0022-3565/07/3221-265–273$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 322:265–273, 2007

Vol. 322, No. 1
121335/3219287
Printed in U.S.A.

Modulation of Sympathetic Activity by Tissue Plasminogen Activator Is Independent of Plasminogen and Urokinase

Ulrich Schaefer,[1] Sandra Vorlova, Takuji Machida, Jerry P. Melchor, Sidney Strickland, and Roberto Levi

Department of Pharmacology, Weill Cornell Medical College, New York, New York (U.S., T.M., R.L.); and Laboratory of Neurobiology and Genetics, Rockefeller University, New York, New York (S.V., J.P.M., S.S.)

Received February 12, 2007; accepted April 10, 2007

ABSTRACT

Sympathetic neurons synthesize, transport, and release tissue-type plasminogen activators (t-PAs) and urinary-type plasminogen activators (u-PAs). We reported that t-PA enhances sympathetic neurotransmission and exacerbates reperfusion arrhythmias. We have now assessed the role of u-PA and plasminogen. Neurogenic contractile responses to electrical field stimulation (EFS) were determined in vasa deferentia (VD) from mice lacking t-PA (t-PA$^{-/-}$), plasminogen activator inhibitor-1 (PAI-1$^{-/-}$), plasminogen (plgn$^{-/-}$), u-PA (u-PA$^{-/-}$), and wild-type (WT) controls. Similar levels of t-PA were present in VD and cardiac synaptosomes of WT, PAI-1$^{-/-}$, plgn$^{-/-}$, and u-PA$^{-/-}$ mice, whereas t-PA was undetectable in t-PA$^{-/-}$ tissues. EFS responses were potentiated and attenuated in VD from PAI-1$^{-/-}$ and t-PA$^{-/-}$ mice, respectively, but indistinguishable from WT responses in VD from plgn$^{-/-}$and u-PA$^{-/-}$ mice. Moreover, t-PA inhibition with t-PA$_{stop}$ decreased EFS response in WT mice, whereas u-PA$_{stop}$ did not. VD responses to ATP, norepinephrine, and K^+ in t-PA$^{-/-}$, PAI-1$^{-/-}$, plgn$^{-/-}$, and u-PA$^{-/-}$ mice were similar to those in WT, whereas t-PA$_{stop}$ did not modify VD responses to norepinephrine in WT, t-PA$^{-/-}$, and PAI-1$^{-/-}$ mice, indicating a prejunctional site of action for t-PA-induced potentiation of sympathetic neurotransmission. Indeed, K^+-induced norepinephrine exocytosis from cardiac synaptosomes was potentiated in PAI-1$^{-/-}$, attenuated in t-PA$^{-/-}$ and not different from WT in u-PA$^{-/-}$ and plgn$^{-/-}$ mice. Likewise, ATP exocytosis was decreased in t-PA$^{-/-}$ and attenuated by t-PA$_{stop}$ in WT mice. Thus, t-PA-induced enhancement of sympathetic neurotransmission is a prejunctional event associated with increased transmitter exocytosis and independent of u-PA and plasminogen availability. This novel t-PA action may be a potential therapeutic target in hyperadrenergic states.

Tissue plasminogen activator (t-PA) and urinary-type plasminogen activator (u-PA, urokinase) are key components of endogenous fibrinolysis (Collen, 1999; O'Rourke et al., 2005). Both enzymes activate the zymogen plasminogen to plasmin, the principal circulating thrombolytic agent; indeed, functional disruption of the t-PA and u-PA genes in mice impairs clot lysis (Carmeliet et al., 1993b, 1994). The activity of t-PA and u-PA in plasma is regulated by specific inhibitors. Of these, plasminogen activator inhibitor type 1 (PAI-1) is considered the main inhibitor (Potempa et al., 1994). The two plasminogen activators have unique biological properties; t-PA, through its fibrin-binding function, is of primary importance in cell-independent fibrinolysis (Collen, 1999; Yakovlev et al., 2000; Medved and Nieuwenhuizen, 2003), whereas u-PA, through its high-affinity cell surface receptor, participates mainly in cell-mediated fibrinolysis, such as fibrin solubilization in organizing wound fields (Blasi et al., 1986; Ellis et al., 1991).

Prominent u-PA immunoreactivity has been demonstrated in the vas deferens and other urogenital organs (Larsson et al., 1984). Furthermore, u-PA mRNA expression was shown in murine gastrointestinal and urogenital tracts, including vas deferens (Kristensen et al., 1991), where the amounts of u-PA mRNA were as high as those of t-PA (Rickles and Strickland, 1988). Interestingly, the expression of t-PA mRNA in the heart was found to be 5-fold greater than in the vas deferens, whereas much lower t-PA mRNA was expressed in the gastrointestinal tract (Rickles and Strickland, 1988).

Recently, the presence of both t-PA and u-PA has been

This study was supported by the National Institutes of Health Grants HL34215, HL73400, HL47073, and HL46403 (to R.L.) and NS35704 and NS38472 (to S.S.). The authors have no conflicting financial interests.
[1] Current affiliation: Medizinische Klinik II, Universitätsklinikum Schleswig Holstein, Campus Lübeck, Germany.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.107.121335.

ABBREVIATIONS: t-PA, tissue plasminogen activator; u-PA, urinary-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; NE, norepinephrine; PAS, plasminogen activator system; $^{-/-}$, knockout; plgn, plasminogen; KH, Krebs-Henseleit; EFS, electrical field stimulation; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid; α,β-MeATP, α,β-methylene-ATP; ANOVA, analysis of variance; WT, wild type.

demonstrated in sensory neurons, and enhanced expression of t-PA, u-PA, PAI-1, and PAI-2 has been reported after neuronal injury (Yamanaka et al., 2005). In addition to its endothelial source, t-PA is synthesized in neural tissue (Peng et al., 1999; Jiang et al., 2002; O'Rourke et al., 2005). A recent report using a transgenic mouse model demonstrated a highly confined expression of t-PA in embryonic cells derived from the neural crest, with most prominent staining in the adrenal medulla and cervical ganglia, the sympathetic chain, axons within the myocardium, and the adventitial surface of several arteries (Hao et al., 2006). Importantly, Pittman and coworkers (Krystosek and Seeds, 1981; Pittman et al., 1989) found that sympathetic neurons are capable of releasing both u-PA and t-PA, a process perhaps involved in neurite outgrowth. Notably, physical and mental stress, electrical sympathetic stimulation, and coronary artery ligation all cause the acute release of t-PA into the circulation (Jern et al., 1994; Björkman et al., 2003; Aspelin et al., 2005).

We recently reported that endogenous t-PA enhances norepinephrine (NE) release from sympathetic nerves, whereas lack of t-PA greatly reduces it (Schaefer et al., 2006). We believe these findings to be relevant in conditions of exaggerated NE release, such as hypertension, myocardial ischemia, and congestive heart failure. Because sympathetic activity is a key player in cardiovascular disease, the aim of the present study was to identify the specific components of the plasminogen-activating system (PAS) (i.e., t-PA, u-PA, PAI-1, and plasminogen) critically involved in the modulation of NE release. In the present study, we find that t-PA is the only PAS component capable of influencing sympathetic tone and that this action is independent of u-PA and plasminogen availability.

Materials and Methods

Tissue Harvest. Mice with single-gene deletions ($tPA^{-/-}$, $plasminogen^{-/-}$, $u\text{-}PA^{-/-}$, $PAI\text{-}1^{-/-}$) have all been described elsewhere (Carmeliet et al., 1993b, 1994). Mice deficient in PAI-1, plgn, t-PA, and $u\text{-}PA^{-/-}$ were purchased from Jackson Laboratories (Bar Harbor, ME). All the mice used have been back crossed to C57/BL6 mice for at least nine generations. All studies were performed on male young adult mice at 4 to 5 months of age. After pretreatment with heparin (100 I.U. i.p.), mice were anesthetized with CO_2 and killed by cervical dislocation (approved by the Weill Cornell Medical College Institutional Animal Care Committee). Hearts and vasa deferentia were quickly excised and cooled in ice-cold modified Krebs-Henseleit (KH) solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 24 mM $NaHCO_3$, 1.1 mM KH_2PO_4, 10 mM glucose, 0.5 mM pyruvic acid, and 2.5 mM $CaCl_2 \cdot 2H_2O$). KH solution was continuously equilibrated with 95% O_2 + 5% CO_2, as described previously (Schaefer et al., 2006).

