-PA is present in neuroendocrine tissues (15–19), including the adrenal medulla (20). Therefore, we tested the hypothesis that t-PA is packaged in and released directly from catecholamine storage vesicles, by investigating t-PA expression, subcellular localization, and secretagogue-mediated t-PA release from several chromaffin cell sources. These included rat PC-12 cells, a well-established chromaffin cell line with abundant catecholamine storage vesicles (21), as well as primary bovine adrenal chromaffin cells, and human pheochromocytoma, a catecholamine-producing tumor of the adrenal medulla, and hence a source of human chromaffin cells. Our results demonstrate that t-PA is expressed in chromaffin cells where it is targeted to the regulated pathway of secretion (into catecholamine storage vesicles) and is co-released with catecholamines by chromaffin cell stimulation. These results suggest that catecholamine storage vesicles may serve as a

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‡ This work was done during the tenure of an Established Investigators award from the American Heart Association. To whom correspondence should be addressed: Dept. of Medicine (9111-H), University of California, San Diego, CA 92161. Tel.: 619-552-8585 (ext. 7373); Fax: 619-552-7549. E-mail: rparmer@ucsd.edu.

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† The abbreviations used are: t-PA, tissue plasminogen activator; ELISA, enzyme-linked immunosorbent assay; kb, kilobase pair(s); PAI, plasminogen activator inhibitor.
reservoir, and sympatheo-adrenal activation may be an important physiologic mechanism, for the rapid release of t-PA.

EXPERIMENTAL PROCEDURES

Cells and Tissues—PC-12 cells (21) were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum, 10% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Bovine chromaffin cells were isolated from bovine adrenal glands as described (22). The cells were cultured in minimal essential media containing 1% non-essential amino acids, 1% t-glutamine, 10% fetal calf serum, 1% Penicillin-Streptomycin, 100 units/ml penicillin, and 100 μg/ml streptomycin for 3–5 days prior to assay.

Human umbilical vein endothelial cells were isolated from human umbilical veins as described (23).

Human chromaffin granules were isolated from fresh pheochromocytoma tissue as described (24). Briefly, the tissue was minced and homogenized in isotonic (0.3 μ) sucrose. Nuclei were sedimented by centrifugation at 1,000 g for 10 min. The supernatant was removed and centrifuged at 10,000 × g for 20 min. The pellet was centrifuged over a sucrose step gradient (0.3–2.5 M) and centrifuged at 100,000 × g for 30 min to sediment granule membranes. Chromaffin vesicle lysates were obtained in the supernatant.

mRNA Isolation and Northern Blot Analysis—Poly(A) RNA was isolated from cells and tissues using guanidinium isothiocyanate and centrifugation through 5.7 M cesium chloride (25) followed by poly(A) selection on an oligo(dT) column.

For Northern blot analysis, 5 μg of poly(A)-enriched RNA were electrophoresed through 1% agarose, 0.66 M formaldehyde (26) and transferred to nylon filters. Hybridization was performed using a 32P-labeled 1.9-kb KpnI-XbaI fragment of human t-PA cDNA probe as described (27, 29–32). Fractions (1 ml) were collected and assayed for t-PA using an ELISA kit (Corvas Biopharmaceuticals, Inc.) for 30 min at 4°C on a shaker, to reduce nonspecific binding. The mixtures were centrifuged and the supernatants were collected and assayed for [3H]norepinephrine by liquid scintillation counting and t-PA by ELISA. Percent secretion was calculated as the amount in release buffer/total (amount in release buffer + amount in cell lysate), and the results were expressed as fold stimulation compared with basal (unstimulated) values.

Fibrin Zymography—Fibrin zymography was performed as described previously (33). Briefly, samples were electrophoresed on 10% SDS-polyacrylamide gels according to the system of Laemmli (34). After removal and neutralization of the SDS by soaking in 2.5% Triton X-100, the gels were incubated on fibrin-agar indicator films containing 2 mg/ml bovine fibrinogen (Calbiochem), 0.5 units/ml human α-thrombin, and 0.01 mg/ml human plasminogen (purified as described (35)).

Assays—Protein content was determined in chromaffin vesicle lysates and pheochromocytoma homogenates using the bicinchoninic acid protein assay reagent (Pierce) in the microtiter plate protocol according to the manufacturer’s instructions.

Catecholamines were measured by differential fluorometry (24, 36).

Reagents—Human recombinant single chain t-PA (rt-PA, Activase) was from Genentech, Inc. (South San Francisco, CA).

Statistics—Results are reported as mean ± standard error of the mean. Statistical significance was determined by Student’s t test or by analysis of variance followed by Student-Newman-Keuls post-hoc tests for multiple comparisons.

