*Review Article*

# The Plasminogen Activation System and the Regulation of Catecholaminergic Function

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The local environment of neurosecretory cells contains the major components of the plasminogen activation system, including the plasminogen activators, tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), as well as binding sites for t-PA, the receptor for u-PA (uPAR), and also the plasminogen activator inhibitor, PAI-1. Furthermore, these cells express specific binding sites for plasminogen, which is available in the circulation and in interstitial fluid. Colocalization of plasminogen and its activators on cell surfaces provides a mechanism for promoting local plasminogen activation. Plasmin is retained on the cell surface where it is protected from its inhibitor, $\alpha_2$-antiplasmin. In neurosecretory cells, localized plasmin activity provides a mechanism for extracellular processing of secreted hormones. Neurotransmitter release from catecholaminergic cells is negatively regulated by cleavage products formed by plasmin-mediated proteolysis. Recently, we have identified a major plasminogen receptor, Plg-R$_{KT}$. We have found that Plg-R$_{KT}$ is highly expressed in chromaffin cells of the adrenal medulla as well as in other catecholaminergic cells and tissues. Plg-R$_{KT}$-dependent plasminogen activation plays a key role in regulating catecholaminergic neurosecretory cell function.

## 1. Introduction

Chromaffin cells of the adrenal medulla and other neurosecretory cells contain specific binding sites for plasminogen [1, 2], which is available at high concentration in the circulation and in interstitial fluid. Furthermore, these cells secrete major components of the plasminogen activation system, including the plasminogen activators tissue plasminogen activator (t-PA) [3–7] and urokinase-type plasminogen activator (u-PA) [4, 8], and also the plasminogen activator inhibitors, PAI-1 [9] and neuroserpin [10]. Binding sites for t-PA [1, 8] and the receptor for u-PA (uPAR) are also expressed in these cells [8, 11–13]. Thus, the major components of the plasminogen activation system are present in the local neurosecretory cell environment.

Colocalization of plasminogen and plasminogen activators on cell surfaces results in promotion of local plasminogen activation [14]. In addition, cell-associated plasmin is protected from its major inhibitor, $\alpha_2$-antiplasmin [15, 16]. In neurosecretory cells, localized plasmin activity provides a mechanism for extracellular processing of secreted hormones [1, 2]. For example, neurotransmitter release from catecholaminergic cells is negatively regulated by cleavage products formed by plasmin-mediated proteolysis of the major secretory vesicle core protein, chromogranin A (CgA) [1, 17]. Recently, we have identified a major plasminogen receptor, Plg-R$_{KT}$ [18]. In this paper we summarize the interplay between components of the local neurosecretory cell plasminogen activation system, the functional consequences of the interaction of plasmin with catecholaminergic cells, and our recent studies demonstrating the expression and function of Plg-R$_{KT}$ in catecholaminergic cells.

## 2. Components of the Local Chromaffin Cell Plasminogen Activation System: Plasminogen Activators, Plasminogen Activator Inhibitors, and Binding Sites for Plasminogen Activators

*2.1. t-PA Expression and Its Targeting to the Regulated Secretory Pathway.* The expression of t-PA, its subcellular localization, and its release in response to secretagogues have been investigated in several chromaffin cell sources.

t-PA synthesis and expression were demonstrated in rat PC12 cells [4–7], bovine adrenal chromaffin cells [7], human pheochromocytoma (a catecholamine producing tumor of the adrenal gland) [7], and adrenal medulla [3]. A variety of methods including Northern blotting, Western blotting, metabolic labeling and immunoprecipitation with specific anti-t-PA antibody, immunoassays, and enzymatic activity assays have been used to demonstrate the presence of authentic t-PA in these cells [7].

Within neuroendocrine cells, secretory proteins are sorted into one of two pathways, either the regulated secretory pathway or the constitutive secretory pathway [19, 20]. Proteins that enter the constitutive pathway are not stored but are transported directly to the cell surface and secreted in the absence of an extracellular signal. In contrast, proteins that enter the regulated pathway are concentrated and stored in vesicles and subsequently released upon stimulation with a secretagogue or other specific extracellular stimuli. Catecholamine storage vesicles within the chromaffin cell are prototypic examples of regulated secretory vesicles [20–23].

We examined the potential targeting of t-PA to the regulated secretory pathway by evaluating the subcellular localization of chromaffin cell t-PA in functional secretagogue release studies and using subcellular fractionation methods. In functional secretagogue-release studies, in which PC12 cells and bovine adrenal chromaffin cells were stimulated with a panel of secretagogues including nicotine (acting through nicotinic cholinergic receptors), KCl (a membrane depolarizing agent), and BaCl$_2$ (a calcium agonist), each of which causes exocytotic release of catecholamine storage vesicle content, significant increases in t-PA secretion occurred (Figure 1). Moreover, t-PA release occurred in parallel with release of catecholamines, consistent with release from the same subcellular pool, the catecholamine storage vesicle. The release of t-PA from PC12 cells also occurs in response to membrane depolarization with tetraethylammonium chloride and depends on the influx of calcium ion [6]. This rapid release is also consistent with release of presynthesized t-PA from a storage pool because no changes in gene expression or protein synthesis are required [6].

Subcellular fractionation studies using sucrose density gradients also demonstrated trafficking of t-PA to catecholamine storage vesicles [7]. Furthermore, catecholamine storage vesicle lysates isolated from human pheochromocytoma tumors were enriched 30-fold in t-PA antigen, compared with tumor homogenate [7]. The enrichment in t-PA antigen paralleled the enrichment in catecholamines, consistent with colocalization in the same subcellular fraction.

The localization of t-PA in secretory vesicles of PC12 cells has provided a key tool to study the mechanisms and kinetics of exocytosis. GFP-tagged t-PA has been used as a marker to understand axonal transport in nerve-growth-factor-(NGF-)treated PC12 cells. (When exposed to NGF, PC12 cells differentiate into cells that morphologically, biochemically, and electrophysiologically closely resemble sympathetic neurons [26].) Scalettar's group demonstrated that GFP-t-PA was targeted for regulated secretion from growth cones of NGF-differentiated PC12 cells and released in response to the calcium ionophore A23187 or the cholinergic agonist,

carbachol [27], and used GFP-t-PA to demonstrate that secretory granules are mobile in growth cones of these cells [28]. In other studies with undifferentiated PC12 cells, fluorophore-tagged t-PA has been used as a marker to demonstrate: that most granules in PC12 cells reseal after exocytosis, resulting in the differential release of cargo [29], that synaptotagmin VII modulates kinetics of dense-core vesicle exocytosis in PC12 cells [30], that actin rearrangement [31] and myosin II [32] influence the time course of secretory granule release, and that newly synthesized dense-core vesicle cargoes are released preferentially compared to aged vesicle cargo [33].

Thus, in response to specific secretagogue stimulation, chromaffin cells release t-PA into the extracellular space. In addition, t-PA can be rapidly released into the circulation in response to stress [34–37]. Studies employing adrenergic stimulation and sympathectomy have demonstrated that sympathoadrenal and sympathoneural tissues may represent substantial sources contributing to changes in plasma t-PA concentrations [38, 39].