Vas Deferens. The midportion of the vas deferens was rapidly mounted into vessel chambers containing Krebs-Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 8.3 mM glucose, 2.5 mM $CaCl_2 \cdot$and $2H_2O$, gassed with 95% O_2 + 5% CO_2) with one end connected to a force transducer. Dissected vasa were allowed to equilibrate for at least 1 h at a resting tension of 250 mg at 37°C. In general, aerated Krebs-Ringer solution was replaced every 30 min. At the beginning of each experiment, a brief contraction with K^+ (80 mM) was induced, and the developed tension was recorded with PowerLab/8SP (ADInstruments, Colorado Springs, CO). After washout and an additional equilibration period of 60 min, sympathetic nerves were stimulated by electrical field stimulation (EFS) at 4 to 32 Hz for 15 s at 5-min intervals with a pulse width of 1 ms and supramaximal voltage. EFS was applied using a stimulator (model S48; Grass Instruments, West Warwick, RI) and a stimulation isolation unit (model SIU5; Grass Instruments). Contractile responses [first phase, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) sensitive, purinergic; second phase, prazosin sensitive, adrenergic] were individually analyzed and expressed as percentage of the response to K^+ (80 mM). Drugs were incubated for at least 15 min, and the resultant response to EFS was analyzed and compared with control stimulation. Responses were stable for at least three to four consecutive stimulations. To differentiate between pre- and postsynaptic sites of action, concentration-response curves for ATP (0.1–100 μM), phenylephrine (0.01–100 μM), and NE (0.01–100 μM) were constructed and expressed as percentage of the response to K^+.

Cardiac Synaptosomes. After excision of the heart, the aorta was cannulated with an 18-gauge custom-made steel cannula, and the heart was perfused at constant pressure (100 cm of H_2O) with KH buffer at 37°C. Two hearts per each mouse type were perfused for 20 min to ensure that blood components were removed from the coronary vasculature. Both hearts were minced together in ice-cold sucrose (0.32 M), and synaptosomes were prepared as described previously (Seyedi et al., 1997). Synaptosomes were depolarized with K^+, and the release of NE and ATP was analyzed in the supernatant and related to the pellet protein content (Sesti et al., 2003).

Inhibition of t-PA Activity. The activity of $u\text{-}PA_{stop}$ and $t\text{-}PA_{stop}$ was determined by their ability to inhibit recombinant t-PA, using a colorimetric substrate for the protease. The assay was performed in 96-well microtiter plates. Recombinant t-PA was diluted in a buffer containing 50 mM Tris-imidazole and 300 mM NaCl, pH 8.4, incubated with various concentrations of both inhibitors for 15 min at room temperature. The kinetics of substrate cleavage (20 μl of Pefachrome t-PA; concentration 2 mM; Pentapharm Ltd., Basel, Switzerland) were monitored spectrophotometrically at 405 nm for 5 min (Schaefer et al., 2006).

NE Assay. Synaptosomal supernatants were assayed for NE by HPLC with electrochemical detection as described previously (Seyedi et al., 1997). The detection limit was ~0.05 pmol.

ATP Assay. ATP levels were measured with a firefly luciferin-luciferase assay-based commercial kit (ATP bioluminescence assay kit HS II; Roche Diagnostics, Indianapolis, IN). Samples (50 μl) of each supernatant were pipetted into appropriate test tubes, placed in a luminometer (model 20/20; Turner Designs, Sunnyvale, CA), and processed by autoinjection of 50 μl of luciferin-luciferase reagent. ATP concentrations were calculated from a calibration curve constructed the same day using ATP standards included in the kit. The amount of ATP was expressed as femtomoles per milligram of synaptosomal protein (Sesti et al., 2003).

t-PA Antigen Assay. A 96-well strip format enzyme-linked immunosorbent assay kit (MTPAKT-TOT) was obtained from LOXO-GmbH (Dossenheim, Germany). The detection limit was below 0.05 pg/μg protein.

Drugs and Chemicals. Adenosine 5′-triphosphate (disodium salt), norepinephrine HCl, prazosin HCl, phenylephrine, PPADS, and α,β-methylene-ATP (α,β-MeATP) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Plasminogen, $t\text{-}PA_{stop}$, and $u\text{-}PA_{stop}$ were purchased from American Diagnostics, Inc. (Stamford, CT). Pefachrome t-PA (Pefa-5037) was purchased from Pentapharm.

Statistics. Values refer to means ± S.E.M. One-way ANOVA followed by Dunnett's post-test, one-sample Student's t test, and unpaired Student's t test were used as indicated. $p < 0.05$ was considered significant.

Results

The Neurogenic Response of Mouse Vas Deferens Is Enhanced by Tissue Plasminogen Activator: A Prejunctional Event Independent of Plasminogen and Urokinase. Two typical phases are recognizable in the contractile response of the isolated mouse vas deferens to electrical field

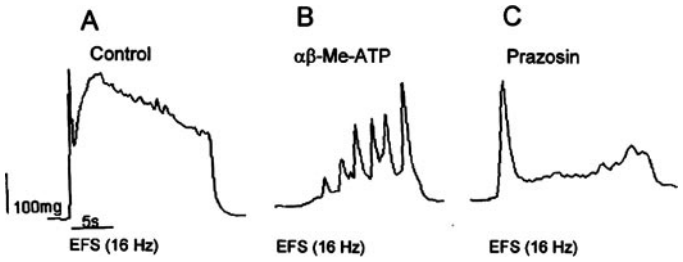

Fig. 1. Biphasic response of the C57/BL6-mouse vas deferens to EFS (representative tracings). A, EFS (16 Hz, supramaximal voltage, 1 ms for 15 s) elicits a biphasic contraction characterized by an initial spike followed by a second, prolonged, and slow-decaying contractile response. B, P2X-receptor desensitization with α,β-MeATP (10 μM for 15 min) selectively blocks the initial spike, characterizing it as a purinergic response. C, α_1-adrenoceptor antagonist prazosin (0.1 μM for 15 min) markedly attenuates the second contractile phase, identifying it as a predominantly adrenergic response.

stimulation (0–32 Hz, supramaximal voltage, 1 ms, 15 s) (Fig. 1A). The initial phase consists of a sharp spike attributed to a rapid smooth muscle contraction upon activation of postjunctional P_2X-receptors by ATP released from sympathetic nerves (Todorov et al., 1996). The second contractile phase is due to activation of postjunctional α_1-adrenoceptors by released NE. A certain degree of overlap exists between both phases, in that ATP partially influences the second phase and NE the first phase (Todorov et al., 1996). Indeed, desensitization of P2X-receptors with a high concentration of α,β-Me-ATP (10 μM) or blockade with the P2X-receptor antagonist PPADS (10 μM; data not shown) abolished the initial contractile phase but also slightly attenuated the second phase (Fig. 1B). Conversely, preincubation with the α_1-adrenoceptor antagonist prazosin (0.1 μM) attenuated the second phase and slightly the first (Fig. 1C). At frequencies between 4 and 32 Hz, EFS induced a progressive increase in the amplitude of both purinergic and adrenergic phases (Fig. 2, A and B). These responses persisted unabated for at least three to four subsequent stimulation cycles (i.e., time controls; data not shown). Frequency-response curves for vasa deferentia isolated from plgn$^{-/-}$ and u-PA$^{-/-}$ mice were superimposable on the respective curves obtained in vasa from WT mice (Fig. 2). In contrast, the frequency-response curves for the vasa deferentia isolated from PAI-1$^{-/-}$ and t-PA$^{-/-}$ mice were shifted to the left and right of the WT curve, respectively. Indeed, at 4 and 32 Hz, peak contractile responses of vasa deferentia were greater in PAI-1$^{-/-}$ than in WT mice and smaller in t-PA$^{-/-}$ (Fig. 2).