RESULTS

Expression of t-PA by Chromaffin Cells—Fig. 1 shows Northern blot results in which we investigated t-PA expression in rat PC-12 cells and in tissue from human pheochromocytoma. Hybridization was performed with a 32P-labeled 1.9-kb human t-PA cDNA KpnI-XbaI fragment as probe. The prominent 2.7-kb band typical of t-PA is present in PC-12 cells and to a lesser extent in rat brain, rat liver, and rat heart as positive controls (37). t-PA message was not detected in rat skeletal muscle as a negative control. Of note, there was marked expression of t-PA mRNA in both PC-12 cells and bovine adrenal chromaffin cells labeled for 2 h with [3H]norepinephrine at 1 μM/ml in cell culture medium, washed twice with release buffer (10 mM HEPES, pH 7, 150 mM NaCl, 5 mM KCl, 2 mM CaCl2) and incubated at 37°C for 30 min in release buffer with or without the following secretagogues: 60 μM nicotine, 55 mM KCl, or 2 mM BaCl2 as described (27). Release buffer for experiments with KCl as secretagogue included NaCl at 100 mM and release buffer was devoid of CaCl2 when BaCl2 was used as secretagogue. After aspirating the release buffer, cells were harvested and lysed in cell lysis buffer (release buffer containing 0.1% Triton X-100). Release buffer and cell lysates were assayed for [3H]norepinephrine by liquid scintillation counting and t-PA by ELISA. Percent secretion was calculated as the amount in release buffer/total (amount in release buffer + amount in cell lysate), and the results were expressed as fold stimulation compared with basal (unstimulated) values.

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thionine, harvested, and incubated with an IgG fraction of either a polyclonal anti-t-PA antiserum (Fig. 2, lane 1) or control normal rabbit serum (Fig. 2, lane 2). Immunoprecipitated proteins were electrophoresed through SDS-polyacrylamide gels and subjected to autoradiography. Two major radiolabeled bands were specifically immunoprecipitated by the anti-t-PA antibodies, one with an apparent molecular weight of 70,000, consistent with the molecular weight of free t-PA. Interestingly, a second band with an apparent molecular weight of 110,000 was present, consistent with t-PA in a complex with inhibitor, plasminogen activator inhibitor 1 (PAI-1), or plasminogen activator inhibitor 2 (PAI-2) (38). The t-PA content (determined by ELISA) of the PC-12 cells was 9.4 ± 0.5 ng/10^6 cells (n = 6), a value ~2-fold greater than the level we determined for human umbilical vein endothelial cells, 4.8 ± 0.4 ng/10^6 cells (n = 3).

Subcellular Localization of t-PA within Chromaffin Granules—To explore the subcellular localization of the t-PA expressed in chromaffin cells, we first performed sucrose gradient fractionation of PC-12 cell homogenates. PC-12 cells were labeled with [3H]norepinephrine, homogenized, and layered over a continuous sucrose density gradient (0.3–2.5 M sucrose). Following centrifugation, fractions were collected, and t-PA antigen, [3H]norepinephrine, and sucrose concentration were determined. [3H]Norepinephrine and t-PA antigen were colocized to the same subcellular fractions with a major peak at 1.4 M sucrose (Fig. 3). The 1.4 M sucrose peak is consistent with the buoyant density which we and others (27, 30, 31) have demonstrated previously for chromaffin granules isolated from PC-12 cells. In addition, some [3H]norepinephrine and t-PA antigen were found at the top (fractions 18–22, ~0.3–0.5 M sucrose) and bottom (fraction 5, ~1.9 M sucrose) of the gradient. These results are similar to those of our own previous sucrose gradient studies (27) as well as to those of other investigators (31, 32). The additional peak toward the top of the gradient is consistent with release of granular components from vesicles lysed during the homogenization step (before application of the sample to the gradient) (31, 39). The additional peak toward the bottom of the gradient (at ~1.9 M sucrose) is consistent with vesicles trapped in undisturbed cell debris (39).

We also investigated the presence and subcellular localization of t-PA antigen in homogenates of chromaffin tissue obtained from human pheochromocytoma. We compared t-PA concentration in pheochromocytoma tumor homogenates with t-PA concentration in lysates of catecholamine storage vesicles (Table I). t-PA antigen (by ELISA) was present in human pheochromocytoma and was markedly enriched in the chromaffin vesicle fraction (approximately 30-fold enrichment compared with tumor homogenates). The enrichment in t-PA antigen paralleled the enrichment in catecholamines, suggesting that both are localized in the same subcellular compartment, the storage vesicle. Whereas the t-PA was enriched about 30-fold in the vesicle fraction over homogenates, the catecholamines were enriched about 15-fold. This potential difference in enrichment possibly could have arisen as a result of differential leakage of vesicle constituents during the tissue preparation and homogenization steps, with leakage of catecholamines occurring to a greater extent than leakage of larger vesicle protein components (including t-PA). Trapping of catecholamines within the granule requires the activity of the vesicular monoamine transporter (40), the activity of which may be impaired during the tissue preparation and homogenization steps.