*2.2. PAI-1 Is also Targeted to Catecholamine Storage Vesicles.* As a potential mechanism for regulating t-PA activity, inhibitors of t-PA are present in catecholaminergic cells. We have recently demonstrated that plasminogen activator inhibitor-1 (PAI-1) is present in PC12 cells and bovine adrenal medullary chromaffin cells [9]. Secretagogue stimulation led to co-release of PAI-1 with catecholamines, consistent with storage in the same subcellular vesicle. Furthermore, immunoelectron microscopy and sucrose gradient fractionation studies demonstrated localization of PAI-1 in catecholamine storage vesicles [9]. In addition, parallel increases in plasma PAI-1 and catecholamines were observed in response to acute sympathoadrenal activation by restraint stress in mice *in vivo* [9]. Thus, the overall effect on plasminogen activation and fibrinolysis (both systemically and locally) from catecholamine storage vesicles will depend on a variety of factors that affect local t-PA/inhibitor balance, including the relative rates of synthesis of PAI-1 and t-PA, relative rates of trafficking to the vesicles, and the potential formation of t-PA/inhibitor complexes both within the vesicle and on release. Differential rates of exocytotic release of t-PA compared to PAI-1 could potentially take place as t-PA has been demonstrated to be released more slowly than other granule components [31, 32, 40]. In addition to PAI-1, the t-PA inhibitor, neuroserpin, is present in dense-core secretory vesicles within PC12 cells [10] and a targeting sequence for regulated secretion of neuroserpin has been identified [41].

*2.3. t-PA Binding Sites on Catecholaminergic Cells.* An additional key mechanism by which local t-PA function is regulated is by the presence of binding sites for t-PA on catecholaminergic cells, as initially demonstrated by Pittman and colleagues [8]. We found that the interaction of t-PA with PC12 cells was saturable and of high capacity [1]. Furthermore, the lysine analog, $\varepsilon$-aminocaproic acid (EACA), also inhibited the interaction [1]. Thus, the recognition
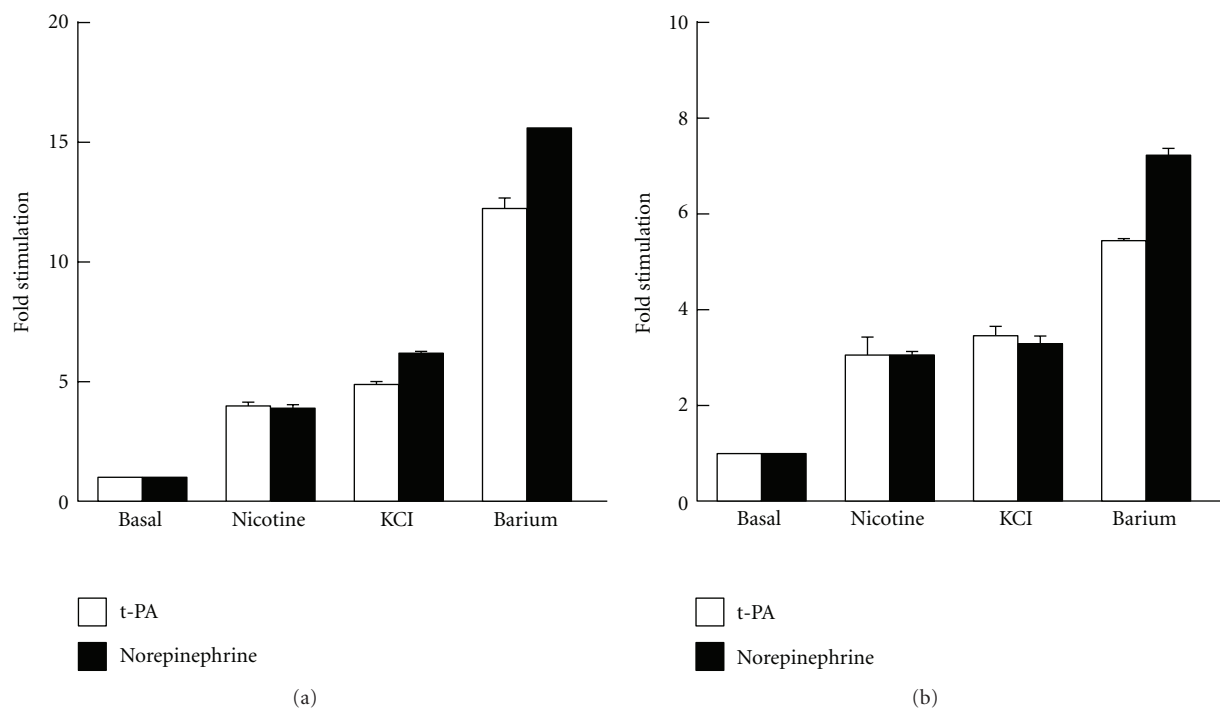
(a)

(b)

FIGURE 1: Corelease of t-PA with norepinephrine from catecholaminergic cells. PC12 cells (a) or bovine adrenal chromaffin cells (b) were labeled with [³H]-norepinephrine and incubated at 37°C for 30 min in either the absence or presence of 60 $\mu$M nicotine, 55 mM KCl, or 2 mM BaCl$_2$. The release buffer was aspirated, cells were harvested and lysed, and the release of t-PA antigen (open bars) and [³H]-norepinephrine (filled bars) was determined. [7]. ©The American Society for Biochemistry and Molecular Biology.

specificity of t-PA for chromaffin cells is likely to be dependent on receptors that express C-terminal lysines. Thus, t-PA, released in response to secretagogue stimulation, can bind to its receptors for further amplification of plasminogen activator activity in the local environment of the chromaffin cell.

*2.4. uPA and uPAR Expression in Chromaffin Cells.* The synthesis and secretion of uPA by chromaffin cells has not been addressed extensively in the literature. uPA secretion by PC12 cells has been detected but accounts for only 5% of the plasminogen activator activity in the long-term conditioned media of these cells; the remaining 95% of plasminogen activator activity is contributed by t-PA [4]. In studies of sympathetic neurons, uPA appears to be released as soon as it is synthesized [8]. Thus, the constitutive release of u-PA may be the predominant source of plasminogen activator activity under basal conditions (on unstimulated chromaffin cells) while the action of t-PA, released in response to secretagogue stimulation, may predominate following its exocytotic release and subsequent binding to the chromaffin cell surface.

The urokinase receptor (uPAR) is also present on PC12 cells. We characterized the binding of single chain u-PA (scu-PA) to untreated PC12 cells. Human scu-PA bound specifically and saturably to PC12 cells (Figure 2(a)) with a Kd of 4.3 ± 0.54 nM and 1.1 ± 0.26 × 10⁴ sites/cell [11]. A representative binding isotherm and Scatchard analysis are shown in Figures 2(a) and 2(b). Thus, the species specificity that was initially observed (human u-PA does not interact with murine cells and murine u-PA does not interact with

human uPAR [42, 43]) does not apply to all interspecies interactions. (Human scu-PA also bound specifically to hamster CHO cells, but minimally to bovine aortic endothelial cells [11, 44]). The affinity of scu-PA for the PC12 cell surface that we determined is consistent with the presence of a functional uPAR on the surface of undifferentiated PC12 cells. In support of this, uPAR mRNA was prominently expressed in PC12 cells as assessed by Northern blotting (Figure 2(c)). Furthermore, we cloned rat uPAR from PC12 cells [11] (Figure 2(d)), and the sequence of the clone we obtained was identical to the sequence published for rat osteoclast uPAR [45]. In addition, using a specific uPAR ELISA, we found substantial expression of uPAR in human pheochromocytoma tissue samples [48 ± 8.2 pg/mg protein ($n = 6$)] [11].