The contractile response elicited by EFS in the vas deferens from WT mice was not different from that of urokinase-deficient mice (see Fig. 2), suggesting that the PAS-induced modulation of sympathetic activity is independent of u-PA. To confirm this result, we used u-PA$_{stop}$, a selective u-PA inhibitor (Stürzebecher et al., 1999). Concentration-response curves for the inhibition of rt-PA activity by u-PA$_{stop}$ (30 nM–300 μM) and t-PA$_{stop}$ (3 nM–300 μM) are shown in Fig. 3A. IC_{50} values were 3.1 $\pm$ 0.08 and 0.13 $\pm$ 0.02 μM for u-PA$_{stop}$ and t-PA$_{stop}$, respectively. Having determined the relative inhibitory potency of u-PA$_{stop}$ and t-PA$_{stop}$, we next assessed the effect of pretreatment with these inhibitors on the response of the WT vas deferens to EFS. At 10 μM (i.e., a concentration at which u-PA$_{stop}$ and t-PA$_{stop}$ inhibited t-PA activity by ~80 and 100%, respectively; see Fig. 3A), both purinergic and adrenergic frequency-response curves were markedly shifted downwards by t-PA$_{stop}$ and only slightly, but not significantly, by u-PA$_{stop}$ (Fig. 3B). This indicated that the response of the vas deferens to EFS is independent of u-PA but dependent on t-PA.

To confirm that the response of the vas deferens to EFS is independent of u-PA, we compared the effects of t-PA$_{stop}$ on the vas deferens of WT mice with the effects of t-PA$_{stop}$ on the

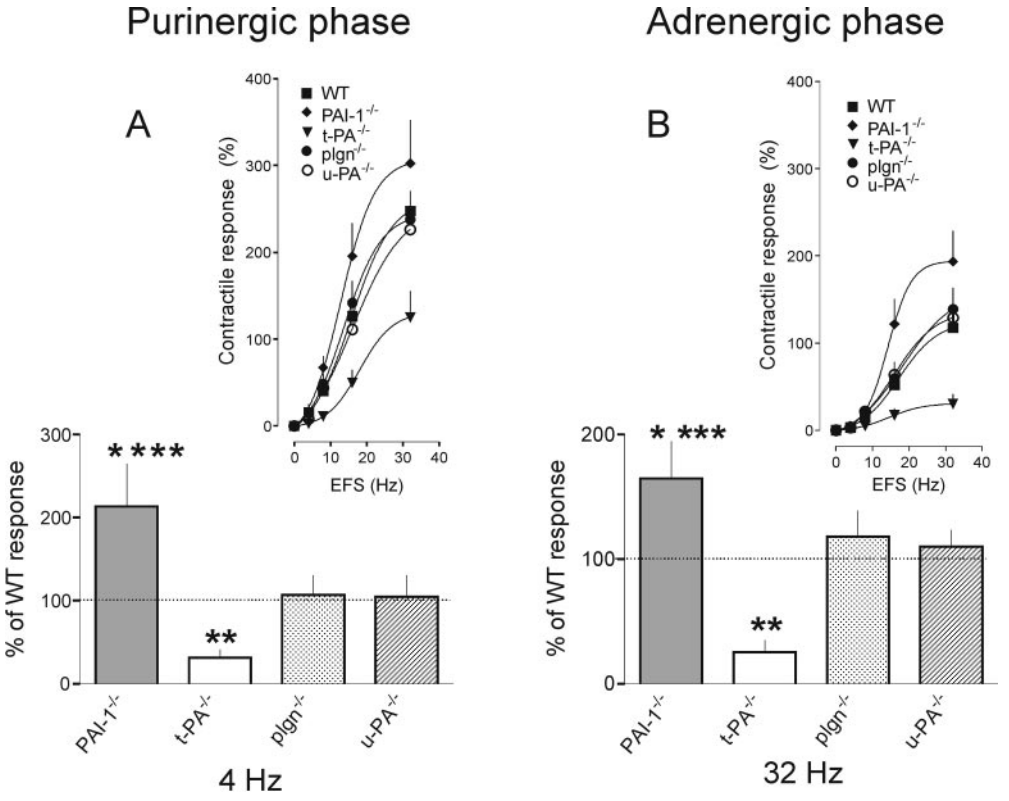

Fig. 2. t-PA gene deletion, but not deletion of u-PA or plasminogen, attenuates the sympathetic response of the vas deferens to EFS. Top graphs in A and B, frequency-response curves for the contractile responses of mouse vas deferens to EFS (0–32 Hz, supramaximal voltage, 1 ms, 15 s). Peak response amplitudes (means $\pm$ S.E.M.; n = 8–10) of various gene-deleted mice (t-PA$^{-/-}$, PAI-1$^{-/-}$, plgn$^{-/-}$, and u-PA$^{-/-}$) and their WT controls are expressed as percentage of the individual response to K^+ (80 mM). Lower bar graphs in A and B, peak contractile responses to EFS (4 and 32 Hz in A and B, respectively) expressed as percentage change from their WT controls. Bars are means ($\pm$S.E.M.; n = 8–10). * and **, $p <$ 0.05 and 0.01, respectively, from WT, u-PA$^{-/-}$ and plgn$^{-/-}$; ***, $p < 0.001$ from t-PA$^{-/-}$, by one-way ANOVA and Dunnett's test.

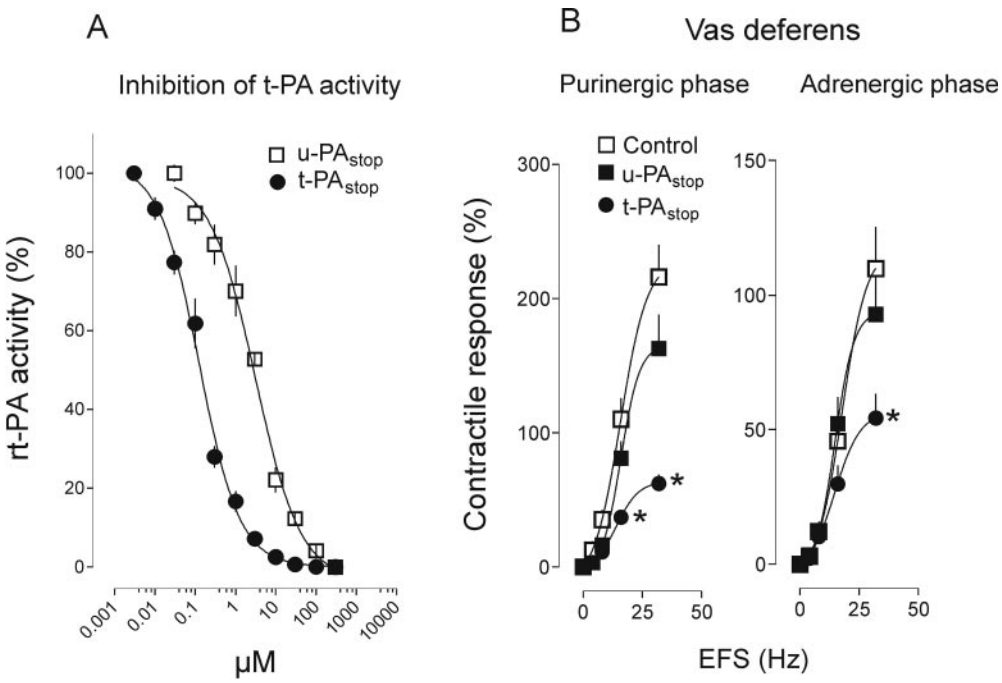

Fig. 3. Lack of u-PA involvement in the neurogenic contraction of the mouse vas deferens. A, comparison of concentration-response curves for the inhibition of rt-PA activity by u-PA$_{stop}$ and t-PA$_{stop}$. rt-PA (0.3 μg/ml), in combination with the colorimetric t-PA substrate Pefa-T (2 mM), was incubated with increasing concentrations of t-PA$_{stop}$ (3 nM-300 μM) or u-PA$_{stop}$ (30 nM–300 μM). IC$_{50}$s were 0.127 ± 0.02 and 3.13 ± 0.08 μM for t-PA$_{stop}$ and u-PA$_{stop}$, respectively. Points are means (±S.E.M.; n = 6). B, frequency-response curves for the contractile responses of mouse vas deferens to EFS (0–32 Hz, supramaximal voltage, 1 ms, 15 s) before (control) and after coincubation with u-PA$_{stop}$ or t-PA$_{stop}$, each at 10 μM. t-PA$_{stop}$ significantly attenuates neurogenic contraction to EFS (*, $p < 0.01$, unpaired Student's t test), whereas the response was only slightly reduced, but not significantly, with u-PA$_{stop}$. Peak response amplitudes are expressed as percentage of the individual response to K^+ (80 mM). Points are means (±S.E.M.; n = 8–12).

