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PLASMINOGEN ACTIVATION ON THE CELL SURFACE

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1. ABSTRACT

The plasminogen activation system appears to be widely involved in many biological processes in health and disease, but the regulation of plasmin generation or the mechanisms of stimulation by cell surface receptors are not well understood. Cell surface plasminogen activation requires binding sites for plasminogen substrate and activator enzyme before enhancement of plasmin generation rate is observed. The cell surface moieties involved in binding these reactants appear to be a mixed group of proteins and other molecules, many of which have been extensively investigated. The binding of plasminogen in particular is characterized by heterogeneous receptor molecules, present in high number but generally with low affinity for plasminogen. The low affinity of the interaction, with K_d values around 10^{-6} M, presents considerable technical difficulties when studying and quantitating plasminogen binding to cells or isolated receptors. Studying plasminogen activation kinetics in the presence of cells also presents technical difficulties and raises difficult questions on interpretation of results. However, approaches developed to study enzyme activation systems in other areas of hemostasis may also be applied to the problems associated with pericellular proteolysis. Models should be developed that match *In vitro* experimental data and help us understand the meaning of kinetic constants derived from these systems. In this way it should be possible to better understand the regulation of plasminogen activation around the cell under normal conditions and in a variety of disease states where cell-associated plasminogen activation is believed to be up-regulated. Ultimately, a sound understanding of theses regulatory mechanisms will enable us to devise strategies for modulating proteolytic activity, test these approaches in well designed *In vitro* systems and relate these results to the *in vivo* situation.

2. INTRODUCTION

Plasminogen receptors are a heterogeneous family of proteins and non-protein molecules. Molecules identified so far include alpha-enolase (1), annexin II (2), cytokeratin 8 (3), gp330 (4), gangliosides (5), and other proteins (6, 7). It is noticeable that plasminogen binding appears to be a secondary function to the more usual role

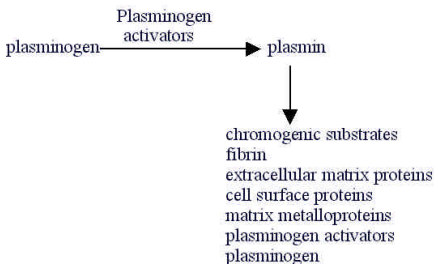
assigned to these biomolecules. This fact, coupled with the relative low affinity for plasminogen and the general "stickiness" of plasminogen, makes the identification of physiologically relevant receptors a contentious business. Hence, functional studies on plasminogen receptors, including kinetic studies, are especially important in the process of identification. The purpose of this review is to consider plasminogen activation on cell surfaces, primarily from the practical standpoint of *In vitro* assay systems. Assay design and the meaning and interpretation of results will be considered and the significance of cell surface plasminogen activation *in vivo* will be discussed.

3. PLASMINOGEN ACTIVATION KINETICS

3.1. Background

Plasminogen is the zymogen precursor of plasmin, a broad specificity serine protease (scheme 1). Plasminogen circulates as an inert protein of 92 kDa molecular weight, known as Glu-plasminogen, from the N-terminal glutamine residue. A number of physical studies have shown that Glu-plasminogen has a compact, globular structure, which appears to be relatively resistant to activation by plasminogen activators (8, 9). Another form of plasminogen, known as Lys-plasminogen, is also known and can be prepared in vitro. This form has been cleaved by plasmin and is lacking the first 68 to 78 amino acids (the N-terminal residue is most often lysine) (10). Lys-plasminogen is more rapidly activated by some plasminogen activators (11-13). However, the significance of lys-plasminogen *in vivo* has been debated (14-16). Recent evidence has suggested that lys-plasminogen may be more important in the plasminogen activation pathway on cell surfaces (17). The major plasminogen activators in vivo are also serine proteases namely, urokinase (uPA) and tissue plasminogen activator (tPA), although other proteases may also perform this role. uPA circulates in a single chain zymogen form scuPA, or pro-uPA, while tPA is active in the single chain form. tPA can be converted into a two chain form with slightly different kinetic properties. tPA is continually reacting with plasminogen activator inhibitor 1 (PAI-1) in the circulation and active tPA levels are dependent on PAI-1 concentrations (18). The normal concentrations circulating in vivo are around 2×10^{-6} M for plasminogen, whereas active tPA and uPA are subnanomolar. It should be noted that many *In vitro* studies on plasminogen activation are performed using much higher concentrations of activator enzymes, which could potentially give some misleading results.