With NGF-treated PC12 cells, binding sites for u-PA that did not require an active site in u-PA for the interaction were first detected immunochemically by Pittman and colleagues on the bottom surface of the cells [8]. Subsequently, Herschman and colleagues identified uPAR as a neurotrophin responsive gene (preferentially induced by NGF versus EGF) in PC12 cells [12, 46] and demonstrated that NGF-induced uPAR expression is required for NGF-driven PC12 cell differentiation [12, 46, 47]. Induction of uPAR requires ERK/JNK signaling [48], binding of specific Fos and Jun family members to a specific AP-1 site [49, 50], and is regulated by SH2B1$\beta$ [51].

Thus, taken together, these studies suggest that multiple plasminogen activator receptors can be present simultaneously on the neurosecretory cell surface.
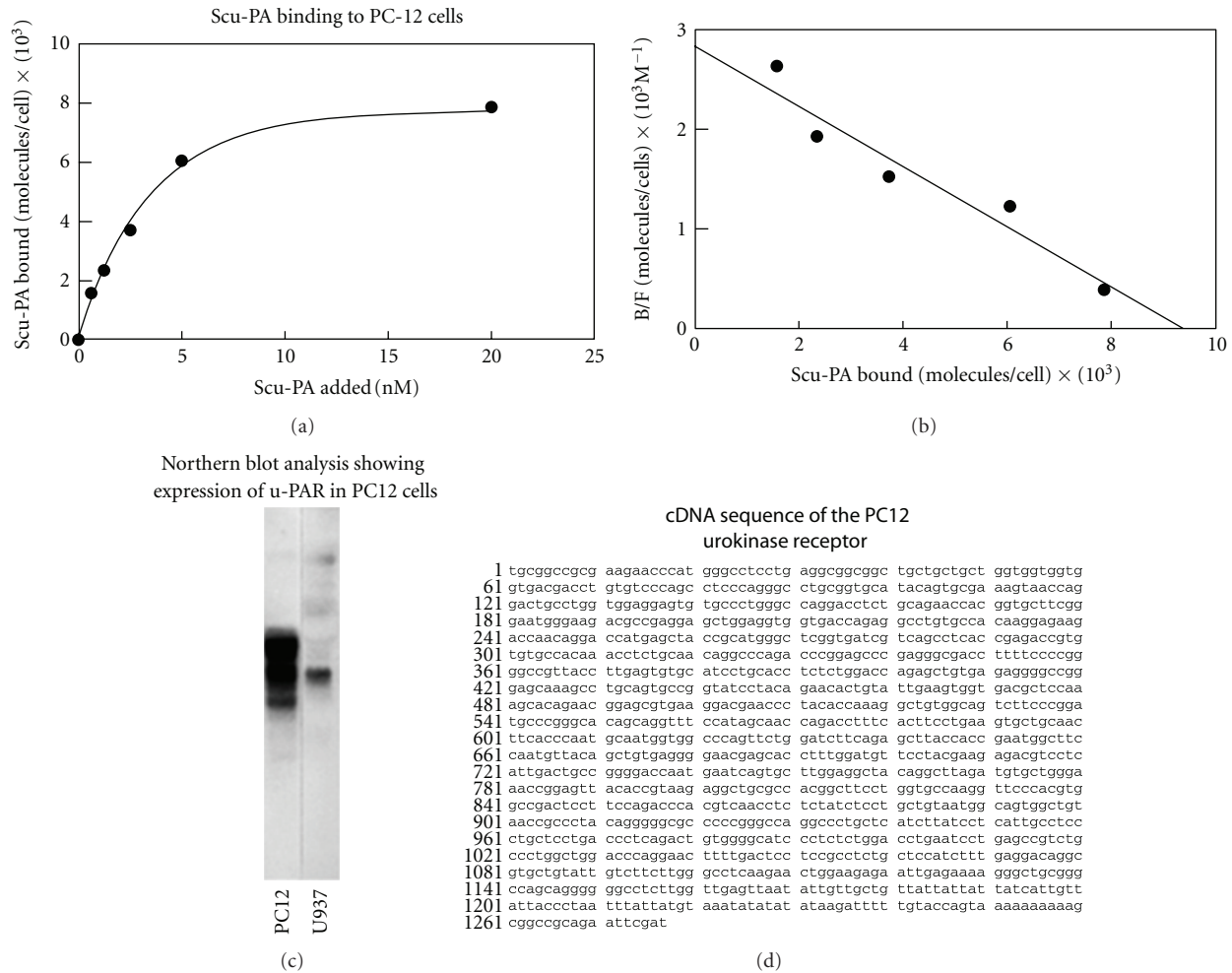
Scu-PA binding to PC-12 cells



(a)



(b)

Northern blot analysis showing
expression of u-PAR in PC12 cells



(c)

cDNA sequence of the PC12
urokinase receptor

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   1 tgcggccgcg aagaacccat gggcctcctg aggcggcggc tgctgctgct ggtggtggtg
  61 gtgacgacct gtgtcccagc ctcccagggc ctgcggtgca tacagtgcga aagtaaccag
 121 gactgcctgg tggaggagtg tgccctgggc caggacctct gcagaaccac ggtgcttcgg
 181 gaatgggaag acgccgagga gctggaggtg gtgaccagag gcctgtgcca caaggagaag
 241 accaacagga ccatgagcta ccgcatgggc tcggtgatcg tcagcctcac cgagaccgtg
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 481 agcacagaac ggagcgtgaa ggacgaaccc tacaccaaag gctgtggcag tcttcccgga
 541 tgcccgggca cagcaggttt ccatagcaac cagacctttc acttcctgaa gtgctgcaac
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1141 ccagcagggg ggcctcttgg ttgagttaat attgttgctg ttattattat tatcattgtt
1201 attaccctaa tttattatgt aaatatatat ataagatttt tgtaccagta aaaaaaaag
1261 cggccgcaga attcgat
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(d)

FIGURE 2: Expression of uPAR by PC12 cells. (a) Binding isotherm of ^{125}I-scu-PA to PC12 cells. Cells in suspension were incubated with ^{125}I-scu-PA, and bound ligand was separated from free by centrifugation over sucrose. Nonspecific binding was determined in the presence of unlabeled scu-PA. (b) Scatchard analysis of the binding isotherm in Panel A. (c) Northern blot analysis showing expression of uPAR in PC12 cells. Poly(A)-enriched RNA was isolated from PC12 cells and Northern blotted with a 458 bp SMaI + BamHI (nuc. 41–493) human uPAR cDNA probe. Northern blotting of U937 cell poly(A) RNA is shown for comparison. (d) cDNA sequence of PC12 uPAR. The rat uPAR sequence was obtained by screening a PC12 cDNA library using a 609 bp EcoRI + NcoI (nuc. 110–718) human uPAR cDNA probe labeled with $[\alpha\text{-}^{32}P]$ dCTP.