vasa of t-PA$^{-/-}$, u-PA$^{-/-}$, and PAI-1$^{-/-}$ mice. t-PA$_{stop}$ (10 μM) inhibited the purinergic and adrenergic phases of the response to EFS (4–32 Hz) to a similar extent in the vasa of WT and u-PA$^{-/-}$ mice, indicating that the absence of u-PA does not influence the response of the vas deferens to EFS and reinforcing the role of t-PA (Fig. 4). In fact, t-PA$_{stop}$ failed to influence the response to EFS of vasa of t-PA$^{-/-}$ mice, whereas it markedly inhibited the response of vasa of PAI-1$^{-/-}$ mice, demonstrating that a higher t-PA activity due to lack of endogenous PAI-1 enhances the sensitivity to t-PA inhibitors (Fig. 4). Collectively, the data obtained with t-PA$_{stop}$ (see Fig. 4) indicated that t-PA, but not u-PA, plays a potentiating role in the neurogenic response of the mouse vas deferens to EFS.

Because the neurogenic response of the isolated vas deferens to EFS is the result of pre- and postjunctional components (Todorov et al., 1996), we questioned whether the potentiating role of t-PA also involves postsynaptic sites. Thus, we investigated the contractile response of the vas deferens to exogenous ATP and NE in WT mice and in mice with various PAS deletions. Concentration-response curves for ATP (0.1–100 μM; Fig. 5A) and NE (0.01–100 μM; Fig. 5B)

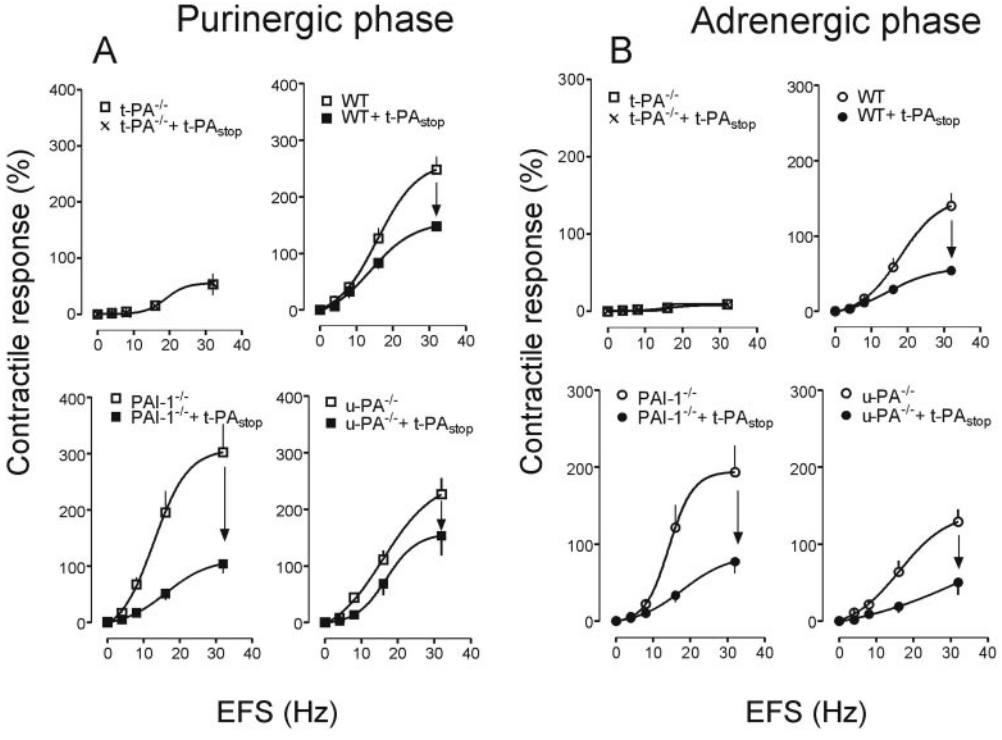

Fig. 4. Gene deletion of t-PA, but not of u-PA, prevents t-PA$_{stop}$ from inhibiting the neurogenic contractile response of mouse vas deferens. A, frequency-response curves for the purinergic contractile responses to EFS (0–32 Hz, supramaximal voltage, 1 ms, 15 s) of mouse vas deferens isolated from various gene-deleted mice (tPA$^{-/-}$, PAI-1$^{-/-}$ and u-PA$^{-/-}$) and their WT controls, before and after coincubation of t-PA$_{stop}$ (10 μM). Peak response amplitudes are expressed as percentage of the individual response to K^+ (80 mM). Points are means (±S.E.M.; n = 4–12). B, frequency-response curves for the adrenergic contractile responses to EFS.

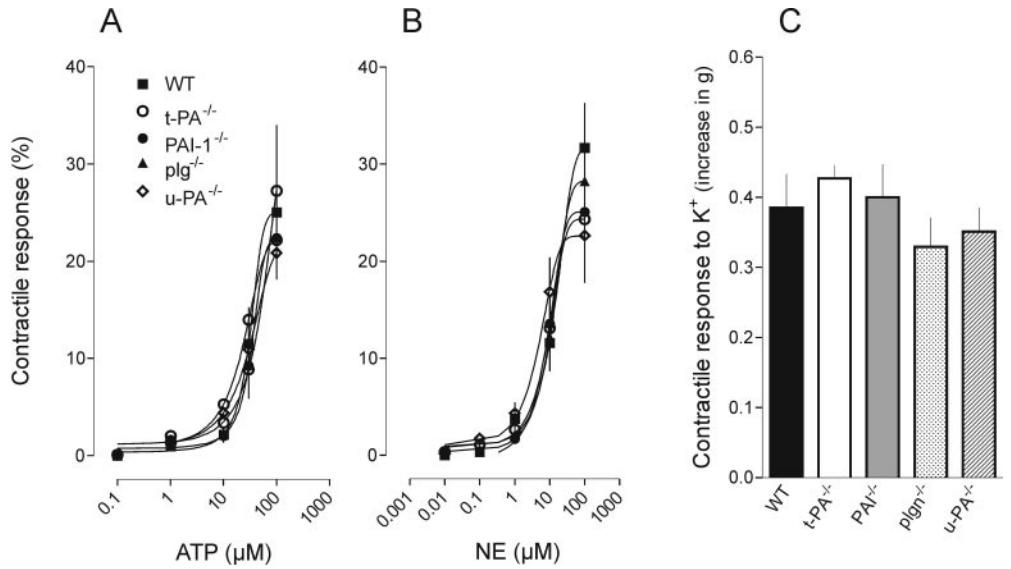

Fig. 5. PAS gene deletions do not modify the postjunctional contractile response of mouse vas deferens to the administration of ATP, NE, and K^+. A, noncumulative concentration-response curves for the contractile effect of ATP in vasa deferentia isolated from WT, t-$PA^{-/-}$, PAI-$1^{-/-}$, plasminogen$^{-/-}$, and u-$PA^{-/-}$ mice. The maximum response for each increment in ATP concentration was measured within 30 s, and ATP was quickly washed out to prevent receptor desensitization. The next higher concentration of ATP was added after a 30-min equilibration interval. The same procedure was followed for each of the five ATP concentrations tested. Contractile responses are expressed as percentage of the response to K^+ (80 mM) (±S.E.M.; $n = 4–10$). B, cumulative concentration-response curves for the contractile effect of NE in vas deferens of WT, t-$PA^{-/-}$, PAI-$1^{-/-}$, plasminogen$^{-/-}$, and u-$PA^{-/-}$ mice. Contractile responses are expressed as percentage of the response to K^+ (80 mM) (±S.E.M.; $n = 4–10$). C, contractile response to K^+ (80 mM) of vasa deferentia from WT, t-$PA^{-/-}$, PAI-$1^{-/-}$, plasminogen$^{-/-}$, and u-$PA^{-/-}$ mice. Contractile responses are expressed as increases in g above baseline tension (200 mg). Points are means (±S.E.M.; $n = 6–10$).

