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Research Article

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The Perturbation of Thrombin Binding to Human Platelets by Anions

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ABSTRACT Thrombin binds with high affinity to specific cell-surface receptors on washed human platelets. We present experiments indicating that thrombin binding correlates with the release reaction when binding is perturbed by anions. Marked differences in the affinity of human ^{125}I-thrombin for platelets were observed in various isotonic buffers at pH 7.4. At low concentrations of thrombin (0.001–0.01 U/ml), binding was 5-fold greater in Tris-sodium acetate and 12-fold greater in Tris-sodium cacodylate than in Tris-sodium chloride. These anion-induced changes in ^{125}I-thrombin binding paralleled changes in [^{14}C]serotonin release when both parameters were measured in the same platelets. Thus, equivalent release occurred for equal amounts of thrombin bound in all buffers, even though the thrombin concentration varied by up to 30-fold. After approximately 100 molecules of thrombin bound per platelet, complete release occurred in all buffers in 2 min. The effect of anions was specific for the thrombin-receptor interaction as there was no corresponding effect on the binding of erythroagglutinating phytohemagglutinin (E-PHA) to platelets nor on E-PHA or collagen-induced serotonin release. The various anions did not alter platelet morphology as judged by electron microscopy. The anions had no effect on thrombin esterase catalytic activity. In addition, the total number of thrombin receptors per platelet was approximately the same in all buffers. Thus anions alter the affinity between platelet thrombin receptors and a site on thrombin distinct from the catalytic site. We conclude that the thrombin receptor is essential for thrombin-induced platelet reactions.

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INTRODUCTION

We have previously reported that washed human platelets bind both human and bovine thrombin with high affinity[1] (1). Two types of binding were observed with thrombin of both species; high affinity binding ($K_{diss} \cong$ 0.02 U/ml) of a small amount of thrombin ($\cong$ 500 molecules/platelet) and a larger amount of thrombin binding of lower affinity ($K_{diss} \cong 2$ U/ml) with 30,000–50,000 thrombin molecules binding per platelet at saturation. The amount of thrombin required to induce 50% [^{14}C]serotonin release in this system is about 0.03–0.1 U/ml while the amount of thrombin required for 50% inhibition of adenylate cyclase activity or release of the thrombin-sensitive protein (2, 3) or for maximal stimulation of protein phosphorylation (4) is about 0.2–0.4 U/ml. Thus, full saturation of thrombin receptor sites occurs at concentrations 10-fold higher than required to elicit the physiological response of platelets. The explanation for this discrepancy is unknown, but a similar situation exists with respect to receptors for glucagon and insulin. It remains to be established that the binding which we have observed actually reflects a part of the physiologic response of platelets to thrombin.

In the present study we have attempted to correlate changes in thrombin binding with changes in the release of [^{14}C]serotonin. We have found striking alterations in thrombin binding when binding experiments were carried out in buffers containing different anions. The changes in binding correlate closely with changes in the sensitivity of platelets to thrombin-induced serotonin release. The anion-induced changes are specifically related to thrombin's interaction with the thrombin receptor of platelets since anions have no effect on serotonin release

[1] Shuman, M. A., D. M. Tollefsen, and P. W. Majerus. 1975. The binding of human and bovine thrombin to human platelets. *Blood.* In press.

induced by either erythroagglutinating phytohemagglutinin (E-PHA)[2] of kidney beans or collagen.

METHODS

Radioisotopes were purchased from the following sources: carrier-free [^{125}I]sodium iodide, Mallinckrodt Chemical Works, St. Louis, Mo.; [^{14}C]serotonin binoxalate (22.1 μCi/μmol), New England Nuclear, Boston, Mass. Bovine serum albumin (crystallized and lyophilized), bovine Achilles tendon collagen (type I) and Australian Taipan snake venom (*Oxyuranus scutellatus scutellatus*) were obtained from Sigma Chemical Co., St. Louis, Mo.

Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) was purchased from Mann Research Labs., Inc., New York. Polyethylene glycol (Carbowax 6000) was obtained from Union Carbide Corp., New York. Membrane filters (Metricel GA-3, 1.2 μm pore size) were obtained from Gelman Instrument Co., Ann Arbor, Mich. Bovine and human fibrinogens (grade L) were purchased from A. B. Kabi, Stockholm, Sweden. E-PHA was purified from Bacto phytohemagglutinin-P (Difco Laboratories, Detroit, Mich.) and labeled with [^{125}I]sodium iodide as previously described (5).

Preparation of platelets. Platelets were prepared from freshly drawn blood as described (1) and used within 6 h.

Purification and iodination of thrombin. Human thrombin was purified from Cohn fraction III paste (American Red Cross, National Fractionation Center, Bethesda, Md.) by the method of Fasco and Fenton (6), except that Australian Taipan snake venom was used to activate prothrombin (7). The thrombin was homogeneous based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Clotting activity was 2,300–2,700 U/mg of protein and was determined by the method of Fenton and Fasco (8).

The thrombin concentration was estimated by measuring ultraviolet absorbance at 280 nm assuming $E_{280}^{1\%} = 16.2$ (9). The molecular weight of thrombin was assumed to be 36,000 (10).

Thrombin was iodinated with [^{125}I]sodium iodide using a modification (1) of the chloramine-T method as described by Hunter (11). Iodinated thrombin was stored at −20°C and used within 4 days except when the specific activity was high (5×10^5–1.5×10^6 cpm/U) in which case it was used on the same day that it was iodinated.

Binding of thrombin to platelets. Platelets (0.5×10^8) were incubated with ^{125}I-thrombin ($2 \times 10^4 - 1.5 \times 10^6$ cpm/U) in a total volume of 0.5 ml of isotonic buffer solution (pH 7.4). All buffers contained 0.015 M Tris-HCl, 0.0055 M glucose, and 5 mg/ml bovine serum albumin, plus one of the following salts mixtures: (*a*) 0.14 M sodium acetate, (*b*) 0.105 M dibasic sodium arsenate and 0.018 M monobasic sodium arsenate, (*c*) 0.14 M sodium cacodylate (dimethyl arsenate), (*d*) 0.14 M sodium chloride, or (*e*) 0.0975 M dibasic sodium phosphate and 0.0325 M monobasic sodium phosphate. After incubation for 20 min at room temperature, an additional 5 ml of incubation buffer at 0°C was added, and the platelets were collected and counted by the membrane filtration technique previously described (1). Time-course studies indicated that binding was complete within 20 min in all buffer systems.

[2] *Abbreviations used in this paper:* E-PHA, erythroagglutinating phytohemagglutinin from *Phaseolus vulgaris;* DIP-thrombin, diisopropyl phosphoryl-thrombin; BAEE, benzoyl-L-arginine ethyl ester hydrochloride.

Thrombin esterase activity. Reaction mixtures contained 10 U of thrombin, 0.25 μmol of BAEE, and one of the isotonic Tris-buffered salts (pH 7.4) described above in a total volume of 1 ml. The rate of ester hydrolysis was determined by measuring the increase in absorbance at 253 nm (12) assuming that 0.1 mM benzoyl arginine reads 0.21 absorbance units.

Collagen-mediated [^{14}C]serotonin release. Platelets (5×10^7) were incubated for 20 min at 37°C in 0.5 ml of isotonic buffer containing 0.175 mg of fibrinogen and 0.4–1.34 mg/ml of acid-soluble bovine tendon collagen prepared by the method of Cazenave et al. (13). After incubation for 20 min, serotonin release was measured as described (1).