## 3. Characteristics of the Interaction of Plasminogen with Chromaffin Cells

As a mechanism for concentrating the activity of plasminogen in the plasminogen activator-rich local environment of the chromaffin cell, we found that plasminogen bound to PC12 and bovine adrenal chromaffin cells in a specific, saturable, and reversible manner [1, 2]. Plasminogen activation is markedly enhanced when plasminogen is bound to chromaffin cells. In the presence of PC12 cells, plasminogen activation by t-PA is markedly increased in a cell-dependent fashion, and at the saturating cell density, the enhancement caused by the presence of cells is 6.5-fold [24]. Primary bovine chromaffin cells also markedly stimulate t-PA-dependent plasminogen activation up to 10-fold in a cell concentration-dependent manner [24].

The interactions of plasminogen with substrates and regulatory molecules depend on the lysine binding sites within the disulfide-bonded kringle structures of plasminogen (reviewed in [52]). The interaction of plasminogen with PC12 and bovine adrenal chromaffin cells is blocked by the lysine analogues, EACA and 8-aminooctanoic acid [1, 2], suggesting that proteins with C-terminal lysines, exposed on the cell surface serve as plasminogen binding sites. To explore the relationship between plasminogen binding and stimulation of plasminogen activation, PC12 cells were treated with increasing concentrations of carboxypeptidase B (CpB) that has a preference for cleavage at the carboxyl side of basic amino acids. A dose-dependent decrease in plasminogen binding to PC12 cells was observed, reaching a plateau at 70% inhibition in the presence of CpB (Figure 3(a)) [24]. In addition,
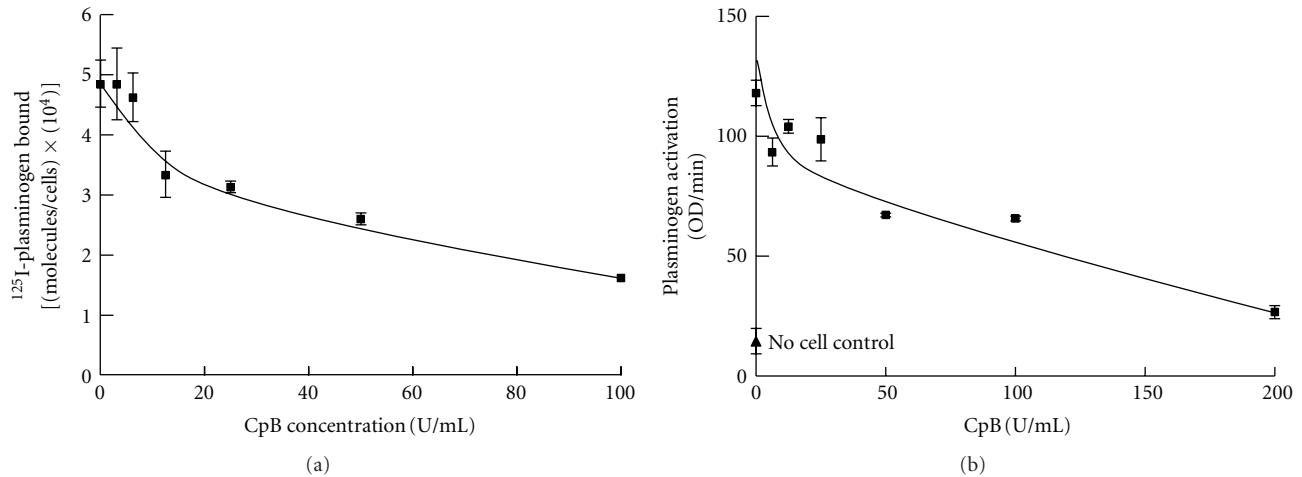
(a)



(b)

FIGURE 3: Effect of treatment of PC12 cells with CpB on the interaction with plasminogen. PC12 cells were incubated with increasing concentrations of CpB and specific binding of 0.2 $\mu$M $^{125}$I-plasminogen (a) and plasminogen activation (b) were assessed as described in [24].
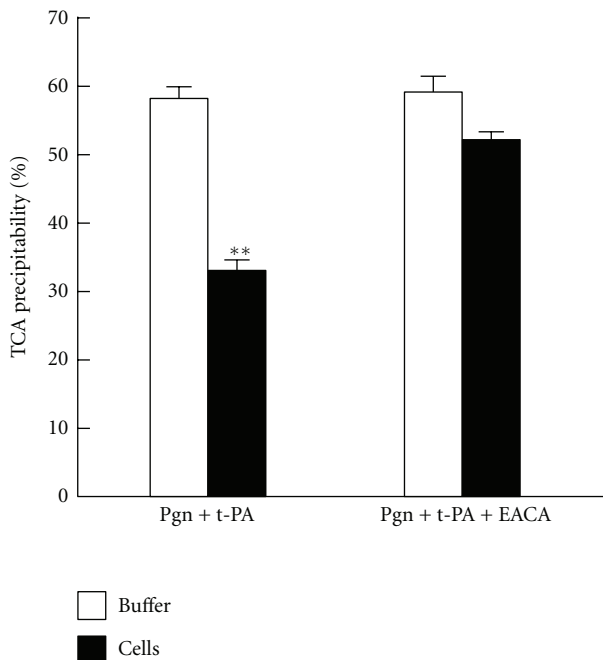


FIGURE 4: Effect of cells on cleavage of $^{125}$I-CgA by plasmin. Either PC12 cells (filled bars) or buffer (open bars) was incubated with unlabeled plasminogen. Then, $^{125}$I-CgA and t-PA were added in either the presence or the absence of EACA. The reactions were stopped by addition of aprotinin and centrifuged. The supernatants were analyzed for % TCA precipitability. Results are as mean $\pm$ SEM; $n = 6$ for each experimental group. **$P < 0.001$ compared to each of the experimental groups. [1].

CpB treatment decreased the stimulating effect of the cells on plasminogen activation in a dose-dependent manner, approaching a plateau at 88% inhibition (Figure 3(b)) [24]. These results suggest that the chromaffin cell plasminogen receptors that are sensitive to CpB (i.e., proteins exposing C-terminal basic residues on the extracellular face of the cell membrane) are primarily responsible
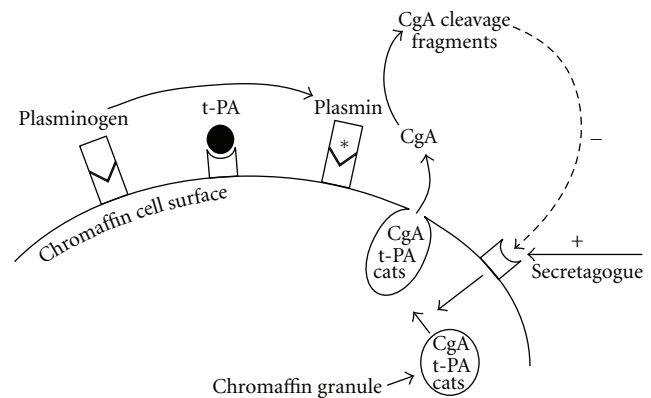


FIGURE 5: Working model for a local (autocrine/paracrine) chromaffin cell plasminogen/plasmin system and its interactions with CgA. Upon stimulation of the chromaffin cell by a secretagogue, CgA and catecholamines (cats) are coreleased by exocytosis. Plasminogen (from circulating sources) and its activator t-PA (synthesized and secreted from the chromaffin cell) bind to the chromaffin cell surface, with resultant activation of plasminogen to plasmin. CgA subsequently is cleaved by plasmin to liberate peptide fragments, which provides a negative feedback loop to modulate subsequent catecholamine release. [1].

for the promotion of plasminogen activation by these cells.