Scheme 1



Plasmin cleaves a number of chromogenic (and fluorogenic) ester substrates in *In vitro* studies, and peptide bonds in proteins where there is a basic Lys- or Arg-residue in the P_1 site. The list of substrates for plasmin indicates that it is involved in a number of positive feedback pathways, on plasminogen and the plasminogen activators (19). Active plasmin can also stimulate other proteolytic pathways involving matrix metalloproteinases (20). Furthermore, plasmin activity generates additional C-terminal lysine residues in proteins such as fibrin and cell surface proteins that act as additional binding sites for plasminogen (21, 22). Bound plasminogen is more readily activated to plasmin (see below) and also results in bound plasmin, which is more resistant to inhibition by naturally occurring plasmin inhibitors (23).

3.2. The kinetics of the plasminogen activation system

3.2.1. Exponential rates

From this brief description of plasminogen activation it is already clear that studying the kinetics of activation can be a complex operation. In theory, the action of plasminogen activators on plasminogen could follow simple Michaelis-Menten kinetics and could be characterized by K_m and k_{cat} parameters. However, the feedback activities of plasmin on plasminogen, plasminogen binding sites and plasminogen activators, will obviously complicate the analysis. In addition, plasminogen activators and plasmin(ogen) are complex molecules with many binding sites available for interaction with proteins, other biomolecules and ions. For example chloride ions are known to bind to plasminogen and suppress its activation by uPA (24, 25). Other interactions between plasminogen activators and plasminogen, and effector molecules, are also possible, which can influence the activation rate in a way that will depend on the precise experimental conditions (26-29). Plasmin and other enzymes are able to generate free C-terminal lysine residues on cell surfaces, thus increasing plasminogen binding, and up-regulate the plasmin generation rate over time (22, 30). The conclusion from all these observations is that K_m and k_{cat} parameters determined in different laboratories under different conditions are unlikely to be comparable. Indeed, caution is needed in comparing results within a single laboratory if great care is not taken to standardize experimental conditions. These factors may help to explain the wide variation for kinetic parameters of the plasminogen activation system in the literature.

Having said all this, the basics of plasminogen activation kinetics are not too difficult to understand, and have been outlined many times previously (24, 31-33). In the case where plasminogen is being activated and plasmin generated at a linear rate, any products generated by the activity of plasmin should increase exponentially. Thus, for example, when plasmin activity is being monitored using a chromogenic substrate the increase in optical density should be described by equation 1:

$$Absorbance = \frac{ext \,.\, B \,.\, k_{cat} \,.\, [E] \,.\, [S] .\, time^2}{2\,(K_m + [S])} \qquad (1)$$

Where *ext* is the molar extinction coefficient for the p-nitoanilide from the chromogenic substrate under these conditions (units of *Absorbance*/M), [E] is the

concentration of activator, [S] is the concentration of plasminogen and *B* is the term describing the activity of the plasmin product on the chromogenic substrate (e.g., S-2251),as shown in equation 2:

$$B = \frac{k_{catS\text{-}2251} . [\text{S-2251}]}{K_{mS2251} + [\text{S2251}]} \quad (2)$$

The rate of plasmin generation, *v*, may be described by the Michaelis-Menten equation (equation 3) so may be determined from direct fitting of data for change in *Absorbance* with time using equation 1.

$$v = \frac{k_{cat} . [\text{E}] . [\text{S}].}{K_m + [\text{S}]} \quad (3)$$

Alternatively, the data may be linearly transformed in plots of *Absorbance* versus time^2 where the slope will be:

$$slope = v . B . ext/2 \quad (4)$$

Hence, *v* is readily calculated since *B*.ext/2 is a constant. *v* will be in units of M/s. Replotting data as the linear transformation of *Absorbance* versus time^2 is recommended, as deviations from linearity are indicators that the system is not behaving well.

It is thus possible to determine *v* over a range of concentrations of plasminogen (or [E], or other effectors, such as cellular receptors) and determine apparent kinetic parameters k_{cat} and K_m by replotting *v* against plasminogen concentration. Where many different conditions or concentrations are being investigated it is advantageous to perform experiments in microtitre plates. Data manipulations, such as squaring the time, should also be automated as far as possible by using spreadsheets or bespoke software. High throughput methods are necessary since many variables need to be investigated if the regulation of plasminogen activation is to be fully understood.

3.3. Heterogeneous catalysis

Superimposed on the general problems of consistency in plasminogen activation studies is the question of heterogeneous catalysis that arises when plasminogen activation kinetics are conducted in a milieu of cells (or in a fibrin matrix). Under these conditions plasminogen substrate and activator enzyme are not evenly mixed in solution but can bind to insoluble binding sites and react in distinct compartments. Not surprisingly, the kinetics of plasminogen activation may deviate from the Michaelis-Menten model, which was originally developed in a very simple system of single enzyme reacting with a single substrate. Hence, this model does not take into account additional interactions, such as plasminogen binding to a receptor, thus redistributing the substrate in two compartments with different kinetics profiles. Clearly, the equilibrium distribution of plasminogen free in solution and bound to receptors will vary as the plasminogen concentration is increased in a kinetic experiment designed to determine K_m and k_{cat}. For these reasons, any kinetic parameters that are determined in such a heterogeneous system, should be denoted *apparent* K_m and *apparent* k_{cat}. This applies even where the data appear to follow Michaelis-Menten kinetics, for example exhibiting a linear Lineweaver-Burk plot (see section 5.3).

