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PPACK–THROMBIN IS A NONCOMPETITIVE INHIBITOR OF α-THROMBIN BINDING TO HUMAN PLATELETS.

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ABSTRACT: Recent studies from our laboratory indicate that purified kininogens are noncompetitive inhibitors of human α-thrombin but not PPACK-thrombin, binding to human washed platelets. In order to understand the mechanism by which the kininogens inhibit α-thrombin binding, investigations were initiated to determine if α-thrombin and PPACK-thrombin bound to the same site on human platelets. Initial investigations reveal that α-thrombin is a more potent inhibitor of ^{125}I-PPACK-thrombin binding than PPACK-thrombin. Further studies show that PPACK-thrombin is a noncompetitive inhibitor of ^{125}I-α-thrombin binding to platelets. These studies suggest that human α-thrombin binds on the platelet surface to a different site or binds differently to the same site from PPACK-thrombin. These data indicate that the ability of the kininogens to block α-thrombin binding to platelets but not PPACK-thrombin binding results from these thrombins having either two different binding sites or one binding site on the platelet surface which they interact with differently.

Key Words: α-thrombin, PPACK-thrombin, kininogen, platelets, thrombin receptor

INTRODUCTION

Recent studies from our laboratory have shown that purified plasma kininogens, both high and low molecular weight kininogen, are noncompetitive inhibitors of α-thrombin (IIa) binding to human platelets (1). This inhibition is observed with proteolytically active thrombin alone. The binding of radiolabeled D-phenylalanyl-prolyl-arginine chloromethyl ketone-treated α-thrombin (PPACK-IIa) to platelets is not influenced by either of the kininogens (1). These results suggest that the binding of

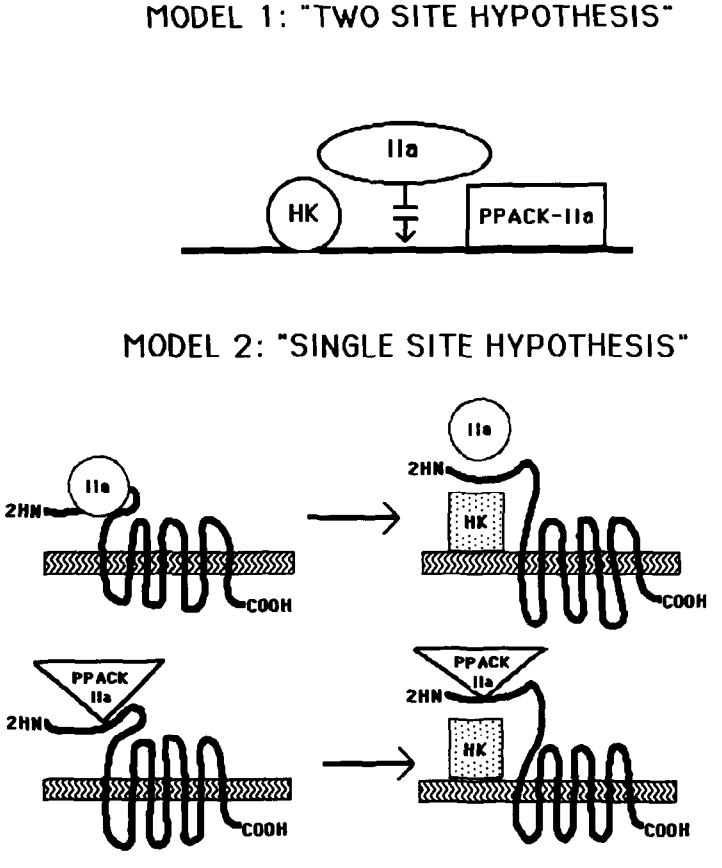

Figure 1: **Mechanisms of kininogen's inhibition of thrombin's binding to platelets:** HK: high molecular weight kininogen; IIa: α-thrombin; PPACK-IIa: D-phenylalanyl-prolyl-arginine chloromethyl ketone-treated α-thrombin. In Model 1, IIa and PPACK-IIa are

hypothesized to bind to different sites. In Model 2, IIa and PPACK-IIa can bind to the same site. However, in the presence of HK, IIa is both unable to bind and subsequently cleave its receptor, but PPACK-IIa is able to bind normally to its site on platelets.