## 4. Role of the Local Chromaffin Cell Plasminogen Activation System in Prohormone Processing

Plasmin functions as a prohormone-processing protease within the neuroendocrine system [1, 17, 53–57]. We used the prototypical prohormone chromogranin A (CgA) to study the role of the local chromaffin cell plasminogen activation system in prohormone processing. CgA is the
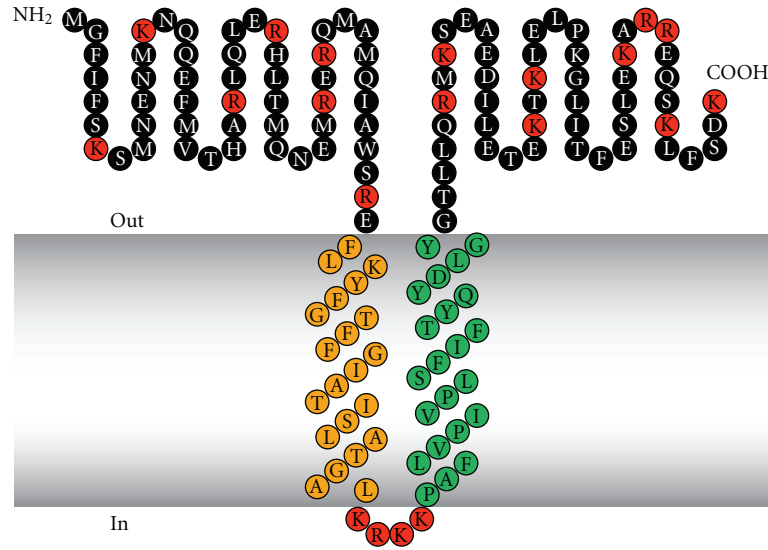
FIGURE 6: Structural model of Plg-R$_{KT}$. Green indicates amino acids within the predicted primary transmembrane helix. Orange indicates amino acids within the predicted secondary transmembrane helix. Red indicates basic amino acids. [18].
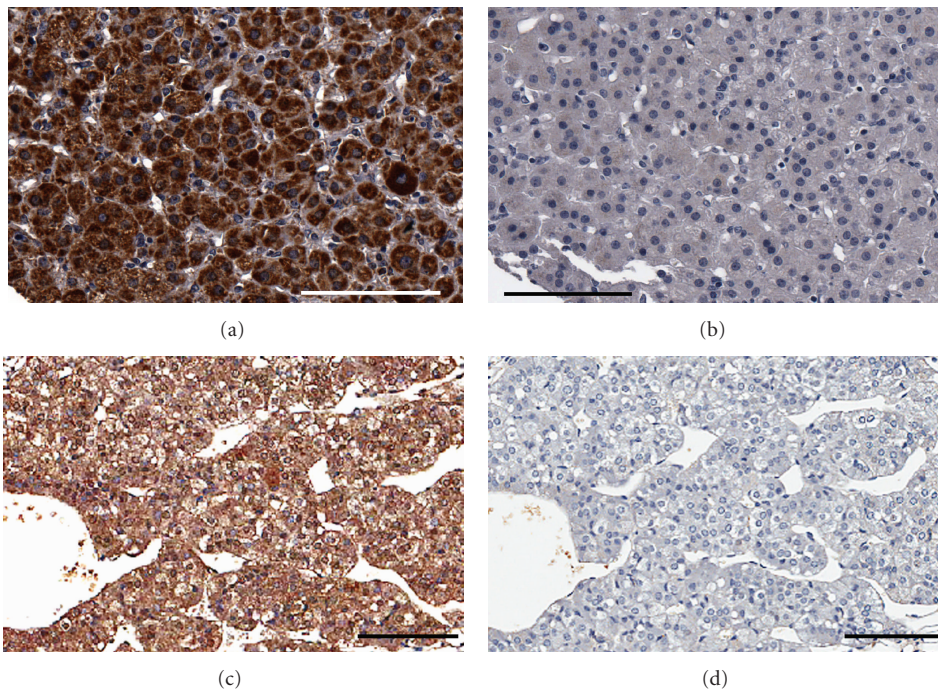


(a)

(b)

(c)

(d)

FIGURE 7: Expression of Plg-R$_{KT}$ in adrenal medulla. Human (a, b) and mouse (c, d) adrenal medulla tissue sections were stained with anti-Plg-R$_{KT}$ mAb, in the absence (a, c) or the presence (b, d) of the decapeptide used for immunization, CEQSKFFSDK (corresponding to the nine C-terminal amino acids of rat Plg-R$_{KT}$ with an amino terminal cysteine added for coupling). Positive tissues are indicated by brown staining with DAB. Scale bar = 100 $\mu$m. [25]. ©The American Society for Biochemistry and Molecular Biology.

major soluble protein in the core of catecholamine storage vesicles and is released with catecholamines in response to secretagogue stimulation of chromaffin cells of the adrenal medulla and from sympathetic neurons [58]. CgA is present ubiquitously in secretory vesicles of neuroendocrine cells [59] and serves as a precursor of several small, biologically active, secretion-inhibitory peptides that play a key autocrine regulatory role in neuroendocrine secretion from

a variety of cell types [60–67]. In the case of chromaffin cells and other catecholaminergic cells, CgA serves as a precursor from which peptides are released that modulate catecholamine secretion, contributing to an autocrine, homeostatic, negative-feedback mechanism for regulating catecholamine responses during stress [65–67].

We found that plasmin processing of CgA produces regulatory secretion-inhibiting peptides. Plasmin specifically
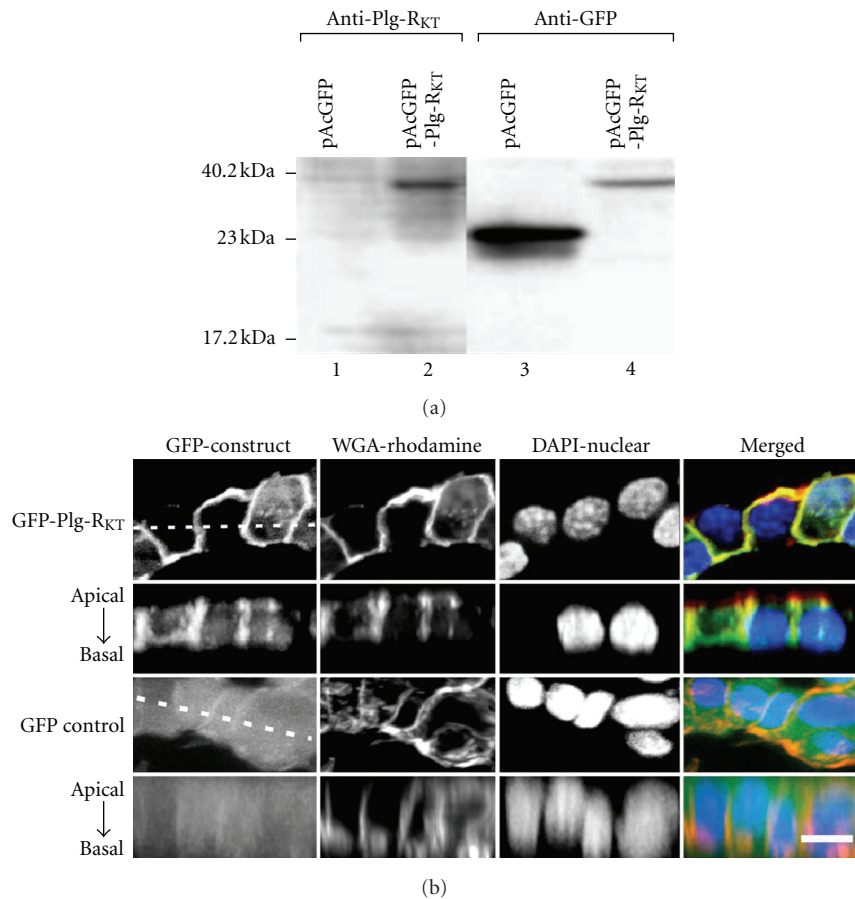
(a)