4. RECEPTORS

4.1. High and low affinity receptors

The definition of a receptor is not entirely straightforward. Receptors are easy to recognize and study when they bind tightly and specifically to a particular ligand. In these cases it is often relatively easy to use radiolabelled ligands to investigate the affinity of binding and the number of binding sites per cell (34), making appropriate corrections for non-specific binding. However, the occurrence and significance of low affinity receptors (or binding sites as they are sometimes referred to) is becoming increasingly appreciated (35). Low affinity receptors may be present in high numbers and be designed to increase reactant concentrations in the pericellular space and for funneling reactants to high affinity receptors (35). In simplistic terms we may use a rule of thumb for categorizing high, medium and low affinity ligands as having K_d values in the ranges $\leq 10^{-9}$ M , 10^{-8} to 10^{-7} M and $> 10^{-7}$ M, respectively. However, it should be stressed that the dissociation constant of a receptor for a ligand is only part of the story when deciding how significant an interaction is. For example, consider the reversible interaction of a receptor R with a soluble ligand analyte A, in the simplest terms, as in scheme 2:

Scheme 2

$$R + A \underset{k_{off}}{\overset{k_{on}}{\leftrightarrows}} RA$$

Thus, at equilibrium $$[ra] = \frac{[r][a]}{K_d} \quad (5)$$

Where [ra], [r] and [a] are the equilibrium concentrations of complex, free receptor and free ligand, respectively. Therefore it is possible to increase [ra] by having a low K_d value and/or have high concentrations of reactants R or A in the system. In other words, even if a ligand is at a low concentration, below the K_d value, a significant amount of complex formation is possible given a large enough concentration of receptor. This important relationship between concentrations and affinity is not always fully appreciated and low affinity binding sites may be erroneously dismissed as irrelevant. Furthermore, there may be situations where a high concentration of low affinity receptors are to be preferred over a low number of high affinity receptors and this is discussed below (section 5.4).

Another feature of low affinity receptors is that they are technically more difficult to study than high affinity receptors (34). This may be appreciated by considering the kinetics of the interaction shown in scheme 2 where k_{on} and k_{off} are the forward and reverse rate constants:

$$K_d = \frac{k_{off}}{k_{on}} \quad (6)$$

It is frequently observed that k_{off} for biochemical interactions is the rate constant that largely determines the strength of an interaction since it can vary over many orders of magnitude. k_{on} tends to remain more consistent, observed within 1 or 2 orders of magnitude of 10^5 M^{-1} s^{-1} (36). The kinetics of association and dissociation of the complex determine the stability. Thus, it is simple to calculate that our typical high affinity interaction, with a K_d of 1.10^{-9} M and a typical kon of 10^5 M^{-1} s^{-1}, would have a half-life of around 2 hours and would thus be stable during washing steps and work up procedures employed to determine affinity and numbers of binding sites. In contrast the half-life of a low affinity receptor with a K_d of 1.10^{-6} M would be less than 10 s, making it more difficult to study by the common radioligand binding techniques.

4.2. Receptors for plasminogen and plasminogen activators

4.2.1. Plasminogen receptors

As mentioned in section 2, plasminogen binding sites are characterised by a relatively low affinity, typically with K_d values around 1.10^{-6} M, although it may be noteworthy that Lys-plasminogen and plasmin are frequently observed to have lower K_d values. The number of plasminogen binding sites observed on a range of cell types is high, often 10^7/cell or greater (21). Furthermore, this may be an underestimate in some cases due to the difficulties of measuring binding of low affinity receptors as discussed above (section 4.1). Therefore, the combination of high receptor numbers and the relatively high circulating concentration of plasminogen means that the extent of plasminogen-cell binding is likely to be significant.

The chemical nature of the plasminogen-receptor interaction is due largely to a low affinity lysine binding sites on one or more of the 5 Kringle domains of plasminogen (37). In native plasminogen, or using isolated domains, a number of studies have found lysine, or lysine analogue, affinities with K_d values in the expected range of 10^{-6} M for the low affinity sites. The significance of plasminogen binding for the stimulation of kinetics of plasminogen activation is readily demonstrated when lysine analogues such as aminohexanoic acid or tranexamic acid are included in the incubation mixtures. Plasminogen binding and kinetic stimulation are both simultaneously abolished in this way (38, 39).

