Plasma contact activation: A revised hypothesis

ALVIN H SCHMAIER*

Division of Hematology and Oncology, Department of Internal Medicine and Pathology, University of Michigan, Ann Arbor, MI 48109-0640, USA

A new hypothesis for activation of the contact system of plasma proteolysis (i.e., the plasma kallikrein/kinin system) is presented. Kininogens have a multiprotein receptor on endothelial cells which consists of at least cytokeratin 1, urokinase plasminogen activator receptor, and gC1qR. When contact proteins (high molecular weight kininogen followed by prekallikrein) assemble on the kininogen receptor on endothelial cells, an endothelial cell membrane cysteine protease is expressed to activate prekallikrein to kallikrein. On endothelial cells, prekallikrein activation is independent of factor XIIa activation. Activation of prekallikrein on endothelial cells results in kallikrein cleaving its receptor high molecular weight kininogen to liberate bradykinin. Bradykinin liberation stimulates release of tissue-type plasminogen activator from endothelial cells. Kallikrein formation also results in kinetically favorable pro-urokinase activation on endothelial cells with subsequent plasminogen activation. In addition to stimulating cellular fibrinolysis, kininogens contribute to the constitutive anticoagulant nature of the intravascular compartment. Kininogens block calpain's participation in forming the heterodimeric complex of platelet integrin $\alpha_{IIb}\beta_3$. Kininogens also block thrombin from binding to the thrombin receptor(s) on platelets. Last, kininogens prevent thrombin from cleaving protease activated receptor 1 after arginine$_{41}$. These combined data indicate a biologic system for activation of the plasma kallikrein/kinin system and physiologic consequences as result of this activation.

Key terms: *antithrombin, bradykinin, contact activation, cytokeratin, factor XII, fibrinolysis, kininogen, kinins, prekallikrein, thrombin*

INTRODUCTION

The contact system of plasma proteolysis, which is the plasma kallikrein/kinin system, has been viewed as a biochemical pathway whose biologic role needs further clarification. Two aspects of this system have served to obfuscate understanding and brand the system as unimportant. The first confounding aspect is that although deficiencies of its constituents, factor XII (FXII), prekallikrein (PK), and high molecular weight kininogen (HK), give striking prolongation of surface-activated coagulation assays, patients with these protein deficiencies do not bleed. The second confounding aspect is that the *in vivo* activator(s) of this system has not been identified. Contact system proteins have been known only to activate when associated with an artificial, negatively charged surface such as glass, kaolin,

* **Correspondence to:** Alvin H Schmaier, MD, Department of Internal Medicine and Pathology, University of Michigan, 5301 MSRB III, 1150 W Medical Center Drive, Ann Arbor, MI 48109-0640, USA. Phone: (1-734) 647-3124. Fax: (1-734) 647-5669. E-mail: aschmaie@umich.edu

celite, *etc*, hence the name "contact system". Although a number of biologic substances, acidic phospholipids, cholesterol sulfate, sulfatides, gout crystals, *etc*, have been shown to function as negatively charged surfaces, none are convincing to serve as the single *in vivo* surface for physiologic activation of this system. Thus a more cogent mechanism for activation of this system needs to be discovered.

For the last twenty years most investigators in the field have accepted the notion that the initiation of activation of the system results from FXII binding to negatively charged surfaces to autoactivate (37, 54). Factor XII's autoactivation leads to PK activation and kallikrein formation amplifies further FXII activation. The rate of initiation and amplification of this system is accelerated by HK and an artificial surface. Amplification of this system's activation by kallikrein is at least 1000-fold faster than the autoactivation phenomena (48). Activation of the zymogens FXII and PK results in enzymes that contribute to factor XI activation (coagulation), complement activation, bradykinin liberation, fibrinolysis and granulocyte activation. Unfortunately, the potential importance of this system to mediate biologic responses has been overshadowed by the untenable explanation for how this system's activation is initiated.

Over ten years ago, my laboratory developed a working hypothesis to serve as an alternative to the factor XII autoactivation phenomena for the initiation of activation of contact system proteins. We reasoned that *in vivo* it is the assembly of a multiprotein complex of contact system proteins on cell receptors that allow for localization and activation of this system. In order to prove that hypothesis, we sought to accomplish three things: First, determine if there is a receptor for the major cofactor of this system, HK, on cell membranes. Second, show how the assembly of contact proteins on cell membranes through HK results in activation of the zymogens PK and FXII. Third, demonstrate if there are important biologic activities associated with contact protein assembly and activation on cell membranes.

CHARACTERIZATION OF THE MULTIPROTEIN KININOGEN RECEPTOR ON ENDOTHELIAL CELLS

Initial investigations to determine the kininogen receptor were spent characterizing the ability of kininogens to bind to various cells in the intravascular compartment. The study by Greengard and Griffin first demonstrated HK binding to activated platelets required Zn^{2+} (10). We followed with the demonstration the HK binds to unstimulated platelets in the presence of Zn^{2+} (15). Additional studies from multiple laboratories have shown that kininogens also bind to granulocytes and endothelial cells in the presence of Zn^{2+} (14, 46, 50). However, the role of zinc ion was not just to associate with the light chain of HK (9). Rather, it was essential for the expression of the kininogen receptor because low molecular weight kininogen (LK), which does not have HK's light chain, required Zn^{2+} as well for binding to platelets (36).