(b)

FIGURE 8: Plg-R$_{KT}$ re-directs GFP to the cell membrane. PC12 cells were transiently transfected with pAcGFP-Plg-R$_{KT}$ (in which the Plg-R$_{KT}$ cDNA was inserted in frame for expression of a GFP-Plg-R$_{KT}$ fusion protein, with Plg-R$_{KT}$ at the C-terminus) or with control pAcGFP vector. (a) Cell lysates were Western blotted with polyclonal anti-Plg-R$_{KT}$ IgG (lanes 1 and 2) or anti-GFP IgG (lanes 3 and 4). (b) The transfected cells were stained with a combination of DAPI and WGA-rhodamine. The first and third panels of images represent maximum projections of a series of optical slices through the cells. The GFP-Plg-R$_{KT}$ signal was localized primarily to the plasma membrane. The second and fourth panels of images represent sagittal (apical-basal) slices through the same cells along the white dotted line indicated. Here the peripheral plasma membrane localization of GFP-Plg-R$_{KT}$ is also evident throughout the vertical stacks of images that were acquired. Scale bar in bottom right corner of image represents 10 microns. [25]. ©The American Society for Biochemistry and Molecular Biology.

cleaved CgA and decreased the trichloroacetic acid (TCA) precipitability of $^{125}$I-CgA, indicating that fragments with Mr < 6,000 had been produced [1, 17]. Most importantly, the peptide fragments generated by plasmic cleavage of CgA inhibited secretagogue-stimulated catecholamine release from PC12 and primary bovine adrenal cells [1]. We identified the specific bioactive CgA peptide produced by plasmin proteolysis [using matrix-assisted laser desorption/ionization mass spectrometry (MALDI)] as a major peptide with a mass/charge ratio ($m/z$) of 1546 corresponding uniquely to hCgA-(360–373) [17]. In functional studies the hCgA-(360–373) peptide markedly inhibited nicotine-stimulated catecholamine release from pheochromocytoma cells [17].

Chromaffin cells had a marked effect on CgA cleavage by plasmin [1]. In the presence of PC12 cells, the TCA precipitability of $^{125}$I-CgA was markedly decreased, indicative of extensive CgA processing (Figure 4) [1]. EACA inhibited

processing in the presence, but not in the absence of cells (Figure 4) [1]. Because EACA inhibits plasminogen binding to chromaffin cells, these data suggest a significant contribution of cellular plasminogen receptors to CgA processing.

We examined the effect of local modulation of the plasminogen activation system on nicotine-mediated catecholamine secretion. Overexpression of t-PA in PC12 cells resulted in a marked (81 ± 2%) inhibition of nicotine-stimulated catecholamine release compared with control cells [1]. In the presence of anticatalytic antiplasminogen mAb, catecholamine secretion was markedly increased, compared with isotype control [1]. Thus, the effect of the anticatalytic anti-plasminogen mAb was to restore nicotine-mediated secretion in these cells.

We present a working model for the function of the local chromaffin cell plasminogen activation system in the processing of CgA in Figure 5 [1]. Upon stimulation of the chromaffin cell by a secretagogue, CgA and catecholamines
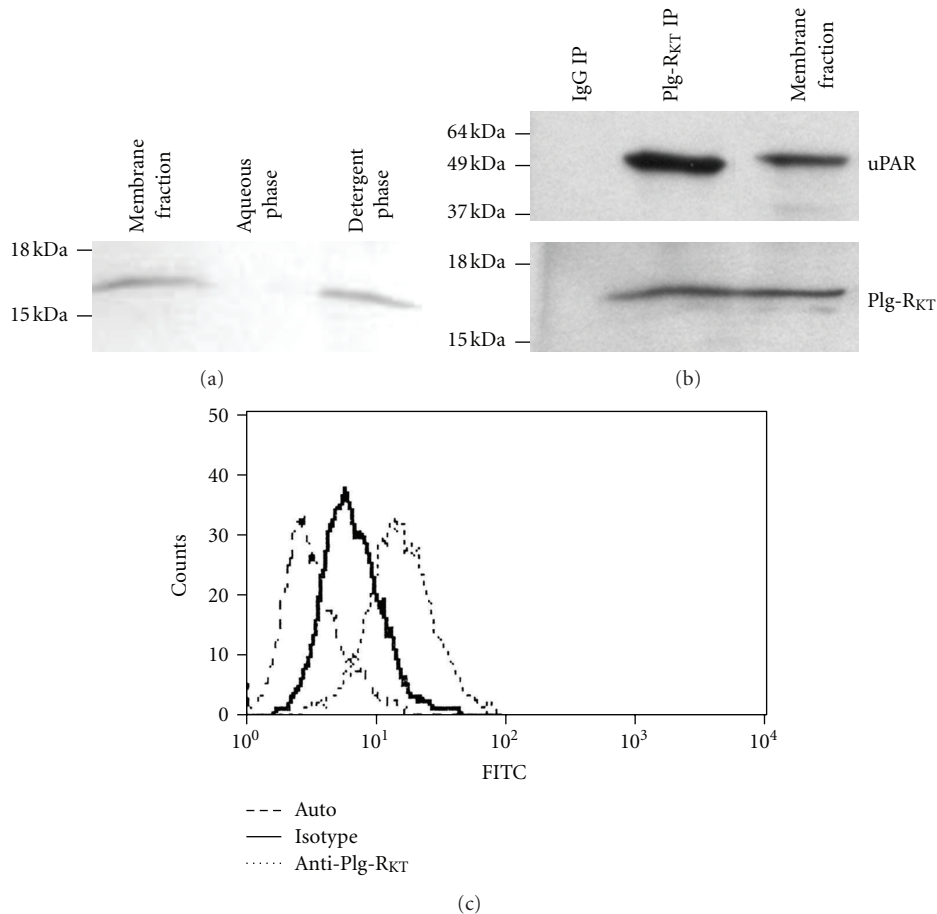
(a)



(b)



(c)

FIGURE 9: Plg-R$_{KT}$ is an integral plasma membrane cell surface protein. (a) Phase partitioning of Plg-R$_{KT}$. An immunoreactive band corresponding to the Mr$_{app}$ of Plg-R$_{KT}$ was detected in the detergent phase, but not in the aqueous phase. (b) Coimmunoprecipitation of Plg-R$_{KT}$ with uPAR. (c) FACS analysis of Plg-R$_{KT}$ expression on intact PC12 cells. Viable cells were gated from nonviable cells and histogram plots of viable cells are shown. Dotted tracings: anti-Plg-R$_{KT}$ mAb IgG. Black tracings: isotype control IgG. Dashed tracings: autofluorescence. [25]. ©The American Society for Biochemistry and Molecular Biology.

are coreleased by exocytosis. Plasminogen (from circulating sources) and t-PA (synthesized and secreted from the chromaffin cell) bind to the chromaffin cell surface, resulting in activation of plasminogen to plasmin. CgA is subsequently cleaved by plasmin to liberate a peptide fragment that provides a negative feedback loop to modulate subsequent catecholamine release.