radiolabeled IIa and PPACK-IIa to human platelets is different. Studies by other investigators support this assessment since PPACK-IIa inhibits α-thrombin induced platelet aggregation and secretion, but does not alter α-thrombin-induced shape change (2). In order to investigate these observations further, two hypothetical models were proposed to serve as guides to characterize the possible influence of kininogen binding on radiolabeled IIa and PPACK-IIa binding to human platelets (Figure 1). In the first model, entitled the "Two Site Hypothesis", the kininogens bind to a site on the platelet membrane that is adjacent to the binding site of IIa, but not PPACK-IIa. In the second model, entitled the "Single Site Hypothesis", both IIa and PPACK-IIa bind to the same region on the thrombin receptor, but the presence of the kininogens prevents α-thrombin but not PPACK-IIa from binding (3). For the first model to be correct, PPACK-IIa would have to be a noncompetitive inhibitor of IIa binding to platelets. Alternatively, if PPACK-IIa were a competitive inhibitor of IIa binding, the second model would better characterize the influence of kininogen on IIa but not PPACK-IIa binding to platelets. The studies presented in this report tested the above hypotheses by determining the nature of PPACK-IIa's inhibition of IIa binding to washed, human platelets.

METHODS

Materials: $Na^{125}I$ (50 mCi/mmol) was obtained from ICN, Irvine CA. IODOGEN (chloramide-1,3,4,6-tetrachloro-3α-6α-diphenylglycouril) was obtained from Pierce Chemical Co. Apiezon A oil was purchased from Biddle Instruments, Blue Bell, PA. Extended tip microsediment tubes were purchased from Sarstedt Inc., Princeton, NJ. *N*-Butylphtahate was purchased from Fisher Scientific, King of Prussia, PA. D-phenylalanyl-prolyl-arginine chloromethyl ketone (PPACK) and diisopropyl fluorophosphate (DFP) were obtained from Calbiochem, SanDiego CA. All other reagents were purchased from Sigma Chemical Corp., St. Louis MO.

Plasmas and Platelets: Pooled normal human plasma, lot 313D, was purchased from George King Biomedicals Inc., Overland Park, KS. Fresh platelets were obtained from medication-free donors after obtaining their informed consent, and blood was collected by venipuncture into 1/9 vol 3.8% sodium citrate. Platelet-rich plasma was prepared by centrifugation at 110 x g for 15 min at room temperature. Platelets were washed by gel

filtration on a 60 ml Sepharose 2B-300 column equilibrated with Hepes-Tyrode's buffer [0.137 M NaCl, 3 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 14.7 mM Hepes (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) and 20 mM glucose, pH 7.35] containing 0.2% bovine serum albumin (4,5). Previous studies have shown that Hepes-Tyrode's buffer contains less than 1 μM free Zn^{++}, Ca^{++} or Mg^{++} by atomic absorption spectroscopy (6).

Functional Assays: A thrombin coagulant assay was performed using a fibrometer (Fibro-Systems Model 5, Becton-Dickinson, Towson, MD) according to a modification of the method of Baughman (7). Briefly, fibrinogen (KabiVitrum Fibrinogen Grade L, Helena Laboratories, Beaumount, TX) was dialyzed against 0.05 M NaH_2PO_4, 0.1 M NaCl, pH 7.0 buffer and quantitated by absorbance at 280 nm and by coagulation assay using a standardized fibrinogen solution (Data-Fi Reference human fibrinogen, Baxter Healthcare Corp., Dade Div., Miami, FL). A stock solution of 2.6 mg/ml clottable fibrinogen was prepared, and thrombin was diluted in the same buffer to 4 U/ml. In a polypropylene clotting cup (Sarstedt), 0.1 ml fibrinogen and 0.1 ml of an addition or buffer were equilibrated for 3 min at 37°C, and then 0.1 ml of thrombin was added and the clotting time was recorded. Thrombin's amidolytic activity was determined spectrophotometrically using the chromogenic substrate H-D-Phe-Pip-Arg-pNA (S2238; Helena Laboratories, Beaumont, TX) (8). In this assay, thrombin activity in the absence or presence of additions was measured by determining the hydrolysis of 0.28 mM S-2238 in 0.01 M NaH_2PO_4, 0.15 M NaCl, pH 7.4.