4.2.2 uPA receptors

Receptors for uPA have been studied in great detail, particularly the GPI-linked receptor uPAR or CD87 (40). This receptor is known to bind uPA and inhibited complex with PAI-1, (uPA-PAI-1) with high affinity (10^{-9} M range). The number of uPAR binding sites per cell is typically around 50 000. Other types of complexes have been observed (41), including low affinity uPA receptors (42) where the K_d value appeared to be in the 10^{-6} M range although it was not possible to quantitate the numbers of binding sites per cell for technical reasons (section 4.1). These receptors were able to stimulate the activation of plasminogen by uPA in the presence of cells and it was clearly demonstrated that this mechanism was independent of the uPAR receptor. Significantly, stimulation of kinetics was observable in the presence of sub-nanomolar uPA (i.e., at physiological concentrations). This non-uPAR-dependent mechanism may be operative in some studies using knockout mice lacking uPAR (43, 44). Because of the low affinity and high numbers of binding sites it was proposed that low affinity binding sites may be formed by cell surface glycosaminoglycans. uPA has been shown to bind heparin (45), possibly via the Kringle domain, and cell surface glycosaminoglycans have been ascribed a similar binding role in other systems (35).

4.4.3. tPA receptors

Receptors for tPA have also been identified and appear to fall into the category of medium to low affinity (39, 46-50). Previously high affinity binding was studied, but later work has suggested this was extracellular matrix-associated PAI-1 (51). Consequently this interaction would not enhance the kinetics of plasminogen activation. Another interesting facet of some tPA-receptor interactions has been the competition for the same binding sites by plasminogen and tPA (50, 52). This is not too surprising from a structural viewpoint as tPA and plasminogen both have similar Kringle domains containing lysine binding sites. Consequently, low affinity interactions would be expected to stimulate plasminogen activation kinetics where tPA is activator since there will be a large number of binding sites. Having said this, the finger domain of tPA is clearly important in receptor binding and kinetics as variants lacking this domain are poorly stimulated in the presence of cells (33, 53). Binding site competition does have some interesting implications for the mechanisms of activation and introduces the opportunity for substrate inhibition, which has been observed (53).

5. MODELING PLASMINOGEN ACTIVATION KINETICS

5.1. Background: templates

As discussed above (section 3.2), simple Michaelis-Menten analysis of plasminogen activation rates, in the presence of a fixed concentration of cells will not give a complete picture of the kinetics of activation. Furthermore, the K_m and k_{cat} values that may be produced will not reflect the real intrinsic constants associated with the activator enzyme. Therefore, it is helpful to look at alternative approaches used to study kinetics in complex systems.

The concept of the "Template" has been widely applied to various systems within the field of hemostasis. In particular, the action of heparin as a stimulator of the reaction of thrombin with antithrombin has been intensively studied and models developed which help explain the mechanism of action (54). In this case heparin acts as a catalyst, binding both enzyme and inhibitor and enhancing their interaction rate many fold. The template action of heparin can be identified in kinetic experiments by the bell shaped curve observed when reaction rate is plotted against the log of the heparin concentration. Since this bell shaped relationship is also observed when cellular receptors, and indeed fibrin, are included as the stimulator

in plasminogen activation reactions, it seems likely that template models can be applied in kinetic studies involving these systems. The reason for the bell shaped curve is quite simple and has been explained elsewhere (55).

5.2. Local Concentration effects (the "shell" model)

Models developed using heparin are potentially helpful when looking at cell surface activation kinetics, but heparin is a soluble family of molecules with a concentration that can be (fairly) easily calculated. Cells introduce extra problems of heterogeneous catalysis (see section 3.3). However, a potentially valuable approach has been used previously in the study of the activation of coagulation enzymes in the presence of phospholipid vesicles. Many coagulation factors bind to membranes containing anionic phospholipids resulting in the stimulation of activation kinetics. The *shell* model originally proposed that phospholipid binding resulted in increased local concentrations of reactants in a compartment around the vesicle, which could be helpful in understanding the mechanism of stimulation (56).

This approach was extended to the study of cells as templates and the activation of plasminogen by tPA and a range of deletion variants with different binding and kinetic characteristics (53). A range of cell types was studied and all were effective as promoters of activation, including U937, THP1, K562, Nalm6 and Molt4. Stimulation of tPA activation of Glu-plasminogen by cells was around 80-fold under optimum conditions with a large decrease in the apparent K_m. Details of the assumptions behind the model, the derivation of the model equations (55), and the results (53) are presented elsewhere. Significantly, the model was able to replicate the bell shaped curve of cell concentration versus activation rate associated with templates and observed in kinetic experiments. Key features of this model were that tPA and plasminogen interact with low affinity receptors present in high numbers, leading to a localised increase in reactant concentration. The intrinsic K_m and k_{cat} of tPA were not affected, rather the observed rate enhancement resulting from local concentration of reactants was entirely consistent with the kinetic results obtained. An alternative model was also considered where bound tPA had drastically altered intrinsic K_m and k_{cat} values to account for the observed stimulation. This model could also fit the data, but only if bound tPA reacted with unbound plasminogen (this model has been proposed to account for results with phospholipid vesicles and coagulation proteins (57)). However, this scenario seems unlikely in the case of cell surface plasminogen activation by tPA for a variety of reasons (53).