Investigations also revealed that kininogen must be binding to a physicochemical structure. The kininogen binding site, putative receptor, on endothelial cells appears to be a structure that can be regulated. First, treatment of endothelial cells with metabolic inhibitors to aerobic and aerobic metabolism and the hexose monophosphate shunt abolish the ability of HK to bind to cells (18). Cycloheximide has no effect on HK binding to endothelial cells. Second, temperature or the bradykinin sequence in kininogens contributes to the level of kininogen binding to endothelial cells (18, 19, 55). Third, bradykinin treatment of endothelial cells results in increased HK and LK binding and this pathway is mediated by protein kinase C and the endothelial cell B1 bradykinin receptor (55). Fourth, heavy chain and LK have a Ca^{2+} requirement for phorbol 12-myristate 13-acetate 4-0 methyl ether up-regulation of their endothelial cell binding site, whereas HK does not (55). Fifth, angiotensin-converting enzyme inhibitors potentiate the effect of bradykinin on up-

regulating the HK binding site on endothelial cells (55). Last, when HK binds to endothelial cells, it initiates a series of events that allow for an endothelial cell-associated enzyme to activate PK bound to HK (38). This last action will be discussed in detail below. Thus, bradykinin up-regulates kininogen binding on endothelial cells and kininogen can influence bradykinin formation (35). These data indicate that this system is tightly controlled in an autocrine-like manner.

The combined information given above indicated that there must be a physicochemical kininogen receptor(s) on endothelial cells. Using an HK affinity column, the first binding protein reported to be isolated from endothelial cell lysates was a 33 kDa protein which upon amino-terminal sequencing was identified as gC1qR, a known receptor for the macromolecular complement protein (23). This protein only bound HK, not LK. Further, the initial report stated that Zn^{2+} was not required for binding (23); a second report stated that Zn^{2+} was a requirement for binding (29). However, there is some controversy as to whether gC1qR is a substantial protein on endothelial cell membranes. It has mostly been described as a mitochondrial protein in endothelial cells (8). CD11b/CD18 on granulocytes also was proposed as binding protein, putative receptor, for kininogen because a monoclonal antibody to this structure partially blocked kininogen binding (51). We found this interpretation untenable because in our own studies, we found that ^{125}I-HK and ^{125}I-LK bound normally to leukocyte adhesion deficiency granulocytes, *i.e.* granulocytes absent in CD11b/CD18 (unpublished). More recently, the urokinase plasminogen activator receptor (uPAR) has been shown to be a kininogen binding site on endothelial cells (6). Since uPAR is linked to CD11b/CD18, it is possible that antibodies to Mac-1 that partially blocked HK binding could have been doing so by interfering with expression of uPAR (52). However, the fact that uPAR is not present on platelets indicates that this protein cannot be a single kininogen receptor on all cells. Additional binding proteins must exist.

Work performed in our own laboratory revealed that the major protein band purified on a HK affinity column from endothelial cell lysates was 54 kDa which on amino acid sequencing was identified as cytokeratin 1 (CK1) (20). Cytokeratin 1 antigen was found on the membrane of endothelial cells by laser scanning confocal microscopy, flow cytometry, and direct anti-CK1 $F(ab)_2$' binding. HK specifically bound to native or recombinant CK1 only in the presence of Zn^{2+}. Further, all three binding domains of HK (domains 3, 4 and 5) blocked HK binding to cytokeratin (17, 19, 24). Last CK1 antigen was found on platelets and granulocytes indicating that this protein can serve as a kininogen receptor on all of these cells. However, the number of CK1 binding sites on each of these cells is not sufficient to account for the total number of kininogen binding sites, indicating that other proteins, those above as well as possible others, may serve in a multiprotein assembly as the kininogen receptor. It is quite unexpected to find CK1 as a kininogen binding protein, putative receptor. Recent studies have indicated that other cytokeratins, CK8 and 18, serve as binding proteins for plasminogen and thrombin-antithrombin III complexes, respectively (21, 22, 53). Kininogens' multiprotein receptor complex is shown in Figure 1. Last, both CK1 and uPAR's link to CD11b/CD18 provide potential mechanisms for signaling when HK assembles on the endothelial cell membrane. These potential mechanisms for cell activation may be important when contact proteins assemble on cell surfaces.

CHARACTERIZATION OF PREKALLIKREIN ACTIVATION ON THE ENDOTHELIAL CELL MEMBRANE

It is well accepted that the majority of plasma PK and factor XI circulates in plasma in complex to HK (34, 49). Since HK serves as the cell receptor for factor XI, we reasoned that it also serves as the prekallikrein binding site on endothelial