Proteins: Human α-thrombin (3250 U/mg) was a generous gift of Dr. John Fenton, Division of Laboratories and Research, N.Y. State Department of Health, Albany, NY. PPACK-thrombin and DFP-thrombin were prepared by incubating α-thrombin with 5-fold molar excess PPACK dissolved in dimethylsulfoxide (<0.02% final concentration) or with DFP. After incubation for 1 h at room temperature, the PPACK-thrombin and DFP-thrombin were dialyzed for 48 hrs with frequent buffer changes. PPACK-thrombin was unable to hydrolyze S2238 and 200-fold molar excess PPACK-IIa did not neutralize α-thrombin's ability to hydrolyze this substrate. Purified protease nexin I was the generous gift of Dr. William Van Nostrand, University of California at Irvine, Irvine CA.

Iodination of Thrombin: Alpha thrombin was radioiodinated by the Iodogen method (1,6,9) The specific radioactivity ranged from 6.9 to 10.0 μCi/μg protein. Greater than 95% of the radioactivity was precipitated by 10% trichloroacetic acid, and 10 to 15% of the protein molecules were iodinated. The percentage of iodinated thrombin molecules in each batch of the radiolabeled material was determined by the ratio of atoms of iodide incorporated into the protein to the number of molecules of thrombin in the radiolabeled preparation. Radiolabeled

thrombin retained 90% of its coagulant activity when measured against purified fibrinogen, and greater than 90% of the iodinated protein was able to complex with various proteins in plasma, as seen as higher molecular mass structures on non-reduced SDS PAGE. Further, greater than 90% of the radiolabeled thrombin complexed (M_r = 77 kDa) with 10-fold molar excess purified protease nexin I. Active-site inhibited ^{125}I-α-thrombin was prepared by incubation of radiolabeled thrombin for 1 hour at room temperature with a 5-fold molar excess of PPACK dissolved in dimethylsulfoxide (<0.02% final conc.). ^{125}I-PPACK-thrombin did not complex with any proteins when added to plasma.

Binding Experiments: In all binding experiments gel-filtered platelets were placed into polypropylene tubes, diluted with Hepes-Tyrode's buffer supplemented with zinc chloride (50 μM), calcium chloride (2 mM), and radiolabeled thrombin in the absence or presence of unlabeled thrombin to a final concentration of 2 x 10^8 platelets/ml. Preliminary experiments revealed that 50 μM $ZnCl_2$ had no influence on ^{125}I-IIa or ^{125}I-PPACK-IIa binding to platelets. ^{125}I-IIa or ^{125}I-PPACK-IIa were added to platelets at a final concentration of 1 nM. Nonspecific binding was measured in the presence of 200-fold molar excess unlabeled IIa or PPACK-IIa. Incubations were performed at 37°C for specified times with various additions. After incubation, 50 μl aliquots were removed in triplicate for each experimental point and placed in polypropylene microcentrifuge tubes with an extended tip containing 200 μl of an oil mixture which consisted of 1 part Apiezon A oil to 9 parts N-butylphthalate (6), and centrifuged at room temperature for 2 min at 12,000 x g in a microcentrifuge (Model E, Beckman Instruments, Palo Alto, CA). The supernatant was removed, the tips amputated, and the radioactivity present in the cell pellet was determined by using a gamma counter.