5.3. Simulation of activation kinetics using *Gepasi*

Gepasi is a freely available program used for simulating metabolic pathways (58, 59) and it has potential as a tool in understanding the regulation of cell surface kinetics. The operation of the program is quite simple whereby a series of chemical reactions in a pathway are entered in a standard notation along with initial concentrations and kinetic constants for the reactions. The program then simulates the progress of the system, calculating reactant concentrations at a predefined frequency and for a set time. An advantage of this program is that scanning of parameters (initial concentrations, kinetic constants, binding affinities, etc) is simple and it is easy to explore parameter space to investigate the important factors in the regulation of a biochemical system.

A very simple system has been entered into *Gepasi* that may conveniently represent the series of 7 reactions taking place in a plasminogen activation assay in the presence of cells, as shown in Scheme 3.

Scheme 3

1 E + S = ES
2 ES → E + P
3 E + R = RE
4 S + R = RS
5 RS + E = RES
6 RE + S = RES
7 RES→ RE + P

Reactions 1 and 2 represent the activation reaction in solution, while 3 to 7 represent the same reaction on receptors (R). The 5 reversible reactions are described by k_{on} and k_{off} rate constants, which are not known, but the ratio is known and represents the affinity of the interaction, which is sufficient for simulation purposes. Thereafter, only the initial concentrations of the reactants need to be entered, and these were chosen to represent typical plasminogen activation kinetics and low affinity interaction (as used previously (42, 53)). It can be seen that in this very simple model there is only one type of receptor, which is intended to represent a category of low affinity receptors able to bind S (plasminogen) and E (uPA or tPA). In this analysis, stimulation of activation rates is not represented by concentration of reactants as in the shell model (section 3.2). Rather, an increased rate is included when E and S are associated with R, which in the example shown below was simply achieved by arbitrarily increasing the k_{cat} for reaction *7* 10-fold, compared with the reaction in solution (reaction *2*). In the example shown, activator enzyme was set to a physiological level (15 pM in this case) and the concentrations of plasminogen and receptors were scanned to cover the ranges used in *In vitro* experiments.

A series of simulations was then performed to determine rates of plasminogen activation over a range of plasminogen concentrations, at a series of receptor concentrations. These rates were then replotted to determine apparent K_m and k_{cat} values at each receptor concentration. The results of these simulations is shown in Figure 1 showing how apparent K_m, k_{cat} and k_{cat}/K_m (i.e. enzyme efficiency) are affected by receptor concentration. A number of conclusions from these results are immediately apparent. First, the patterns observed in Figure 1 are very similar to real observations made when looking at these kinetic parameters for plasminogen activation by uPA in the presence of low affinity receptors on Nalm 6 cells (figure 6 in (42)). Hence the increasing apparent k_{cat} , the apparent K_m that could fall and rise, and the bell shaped pattern of apparent k_{cat}/K_m were observed in real kinetic experiments. It is noteworthy that the large