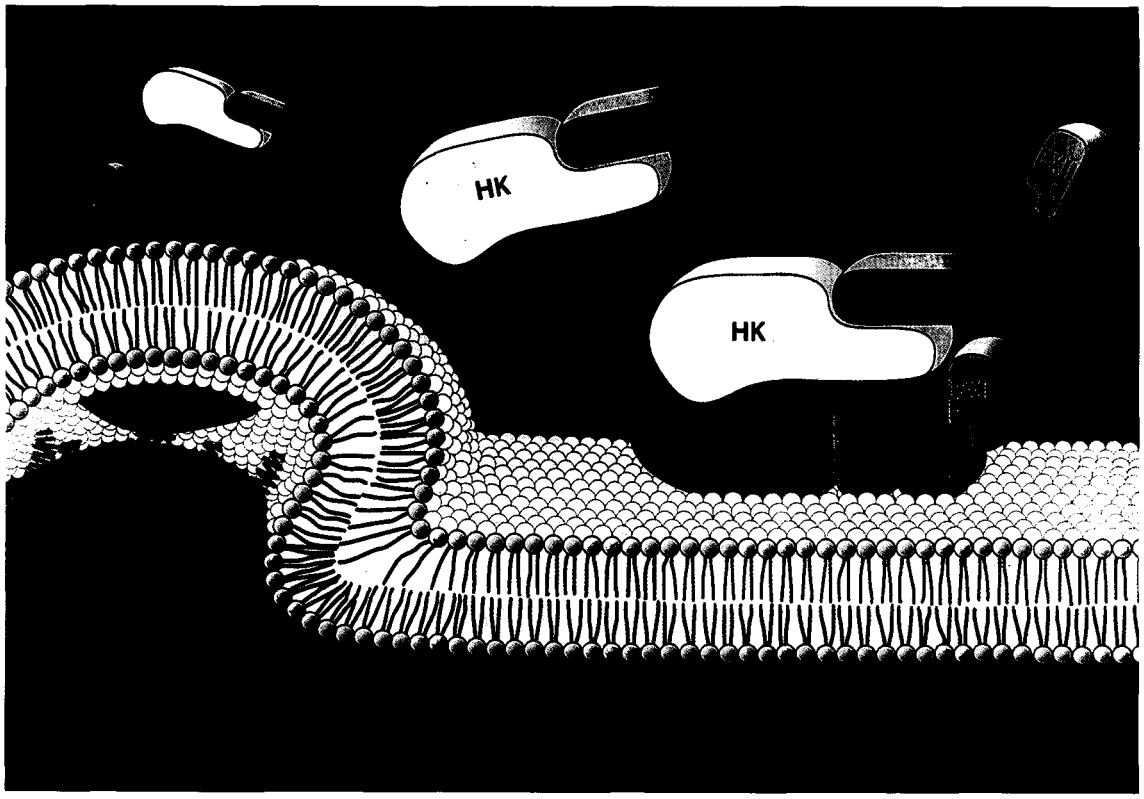

Fig 1. Multiprotein kininogen receptor on endothelial cell membrane. Circulating plasma PK and factor XI are mostly bound to plasma HK. The complex between plasma HK and PK or factor XI binds to a multiprotein kininogen receptor on endothelial cells which consists of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR), and gC1qR. Factor XII (XII) also binds to gC1qR.

cells (11, 38). PK specifically bound to endothelial cells only in the presence of Zn^{2+} and after being saturated with HK (38). The apparent Kd for this interaction was 23 nM, a value similar to that seen with PK binding to HK in solution. PK binding to endothelial cells in the presence of added HK was almost completely inhibited by antibodies to the binding domains for PK on HK and *vice versa* (38).

Further studies were performed to determine if when HK and PK assembled on endothelial cells would the PK become activated to kallikrein (11). Using models from the plasma and artificial surface system, PK is only activated on surfaces in the presence of activated factor XII (4, 12, 43). Initial studies showed that the assembly of HK, PK, or kallikrein alone on endothelial cells did not result in any significant amidolytic activity (Fig 2A). As a control for the addition of activated forms of FXII for the assembly of HK and PK, we added HK followed by PK alone to endothelial cells. The amount of measured kallikrein formed from the HK and PK assembly alone was greater than that seen when zymogen FXII, activated FXII (FXIIa, αFXIIa), or Hageman factor fragment ($FXII_f$, βFXIIa) were added along with the PK (Fig 2A). Further, substituting kallikrein at the same concentration than PK did not result in higher levels of amidolysis (Fig 2A). These results surprised us since they indicated that on endothelial cells there is a PK activating mechanism independent of factor XII and its activated forms. In fact, under the conditions of the assay, addition of the enzyme kallikrein resulted in less measured amidolytic activity.

Further investigations were performed to determine if there was any contaminating activated FXII in the reaction. The ambient tissue culture media contained less than 0.0001 U/ml activated