Secretagogue-stimulated Co-release of t-PA and Catecholamine from Chromaffin Cells—The subcellular localization of t-PA was independently verified in functional secretagogue release studies. In these experiments, t-PA antigen and norepinephrine were measured in both releasates and whole cell lysates from [3H]norepinephrine-loaded PC-12 cells under basal conditions and after 30 min of exposure to several well established chromaffin cell secretagogues: 60 μM nicotine (which acts through nicotinic cholinergic receptors), 55 mM KCl (a membrane depolarizing agent), or 2 mM BaCl2 (a calcium agonist (27)). Significant increases in t-PA secretion were observed in response to each of these secretagogues, approximately 4-fold for nicotine, 5-fold for KCl, and 12-fold for BaCl2 (Fig. 4). Furthermore, secretion of t-PA parallelled secretion of norepinephrine, consistent with release from the same subcellular pool.

We performed similar functional secretagogue release studies using primary cultures of bovine adrenal chromaffin cells as a source of nontransformed chromaffin cells. t-PA antigen was
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FIG. 4. Co-release of t-PA with norepinephrine from PC-12 cells. PC-12 cells were labeled with \[^{3}H\]norepinephrine and incubated at 37°C for 30 min in release buffer in either the presence or absence of 60 \(\mu\)M nicotine, 55 mM KCl, or 2 mM BaCl\(_2\). After aspirating the release buffer, cells were harvested and lysed, and the release of t-PA antigen (filled bars) and \[^{3}H\]norepinephrine (open bars) was determined as described under “Experimental Procedures.” Percent secretion was calculated as the amount in release buffer/total (amount in release buffer + amount in cell lysate), and the results were expressed as fold stimulation compared with basal (unstimulated) values. Basal release values for t-PA and \[^{3}H\]norepinephrine were 4.8 ± 0.5 and 5.2 ± 0.5%, respectively. Values are represented as the mean ± S.E. of six independent determinations for each group (*p < 0.01 compared with corresponding basal values, by analysis of variance followed by Student-Newman-Keuls post-hoc tests for multiple comparisons).

FIG. 5. Co-release of t-PA with norepinephrine from bovine adrenal chromaffin cells. Cultured bovine adrenal chromaffin cells were labeled with \[^{3}H\]norepinephrine. The release of t-PA antigen (filled bars) and norepinephrine (open bars) was determined as described under “Experimental Procedures.” Percent secretion was calculated as the amount in release buffer/total (amount in release buffer + amount in cell lysate), and the results were expressed as fold stimulation compared with basal (unstimulated) values. Basal release values for t-PA and \[^{3}H\]norepinephrine were 5.3 ± 0.9 and 6.4 ± 0.7%, respectively. Values are represented as the mean ± S.E. of six independent determinations for each group (*p < 0.01 compared with corresponding basal values, by analysis of variance followed by Student-Newman-Keuls post-hoc tests for multiple comparisons).

Present (4.7 ± 0.7 ng/10^6 cells (n = 8)) and was released from the bovine chromaffin cells in response to the three chromaffin cell secretagogues (nicotine, KCl, and BaCl\(_2\)) in these cells as well (Fig. 5). Secretagogue-mediated t-PA release paralleled norepinephrine secretion, consistent with exocytotic release of t-PA from catecholamine storage vesicles in this additional chromaffin cell source.

Fibrin Zymography of Chromaffin Cell Releasates and Lyases—To confirm the authenticity of t-PA in chromaffin cells, we employed the technique of fibrin zymography for t-PA enzymatic activity. This technique determines apparent molecular weights of plasminogen activator activity within a sample (33). We examined secretagogue-mediated cell releasates from both PC-12 and bovine adrenal chromaffin cells in culture, as well as human pheochromocytoma chromaffin vesicle lysates, for t-PA activity. Zones of lysis indicating plasminogen activator activity were detected in secretagogue-mediated releasates from both PC-12 and bovine adrenal chromaffin cells (Fig. 6, lanes 1 and 3) but not in conditioned media of cells incubated with buffer alone (lanes 2 and 4). This plasminogen activator migrated with a \(M_r\) of 70,000, consistent with that of the free t-PA standard (lane 6). In addition, plasminogen activator activity, migrating with a \(M_r\) of 70,000 was observed in the lysates from pheochromocytoma chromaffin vesicles (lane 5). Thus, t-PA activity was present in the secretagogue-responsive (releasable) pool from chromaffin cells, and the molecular weight of the activity in each case suggested authentic t-PA.