obtained in vasa from t-$PA^{-/-}$, PAI-$1^{-/-}$, plgn$^{-/-}$, and u-$PA^{-/-}$ mice were superimposable on the curves obtained in vasa from WT mice. Comparable results were obtained with phenylephrine (data not shown). In addition, the amplitude of the contractile response of vasa deferentia to K^+ (80 mM) was the same in WT, t-$PA^{-/-}$, PAI-$1^{-/-}$, plgn$^{-/-}$, and u-$PA^{-/-}$ mice (Fig. 5C). Moreover, t-PA_{stop} (10 μM) failed to modify the concentration-response curves for the contractile response of vasa deferentia to exogenous NE in WT, t-$PA^{-/-}$, and PAI-$1^{-/-}$ mice (Fig. 6). The finding that various PAS deletions and selective inhibition of t-PA with t-PA_{stop} did not influence the response of the vas deferens to stimulation of postjunctional sites suggested that t-PA selectively targets prejunctional sites in its modulation of the neurogenic contractile response and that neither plasminogen nor urokinase is involved in this action of t-PA.

Exocytosis of NE and ATP from Mouse Heart Nerve Endings Is Enhanced by Tissue Plasminogen Activator and Is Independent of Plasminogen and Urokinase. Because our findings suggested a presynaptic site of t-PA action, we chose the cardiac synaptosomal preparation, an optimal model of sympathetic nerve endings, for the investi-

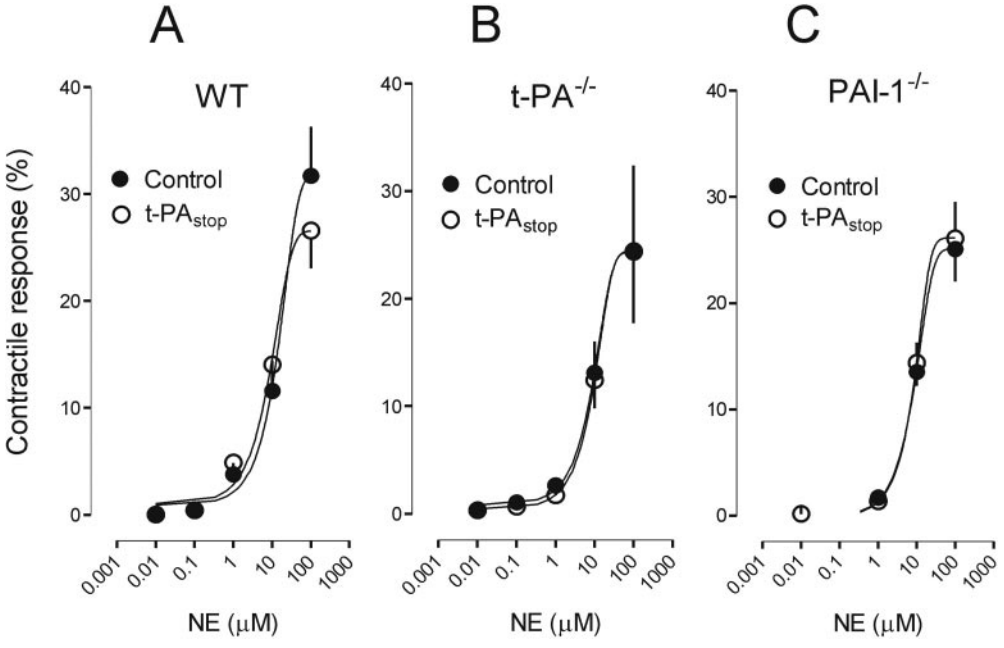

Fig. 6. t-PA_{stop} does not modify the contractile response of mouse vas deferens to the administration of NE. Cumulative concentration-response curves to NE with or without pretreatment with t-PA_{stop} (10 μM) in vasa deferentia isolated from of WT (A), t-$PA^{-/-}$ (B), and PAI-$1^{-/-}$ mice (C). Contractile responses are expressed as percentage of response to K^+ (80 mM). Points are means (±S.E.M.; $n = 4–10$).

gation of NE and ATP exocytosis (Seyedi et al., 1997; Sesti et al., 2003), to determine whether the action of t-PA entails a modulation of transmitter release. Upon depolarization with 50 and 100 mM K^+, NE release from synaptosomes isolated from WT mouse hearts increased by ~15 and 30% over basal level, respectively, and further increased to ~40 and 45% in synaptosomes from PAI-$1^{-/-}$ mouse hearts (Fig. 7). In contrast, K^+-induced NE release in synaptosomes from t-$PA^{-/-}$ hearts was 6-fold lower than WT. In synaptosomes from hearts of $plgn^{-/-}$ and u-$PA^{-/-}$ mice, NE exocytosis was not different from WT synaptosomes (Fig. 7). K^+-induced synaptosomal ATP release increased by ~10 and 50% over basal level with 50 and 100 mM K^+, respectively (Fig. 8). Notably, the concentration-response curve for the K^+-induced ATP release in WT synaptosomes was shifted downwards in the presence of t-PA_{stop} (10 μM; Fig. 8A). Likewise, the concentration-response curve for the K^+-induced ATP release in t-$PA^{-/-}$ synaptosomes was shifted downwards compared with the curve in WT synaptosomes (Fig. 8B). Thus, the t-PA-induced potentiation of neurogenic sympathetic responses is associated with an increase in both ATP and NE exocytosis, which is plasminogen- and urokinase-independent.

Presence of t-PA Antigen in Vas Deferens and Mouse Heart Synaptosomes. t-PA antigen levels in vasa deferentia and cardiac synaptosomes isolated from WT mice were similar to those detected in vasa and synaptosomes from PAI-$1^{-/-}$, u-$PA^{-/-}$, and $plgn^{-/-}$ mice (Fig. 9). In contrast, t-PA antigen was undetectable in vasa deferentia and cardiac synaptosomes isolated from t-$PA^{-/-}$ mice. Interestingly, t-PA antigen level was ~10-fold higher in cardiac synaptosomes than in whole heart (~1.7 pg/mg protein in synaptosomes, see Fig. 9A, versus 0.173 ± 0.04 pg/mg in whole-heart homogenate).