Fibrin monomer polymerization. The rate of fibrin monomer polymerization was determined by a modification of the method described by Mommaerts (14). Reaction mixtures contained 3.3 mg of polyethylene glycol, 0.7 mg of fibrinogen, and 0.5 U of thrombin in 0.5 ml of isotonic Tris-saline (pH 7.4). After clot formation, samples were centrifuged for 10 min at 3,500 *g*, and the supernate was removed. Clots were dissolved by incubating for 10 min in 0.5 ml of 3 M urea at 37°C. The clotting time was then measured after addition of 4.5 ml of isotonic buffer of the various anion types indicated above containing 6.6 mg/ml of polyethylene glycol.

Other methods. Serotonin release from platelets (1, 15), E-PHA binding to platelets (5), fibrinopeptide release from fibrinogen (16), and electron microscopy (15) were performed as described previously.

RESULTS

Effect of buffer anions on binding of thrombin to platelets. We previously measured the binding of bovine and human thrombin to platelets in isotonic Tris-buffered saline[1] (1). When buffers containing anions other than chloride were used for binding experiments, marked changes in thrombin binding were observed. At low concentrations of thrombin (0.001–0.02 U/ml) the amount of thrombin bound varied with the particular buffer used. From the initial linear portion of the binding curves illustrated in Fig. 1, there was 5-fold

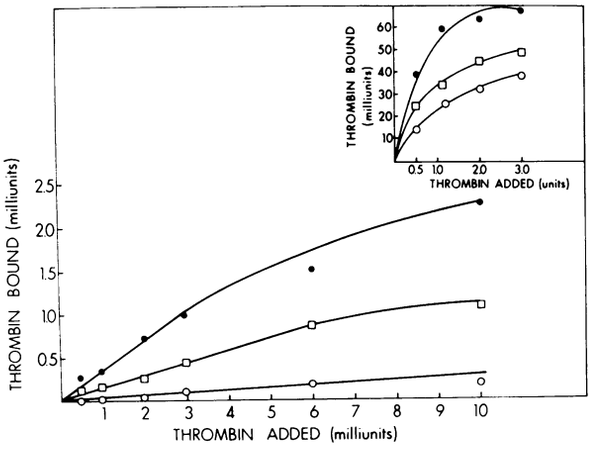

FIGURE 1 Effect of anions on binding of ^{125}I-thrombin to platelets. (○), Tris-buffered saline; (●), Tris-buffered sodium cacodylate; (□), Tris-buffered sodium acetate.

TABLE I

Effect of Anions on Thrombin-Induced Release of [^{14}C]Serotonin from Platelets

Buffer	*Thrombin concentration required for half-maximal release of [^{14}C]serotonin
	U/ml
Tris-buffered $\frac{Na_2HPO_4}{NaH_2PO_4}$	0.45
Tris-buffered sodium arsenate	0.3
Tris-buffered sodium chloride	0.056
Tris-buffered sodium acetate	0.012
Tris-buffered sodium cacodylate	0.006

10-min incubations were conducted as described in Methods, except that platelets were loaded with [^{14}C]serotonin.

* Release sensitivity was measured as the concentration of thrombin required for half-maximal serotonin release as determined by plotting that data obtained in each buffer as shown in Fig. 2.

greater thrombin binding in Tris-buffered sodium acetate and 12-fold greater thrombin binding in Tris-buffered sodium cacodylate as compared to Tris-buffered sodium chloride. At higher concentrations of thrombin (inset Fig. 1) the differences were less marked, and at saturation approximately the same number of binding sites were observed in all buffers (data not shown). The exact magnitude of altered binding varied somewhat with platelets from different donors, but the effects illustrated here were consistently observed. In addition, identical effects were noted on the binding of diisopropyl phosphoryl-thrombin (DIP-thrombin).