## 5. Expression and Subcellular Localization of Plg-R$_{KT}$ in Chromaffin Cells

In earlier studies of chromaffin cell plasminogen receptors, we identified catecholaminergic plasminogen receptors required for enhancing plasminogen activation using targeted specific proteolysis with CpB and a proteomics approach using two-dimensional gel electrophoresis, radioligand blotting, and tandem mass spectrometry. Two major plasminogen-binding proteins that exposed C-terminal lysines on the cell surface contained amino acid sequences corresponding to $\beta/\gamma$ actin [24]. An antiactin monoclonal

antibody inhibited cell-dependent plasminogen activation and also enhanced nicotine-dependent catecholamine release [24], suggesting that cell-surface-expressed forms of actin (with a processed C-terminus to generate a C-terminal lysine) bind plasminogen, thereby promoting plasminogen activation and increased prohormone processing, leading to inhibition of neurotransmitter release. Although cell surface actin accounted for a substantial fraction of plasminogen binding and activation, a critical role for other cell surface plasminogen-binding proteins with C-terminal lysines was also suggested on these cells. Notably, in plasminogen ligand blotting of 2D gels of PC12 membrane fractions, we also detected a major unknown CpB-sensitive protein migrating with an M$_{rapp}$ of 17,200 [24].

Recently, we isolated a structurally unique plasminogen receptor from monocyte progenitor cells, the novel protein, Plg-R$_{KT}$ [18]. The Plg-R$_{KT}$ protein is composed of 147 amino acids and has a molecular mass of 17,261 Da. Plg-R$_{KT}$ is synthesized with and exposes a C-terminal lysine on the cell surface, in an orientation to bind plasminogen and to promote plasminogen activation (Figure 6) [18]. Therefore,
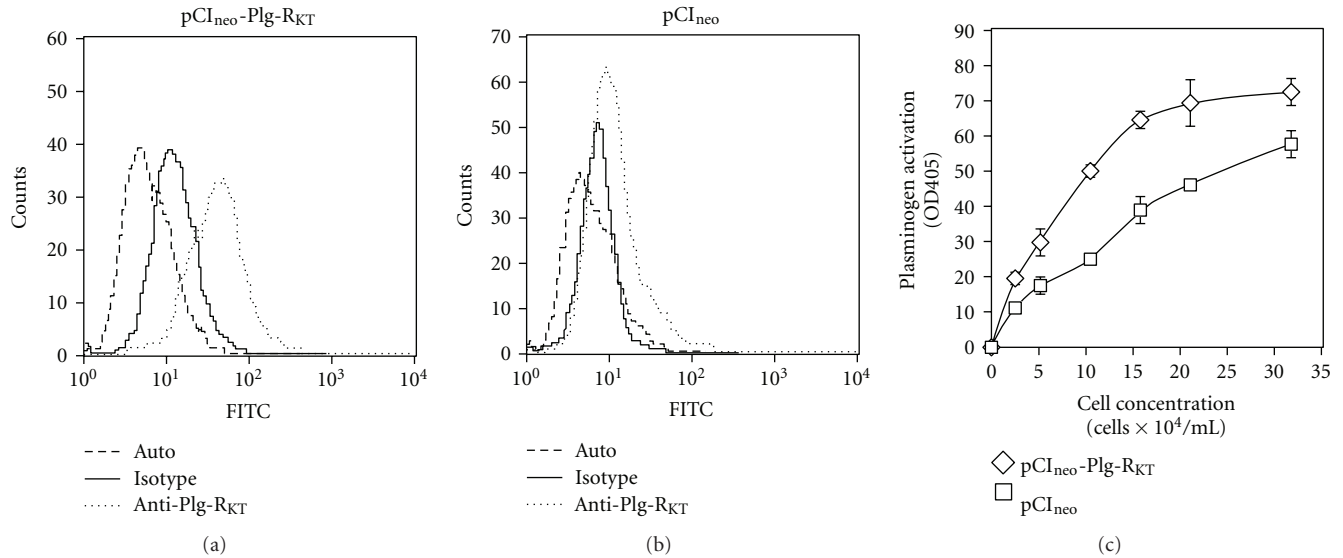
FIGURE 10: Overexpression of Plg-$R_{KT}$ increases cell surface plasminogen activation. PC12 cells stably overexpressing Plg-$R_{KT}$ (pCIneo-Plg-$R_{KT}$) (a) or vector alone (pCIneo) (b) were analyzed by dual color FACS analysis. Viable cells were gated from nonviable cells and histogram plots of viable cells are shown. Dotted tracings: anti-Plg-$R_{KT}$ mAb IgG. Black tracings: isotype control IgG. Dashed tracings: autofluorescence. (c) PC12 cells stably overexpressing Plg-$R_{KT}$ (pCIneo-Plg-$R_{KT}$) or vector alone (pCIneo) were incubated with plasminogen and t-PA was added and plasminogen activation was measured. Cell-mediated plasminogen activation was substantially greater in Plg-$R_{KT}$ overexpressing cells than in control cells ($P < 0.001$ at each cell concentration tested) ($n = 3$). [25]. ©The American Society for Biochemistry and Molecular Biology.

we investigated expression of Plg-$R_{KT}$ in human and murine adrenal tissues. Prominent staining with anti-Plg-$R_{KT}$ mAb was observed in adrenal medullary chromaffin cells in human (Figure 7(a)) and murine (Figure 7(c)) adrenal tissue [25]. Plg-$R_{KT}$ was also prominently expressed in PC12 cells, bovine adrenal chromaffin cells, and human pheochromocytoma and also in murine hippocampus, representing a nonadrenal source of catecholaminergic cells [25].

To assess the subcellular localization of Plg-$R_{KT}$ in catecholaminergic cells and to determine whether the Plg-$R_{KT}$ protein contains a dominant plasma membrane trafficking signal, we transfected PC12 cells with pAcGFP-Plg-$R_{KT}$ (an expression vector in which the Plg-$R_{KT}$ cDNA was inserted in-frame for expression of a GFP-Plg-$R_{KT}$ fusion protein with Plg-$R_{KT}$ at the C-terminus). In confocal microscopy of nonpermeabilized fixed cells, GFP-Plg-$R_{KT}$ showed membrane localization that was highly colocalized with wheat germ agglutinin (WGA) (a well-established cell surface marker) (Figure 8) [25]. In Triton X-114 phase separation experiments [42, 68] Plg-$R_{KT}$ was detected in the detergent phase, but was not detected in the aqueous phase (Figure 9(a)) [25], consistent with Plg-$R_{KT}$ behaving as an integral membrane protein in these cells. Furthermore, anti-Plg-$R_{KT}$ antibody immunoprecipitated both Plg-$R_{KT}$ and uPAR from membrane fractions of PC12 cells (Figure 9(b)) [25], providing further demonstration of the cell membrane localization of Plg-$R_{KT}$. The physical association of these receptors suggests a key mechanism for promoting plasminogen activation via colocalization of uPA activity (bound to uPAR) with the substrate, plasminogen (bound to Plg-$R_{KT}$). Furthermore, t-PA also binds to the C-terminus of Plg-$R_{KT}$

[18]. Thus, binding of t-PA and plasminogen to adjacent Plg-$R_{KT}$ molecules may also serve as a means to promote plasminogen activation on the neurosecretory cell surface.