Since the results of the immunoprecipitation experiment (Fig. 2) suggested that both t-PA and a complex with an electroforetic mobility consistent with a t-PA-plasminogen activator inhibitor complex were synthesized by chromaffin cells, we examined whether the inability to detect t-PA-plasminogen activator inhibitor complexes in the releasates by zymography was due to greater sensitivity of the immunoprecipitation method in detecting these complexes or the absence of t-PA inhibitors from the storage granules. We subjected human chromaffin vesicle lysates to fibrin zymography using a prolonged exposure which yielded a large zone of lysis corresponding to free t-PA that was close to the maximum zone of lysis that could be resolved on the gel (Fig. 7). Under these conditions, a small zone of lysis was detected which migrated with an \(M_r\) corresponding to the presence of t-PA inhibitor complexes. Thus, these data are consistent with the presence of t-PA-inhibitor complexes in addition to free t-PA within the storage granules.

DISCUSSION

The present study demonstrates that t-PA is expressed in and is targeted to the regulated secretory pathway (41) in chromaffin cells. This was demonstrated for chromaffin cells from several sources. Moreover, these data provide the identification of a specific subcellular compartment (the catecholamine storage vesicle) into which the t-PA molecule is sorted and from which t-PA is released by specific stimuli. In addition, these results suggest that catecholamine storage vesicles are a previously unrecognized reservoir and that sympathetic activation, with resultant exocytotic release from these organelles, may be an important physiologic mechanism.
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for the rapid release of t-PA.

We examined the expression of t-PA in three different chromaffin cell sources, including the rat PC-12 cell line, primary bovine chromaffin cells, and human pheochromocytoma. t-PA was expressed in these tissues as assessed by Northern blotting, immunoprecipitation of cells labeled with [35S]methionine, by a specific ELISA for t-PA, and by fibrin zymography. Previous reports have shown expression of t-PA in PC-12 cells (42–44).

Endothelial cells are a well-recognized source of circulating t-PA under basal conditions (3, 4). We compared the t-PA content of chromaffin cells with that of human umbilical vein endothelial cells. The t-PA content of chromaffin cells was quantitatively equal to (4.7 ± 0.7 ng/106 cells for bovine chromaffin cells) or greater than (9.4 ± 0.5 ng/106 cells for PC-12 cells) the t-PA content of human umbilical vein endothelial cells (4.8 ± 0.4 ng/106 cells). Indeed, the t-PA antigen content of the bovine chromaffin cells may have been underestimated by the ELISA (perhaps due to lower cross-reactivity of the anti-human t-PA antiserum with bovine than with human t-PA) since bovine samples yielded much larger zones of lysis on fibrin zymography than human samples with the same apparent t-PA antigen concentration as determined by the ELISA (data not shown).

Secretory proteins expressed in neuroendocrine cells are targeted into one of two pathways, constitutive and regulated (41, 45). Proteins entering the regulated pathway are concentrated and stored in vesicles and subsequently released upon stimulation by a secretagogue. In the constitutive pathway, newly synthesized protein is not stored but is transported directly to the cell surface and secreted even in the absence of any extracellular signal. Catecholamine storage vesicles within chromaffin cells are prototype examples of regulated secretory vesicles (14, 27, 39, 46, 47).

Therefore, to investigate whether chromaffin cell t-PA is sorted into catecholamine storage vesicles, we examined the subcellular localization of chromaffin cell t-PA, using both sucrose gradient fractionation and functional secretagogue-mediated release studies.

Sucrose gradient studies demonstrated co-localization of PC-12 cell t-PA and catecholamines to the same subcellular fraction at 1.4 M sucrose. The peak at 1.4 M is consistent with the buoyant density which we and others (27, 30, 31) have demonstrated previously for chromaffin granules isolated from PC-12 cells. In addition, lysates of human chromaffin granules, isolated from pheochromocytoma tissue on a sucrose gradient, were enriched in t-PA antigen compared with the whole tumor homogenate. This enrichment in t-PA antigen paralleled the enrichment in catecholamine content, suggesting co-localization of t-PA and catecholamines to the same subcellular compartment, the catecholamine storage vesicle.