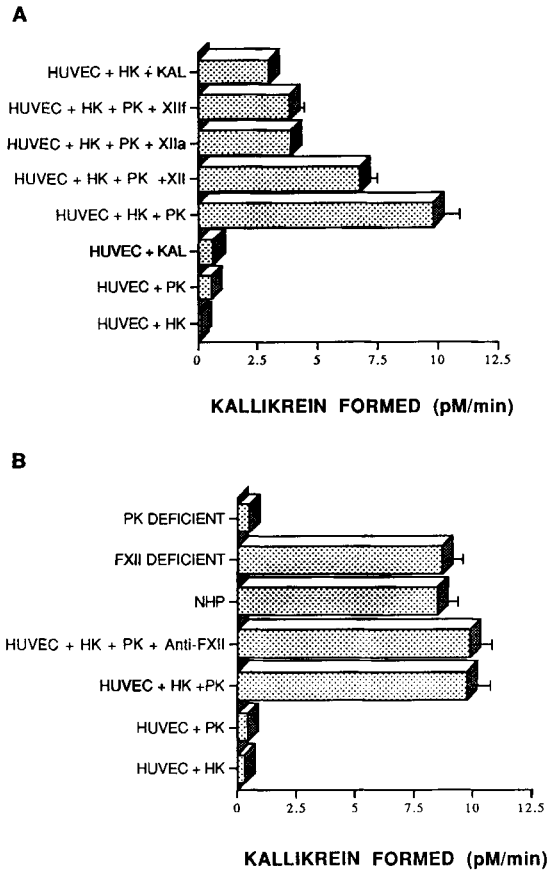

Fig 2. Activation of PK on HUVEC. Panel **A:** Endothelial cell monolayers (HUVEC) preincubated with 200 µl of a solution containing 2% bovine serum albumin. HK (20 nM) or buffer then added for 1 h at 37°C; unbound protein removed by washing, and 20 nM PK or 20 nM plasma kallikrein (Kal) incubated for an additional h. Wells were then washed and 0.4 mM S2302 added in absence or presence of 20 nM FXII (XII), 3.4 nM αFXIIa (XIIa), or 3.4 nM βFXIIa (XIIf), as indicated. Data, means ± SEMs of 3 experiments. Absence of standard error bars in some columns indicates that variation was too little to portray visually. Panel **B:** Endothelial cell monolayers (HUVEC) preincubated with 200 µl of a solution containing 2% bovine serum albumin. HK (20 nM) or buffer then added for 1 h at 37°C; unbound protein removed by washing, and 20 nM PK incubated for an additional h in absence or presence of 0.4 mg/ml of an anti-FXII antibody (Anti-FXII). In other experiments, HUVEC saturated with HK (20 nM) incubated with 50 µl of pooled normal plasma (NHP), FXII-deficient plasma, or PK-deficient plasma for 1 h at 37°C. After washing, 0.4 mM S2302 added and hydrolysis monitored for 1 h. Data, means ± SEMs of 3 experiments. Absence of standard error bars in some columns indicates that the variation was too little to portray visually.

FXII coagulant activity. In the presence of a neutralizing concentration of an antibody to FXIIa, PK activation was to the same extent when no antibody was present (Fig 2B). Soybean trypsin inhibitor, a kallikrein inhibitor, abolished the measured activity, but corn trypsin inhibitor, an activated FXII inhibitor, did not abolish the generated kallikrein from PK assembled on HK on endothelial cells. Moreover, an antibody to the PK binding site on HK and a peptide to compete PK binding to HK abolished the ability of PK to be activated on the endothelial cell membrane. Last, when normal human plasma or plasma deficient in FXII were incubated over endothelial cells, kallikrein activity was demonstrable; no activity was measured when PK deficient plasma was incubated over endothelial cells. These combined data indicated that the PK activation mechanism was dependent on HK and PK and independent of FXII.

Investigations next were performed to determine the role, if any, for FXII activation on endothelial cells. Using optimal Zn^{2+} and substrate concentrations, we found no evidence for FXII autoactivation on the endothelial cell membrane (44). PK activation is the initial and central event in contact proteins' activation on endothelium. FXII contributes to the rate and extent of enzymatic activity generated on the endothelial cell membrane, but not the initiation of activity. This point was made by determining the kinetics of PK activation. In the absence of FXII, the K_m (20 ± 8 nM) and V_{max} (12 ± 3 pM/min) of PK activation was virtually the same as that generated in the presence of FXII (K_m = 30 ± 4.2 nM; V_{max} = 9.2 ± 2.1 pM/min) (38). These findings on endothelial cells are just the opposite from that known to occur on artificial surfaces when activation of FXII initiates the system. From normal plasma or FXII deficient plasma, PK bound to endothelial cells becomes fully activated within 4-5 min. Further, using purified contact proteins at plasma concentrations, the full extent of activation also occurred within 5 min. The kinetics of activation of this system on endothelial cells greatly exceeds FXII autoactivation which only results in low levels of activity even after 120 min of activation. These data indicate that PK activation is the critically important

mechanism for activation of this system in a physiologic manner on endothelial cells.

Further studies were performed to determine the mechanism by which PK became activated on endothelial cells. Initial investigations examined the role of HK. In the absence of added HK, there is little change in the structure of 85 and 88 kDa PK bound to endothelial cells when incubated for 2 h. In the presence of HK, bound PK is rapidly cleaved within 1 to 2 min to show its heavy chain (51 kDa) and its light chains at 37 and 34 kDa (38). These data indicate that the presence of HK and PK assembly with HK are critical for the activation of PK. Investigations next proceeded to determine if the activation of PK when bound to HK was an enzymatic process. Initial studies determined if serine protease inhibitors would block PK activation on endothelial cell membranes. Neutralizing antibody to FXIIa, benzamidine, PMSF, soybean trypsin inhibitor, and Pro-Phe-Arg-chloromethylketone did not inhibit the change in structure of zymogen PK to kallikrein as shown on SDS-PAGE. These data indicated that the activation of PK bound to HK was not due to FXIIa, PK autoactivation, or a serine protease (38). Metal chelators like EDTA, EGTA, orthophenanthroline, *etc*, blocked PK activation presumably by blocking HK binding to endothelial cells. Alternatively, antipain (100 μM), cysteine, $HgCl_2$, DTT, 2-mercaptoethanol all fully blocked PK activation. Interestingly, cystatin, n-ethylmaleimide, iodoacetamide did not inhibit this PK activating enzyme(s). Further metalloprotease inhibitors, TIMP-1, TIMP-2, and BB94 were not inhibitory. These combined data indicate that a peculiar cysteine protease associated with the membrane of endothelial cells is responsible for the enzymatic conversion of PK to kallikrein only when bound to HK. These data indicate that endothelial cells have a mechanism by which contact protein assembly allows for PK activation. We may have discovered a fundamental mechanism in cell biology. We have already demonstrated that HK-regulated PK activation by a cysteine protease actually can occur on rat 3T3 fibroblasts (unpublished). Figure 3 shows a cartoon of the multiprotein assembly of PK on HK on its receptor complex on endothelial cells and the role of PK in FXII activation.