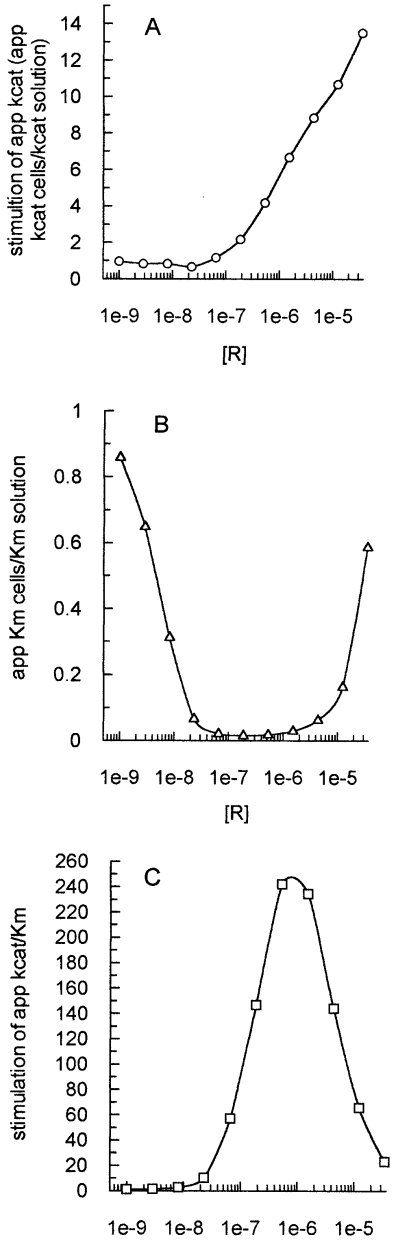


Figure 1. Gepasi simulations of receptor-bound plasminogen activation. Simulations were performed assuming a system of a single species of receptor with low affinity binding for enzyme and substrate according to Scheme 3. The constants used for enzyme activation in solution were K_m=65.10^{-6} M, k_{cat}=0.06 s^{-1}. The reaction on receptors was 10-fold faster (represented here by an increase in k_{cat} to 0.6 s^{-1}). Binding of E to R or RS was given a K_d=5.10^{-7} M and binding of S to R or RE was given a K_d=1.10^{-6} M. The concentration of E was 15.10^{-12} M and receptor and substrate concentrations were both scanned over physiological ranges. Stimulation of apparent k_{cat} (i.e. observed apparent k_{cat} cells/ k_{cat} in solution) is shown in panel A. Panel B shows the stimulation in apparent K_m (i.e. apparent K_m cells/K_m solution) and panel C shows the overall stimulation of apparent k_{cat}/ K_m by receptor binding.

changes plotted in K_m arise purely from the characteristics of the system and have nothing to do with the real K_m of the enzyme for plasminogen, which does not change. Furthermore, detailed analysis of the results showed that Lineweaver-Burk plots of simulated data at each receptor concentration were usually non-linear indicating deviation from simple Michaelis-Menten kinetics. However, a small region of results, around the middle of the range shown in Figure 1, did show linear Lineweaver-Burk plots. Thus, it seems that where examples of linear Lineweaver-Burk plots have been presented in the literature for plasminogen activation reactions in the presence of a particular concentration of cells, these experiments may have been performed in this fortuitous range of cells.

Based on this promising initial evaluation of the simulated results, further simulations were performed to investigate the effect of increasing enzyme concentration and the effect of increased affinity of enzyme for receptor (reactions *3* and *5* in scheme 3). Tighter binding of enzyme to receptor is interesting as high affinity receptors for uPA in particular are well known (section 4.2.2). The results from these studies are shown in Figures 2 to 4. Figure 2 shows a standard set of conditions as used in Figure 1. Figure 2 may be compared with Figure 3 where there was a 10-fold increase in enzyme concentration. This translates directly into a 10 fold increase in activation rates (note the scales of the y axis), with no change in the shape of the curve (as can readily be seen in the contours in the plot). A direct linear relationship between activation rate and enzyme concentration in the presence of Nalm6 cells was also observed in experimental data (Figure 3 in (42)), again suggesting the model is behaving well. Figure 4 was generated using the standard conditions from Figure 1 but assuming a high affinity enzyme-receptor interaction (K_d=2.10^{-9} M in this case). Here, the shape of the curve has obviously changed such that plasminogen activation is enhanced at low receptor and low plasminogen concentrations. However, the highest levels of activation are unchanged from Figure 2.

5.4. Limitations of kinetic models

Kinetic modeling studies are aimed at understanding the regulation of plasminogen activation and will assist in efforts to modulate cell surface kinetics. The modeling approaches used above in sections 5.2 and 5.3 are of course simplifications of reality and may not always be appropriate, so should be used with caution. It is advisable to check real data against model predictions over a large range of conditions. Where models do appear to match real data then they may be useful in understanding the regulation of kinetics and may be used to make predictions under other conditions and *in vivo*.

From a theoretical point of view, modeling the behavior of low affinity receptors may be the simplest situation. For example, using the *shell* approach a simple stimulatory mechanism is invoked relying on increased concentrations of reactions in a zone around the cell where the reactants are free in solution and behave normally. In other words, kinetics are taking place fully in 3 dimensions (3D/3D (60)), as in free solution. In these circumstances it