Expression and Calculation of Binding Experiment Data: Determination of the amount of bound radioligand was based upon its specific radioactivity and expressed as nanograms bound/10^8 platelets per ml or converted to pmoles bound. ^{125}I-thrombin concentration-dependent binding experiments in the absence or presence of PPACK-IIa were analyzed according to the method of Eadie-Scatchard (10) using a weighted linear regression analysis (11). Briefly, the weighted linear regression was determined using the formula, $n-2/(SEM)^2$ (12), where n = the number of determinations of added radioligand at one concentration and the SEM is determined from all the experiments of the specifically bound radioligand at that concentration. In these experiments increasing concentrations of ^{125}I-α-thrombin (0.17 - 1.37 nM) were incubated with platelets at 37°C for 20 min in the absence or presence of 114 or 172 nM PPACK-IIa. Nonspecific binding was determined by the presence of 200 nM unlabeled α-thrombin. The

concentration of specifically bound thrombin was calculated in the absence and presence of the competitors by subtracting nonspecific binding from total binding. All data were plotted on a graph of bound/free versus bound using the weighted linear regression analysis described above.

RESULTS

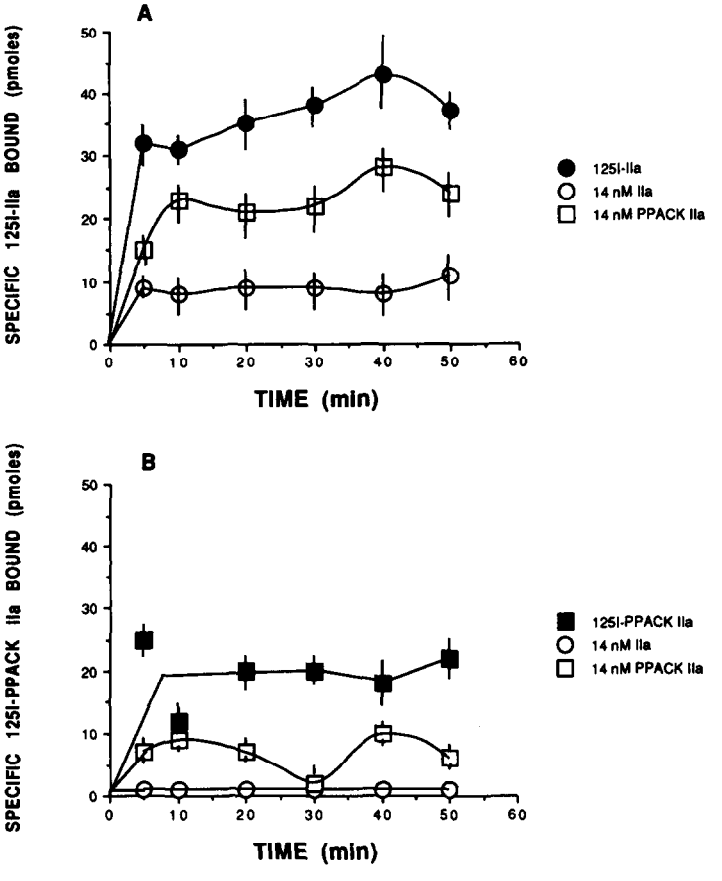

Figure 2: **Inhibition of Radiolabeled IIa and PPACK-IIa Binding to Platelets.** In Panel A, ^{125}I-IIa (1 nM) was bound to platelets in the absence (●) or presence of 14 nM IIa (O) or PPACK-IIa (□). The data are plotted as the mean ± SEM of specific ^{125}I-IIa bound after the binding obtained with 200 fold molar excess IIa was subtracted from all samples. In Panel B, ^{125}I-PPACK-IIa (1 nM) was bound to platelets in

the absence (■) or presence of 14 nM IIa (O) or 14 nM PPACK-IIa (□). The data are plotted as the mean ± SEM of specific ^{125}I-PPACK IIa bound after the binding obtained with 200-fold molar excess PPACK-IIa was subtracted. These data are the mean ± SEM of three experiments.