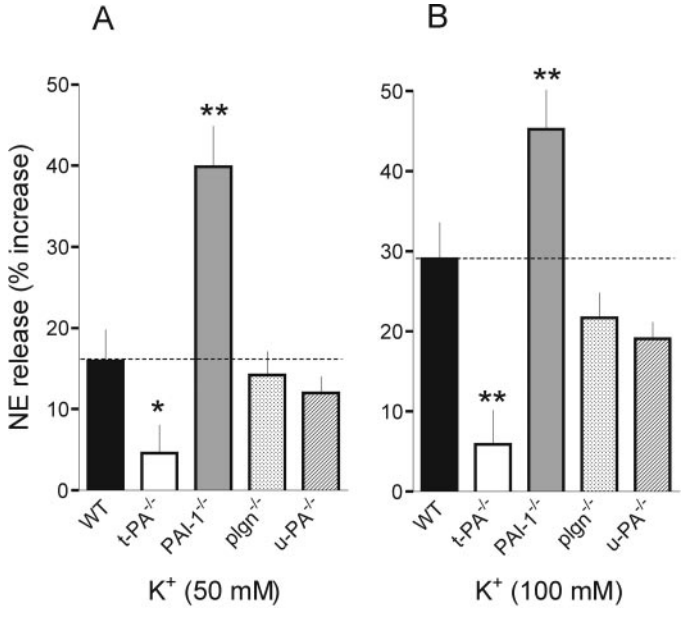

Fig. 7. t-PA and PA-1 gene deletions attenuate and potentiate NE exocytosis from mouse heart synaptosomes, respectively, whereas plgn and u-PA deletions do not affect it. A and B, NE exocytosis evoked by K^+ (50 and 100 mM, respectively) from synaptosomes isolated from hearts of WT and various PAS gene-deleted mice. Points are means of percent increases in NE release above basal levels (±S.E.M.; n = 12–16). Basal NE release levels were 0.60 ± 0.03 pmol/pg protein WT, 0.53 ± 0.02 pmol/pg protein t-$PA^{-/-}$, 0.66 ± 0.04 pmol/pg protein PAI-$1^{-/-}$, 0.61 ± 0.03 pmol/pg protein $plgn^{-/-}$, and 0.58 ± 0.01 pmol/pg protein u-$PA^{-/-}$. * and **, significantly different from WT ($p < 0.05$ and 0.01, respectively) by one-way ANOVA and Dunnett's test.

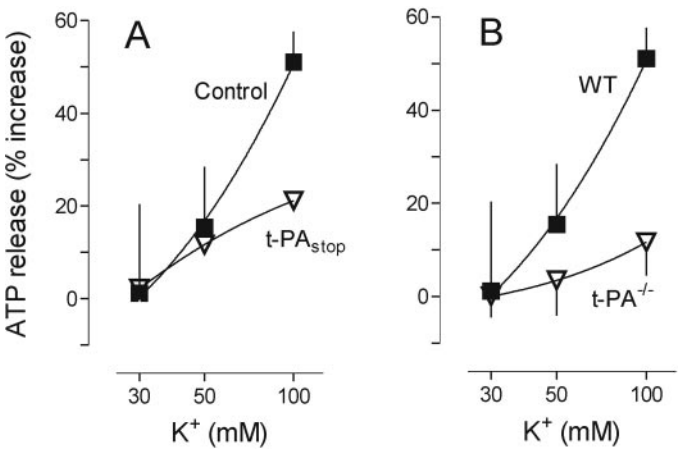

Fig. 8. t-PA_{stop} and t-PA gene deletion attenuate ATP exocytosis from mouse heart synaptosomes. Concentration-response curves for the K^+-induced ATP release from mouse heart synaptosomes. A, cardiac synaptosomes were isolated from WT mice and depolarized with K^+ in control conditions (control) or after pretreatment with t-PA_{stop} (10 μM). Points are means (±S.E.M.; n = 4–6) of increases in ATP release above basal levels. Basal ATP release: 1.00 ± 0.07 fmol/mg protein control (n = 4) and 0.9 ± 0.04 fmol/mg protein t-PA_{stop} (n = 6). B, synaptosomes were isolated from WT or t-$PA^{-/-}$ mice and depolarized with K^+. Points are means (±S.E.M.; n = 4–8) of increases in ATP release above basal levels. Basal ATP release: WT, 1.00 ± 0.07 fmol/mg protein (n = 4); and t-$PA^{-/-}$, 0.21 ± 0.04 fmol/mg protein (n = 8, $p < 0.001$ from WT by unpaired Student's t test).

Discussion

Due to its dense sympathetic plexus, the vas deferens is frequently used as a functional model to assess drug effects at the sympathetic neuroeffector junction. When sympathetic nerves of the vas deferens are stimulated, ATP and NE are released, thereby activating postjunctional purinergic $P2X_1$ and adrenergic α_1 receptors, respectively (Todorov et al., 1996; Westfall et al., 1996). Efficient control of synaptic transmission requires a rapid mechanism for terminating the actions of neurotransmitters. Amino acids and monoamines are taken back up into neurons by specific high-affinity transporters, whereas ATP is broken down in the extracellular space (Amara and Kuhar, 1993; Zimmermann and Braun, 1999). Quantities of released transmitters determine signaling strength. Typically, EFS of vas deferens elicits a biphasic twitch response. Most ATP is released during the first phase of contraction. Neuronally released ATP elicits excitatory junction potentials, which in turn initiate action potentials, resulting in the phasic component of the neurogenic response (Allcorn et al., 1986). Seconds later, NE is released into the sympathetic junction, leading to the tonic component of contraction. The first phase is blocked by α,β-MeATP, and the second phase is blocked by prazosin (see Fig. 1). It is well known that with increasing stimulation frequencies, NE release quantitatively exceeds that of ATP; therefore, particularly during low-stimulation frequencies (≤4 Hz), purinergic responses can be easily separated from adrenergic responses (>8 Hz). Numerous presynaptic modulators of the sympathetic nervous system have been described previously (Boehm and Kubista, 2002), but PAS components

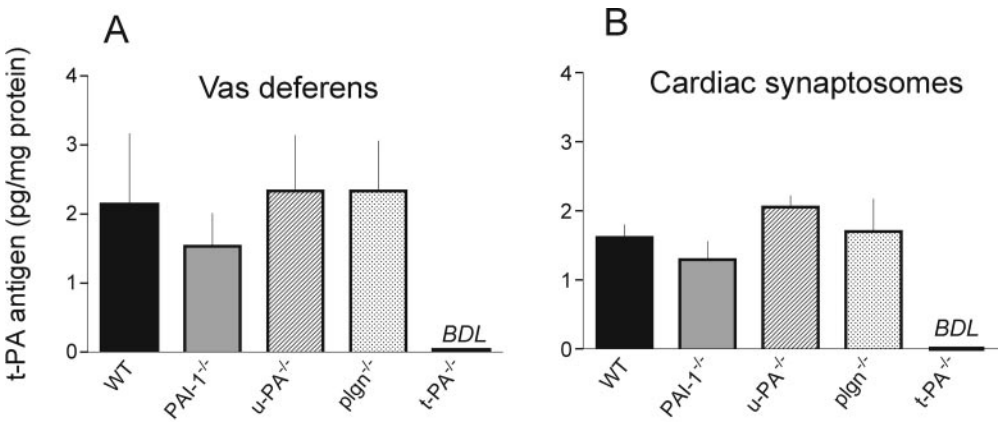

Fig. 9. t-PA antigen levels in vas deferens and cardiac synaptosomes from WT and various PAS gene-deleted mice. A, t-PA antigen levels in freshly dissected vasa deferentia. Points are means (±S.E.M.; $n = 3$). B, t-PA antigen levels in nondepolarized mouse heart synaptosomes. Points are means (±S.E.M.; $n = 6–11$). No significant difference was observed among t-PA antigen levels detected in vasa and synaptosomes from WT, PAI-$1^{-/-}$, u-$PA^{-/-}$, and $plgn^{-/-}$ mice. Notably, t-PA antigen was below detection limit (BDL) in vasa and synaptosomes from t-$PA^{-/-}$ mice.

were not part of the list. We recently demonstrated that PAS plays a major role in sympathetic neurotransmission (Schaefer et al., 2006). In addition, there is increasing evidence that sympathetic neurons synthesize, transport, and release t-PA, thereby contributing significantly to plasmatic fibrinolytic capacity. Prominent t-PA immunostaining is present in sympathetic axons of small arteries, arterioles, and vasa vasorum (Lochner et al., 1998; O'Rourke et al., 2005). Evidence for an axonal source of t-PA activity includes: 1) a decreased blood fibrinolytic activity following guanethidine sympathectomy (Peng et al., 1999), 2) an increased regional blood t-PA activity during in vivo electrical stimulations of sympathetic nerves (Jern et al., 1994; Björkman et al., 2003), 3) much slower t-PA-releasing kinetics from resting cultured endothelial cells than from sympathetic neurons, and 4) very prominent arteriolar t-PA immunostaining in striated muscle arterioles, but not in venules (Huber et al., 2002). In addition, u-PA has been found to be present within the sympathetic nervous system (Pittman et al., 1989).