Effect of anions on thrombin-induced serotonin release. We next measured whether the difference in thrombin binding was reflected in alterations in thrombin-induced [^{14}C]serotonin release. As seen in Table I, a striking variation in response to thrombin was noted depending on the anion present. In this experiment there was a 70-fold difference between the amount of thrombin required for half-maximal [^{14}C]serotonin release in phosphate buffer as compared to cacodylate buffer. While release sensitivity (as defined in Table I) varied with anions, the maximal serotonin release obtainable was the same in all anions with any experiment. The full thrombin-response curve from a different experiment is shown for chloride and cacodylate in Fig. 2 and illustrates that the total amount of release obtainable was the same in both buffers but the sensitivity varied by 33-fold in this experiment. The variation with different platelets mentioned above is also illustrated since the difference between chloride and cacodylate in

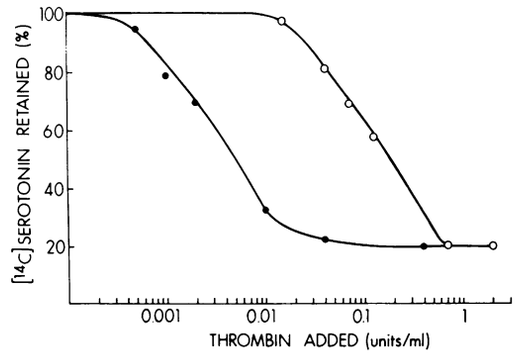

FIGURE 2 Effect of anions on thrombin-induced [^{14}C]serotonin release. (○), Tris-buffered saline; (●), Tris-buffered sodium cacodylate.

this experiment was greater than seen in the experiment in Table I (9.3-fold). We have no clues as to the nature of this individual variation between platelets from different donors. When isotonic buffers were prepared by mixing various proportions of Tris-sodium cacodylate and Tris-sodium chloride, an increasing sensitivity to thrombin-induced serotonin release was seen as the proportion of sodium cacodylate increased (Fig. 3). This requirement for relatively high concentrations of cacodylate (> 0.1 M) for maximal effect suggests that the mechanism is related to the anion itself rather than to some metabolic effect which might be seen with lower concentrations of cacodylate. Since the greatest sensitivity to thrombin was seen using cacodylate buffer and since this compound is an arsenic salt, we considered the possibility that the effects observed were due to toxicity of sodium cacodylate buffer to platelets. As measured by serotonin release, platelet response to thrombin was less in sodium arsenate than in cacodylate or even chloride (Table I). The response in arsenate

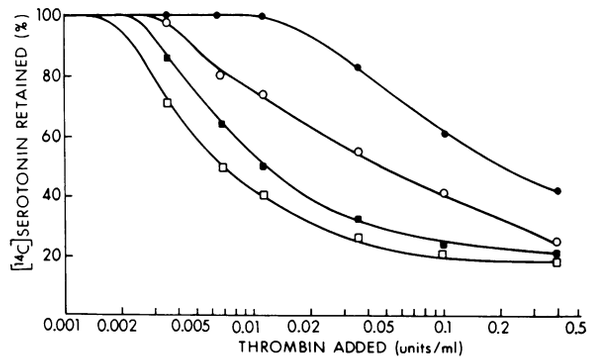

FIGURE 3 Effect of concentration of sodium cacodylate on thrombin-induced [^{14}C]serotonin release. (●), Tris-buffered saline; (○), Tris-buffered saline—Tris-buffered sodium cacodylate, 80:20; (■), equal mixture of Tris-buffered saline and Tris-buffered sodium cacodylate; (□), Tris-buffered sodium cacodylate.