DETERMINATION OF BIOLOGICALLY IMPORTANT ACTIVITIES ASSOCIATED WITH PREKALLIKREIN ACTIVATION

Fibrinolysis.

The consequences of PK activation on endothelial cells needs to be enumerated. First and foremost after very rapid PK activation on HK on endothelial cells, kallikrein cleaves its receptor, HK, to liberate bradykinin (38). The local biologic effects of bradykinin on blood pressure regulation, prostaglandin formation, NO formation, superoxide formation, stimulation of smooth muscle hyperpolarization factor, and tissue plasminogen activator liberation are important alone (3, 7, 25, 26, 39, 40, 47). Bradykinin has been demonstrated to be the most potent stimulator of tissue-type plasminogen release activator *in vivo* in rabbits and man (3, 47). This fact alone is an important contribution to fibrinolysis. However, the kallikrein/kinin system has been shown to have other roles in fibrinolysis. Plasma kallikrein is known to be the most favorable kinetic activator of pro-urokinase (single chain urokinase) *in vitro* (27). Gurewich and his coworkers (13, 31, 33) performed an important series of experiments where they showed that HK and PK assembly on platelets or endothelial cells, after the addition of activated FXII, led to kinetically favorable pro-urokinase activation. This mechanism of single chain urokinase activation was kallikrein, HK, and cell-dependent (platelet,endothelial cell) after the addition of a PK activator, *i.e.* FXIIa.

We asked the question if our FXII-independent, PK activation system on endothelial cells also could result in kinetically favorable pro-urokinase and, subsequently, plasminogen activation (Fig 4). The ability of HK, PK, pro-urokinase, or HK + PK alone to generate enzymatic activity to cleave a chromogenic substrate

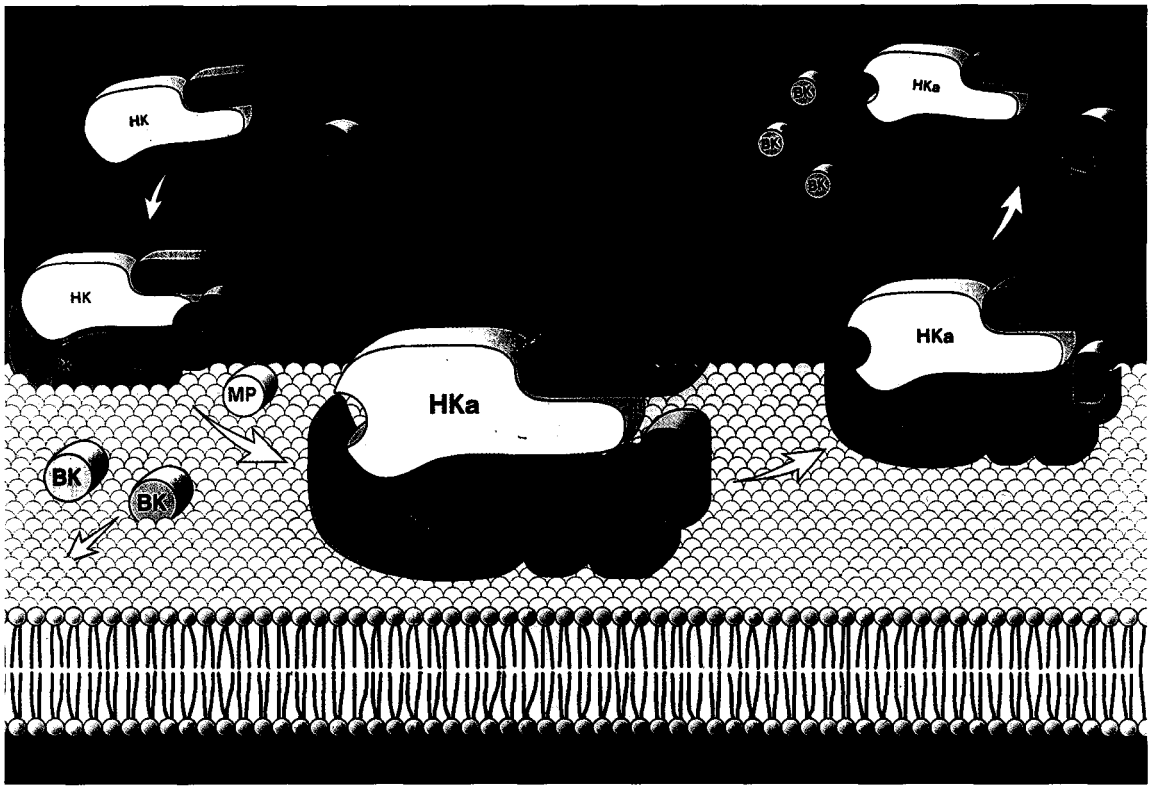

Fig 3. Mechanism of FXII activation on endothelial cells. Plasma complex of HK and PK binds to endothelial cell multiprotein kininogen receptor. Binding of PK to HK on this receptor allows for expression of an endothelial cell membrane cysteine protease (MP) that activates PK to kallikrein (K). FXII (XII) bound to the multiprotein kininogen receptor is then activated by kallikrein to activated FXII (XIIa). Kallikrein cleaves its receptor, HK, to liberate bradykinin (BK) and release itself from the membrane.

for two chain urokinase was little (Fig 4A). As previously shown, the addition of pro-urokinase to endothelial cells resulted in more enzymatic activity which was not potentiated by PK alone (1) (Fig 4A). However, the assembly of HK and PK along with pro-urokinase on endothelial cells resulted in increased two chain urokinase activity (Fig 4A). Further, this increased activity was not blocked by an antibody to factor XIIa (38). Additional studies showed that the K_m (135 ± 81 nM) and V_{max} (14.5 ± 8 nM/min) of pro-urokinase activation on endothelial cells in the presence of HK, PK and FXII was the same as that seen in the absence of FXII (K_m = 64 ± 5 nM; V_{max} = 10 ± 0.1 nM/min) (38).