The aim of this study was to identify which PAS components modulate sympathetic neural activity and, in particular, to determine a possible role of u-PA and plasminogen. Using vasa deferentia isolated from gene-targeted mice, we found that only vasa isolated from t-PA-null mice displayed attenuated purinergic and adrenergic neurogenic responses to EFS. In contrast, responses evoked in vasa from u-$PA^{-/-}$ and $plasminogen^{-/-}$ mice were almost identical to those in WT mice. Thus, the interaction between PAS and sympathetic nervous system seems to be restricted to t-PA and most likely independent of u-PA and plasminogen availability. Indeed, as previously reported (Schaefer et al., 2006), we found an enhanced contractile response to EFS in vasa deferentia from PAI-$1^{-/-}$ mice. This enhanced response is probably related to increased t-PA activity, as evidenced by increased fibrinolytic capacity in PAI-$1^{-/-}$ mice (Carmeliet et al., 1993a, b, 1994).

Moreover, we investigated the effects of t-PA_{stop} and u-PA_{stop}, reversible competitive inhibitors of t-PA and u-PA, respectively, on the response to EFS and found additional evidence that only t-PA is involved in the neurogenic contraction. Indeed, only t-PA_{stop} significantly attenuated the contractile response of vasa deferentia isolated from WT mice. Furthermore, t-PA_{stop} failed to influence the contractile response to EFS of vasa from $tPA^{-/-}$ mice but markedly inhibited the response of vasa from PAI-$1^{-/-}$ mice. In contrast, a similar mild inhibition was observed in vasa from WT, u-$PA^{-/-}$, and $plgn^{-/-}$ mice. Thus, our data clearly suggest that t-PA positively modulates sympathetic neurotransmission independently of urokinase and plasminogen availability. Although the potentiation of sympathetic neurotransmission could involve pre- and/or postjunctional sites, we found that the contractile response of the vas deferens to the administration of ATP and NE or to depolarization with K^+ was the same in WT and every single PAS gene-deleted mouse. Moreover, t-PA_{stop} did not modify the contractile response to NE, not only in WT vasa, but also in vasa from t-$PA^{-/-}$ and PAI-$1^{-/-}$ mice. Thus, t-PA modulates sympathetic neurotransmission in the vas deferens exclusively at prejunctional sites.

Prejunctional modulation of sympathetic neurotransmission by t-PA, as evidenced by the enhancement of both purinergic and adrenergic phases of the twitch response of vas deferens to EFS, could involve an enhanced release of ATP and NE and/or an increase in transmitter bioavailability due to reduced NE reuptake and diminished metabolism of released ATP. However, it is unlikely that t-PA would affect both NE reuptake and ATP metabolism, two totally different mechanisms of signal termination. Furthermore, pretreatment with desipramine, a neuronal NE transport inhibitor, diminished rt-PA-induced NE release (Schaefer et al., 2006), discounting the possibility that t-PA or t-PA_{stop} might reduce NE re-uptake. Thus, it would seem that t-PA acts prejunctionally augmenting transmitter release. Indeed, we found that synaptosomes isolated from t-$PA^{-/-}$ mouse hearts released much less ATP and NE upon K^+ depolarization than synaptosomes from WT hearts, whereas synaptosomes from PAI-$1^{-/-}$ hearts released much more NE. Again, this effect was restricted to t-PA because NE exocytosis in cardiac synaptosomes from u-$PA^{-/-}$ and $plgn^{-/-}$ mice was not different from that in WT. In addition, we recently demonstrated that administration of therapeutically relevant concentrations of rt-PA to guinea pig heart synaptosomes and primary cultures of human neuroblastoma SH-SY5Y cells releases NE as a function of its concentration (Schaefer et al., 2006). In agreement with our findings, a recent study demonstrated that the release of dopamine and acetylcholine evoked by depolarization in mouse brain was markedly enhanced by local rt-PA microinjections, whereas it was reduced by PAI-1 administration and in t-$PA^{-/-}$ mice (Ito et al., 2006).

We postulate that the activity level of t-PA will determine the magnitude of NE release. In fact we had found that

pretreatment with t-PA stimulators (CNBrF) enhances r-tPA-induced NE release in guinea pig heart synaptosomes and SH-SY5Y cells (Schaefer et al., 2006), whereas the vas deferens response to EFS and NE release from cardiac synaptosomes are both markedly increased in PAI-$1^{-/-}$ mice (see Figs. 2, 4, and 7). Furthermore, we predict that the neuromodulatory function of the PAS, as observed in our models, is dependent upon the actions of t-PA and independent of u-PA and plasminogen. In fact, t-PA levels were below detection limits in vasa deferentia and cardiac synaptosomes of t-PA$^{-/-}$ mice. Moreover, only t-PA gene deletion, but not deletion of u-PA and plasminogen, caused a marked attenuation of neuronal function in the vas deferens and a striking decrease in NE release from cardiac synaptosomes compared with WT controls.

The mechanism of the prejunctional action of t-PA resulting in the potentiation of sympathetic neurotransmission remains speculative. Until now, a specific t-PA receptor has not been discovered, although different binding proteins for t-PA have been described previously (Melchor and Strickland, 2005). A specific receptor or substrate is likely to exist because the effects mediated by rt-PA clearly involve well known mechanisms of exocytotic and nonvesicular NE release (i.e., intracellular Ca^{2+}, Na^{+}/H^{+} exchanger, and NE transporter), as we previously showed in guinea pig heart synaptosomes (Schaefer et al., 2006). A less likely explanation entails a distinct protein-protein interaction. Multiple possibilities for protein-protein interactions involving different subdomains within the five-domain structure of t-PA have been described previously (Melchor and Strickland, 2005). Future studies using site-directed mutagenesis would be one approach to elucidate a specific t-PA subdomain interacting with the sympathetic nervous system. So far, the serine protease domain seems to be a candidate for the effects of t-PA.

Interestingly, brain and heart contain the highest concentrations of t-PA (Christie et al., 1999); this observation is likely to have important pathophysiological implications. Indeed, we found a 10-fold enrichment in t-PA antigen in cardiac synaptosomes compared with the intact heart. Notably, the vas deferens with its dense sympathetic innervation had t-PA antigen levels similar to those of cardiac synaptosomes. Thus, our observations may be applicable to all sympathetically innervated tissues. Administration of r-tPA for thrombolysis in acute myocardial infarction or stroke is still considered an important treatment option. Inasmuch as vascular patency is the major goal to preserve cardiac function in myocardial infarction, magnification of sympathetic stress with rt-PA thrombolysis carries potential risks. In this regard, we found that NE spillover and ventricular arrhythmias during ischemia/reperfusion were markedly decreased in hearts of t-PA$^{-/-}$ mice compared with WT and PAI-$1^{-/-}$ hearts (Schaefer et al., 2006).

In summary, we have used mouse models with single PAS component deletions (i.e., t-PA$^{-/-}$ or PAI-$1^{-/-}$) to uncover interactions between plasminogen-activating and sympathetic nervous systems. Extending previous data from our laboratory, we offer novel evidence that the neuromodulatory action of PAS is exclusively related to t-PA and independent of u-PA and plasminogen availability. Safeguarding the thrombolytic activity of t-PA and eliminating its prosympathetic effects should be an important goal in preclinical research.

Acknowledgments

We thank Geoffrey Abbott and Christopher Morrey for excellent graphical assistance.

References

Allcorn RJ, Cunnane TC, and Kirkpatrick K (1986) Actions of alpha, beta-methylene ATP and 6-hydroxydopamine on sympathetic neurotransmission in the vas deferens of the guinea-pig, rat and mouse: support for cotransmission. *Br J Pharmacol* **89:**647–659.