Interpretation of sucrose gradient results may be limited by the potential for co-purification of heterogeneous organelles to the same fraction (30–32). Hence, we cannot exclude the possibility that part of the chromaffin cell t-PA may reside in a different subcellular compartment of similar buoyant density to chromaffin granules. Nonetheless, previous sucrose gradient fractionation studies by our group have yielded relatively clear separation of markers for chromaffin granules, lysosomes, and mitochondria, with buoyant densities for these organelles as follows: chromaffin granules > lysosomes > mitochondria (48). Furthermore, the sucrose gradient results coupled with results of functional secretagogue release studies suggest co-localization of t-PA and catecholamines to the catecholamine storage vesicle.

We used several well-established chromaffin cell exocytotic secretagogues to assess the subcellular location of t-PA in PC-12 cells as well as in bovine adrenal chromaffin cells, as a source of nontransformed chromaffin cells. t-PA was released from both cell types in response to either 60 μM nicotine (which acts through nicotinic cholinergic receptors), 55 mM KCl (a membrane depolarizing agent), or 2 mM BaCl2 (a calcium agonist (27)). Moreover, release of t-PA from these chromaffin cell sources was in parallel with that of catecholamines, consistent with release from the same subcellular pool. The values for percent release during secretagogue-mediated stimulation were, in general, less for t-PA compared with those for [3H]norepinephrine. Previously, we noted differences between release of a chromaffin granule protein, chromogranin A, and release of [3H]norepinephrine in response to secretagogues (27). Release of endogenous chromaffin vesicle proteins after their synthesis requires trafficking through the endoplasmic reticulum and Golgi stacks before final localization in the dense core secretory vesicles. Thus, a percentage of the measured total cellular protein may still be in route to the secretory vesicle and, therefore, unavailable for exocytotic release. In contrast, since the [3H]norepinephrine is supplied to the cells as an exogenous label and does not require synthesis or routing through the endoplasmic reticulum or Golgi, a greater percentage of cell [3H]norepinephrine may be immediately available for release in response to secretagogue stimulation. In addition, recent studies have shown that both the chromogranins (49) and t-PA (50) bind to chromaffin cell membranes in a saturable and specific manner, so that some of the chromogranins and t-PA initially secreted upon stimulation into the release medium may be rapidly bound to the chromaffin cell membrane and therefore not measured in the secreted fraction. Another possible explanation is the existence (in addition to the regulated vesicles containing both [3H]norepinephrine and t-PA) of a population of recycled vesicles (51) which, having already released their t-PA, contain only (re-loaded) [3H]norepinephrine. Nonetheless, our secretion studies with a variety of secretagogues (Figs. 4 and 5) showed parallel secretion of [3H]norepinephrine and t-PA such that each increment in [3H]norepinephrine release was associated with a corresponding increase in t-PA release, consistent with co-localization of [3H]norepinephrine and t-PA to the same subcellular pool.

Using fibrin zymography, we confirmed the authenticity of t-PA within the three chromaffin cell sources. Plasminogen activator activity was present and was of the appropriate size for authentic t-PA. In addition, t-PA activity was present in the releasable pool from all three chromaffin cell sources studied. Although a band (M, 110,000) consistent with t-PA complexed with inhibitor was detected following immunoprecipitation of [35S]methionine-labeled PC-12 cells, such a complex was not observed initially in cell releasates by fibrin zymography (Fig. 6). This could have been due to either greater sensitivity of the immunoprecipitation method compared with fibrin zymography in detecting these complexes or the absence of t-PA inhibitors from the storage granules. To further investigate this question and to determine whether t-PA/inhibitor complexes could be detected in the storage granules, we subjected human chromaffin vesicle lysates to fibrin zymography using a prolonged exposure (Fig. 7). Under these conditions, a small lytic zone consistent with the M, (app) of a t-PA/inhibitor complex could be detected in the chromaffin vesicle lysate. Thus, these results suggest the presence of t-PA/inhibitor complexes within the storage granule. The detection of t-PA/inhibitor complexes by fibrin zymography is presumably based on dissociation of some of the complexes during the processing of the zymograms (33) and, therefore, the absolute concentrations of t-PA/inhibitor complexes relative to free t-PA could not be
estimated. Nonetheless, the detection of the free t-PA band in the storage granules as well as in releasates from both bovine and PC-12 cells (Fig. 6) indicates that free t-PA is present within the vesicles and is released upon stimulation. Interestingly, transfection of AtT-20 mouse pituitary cells with a CDNA for PAI-1 results in PAI-1 expression and targeting of this inhibitor to the regulated secretory pathway in this cell type (52). Further studies will be necessary to resolve whether t-PA inhibitors are targeted to the regulated pathway of secretion in the chromaffin cell.