In additional studies to address the cell surface orientation of the C-terminus of Plg-$R_{KT}$, fluorescence activated cell surface (FACS) analysis with anti-Plg-$R_{KT}$ mAb (raised against a synthetic peptide corresponding to the C-terminal peptide of Plg-$R_{KT}$) demonstrated prominent and specific binding of the mAb to the PC12 cell surface, indicating both cell membrane localization and exposure of the C-terminus of Plg-$R_{KT}$ on the cell surface (Figure 9(c)).

The above studies do not exclude the possibility that other plasminogen receptors with C-terminal lysines (including α-enolase [69], S100A10 [70, 71], Histone H2B [72], and TIP49a [73]) may participate in plasminogen binding on these cells.

## 6. Regulation of Catecholaminergic Neurosecretory Cell Function by Plg-$R_{KT}$

Based on our studies reviewed above (Section 3), we investigated whether Plg-$R_{KT}$ played a role in promoting plasminogen activation. We stably overexpressed Plg-$R_{KT}$ in PC12 cells, which resulted in prominent expression of Plg-$R_{KT}$ on the cell surface that was markedly greater than the expression of endogenous Plg-$R_{KT}$ (Figure 10) [25]. When cells were stably transfected with Plg-$R_{KT}$, plasminogen activation was markedly enhanced compared to cells transfected with empty vector (Figure 10(c)) [25]. These results are consistent with a
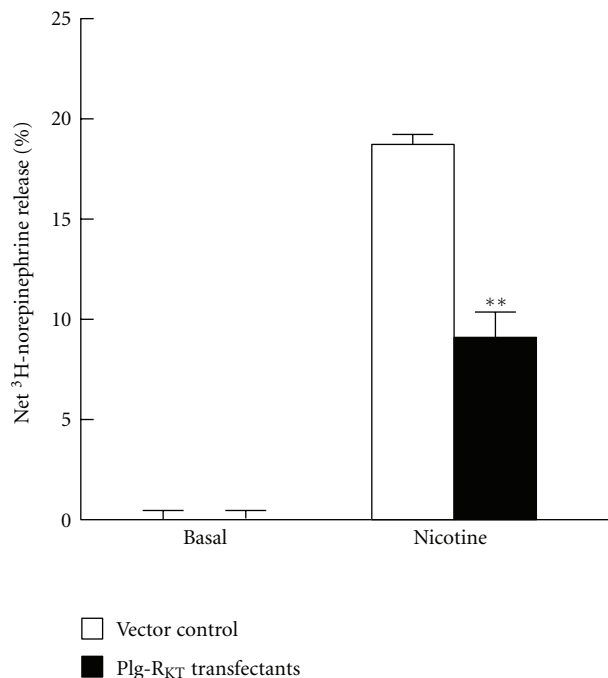
FIGURE 11: Effect of overexpression of Plg-R$_{KT}$ on catecholamine release. PC12 cells stably overexpressing either Plg-R$_{KT}$ (filled bars) or vector alone (open bars) were treated with $60\,\mu$M nicotine (Nicotine) or buffer (Basal) at $37°$C for 15 minutes and catecholamine release was measured by liquid scintillation counting. Percent release was calculated as percentage of secretion [amount released/(amount released + amount in cell lysate)] and results expressed as net release (% secretagogue-stimulated release minus % basal release). Results are represented as mean $\pm$ SEM, $n = 9$ for each experimental group. **$P < 0.001$ for the Plg-R$_{KT}$ transfectants stimulated with nicotine compared with corresponding values for the vector control cells. [25]. ©The American Society for Biochemistry and Molecular Biology.

major role for Plg-R$_{KT}$ in cell surface-dependent stimulation of plasminogen activation.

Based on our studies reviewed above (Section 4), we tested the effect of overexpression of Plg-R$_{KT}$ on secretagogue-stimulated catecholamine release. Norepinephrine release in response to nicotine was markedly sup-pressed in cells overexpressing Plg-R$_{KT}$ when compared with release from control cells (Figure 11) [25]. This result is consistent with processing of prohormones by plasmin to produce peptides that feed back to inhibit catecholamine release as outlined in our model in Figure 5.

## 7. Conclusions

The plasminogen activation system plays a major role in catecholaminergic cell function by processing secreted hormones that feed back to regulate the neurosecretory characteristics of these cells. In this paper, we summarize results suggesting that Plg-R$_{KT}$ is a crucial molecular focal point in the regulation of the cell-surface-dependent mechanism underlying the ability of catecholaminergic cells to promote local plasminogen activation. Expression of Plg-R$_{KT}$ and additional binding sites for plasminogen [1, 24] and t-PA [1, 8], along with trafficking of t-PA to catecholamine storage vesicles [7, 74], constitute a local catecholaminergic cell plasminogen activation system that regulates cell surface-dependent neuroendocrine prohormone processing (after secretagogue-stimulated storage vesicle exocytosis) that plays a key role in the regulation of neurotransmitter release. In addition, constitutive release of low levels of uPA [4] and subsequent binding to the neurosecretory cell uPAR [11] may also contribute to prohormone processing and to the regulation of differentiation of neuronal cells [12, 46, 47]. These processes are, in turn, locally regulated by the presence of PAI-1 [9] and neuroserpin [10], which are secreted in a regulated fashion, concomitantly with t-PA.

Components of the plasminogen activation system are expressed broadly in neuroendocrine sites, including the cerebral cortex [75], cerebellum [75–77], hippocampus [75, 77–81], sympathetic neurons [39, 82] as well as the adrenal medulla [7, 77]. Notably, the transcript for Plg-R$_{KT}$ is expressed in each of these tissues (http://www.ebi.ac.uk/gxa/). Future studies to develop and characterize Plg-R$_{KT}$ deficient mice and studies with mice deficient in other plasminogen receptors are warranted to address the role of Plg-R$_{KT}$ and other plasminogen receptors in key plasminogen- and t-PA-dependent neuronal/neuroendocrine plasminogen-dependent processes, including neurite outgrowth [5, 83, 84]; synaptic transmission, NMDA receptor-mediated signaling and excitotoxin-induced neuronal degeneration [85, 86]; long-term potentiation, learning, and memory [56, 78, 80, 87–90]; cleavage and activation of other neuroendocrine substrates such as the neurotrophin proBDNF (brain-derived neurotrophic factor) [56], $\beta$-endorphin, and $\alpha$-melanocyte-stimulating hormone [57]; and systemic metabolic and cardiovascular physiologic responses under the control of sympathoadrenal and sympathoneuronal activities [1, 17, 39, 55].

## Acknowledgments

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