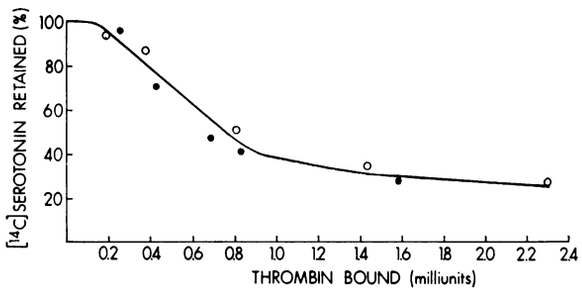

FIGURE 4 Relationship of binding of ^{125}I-thrombin to platelets to [^{14}C]serotonin release. Incubations were performed as described in Methods except that platelets were loaded with [^{14}C]serotonin before incubation with thrombin and that the reactions were for 10 min. ^{125}I-thrombin bound and [^{14}C]serotonin retained were determined by counting each sample in an autogamma scintillation counter for ^{125}I and in a liquid scintillation counter for ^{14}C. The [^{14}C]serotonin radioactivity was calculated by correcting gross counts per minute observed in the liquid scintillation counter for ^{125}I which counted in the liquid scintillation counter as determined using ^{125}I-thrombin standards. (●), Tris-buffered saline; (○), Tris-buffered sodium cacodylate.

was similar to that seen in phosphate, a chemically related anion. Therefore, if toxicity were expressed by cell lysis and release, arsenate ion is nontoxic. We conclude that the effects are most likely related to the properties of the anions and not due to toxicity of arsenate. Furthermore, the effect of anions was independent of the time of exposure of anions to platelets. Thus, when platelets were incubated in sodium cacodylate buffer for 15 s to 30 min before addition of thrombin for an additional 10 s, the time of incubation in cacodylate before addition of thrombin did not affect serotonin release. If platelets were adversely effected by cacodylate, we would have expected to see an exaggeration of the effect with time. Electron micrographs of platelets incubated in cacodylate buffer were very similar to platelets in isotonic phosphate buffer. Platelet granules were intact and only a few platelets showed pseudopod formation.

Correlation of thrombin binding with [^{14}C]serotonin release. We correlated the effects of cacodylate and chloride on thrombin binding to platelets with the effects of these anions on [^{14}C]serontonin release in a double label experiment where ^{125}I-thrombin bound and [^{14}C]-serotonin retained were measured in the same platelets. Although the concentrations of thrombin necessary to induce an equivalent amount of serotonin release were much higher in sodium chloride than in sodium cacodylate (10-fold in this experiment) for a given amount of thrombin bound, the same degree of release was obtained in both anions (Fig. 4). A similar correlation was seen in experiments using other anions (data not shown) in that the quantity of thrombin necessary to induce maximal release was relatively constant in each experiment. Approximately 1 mU of thrombin bound per 5×10^7 platelets (which is about 100 molecules of thrombin bound per platelet) resulted in maximal serotonin release. The absolute amount of thrombin bound per platelet correlates strikingly with serotonin release. This observation suggests that thrombin binding is physiologically important as the first step in the thrombin-induced platelet release reaction.

Effect of sodium cacodylate on E-PHA- and collagen-induced [^{14}C]serotonin release. We next examined whether the effect noted with cacodylate on thrombin-induced serotonin release was specific for the interaction of thrombin with its receptor on platelets or whether the effect applied to other release-inducing agents. E-PHA binds to platelets and induces serotonin release

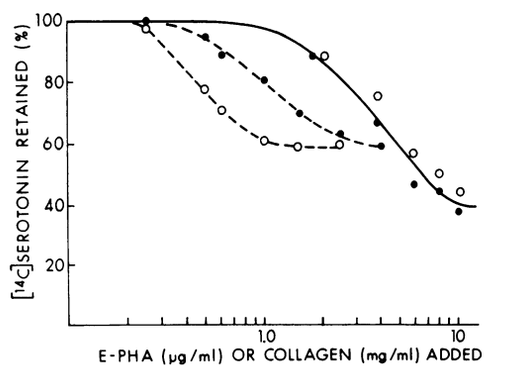

FIGURE 5 Effect of anions on E-PHA- and collagen-induced [^{14}C]serotonin release. Incubations of platelets with E-PHA and with collagen for 20 min. (○), Tris-buffered saline; (●), Tris-buffered sodium cacodylate; (——), E-PHA; (- - - -), collagen.