Since the chromogenic substrate for two chain urokinase has a high K_m, we performed studies with two chain urokinase and its biologic substrate, plasminogen (Fig 4B) (38). HK, PK, plasminogen, or pro-urokinase result in little hydrolysis of the chromogenic substrate for plasminogen (Fig 4B). Further, the assembly of HK + PK, plasminogen alone, plasminogen + pro-urokinase, or PK, plasminogen, + pro-urokinase on endothelial cells also has little activity on the plasmin substrate. However, if we assemble HK and PK on endothelial cells followed by plasminogen and pro-urokinase is added at the time of the adding the chromogenic substrate, there is a marked increase in the amount of plasmin formed (Fig 4B). The generation of this plasmin is not inhibited by an antibody to FXII. These data indicate a mechanism for fibrinolysis independent of FXII, tissue-type plasminogen activator, and fibrin. This work has already been confirmed by studies from another laboratory (32). Figure 5 represents a cartoon on how prekallikrein assembles on endothelial cells to result in kinetically favorable pro-urokinase and

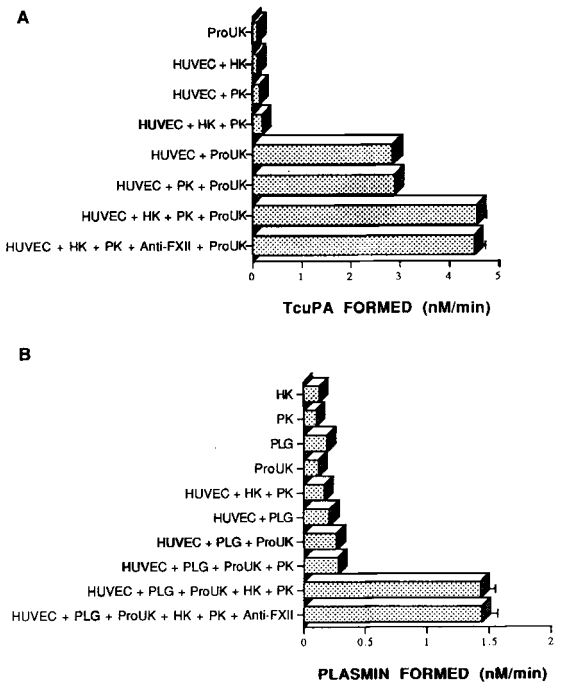

Fig 4. Influence of HK and PK on pro-urokinase and plasminogen activation. Panel **A:** Pro-urokinase activation. Empty microtiter plate wells or wells coated with a monolayer of endothelial cells (HUVEC) incubated with HK (20 nM) or buffer for 1 h. Unbound HK removed and cells incubated with PK (20 nM) for another h and washed. Pro-UK (20 nM) and 0.6 mM S2444 added to empty wells or wells coated with HUVEC, and hydrolysis monitored continuously over 75 min at 37°C. In one set of experiments, 0.4 mg/ml of a neutralizing antibody to FXII added along with the PK. Formation of two chain urokinase plasminogen activator (TcuPA) determined by comparing substrate hydrolysis on cells with known concentrations of soluble TcuPA. Data, means ± SEMs of 3 experiments. Panel **B:** Plasminogen activation. Empty microtiter plate wells or wells coated with a monolayer of HUVEC incubated for 1 h with 1 μM plasminogen (PLG) before 0.3 mM S2251 was added either alone or in presence of 2 nM Pro-UK. In other experiments, HUVEC-coated wells incubated for 1 h with 20 nM HK. After removal of HK, wells incubated with 20 nM PK for another h. After removal of excess PK, cells incubated with 1 μM plasminogen (PLG) for a third h. As indicated, in one case, 0.4 mg/ml of a neutralizing antibody to FXII was added along with the PK. Hydrolysis of substrate measured over 210 min at 37°C. Plasmin formation determined using a standard curve made by adding known amounts of purified plasmin to S2251. Data, means ± SEMs of 4 independent experiments. Absence of standard error bars in some columns indicates that variation was too little to portray visually.

plasminogen activation. Thus, PK activation on endothelial cells results in a mechanism for cellular fibrinolysis. This pathway could result in the initial levels of plasmin which could subsequently amplify pro-urokinase activation.

Thrombin inhibition.

A second biologic activity of kininogens is their ability to function as inhibitors of thrombin activation of platelets. It appears that there are multiple mechanisms by which kininogens function as inhibitors to thrombin activation of platelets (Fig 6). The first mechanism to be described was that kininogens inhibit calpain-induced platelet aggregation (45). When platelets are activated with thrombin, calpain is membrane-expressed and its presence allows for the formation of the heterodimeric complex of $\alpha_{IIb}\beta_3$ integrin (platelet glycoprotein IIb/IIIa) to be formed to support fibrinogen binding and platelet aggregation (41, 45). This mechanism can fully account for kininogen inhibition of thrombin-induced platelet aggregation. However, when thrombin activates platelets, platelets secrete their contents before they aggregate and kininogens inhibit both processes (36). Thus another mechanism(s) of kininogen inhibition of thrombin activation of platelets needed to be sought.