Catecholamines (primarily epinephrine) stimulate t-PA release into the circulation, presumably by their actions on vascular endothelium (10). However, during exercise only ~50% of the increase in plasma t-PA concentrations can be attributed to this mechanism (exercise-induced increases in catecholamines causing endothelial cell t-PA release (53)). Moreover, the exercise-induced release of t-PA is not fully abolished by β-adrenergic receptor antagonists (54–56). Therefore, release of catecholamine storage vesicles during sympathoadrenal activation may enhance the profibrinolytic capability of plasma by the direct release of t-PA and, secondarily, by catecholamine secretion. Further studies will be required to assess the overall importance of chromaffin cell t-PA as a source of t-PA release during stress in vivo.

It has been generally considered that t-PA follows the constitutive secretory pathway (5). The present study identifies the catecholamine storage vesicle as a specific subcellular compartment from which t-PA is released in a regulated fashion. Intriguing data in this regard also are emerging from studies of other cell types. t-PA and parathyroid hormone exhibit parallel calcium-regulated release from 24-h cultures of human parathyroid cells, raising the possibility that t-PA and parathyroid hormone may follow the regulated secretory pathway in parathyroid cells, an additional neuroendocrine cell type (16). Also, a rapid release of t-PA is induced upon stimulation of endothelial cell cultures with products formed during coagulation, thrombin, factor Xa, fibrin, bradykinin, and platelet-activating factor (57), but the identity of a specific subcellular granule that contains t-PA in these cells has not been established (58).

Two possible mechanisms have been proposed for targeting proteins into the regulated pathway of secretion and for sorting regulated secretory proteins away from constitutively secreted proteins at the trans-Golgi cisternae: 1) receptor-mediated protein targeting and 2) selective protein aggregation and condensation. In the first mechanism, correct targeting to the regulated pathway results from specific binding to carrier proteins or receptors in the Golgi membrane (59) which recognize sorting signals on target proteins. Alternatively, in the second mechanism, the sorting of regulated secretory products from other soluble (constitutive) proteins in the pathway results from formation of molecular aggregates triggered by conditions in the trans-Golgi region (acidic pH and millimolar concentrations of calcium ions) (60, 61). The targeting of t-PA to catecholamine storage vesicles could perhaps be mediated or facilitated through either of these mechanisms by a specific region or regions within the primary structure of t-PA. Mutagenesis studies have identified domains within the sequences of secretory proteins, for example, for chromogranin A (27) and P-selectin (62), which may mediate targeting to the regulated pathway of secretion. These types of mutagenesis studies may be useful to elucidate the mechanisms responsible for the targeting of t-PA to catecholamine storage vesicles and perhaps to other regulated secretory granules.

In summary, these results demonstrate that t-PA is expressed in chromaffin cells where it is sorted into the regulated secretory pathway (into catecholamine storage vesicles) and is co-released with catecholamines by chromaffin cell stimulation. These results suggest that catecholamine storage vesicles may serve as a reservoir, and sympathoadrenal activation may be an important physiologic mechanism, for the rapid release of t-PA. In addition to providing a potential source of circulating vascular levels of t-PA, the presence of t-PA within catecholamine storage vesicles has potential implications for neuroendocrine function. t-PA is synthesized by neurons in most areas of the brain (18) and may be important in neuronal migration and regeneration (50, 63–66). t-PA appears to play a role in excitoxotoxin-induced injury to the hippocampus as assessed in mice with t-PA gene inactivation (67). Also, t-PA-deficient mice exhibit differences in synaptic transmission and in long term potentiation (68). Moreover, consistent with our results, administration of KCl into the murine brain in vivo increases t-PA activity within the hippocampus (44). In addition, the widespread distribution of t-PA in neuroendocrine tissue has suggested that t-PA may contribute to prohormone processing (20). Recently, we have obtained evidence that chromaffin cell t-PA can participate in plasmin-dependent processing of chromogranin A (69), the major soluble protein present in chromaffin granules as well as in regulated secretory granules throughout the neuroendocrine system (70), and a prohormone precursor of pancreateatin (71–73) and other bioactive peptides (74), including peptides which modulate catecholamine release (75). The present study, therefore, supports a link between the neuroendocrine and fibrinolytic systems, a link with multiple functional consequences.