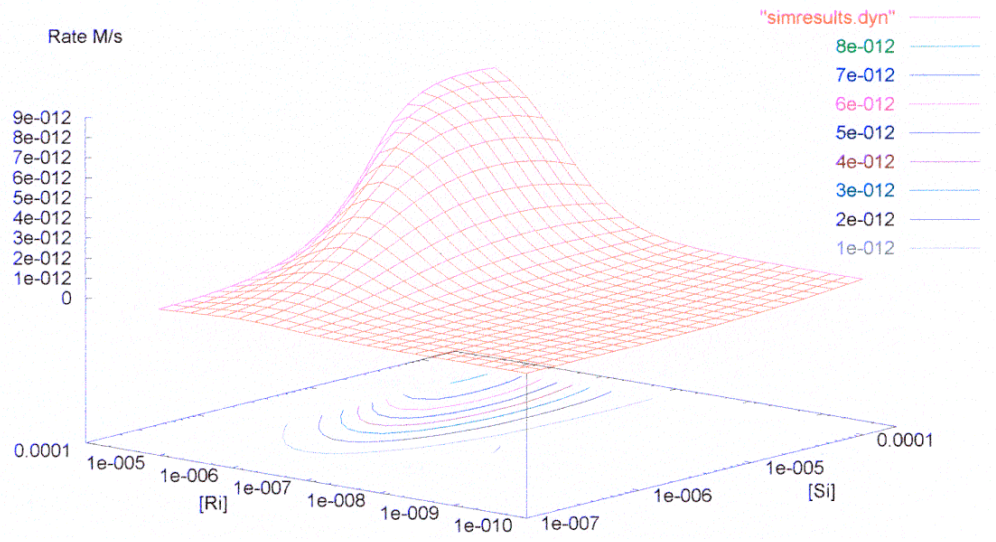


Figure 2. Results from Gepasi simulations of receptor-bound plasminogen activation showing the relationship between activation rate, substrate concentration [Si] and receptor concentration [Ri]. The parameters and concentrations used in these simulations were as given in the legend to Figure 1.

may be anticipated that low affinity interactions are preferred so that diffusion of reactants within the *shell* is not hindered. Hence high numbers of low affinity receptors, rather than fewer high affinity receptors, are appropriate for this mechanism of stimulation. However, the shell model has been criticized on theoretical grounds (61, 62).

When moving to a system with tightly bound enzyme occupying high affinity receptors on the cell surface, then there is a change in dimensionality, such that the system is now 2D/3D (60). At this point additional factors may be introduced which account for stimulation of activity, such as orientation effects and the more efficient searching achieved by reactants as dimensionality is reduced (63). Reactions on the cell surface exclusively involving high affinity receptors may also have a 2D/2D dimensionality. Both these circumstances necessitate more complex models involving geometrical factors to explain increased reaction rates (63).

6. REGULATION OF PLASMINOGEN ACTIVATION *IN VIVO*

Pericellular proteolysis is a vital part of normal biochemistry involving many physiological processes and cell types (64-68). Many disease states are also associated with irregularities in cell surface plasminogen activation. A number of studies have clearly indicated that the plasminogen activation system is heavily involved in wound healing processes (69, 70) and a large number of clinical studies have been carried out showing a correlation between uPA and/or uPAR levels and poor survival in a range of cancers (71-84). The implication from these studies is that increased levels of reactants and/or receptors results in enhanced pericellular proteolysis, accelerated tumor cell invasion and metastasis. This would seem to be consistent with the *In vitro* kinetics studies such as those described above, and others where cell migration rates are measured through Matrigel substrates (85-88).

Fewer studies have investigated tPA or plasminogen binding in relation to disease compared with uPA/uPAR, but this may simply be for technical reasons. However, there are some suggestions that plasminogen binding may be important in regulating the plasminogen activation kinetics (89) and influence the development of some tumors (90). Furthermore, prokaryotic evolution shows that cell surface plasminogen binding and activation are critical regulatory factors for the success of pathogenic organisms (91). Once again alpha-enolase has been identified as an important plasminogen receptor in prokaryotes, for example *Streptococci* (92).

These modeling studies also suggest how affinities and concentrations interact to regulate plasminogen activation. It is tempting to conclude from the simulations in section 5.3 that inhibition of activator may be the most effective method of globally reducing pericellular proteolysis. Nevertheless, inhibiting receptor binding is also likely to be effective and would work in a different way (see Figure 2 to 4). However, alternative mechanisms to proteolysis also need to be considered. It is likely that signalling pathways involving receptor binding and internalisation of inhibited uPA complexes, for example, also play a role in tumor cell growth and movement (93, 94). Other aspects of receptor cell biology that are not considered in the kinetic studies outlined above are mechanisms of focalisation of bound enzymes to a pole of the cell enabling directional migration through the extracellular matrix (49, 95, 96).