Both HK and LK have been shown to inhibit α-thrombin binding to platelets and endothelial cells (16, 17, 36). The actual location on platelets that thrombin binds to is not completely known. Thrombin has been postulated to bind to at least two sites on the platelet surface. In data to be presented below, it is reasonable to consider that kininogens bind to the first cloned thrombin receptor, protease activated receptor 1 (PAR1) to block α-thrombin from binding to this site on platelets (16). Other data suggest that kininogens may interact with platelet glycoprotein Ib-IX-V complex or compete with thrombin's ability to bind to that complex (2, 28). Preliminary studies suggest that glycoprotein Ib may be a zinc dependent binding site for HK (28). However, our own studies have shown that HK binds normally to Bernard-Soulier platelets, *i.e.* platelets deficient in platelet glycoprotein Ib (unpublished). Alternatively, it has been proposed that kininogen with its domain 3 sequence *LNAENNA* may serve as a competing

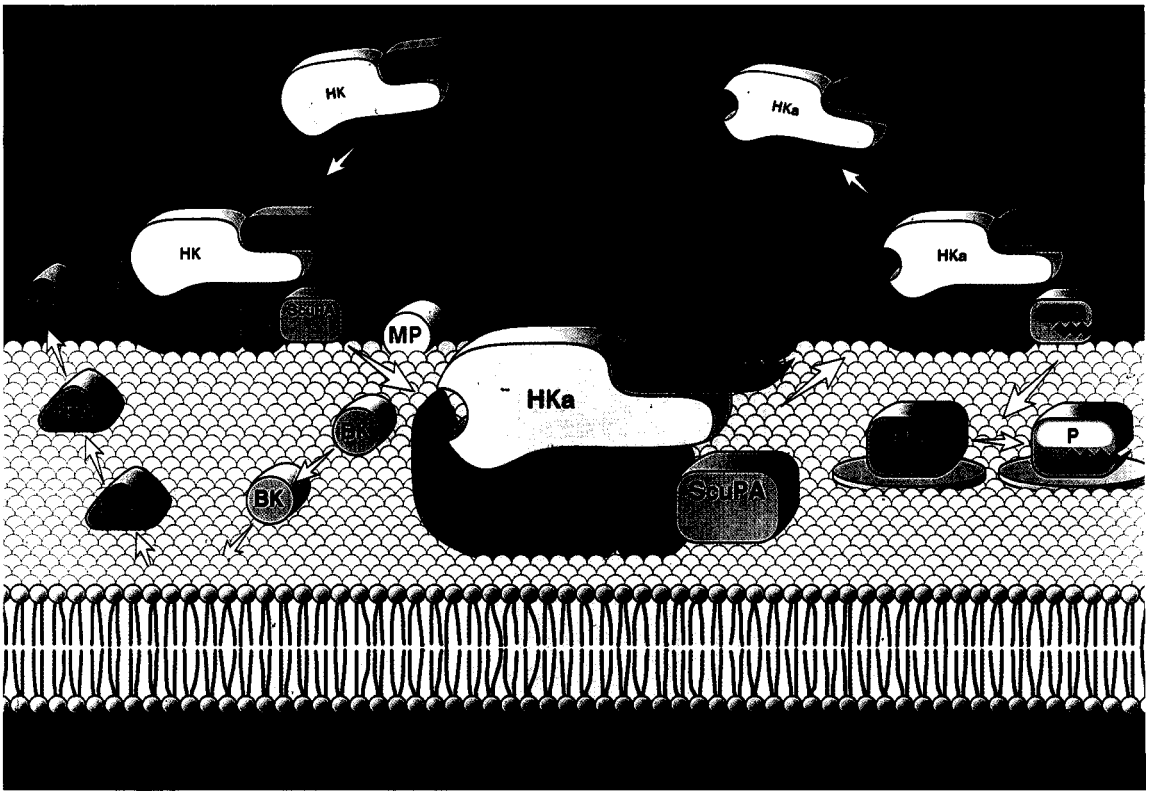

Fig 5. Mechanism of kallikrein-mediated cellular fibrinolysis. PK bound to HK becomes activated by an endothelial cell membrane cysteine protease (MP). Kallikrein (K) bound to HK proteolyzes HK to liberate bradykinin (BK). Bradykinin stimulates the release of endothelial cell tissue-type plasminogen activator (tPA). Kallikrein also activates endothelial cell-bound single chain urokinase (SuPA) bound to the urokinase plasminogen activator receptor (uPAR) to form two chain urokinase plasminogen activator (TcuPA). Two chain urokinase plasminogen activator initiates the conversion of zymogen plasminogen (PLG) to plasmin (P) bound to its endothelial cell receptor.

binding site for α-thrombin binding to glycoprotein-Ib-IX-V complex (2). Glycoprotein Ib contains the sequence *NAEN* at residues 223-226 which is within 24 amino acids of a sequence which is known to inhibit thrombin-induced platelet aggregation (30). Thus, it is possible that kininogen serves as a pseudoglycoprotein Ib, binding available thrombin and keeping it from associating with this platelet glycoprotein. In addition, it is also possible that kininogens could bind to glycoprotein Ib-IX-V at another region and thus interfere with thrombin binding to platelets. Both mechanisms could be operative, but at this time there is insufficient information to say which is actually occurring.