Amara SG and Kuhar MJ (1993) Neurotransmitter transporters: recent progress. *Annu Rev Neurosci* **16:**73–93.

Aspelin T, Eriksen M, Lindgaard AK, Lyberg T, and Ilebekk A (2005) Cardiac fibrinolytic capacity is markedly increased after brief periods of local myocardial ischemia, but declines following successive periods in anesthetized pigs. *J Thromb Haemost* **3:**1947–1954.

Björkman JA, Jern S, and Jern C (2003) Cardiac sympathetic nerve stimulation triggers coronary t-PA release. *Arterioscler Thromb Vasc Biol* **23:**1091–1097.

Blasi F, Stoppelli MP, and Cubellis MV (1986) The receptor for urokinase-plasminogen activator. *J Cell Biochem* **32:**179–186.

Boehm S and Kubista H (2002) Fine tuning of sympathetic transmitter release via ionotropic and metabotropic presynaptic receptors. *Pharmacol Rev* **54:**43–99.

Carmeliet P, Kieckens L, Schoonjans L, Ream B, van Nuffelen A, Prendergast G, Cole M, Bronson R, Collen D, and Mulligan RC (1993a) Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J Clin Invest* **92:**2746–2755.

Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, and Mulligan RC (1994) Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* **368:**419–424.

Carmeliet P, Stassen JM, Schoonjans L, Ream B, van den Oord JJ, De Mol M, Mulligan RC, and Collen D (1993b) Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *J Clin Invest* **92:**2756–2760.

Christie PD, Edelberg JM, Picard MH, Foulkes AS, Mamuya W, Weiler-Guettler H, Rubin RH, Gilbert P, and Rosenberg RD (1999) A murine model of myocardial microvascular thrombosis. *J Clin Invest* **104:**533–539.

Collen D (1999) The plasminogen (fibrinolytic) system. *Thromb Haemost* **82:**259–270.

Ellis V, Behrendt N, and Dano K (1991) Plasminogen activation by receptor-bound urokinase: a kinetic study with both cell-associated and isolated receptor. *J Biol Chem* **266:**12752–12758.

Hao Z, Guo C, Jiang X, Krueger S, Pietri T, Dufour S, Cone RE, and O'Rourke J (2006) New transgenic evidence for a system of sympathetic axons able to express tissue plasminogen activator (t-PA) within arterial/arteriolar walls. *Blood* **108:**200–202.

Huber D, Cramer EM, Kaufmann JE, Meda P, Masse JM, Kruithof EK, and Vischer UM (2002) Tissue-type plasminogen activator (t-PA) is stored in Weibel-Palade bodies in human endothelial cells both in vitro and in vivo. *Blood* **99:**3637–3645.

Ito M, Nagai T, Kamei H, Nakamichi N, Nabeshima T, Takuma K, and Yamada K (2006) Involvement of tissue plasminogen activator-plasmin system in depolarization-evoked dopamine release in the nucleus accumbens of mice. *Mol Pharmacol* **70:**1720–1725.

Jern C, Selin L, and Jern S (1994) In vivo release of tissue-type plasminogen activator across the human forearm during mental stress. *Thromb Haemost* **72:**285–291.

Jiang X, Wang Y, Hand AR, Gillies C, Cone RE, Kirk J, and O'Rourke J (2002) Storage and release of tissue plasminogen activator by sympathetic axons in resistance vessel walls. *Microvasc Res* **64:**438–447.

Kristensen P, Eriksen J, and Dano K (1991) Localization of urokinase-type plasminogen activator messenger RNA in the normal mouse by in situ hybridization. *J Histochem Cytochem* **39:**341–349.

Krystosek A and Seeds NW (1981) Plasminogen activator release at the neuronal growth cone. *Science* **213:**1532–1534.

Larsson LI, Skriver L, Nielsen LS, Grondahl-Hansen J, Kristensen P, and Dano K (1984) Distribution of urokinase-type plasminogen activator immunoreactivity in the mouse. *J Cell Biol* **98:**894–903.

Lochner JE, Kingma M, Kuhn S, Meliza CD, Cutler B, and Scalettar BA (1998) Real-time imaging of the axonal transport of granules containing a tissue plasminogen activator/green fluorescent protein hybrid. *Mol Biol Cell* **9:**2463–2476.

Medved L and Nieuwenhuizen W (2003) Molecular mechanisms of initiation of fibrinolysis by fibrin. *Thromb Haemost* **89:**409–419.

Melchor JP and Strickland S (2005) Tissue plasminogen activator in central nervous system physiology and pathology. *Thromb Haemost* **93:**655–660.

O'Rourke J, Jiang X, Hao Z, Cone RE, and Hand AR (2005) Distribution of sympathetic tissue plasminogen activator (tPA) to a distant microvasculature. *J Neurosci Res* **79:**727–733.

Peng T, Jiang X, Wang YF, Hand A, Gillies C, Cone RE, and O'Rourke J (1999) Sympathectomy decreases and adrenergic stimulation increases the release of tissue plasminogen activator (t-PA) from blood vessels: functional evidence for a neurologic regulation of plasmin production within vessel walls and other tissue matrices. *J Neurosci Res* **57:**680–692.

Pittman RN, Ivins JK, and Buettner HM (1989) Neuronal plasminogen activators: cell surface binding sites and involvement in neurite outgrowth. *J Neurosci* **9:**4269–4286.

Potempa J, Korzus E, and Travis J (1994) The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J Biol Chem* **269:**15957–15960.
Rickles RJ and Strickland S (1988) Tissue plasminogen activator mRNA in murine tissues. *FEBS Lett* **229:**100–106.
Schaefer U, Machida T, Vorlova S, Strickland S, and Levi R (2006) The plasminogen activator system modulates sympathetic nerve function. *J Exp Med* **203:**2191–2200.
Sesti C, Koyama M, Broekman MJ, Marcus AJ, and Levi R (2003) Ectonucleotidase in sympathetic nerve endings modulates ATP and norepinephrine exocytosis in myocardial ischemia. *J Pharmacol Exp Ther* **306:**238–244.
Seyedi N, Win T, Lander HM, and Levi R (1997) Bradykinin B_2-receptor activation augments norepinephrine exocytosis from cardiac sympathetic nerve endings: mediation by autocrine/paracrine mechanisms. *Circ Res* **81:**774–784.
Stürzebecher J, Vieweg H, Steinmetzer T, Schweinitz A, Stubbs MT, Renatus M, and Wikstrom P (1999) 3-Amidinophenylalanine-based inhibitors of urokinase. *Bioorg Med Chem Lett* **9:**3147–3152.
Todorov LD, Mihaylova-Todorova S, Craviso GL, Bjur RA, and Westfall DP (1996) Evidence for the differential release of the cotransmitters ATP and noradrenaline from sympathetic nerves of the guinea- pig vas deferens. *J Physiol* **496:**731–748.
Westfall DP, Todorov LD, Mihaylova-Todorova ST, and Bjur RA (1996) Differences between the regulation of noradrenaline and ATP release. *J Auton Pharmacol* **16:**393–395.
Yakovlev S, Makogonenko E, Kurochkina N, Nieuwenhuizen W, Ingham K, and Medved L (2000) Conversion of fibrinogen to fibrin: mechanism of exposure of tPA- and plasminogen-binding sites. *Biochemistry* **39:**15730–15741.
Yamanaka H, Obata K, Fukuoka T, Dai Y, Kobayashi K, Tokunaga A, and Noguchi K (2005) Induction of plasminogen activator inhibitor-1 and -2 in dorsal root ganglion neurons after peripheral nerve injury. *Neuroscience* **132:**183–191.
Zimmermann H and Braun N (1999) Ecto-nucleotidases-molecular structures, catalytic properties, and functional roles in the nervous system. *Prog Brain Res* **120:**371–385.

Address correspondence to: Dr. Roberto Levi, Department of Pharmacology, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10021. E-mail: rlevi@med.cornell.edu