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REFERENCES
1. Collen, D., and Lijnen, H. R. (1991) Blood 78, 3114–3124
2. Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J. J., Collen, D., and Mulligan, R. C. (1994) Nature 368, 419–424
3. Todd, A. S. (1959) J. Pathol. Bacteriol. 78, 281–283
4. Warren, B. A. (1963) Br. J. Exp. Pathol. 44, 365–372
5. Vassalli, J. D., Sappino, A. P., and Belin, D. (1991) J. Clin. Invest. 88, 1067–1972
6. Emeis, J. J. (1988) in Tissue-type Plasminogen Activator, Physiological and Clinical Aspects (Kluft, C., ed) pp. 21–35, CRC Press, Inc., Boca Raton, FL
7. Higazi, A. A.-R., and Barbour, I. I. (1994) J. Biol. Chem. 269, 25529–25533
8. Schrauwen, Y., DeVries, R. E. M., Kooistra, T., and Emeis, J. J. (1994) Fibrinolysis 2, Suppl. 8, S8–S12
9. Manucci, P. M., Alberg, M., Nilsson, I. M., and Robertson, B. (1975) Br. J. Haematol. 30, 81–93
10. Biggs, R., Macfarlane, R. G., and Pilling, J. (1947) Lancet ii, 402–405
11. Macfarlane, R. G., and Biggs, R. (1946) Lancet ii, 862–864
12. Schneek, A., and van Kuijk, K. A. (1981) Neurology 11, 959–969
13. Fantl, P., and Simon, S. E. (1948) Austr. J. Exp. Biol. Med. Sci. 26, 512–529
14. Winkler, H., and Westhead, E. (1980) Neurosci. 5, 1803–1823
15. Kristensen, P., Nielsen, L. S., Gronohal-Hansen, J., Andresen, P. B., Larssen, L.-I., and Dano, K. (1985) J. Cell Biol. 101, 305–311
16. Banaal, D. D., and MacGregor, R. R. (1992) J. Clin. Endocrinol. & Metab. 74, 266–271
17. Denuxain-Gonzalez, S., Delari, P., Gruftat, D., and Chabaud, O. (1993) In Vitro Cell. & Dev. Biol. 29, 161–164
18. Sappino, A.-P., Madani, R., Haarste, J., Belin, D., Kiss, J. Z., Wohland, A., and Vassalli, J.-D. (1993) J. Clin. Invest. 92, 679–685
19. Friedman, G. C., and Seiden, N. W. (1994) Dev. Brain Res. 81, 41–49
20. Kristensen, P., Hougaard, D. M., Nielsen, L. S., and Dano, K. (1986) Histochemistry 85, 431–436
21. Greene, L. A., and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2429
22. Livett, B. G. (1984) Physiol. Rev. 64, 1103–1161
23. Levine, J. D., Harian, J. M., Harker, L. A., Joseph, L. M., and Counts, R. R. (1982) Blood 60, 531–534
24. Parmer, R. J., and O’Connor, D. T. (1988) J. Hypertens. 6, 187–198
25. Chirgwin, J. M., Prybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5284–5299
26. Sambrook, J., Fritsh, E. P., and Maniatis, T. (1989) Molecular Cloning: A
27. Parmer, R. J., Xi, X.-P., Wu, H.-J., Helman, L. J., and Petz, L. N. (1993) J. Clin. Invest. 92, 1042–1054
28. Medhi, R. D., Santell, L., and Levin, E. G. (1992) Blood 80, 981–987
29. Smith, A. D., and Winkler, H. (1967) Biochem. J. 103, 480–482
30. Schubert, D., and Klier, F. G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5184–5188
31. Roda, L. G., Nolan, J. A., Kim, S. U., and Hogue-Angeletti, R. A. (1980) Exp. Cell Res. 128, 103–109
32. Oberlechner, E., Westhead, E., Neuman, B., Schmidt, W., Fischer-Colbrie, R., Weber, A., Sperk, G., and Winkler, H. (1982) J. Neurochem. 38, 615–624
33. Loskutoff, D. J., and Schleef, R. R. (1988) Methods Enzymol. 163, 293–302
34. Laemmli, U. K. (1970) Nature 227, 680–685
35. Miles, L. A., Dahlberg, C. M., and Plow, E. F. (1988) J. Exp. Biol. 133, 1747–1752
36. Weil-Malherbe, H. (1971) in Methods of Biochemical Analysis (Lick, D. J., ed) pp. 119–152, Wiley Interscience Publishers, New York
37. Abbott, J., Van den Hoogen, C. M., and Emeis, J. J. (1990) Blood Coagul. & Fibrinolysis 1, 601–608
38. Schleef, R. R., Higgins, D. L., Pillemer, E., and Levit, L. J. (1989) Clin. Invest. 83, 1747–1752