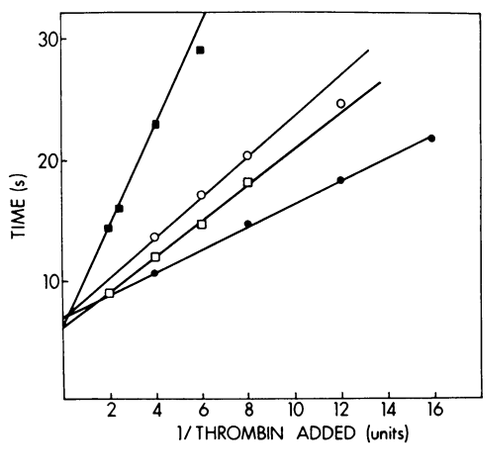

FIGURE 6 Effect of anions on thrombin clotting of fibrinogen. (●), Tris-buffered sodium cacodylate; (○), equal mixture of Tris-buffered saline and Tris-buffered sodium cacodylate; (■), Tris-buffered saline; (□), Tris-buffered sodium acetate.

as described previously (5, 15). We observed no difference in binding of E-PHA to platelets (data not shown) or in E-PHA-induced release of serotonin when these reactions were carried out in Tris-buffered sodium chloride as compared to Tris-buffered sodium cacodylate (Fig. 5). Similarly, anions had little effect on collagen-induced serotonin release. In the experiment shown in Fig. 5, serotonin release was slightly less in Tris-buffered sodium cacodylate than in Tris-buffered sodium chloride. In other experiments, there was no effect of anions on collagen-induced release of serotonin. We conclude that the enhancement of binding of thrombin to platelets and the subsequent increase in serotonin release is a direct result of an alteration in the thrombin-thrombin receptor interaction and that there is not a corresponding alteration in the interaction of other release-inducing agents with platelets. These experiments also provide further evidence that cacodylate does not have any toxic effect on platelets.

Effect of anions on thrombin. The above experiments do not distinguish whether the effect of anions is on thrombin itself or on its receptor on the platelet membrane. Thus we studied the effects of anions on other thrombin-catalyzed reactions. The rate of hydrolysis of BAEE by thrombin in chloride and acetate buffers was 4.3 nmol BAEE/U thrombin per min and in cacodylate was 5.2 nmol BAEE/U thrombin per min. Thus, anions do not have a major effect on thrombin esterase activity. However, significant differences were seen in the clotting of fibrinogen when carried out in these same buffers (Fig. 6). Thus, clotting was enhanced approximately 3.5-fold in cacodylate and 2.5-fold in acetate. Since these effects are qualitatively similar to the effects observed with platelets, we explored the effect of anions on fibrinogen clotting further to see whether the effect was due to enhancement of the rate of fibrinopeptide cleavage by thrombin or, alternatively, to an increased rate of fibrin monomer polymerization. The rate of fibrinopeptide release when thrombin is incubated with fibrinogen in chloride, acetate, and cacodylate is shown in Fig. 7. There were no differences in the rate of release of fibrinopeptides, and this result plus the lack of significant effects of anions on esterase activity indicated that anions did not effect the catalytic interaction between thrombin and fibrinogen. The explanation for the variability of clotting seen with different anions appears to be related to their effect on the rate of fibrin monomer polymerization. We measured the rate of polymerization of fibrin monomers after dissolution of newly formed fibrin clots in 3 M urea. The polymerization was threefold faster in acetate and about fourfold in cacodylate as compared to chloride (Table II). This difference probably accounts for the differences observed in the clotting of fibrinogen, illustrated in Fig. 6. Since the anions tested do not have a general effect on thrombin catalytic activity (BAEE hydrolysis) or an interaction of thrombin with a macromolecular substrate, fibrinogen, we conclude that the anions have a relatively specific effect on the binding of thrombin to platelets.