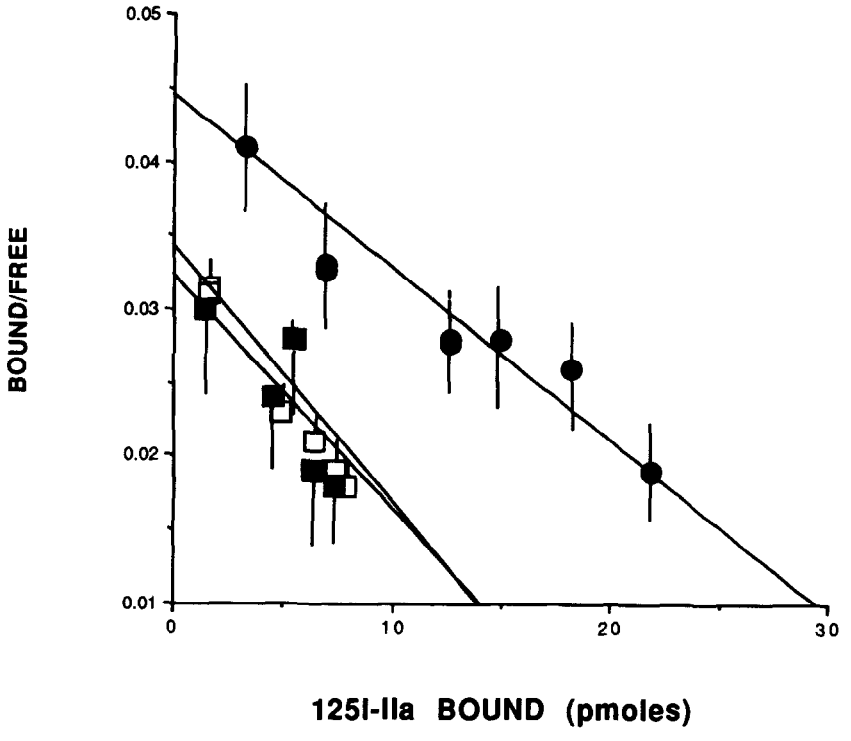

Figure 3: **Eadie/Scatchard plot of PPACK-IIa inhibition of ^{125}I-IIa binding to platelets.** ^{125}I-IIa binding was performed in the absence (●) or presence of 114 nM (▯) and 172 nM (▮) PPACK-IIa. The data presented are a weighted linear regression analysis of the means ± SEM of four independent binding experiments.

Initial investigations sought to determine if there were differences in the nature of radiolabeled α-thrombin and PPACK-treated thrombin binding to platelets. When radiolabeled IIa or PPACK-IIa binding to platelets was competed with lessor quantities of unlabeled ligand, differences in the ability of each ligand to inhibit the tracer were noted

(Figure 2). Fourteen nM IIa was a good inhibitor of ^{125}I-IIa binding but 14 nM PPACK-IIa was a much weaker competitor (Figure 2A). Moreover, 14 nM IIa was a better inhibitor of ^{125}I-PPACK-IIa binding than 14 nM PPACK-IIa (Figure 2B). Alpha thrombin was an equally strong inhibitor of ^{125}I-PPACK IIa binding at concentrations greater than 2 fold molar excess to the radioligand. The ability of IIa to be a more potent inhibitor of ^{125}I-PPACK-IIa binding than PPACK-IIa was not due to proteolysis of the radioligand by the enzyme. Equal molar concentrations of DFP-treated IIa was as good an inhibitor of ^{125}I-PPACK-IIa binding as α-thrombin.

Additional studies were performed to determine the characteristics of ^{125}I-IIa binding to platelets. As previously reported (1,2,13) ^{125}I-IIa bound to platelets with an affinity of about 854 pM and 175 sites per platelet (Figure 3). Similarly ^{125}I-PPACK-IIa bound to platelets with an affinity of 459 pM and 132 sites per platelet (data not shown). However when ^{125}I-IIa was bound to platelets in the presence of 114 and 172 nM PPACK-IIa, the affinity of the radioligand to bind to its receptor remained the same (571 pM, 632 pM, respectively) but the number of binding sites decreased by about half to 84 sites per platelet (Figure 3). These data suggested that PPACK-IIa was a noncompetitive inhibitor of ^{125}I-IIa binding to platelets.