39. Schweitzer, E. S., and Kelly, R. B. (1985) J. Cell Biol. 101, 667–676
40. Henry, J. P., Botton, D., Sagne, E., Isambert, M. F., Debas, C., Blanchard, V., Raisman-Vozari, R., Krejci, E., Massoulie, J., and Gasnier, B. (1994) J. Exp. Biol. 186, 251–262
41. Kelly, R. B. (1985) Science 230, 25–32
42. Leprince, P., Register, B., Delree, P., Rigo, J. M., Andre, B., and Moonen, G. (1991) J. Neurochem. 57, 665–674
43. Pitham, R. N., and Dilts, J. A. (1995) J. Neurochem. 64, 566–575
44. Gualandris, A., Jones, T. E., Strickland, S., and Tairka, S. E. (1996) J. Neurosci. 16, 2220–2225
45. Moore, H. P. (1987) Ann. N.Y. Acad. Sci. 493, 50–61
46. Winkler, H. (1970) Neuroscience 1, 65–80
47. Schweitzer, E. S., and Paddock, S. (1990) J. Cell Sci. 96, 375–381
48. Takii, Y., and Czerchowicz, J. H. (1995) J. Neurosci. 15, 375–381
49. Kirkpatrick, S. W., and Huttner, W. B. (1992) J. Biol. Chem. 267, 4110–4118
50. Pittman, R. N., Ivins, J. K., and Buetter, H. M. (1989) J. Neurosci. 9, 4289–4298
51. Patzak, A., and Winkler, H. (1986) J. Cell Biol. 102, 510–515
52. Gombau, L., and Schleef, R. R. (1994) J. Biol. Chem. 269, 3875–3880
53. Chandler, W. L., Veith, R. C., Fellingham, G. W., Levy, W. C., Schwartz, R. S., Cercor, M. D., Kahn, S. E., Larson, V. G., Cain, K. C., Beard, J. C., Abrass, I. B., and Stratton, J. R. (1992) J. Am. Coll. Cardiol. 19, 1412–1420
54. Cohen, R. J., Epstein, S. E., Cohen, L. S., and Dennis, L. H. (1968) Lancet ii, 1264–1266
55. Korzan, J. and G. and Conradson, T. B. (1974) Scand. J. Haematol. 13, 377–384
56. Britton, B. J., Wood, W. G., Smith, M., Hawkey, C., and Irving, M. H. (1976) Thromb. Haemostasis 35, 386–402
57. Emeis, J. J. (1992) Ann. N.Y. Acad. Sci. 667, 249–259
58. van den Eijnden-Schrauwen, Y., Kooistra, T., de Vries, R. E., and Emeis, J. J. (1995) Blood 85, 3510–3517
59. Chung, K. N., Walter, P., Aponte, G. W., and Moore, H. P. (1989) Science 243, 192–197
60. Huttner, W. B., Gerdes, H. H., and Rosa, P. (1991) Trends Biochem. Sci. 16, 27–30
61. Chanat, E., and Huttner, W. B. (1991) J. Cell Biol. 115, 1505–1519
62. Dandier, M., Morrissey, J. H., Fugate, R. D., Rainton, D. F., and McEver, R. P. (1993) Mol. Biol. Cell 3, 309–321
63. Krystofek, A., and Seeds, N. W. (1981) Science 213, 1532–1534
64. Moonen, G., Grauw-Wagemans, M. P., and Selak, I. (1982) Nature 298, 753–755
65. Seeds, N. W., Verrall, S., Friedman, G., Hayden, S., Gadotti, B., Haflke, S., Christensen, K., Gardiner, P., McGuir, P., and Krystofek, A. (1992) Ann. N.Y. Acad. Sci. 667, 32–40
66. Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel, E. R., and Kuhl, D. (1999) Nature 361, 453–457
67. Tairka, S. E., Gualandris, A., Amaral, D. G., and Strickland, S. (1995) Nature 377, 340–344
68. Frey, U., Muller, M., and Kuhl, D. (1996) J. Neurosci. 16, 2057–2063
69. Parmer, R. J., O’Connor, D. T., and Miles, L. A. (1994) Fibrinolysis Suppl. 1, 53 (abstr.)
70. O’Connor, D. T., Wu, H. J., Gill, B. M., Rozansky, D. J., Tang, K., Mahata, S. K., Mahata, M., Eiskeland, N. L., Videen, J. S., Zhang, X., Takii, Y., and Czerchowicz, J. H. (1994) Ann. N.Y. Acad. Sci. 733, 36–45
71. Tatemoto, K., Endriss, S., Matt, V., Makk, G., Feistner, G. J., and Barchas, J. D. (1986) Nature 324, 476–478
72. Eiden, L. E. (1987) Nature 325, 301
73. Huttner, W. B., and Benedum, U. M. (1987) Nature 325, 305
74. Helle, K. B., and Angeletti, R. H. (1994) Acta Physiol. Scand. 152, 1–10
75. Simon, J. P., Buder, M. P., and Aunis, D. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 1712–1716