A third mechanism by which kininogens or a discreet peptide from domain 4, *RPPGF*, interfere with thrombin activation of platelets has been described (16). *RPPGF* preserves the epitope of the cleavage site on PAR1 after thrombin activation. It does so by preventing α-thrombin from cleaving PAR1 after $arginine_{41}$, a critical site for thrombin activation of this receptor (16). It is incredibly interesting that the angiotensin converting enzyme breakdown product of bradykinin, *RPPGF*, has additional biologic activity to prevent thrombin's actions. In work which is in-progress, *RPPGF* directly binds to platelets to prevent thrombin's activation of this receptor.

Thus, kininogens' interactions with thrombin appear to be multifaceted. One may argue that kininogens may contribute to the constitutive anticoagulant nature of the intravascular compartment. In kininogen deficient platelet-rich plasma, 2.5 times less γ-thrombin is needed to

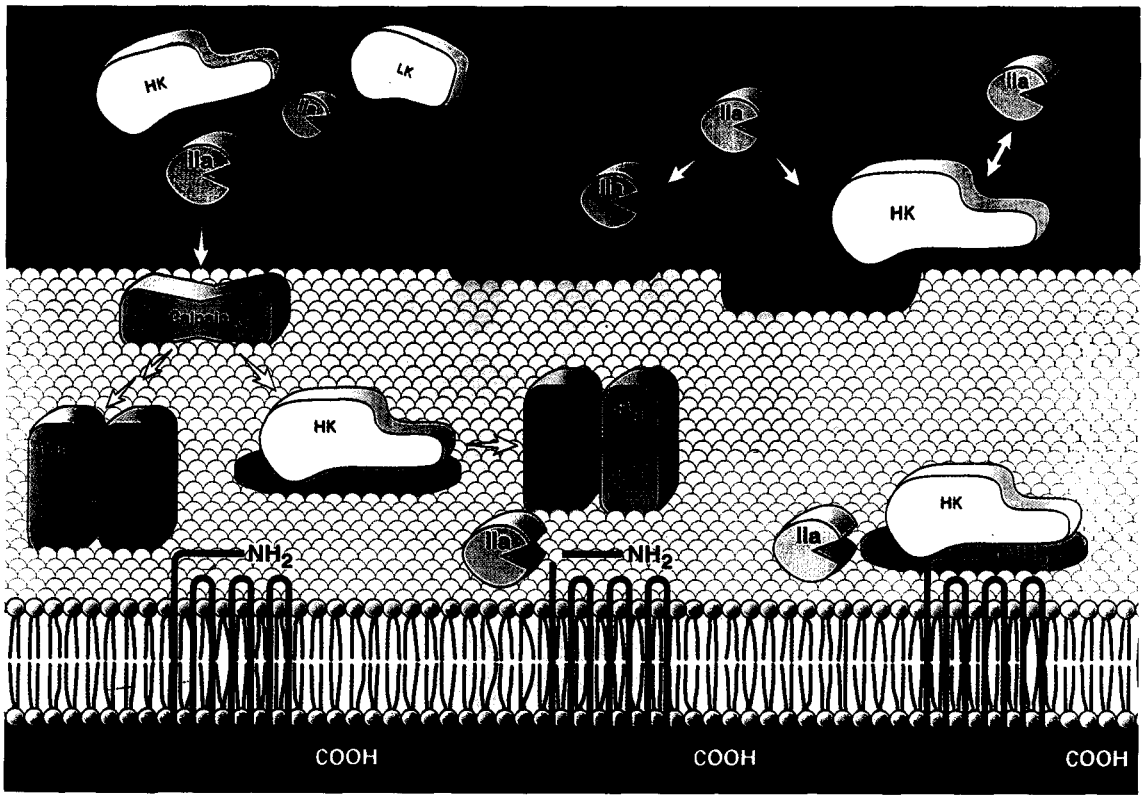

Fig 6. Mechanisms of kininogens' inhibition of thrombin. One mechanism interferes with calpain stimulation of formation of heterodimeric complex between integrin $\alpha_{IIb}\beta_3$. HK or LK blocks calpain which prevents formation of this integrin complex. A second mechanism of kininogen inhibition of thrombin activation of platelets is that HK blocks thrombin (IIa) from binding to thrombin receptor. A third mechanism of inhibition of thrombin-induced platelet activation is that HK and fragments of its domain 4 prevent thrombin (IIa) from cleaving PAR1, the seven transmembrane thrombin receptor, near its amino-terminus.

aggregate platelets than that needed for normal plasma (42). Further, preliminary studies reveal that the time to thrombosis in a minimal injury model of the left iliac artery was much shorter in kininogen deficient rats than in normal rats (6).

SUMMARY

In conclusion, there has been a major evolution in understanding the plasma contact system of proteolysis. Kininogens' assembly on their multiprotein receptor allows for regulated PK activation in a biologic environment. Obviously, regulation of the receptor and kininogen and PK binding will modify the association of these proteins and activation of this system. On endothelial cells, FXII activation is secondary and amplifies PK activation. The immediate consequence of activation of the plasma kallikrein/kinin system is the liberation of bradykinin with its attendant activities. Further, contact proteins probably contribute to cellular fibrinolysis and the constituent anticoagulant nature of the intravascular compartment. It is a paradox that proteins thought to contribute to hemostasis in reality contribute to prevention of thrombosis. In conclusion, I want to convey to you that I believe that we are at the dawn of a new understanding of the physiologic mechanism of activation and importance of the plasma kallikrein/kinin system. Many discoveries on the physiologic role of this system still await our recognition.

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