DISCUSSION

Since the high affinity binding of ^{125}I-IIa and ^{125}I-PPACK-IIa to the platelet surface are similar (854 and 459 pM, respectively) and both radioligands appear to have about the same number of platelet binding sites (132 to 175), it was assumed that both radioligands are binding to the same site on the platelet membrane. However our present investigation indicates that IIa and PPACK-IIa are not equal inhibitors of the other radioligand's binding (Figure 2). IIa is a more potent inhibitor of ^{125}I-PPACK binding than PPACK-IIa itself. Assuming that a uniform class of binding sites was investigated by the addition of 1 nM radioligand, the ^{125}I-IIa concentration-dependent binding experiments in the absence or presence of molar excess PPACK-IIa show that the inhibited thrombin is a noncompetitive inhibitor of radiolabeled, proteolytically active thrombin binding to platelets (Figure 3). We were unable to perform the reciprocal binding experiments to determine whether IIa is a noncompetitive inhibitor of ^{125}I-PPACK binding to platelets because IIa produced 100% inhibition of binding of the radioligand at 2 fold molar concentrations. This fact did not allow for the performance of adequate partial inhibition curves of ^{125}I-PPACK-IIa binding by IIa.

The finding that PPACK-IIa is a noncompetitive inhibitor of radiolabeled IIa binding to platelets appears to be at odds with the data

published by Tollefson et al. (15). In that study DFP-thrombin is shown to be a competitive inhibitor of ^{125}I-IIa binding to platelets. These data, however, are consistent with the present experiments which showed that DFP-thrombin is as good an inhibitor of ^{125}I-PPACK-IIa binding as IIa. The reason why PPACK treatment, but not DFP treatment, alters thrombin's platelet binding abilities is not completely known. The finding that the kininogens block proteolytically active thrombin but not PPACK-treated thrombin binding supports the notion that IIa and PPACK-IIa bind to different sites on the platelet surface (1). The possibility that IIa and PPACK-IIa bind to different platelet sites provides an explanation of the findings of Greco et al. which show that PPACK-IIa inhibits α-thrombin-induced platelet aggregation and secretion but not α-thrombin-induced shape change (2). Shape change is induced by either of these ligands binding to its site but the presence of platelet-bound PPACK-IIa prevents platelet-bound IIa from cleaving its receptor to induce platelet activation (3).

The results of our studies suggest that the ability of the kininogens to inhibit IIa binding, but not PPACK-IIa binding, may be due to each thrombin's binding to "different" sites on the platelets' surface (Figure 1). This interpretation is not necessarily at odds with the characterization of the thrombin receptor by Vu et al (3). It is possible that the two forms of thrombin could be binding to either two different sites on the same receptor or different portions on the same site on the receptor. This latter interpretation suggests that α-thrombin's and PPACK-thrombin's interaction with platelets is an amalgam between the two models in Figure 1. Since the hirudin-like domain on the receptor's extracellular, amino terminal extension is the α-thrombin binding determinant (14), it is difficult to postulate an adjacent, different structure for the binding site for PPACK-IIa (14). How PPACK can alter thrombin to induce it to bind differently to the same site or to another site is not completely known. Since PPACK is such a selective, high affinity, active site inhibitor of α-thrombin, its reaction with thrombin's active site may alter the conformation of the protein such that PPACK-thrombin interacts with its receptor's binding site differently from native thrombin (15,16). This possible difference in the interaction of PPACK-thrombin versus α-thrombin with the thrombin receptor binding site could account for the differing abilities of each form of thrombin to inhibit the other's binding and for the finding that PPACK-thrombin noncompetitively inhibits α-thrombin binding.

ACKNOWLEDGEMENTS

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