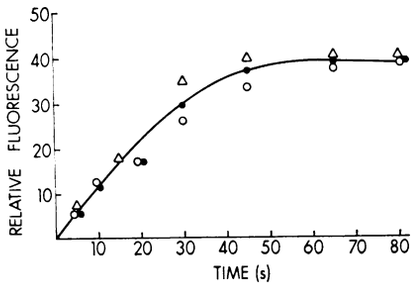

FIGURE 7 Effect of anions on fibrinopeptide release. The reaction mixture contained 300 μg/ml of human fibrinogen in 0.5 ml of the indicated isotonic buffered salt which contained 0.01 M imidiazole-HCl (pH 7.0). Thrombin in a final concentration of 1.0 U/ml was added, and the reaction was terminated after the specified interval by the addition of trichloroacetic acid. Soluble fibrinopeptides released were measured with fluorescamine. (○), Sodium acetate; (●), sodium cacodylate; (△), sodium chloride.

DISCUSSION

Previous studies have shown the specific binding of thrombin to platelets, but the concentration of thrombin for half-maximal saturation of platelets was one to two orders of magnitude greater than the concentration of thrombin required to elicit the physiologic reactions of thrombin on platelets. The question arises, therefore, as to the significance of the binding of thrombin to platelets that we have measured. This same controversy has been raised in other systems where there are apparent inconsistencies between the binding of a hormone or protein to a cell and its physiologic effect. Birnbaumer and Pohl reported stimulation of liver membrane adenylate cyclase by glucagon to be complete when only 10–20% of the glucagon binding sites were occupied (17).

TABLE II
Effect of Anions on Fibrin Monomer Polymerization

Anion	Clotting time	
	s	
Sodium chloride	18.0	18.0
Sodium acetate	6.0	6.7
Sodium cacodylate	4.9	4.5

Fibrinogen was clotted with thrombin in sodium chloride and the supernate removed as described under Methods. The clot was dissolved in 0.5 ml of 3 M urea and the clotting time measured after the addition of 4.5 ml of the indicated isotonic Tris-buffered sodium salt (pH 7.5) containing 6 mg/ml o polyethylene glycol.

This would suggest that 80–90% of the binding sites were unrelated to the biological activity. Generally accepted criteria for specific binding appear to be met by the binding of ^{125}I-insulin to glass tubes, talc, and silica (18). Cuatrecasas and Hollenberg also report similar "specific" binding of ^{125}I-glucagon to Millipore filters (Millipore Corp., Bedford, Mass.) (18). Thus, the physiologic significance of binding studies must be interpreted with caution. The data presented here support the concept that the binding of thrombin to the thrombin receptor on platelets is of physiologic importance since alterations in binding of 10- to 30-fold gave exactly parallel changes in the release of serotonin from platelets. Other experiments have shown that the binding of thrombin to platelets is highly specific since none of the intermediates of prothrombin activation will bind to platelets (19). This includes intermediate-2 which has the same amino acid sequence as thrombin and differs only by a single peptide bond which when cleaved generates two-chain disulfide-linked thrombin.

The sensitivity of platelets to thrombin in cacodylate buffer is striking with half-maximal serotonin release occurring from 0.003 to 0.006 U of thrombin/ml. 1 ng of thrombin can be measured with this assay. This bioassay provides an advantage over present relatively insensitive techniques (fibrinogen clotting, thrombin esterase activity) for measurement of low concentrations of thrombin.

The mechanism by which anions effect thrombin binding to platelets is unclear. The total number of thrombin binding sites is unchanged in each buffer, thrombin catalytic activity is not affected, and the total magnitude of the release reaction is unaltered. It appears that anions affect only binding, the first step in thrombin action on platelets, and that the subsequent effects of thrombin proceed unaltered by anion composition of the buffer. By analogy to studies of anions on the binding of substrates to enzymes (20), it may be that anions compete with various degrees of effectiveness for a positively charged site required for thrombin binding. Although the results presented here do not establish whether the anion effects are on the thrombin molecule or on the platelet membrane, it is clear that anions modify only the interaction of thrombin and the thrombin receptor and have no significant effect on other thrombin-catalyzed reactions.

ACKNOWLEDGMENTS

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