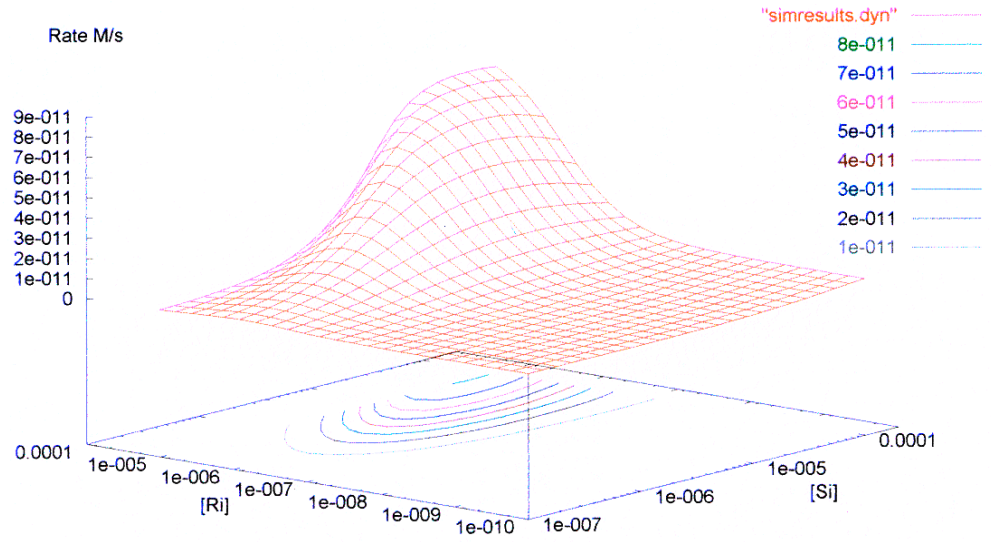


Figure 3. Gepasi simulations of receptor-bound plasminogen activation showing the relationship between activation rate, substrate concentration and receptor concentration. The parameters used in these simulations were as in Figure 2, but enzyme concentration was increased 10 fold to 150.10^{-12} M. The pattern of activation is unchanged from that seen in Figure 2 (note the same pattern on the contours plotted on the x, z plane), however all activation rates are increased 10-fold (note the y scale).

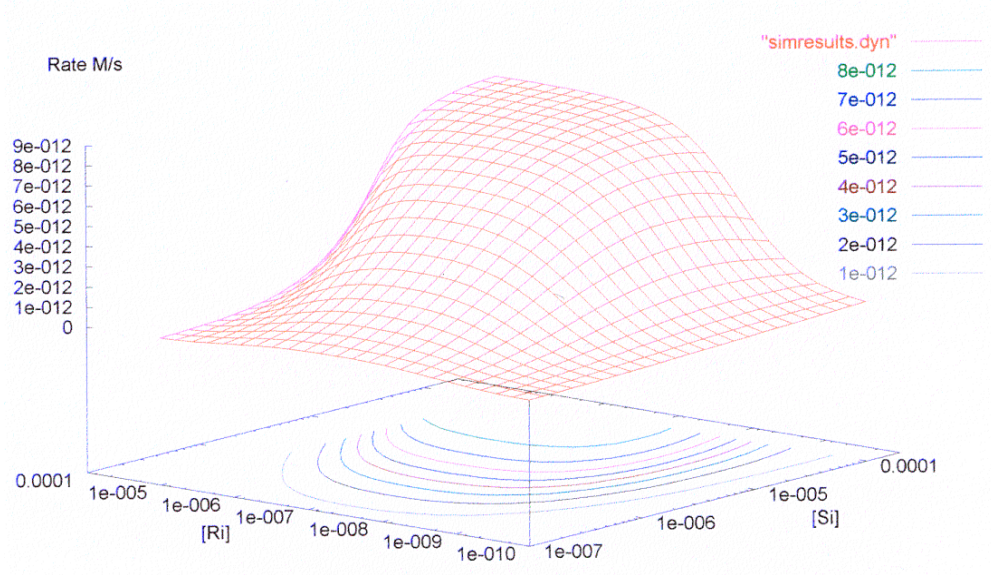


Figure 4. Gepasi simulations of receptor-bound plasminogen activation showing the relationship between activation rate, substrate concentration and receptor concentration. The parameters and concentrations used in these simulations were given in Figure 2, except the affinity of enzyme-receptor binding was increased such that the K_d for R=E and RS=E binding was now 2.10^{-9} M. The maximum activation rate is unchanged from Figure 2, but the pattern is different as can be seen by comparing the contours. Increased activation rates are now observed at lower substrate and receptor concentrations.

7. CONCLUDING REMARKS

In vitro studies of plasminogen activation in the presence of cells should be designed not simply to accumulate data but to understand the fundamental mechanisms regulating activation kinetics. With this knowledge it should be possible to understand how plasminogen activation is regulated *in vivo* and design strategies for modulating activation rates in disease. These strategies could include lowering free enzyme concentrations, or modulating receptor expression or interfering with receptor-ligand binding (97-102). The effectiveness of these approaches needs to be investigated in carefully designed *In vitro* assays. One important message from the studies outlined above is that low affinity receptors involved in plasminogen activation pathways may have been neglected in the past, partly due to the technical difficulties associated with long established binding studies

normally applied to high affinity receptors. Kinetic and modeling studies suggest that low affinity receptors may be very important in regulating plasminogen activation rates *in vivo*.

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