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PLATELET INTERACTION WITH ACTIVE AND TLCK-INACTIVATED α-THROMBIN[1]

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SUMMARY: Interaction of α-thrombin with platelets causes a dose-dependent depolarization of the trans-membrane potential which can be measured by monitoring the fluorescence of a cyanine dye. Inactivation with tosyllysyl-chloromethylketone abolishes the ability of thrombin to either initiate platelet aggregation or to cause the change in potential observed with unmodified thrombin. Preincubation of washed platelets with inactivated thrombin lowers the subsequent membrane potential change in response to 0.005 to 0.05 U/ml of active thrombin. The extent of that competition is a function of quantities of modified and native thrombin used. The data presented here are fully compatible with the concept that two distinct thrombin binding modes, with different affinities, exist on the platelet surface.

INTRODUCTION: Thrombin is a physiologically important activator of platelet release and aggregation during hemostasis, but the mechanism by which thrombin stimulates the platelet response *in vivo* or *in vitro* is not completely understood. One aspect of the mechanism which still awaits complete characterization is the nature of the site of thrombin action at the platelet surface. It has been suggested that thrombin binds to specific receptors on the platelet surface, and a number of thrombin-sensitive platelet proteins have been reported (1-22). There appear to be two distinct binding modes, with different affinities for thrombin: high-affinity (K_d ~2 nM; ~750 sites/platelet) and low-affinity (K_d ~30 nM; 45,000-95,000 sites/platelet). It has been suggested that either the thrombin binding species consist of a single molecular entity which exhibits negative cooperativity (19) or, alternatively, that there are two different molecular species (21).

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Several groups have reported that thrombin which has been covalently modified at the active site binds to platelets without activating them (3, 11, 12, 18-21). Some investigators have reported that such modified thrombins appear to potentiate the aggregating and release-inducing effects of suboptimal concentrations of thrombin (3, 20). We have examined the effect of thrombin inactivated by tosyllysylchloromethylketone (TLCK) on the ability of native thrombin to induce aggregation, release and the change in trans-membrane potential ($\Delta\psi$) recently reported by our laboratory (23).

METHODS: Most of the methods used for these experiments have been previously described (23, 24). To prevent aggregation, Ca^{2+} was omitted from the modified Tyrode's buffer, pH 7.4, used for the gel filtration of the platelets.

α-thrombin and TLCK-thrombin were prepared according to Workman, et al (20, 21).

RESULTS: Active thrombin produces a dose-dependent change in the membrane potential of gel-filtered platelets in Tyrode's buffer, as well as in their release of ^{3}H serotonin (Figure 1). At concentrations greater than 0.005 U/ml, the probe's fluorescence increases rapidly, attaining its maximum value (i.e. greatest decrease in potential) within less than one minute. TLCK-thrombin elicits no change at all in membrane potential.

Preincubation of the platelets with TLCK-thrombin for 5 minutes results in competition for the thrombin reactive site(s) and the response to active α-thrombin is decreased in proportion to the relative ratio of TLCK-thrombin to thrombin (Figure 2). A double reciprocal plot (velocity of membrane potential change)$^{-1}$ vs. (α-thrombin concentration)$^{-1}$, for a number of these ratios (TLCK-thrombin:thrombin = 10:1, 4:1, 1:1, 1:2, 1:10) (Figure 3), shows that the results do not fit the pattern observed for a classical enzyme-substrate-inhibitor system. While the rate of stimulation, and presumably of displacement of bound TLCK-thrombin, increases with

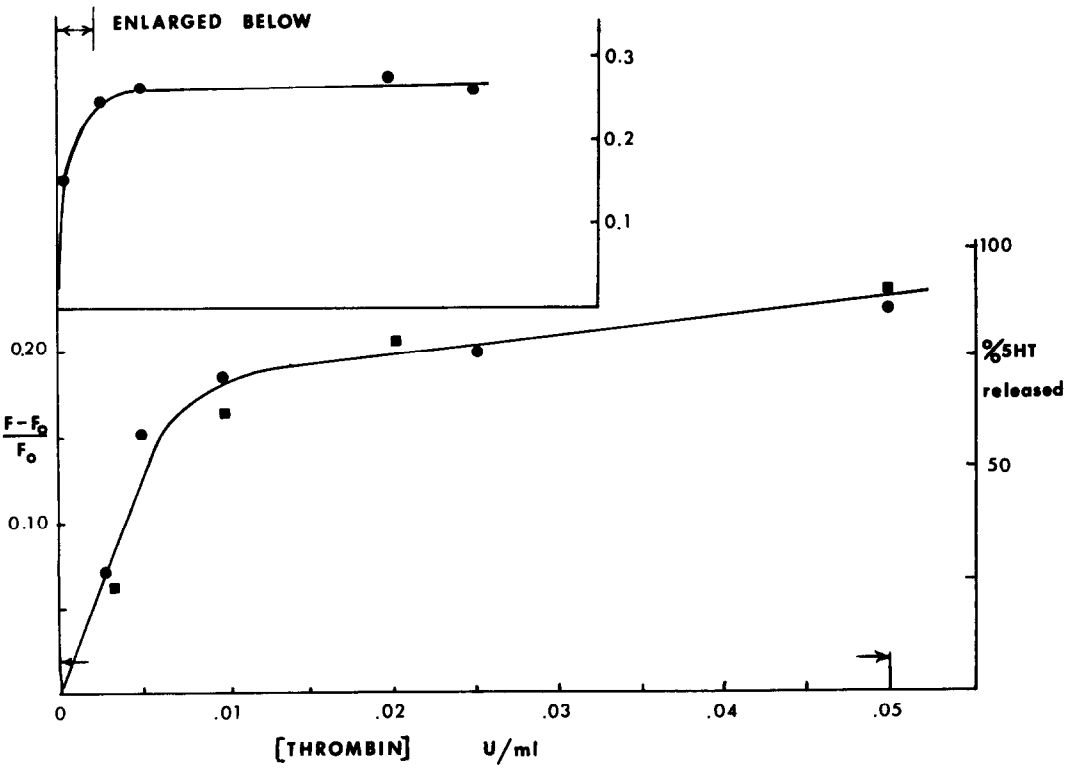

Figure 1. Relative change in fluorescence, $\left(\frac{F - F_o}{F_o}\right)$, (●), and percent of serotonin released (■) after 1 minute, as a function of thrombin concentration.

increasing thrombin concentration up to a thrombin concentration of about 0.01 U/ml for any given TLCK-thrombin:thrombin ratio, little further change in rate is observed at concentrations greater than 0.01 U/ml. The rate of stimulation in the presence of any TLCK-thrombin never attains that observed in its absence, and the platelet-bound TLCK-thrombin is therefore not completely displaced even by a ten fold excess of α-thrombin.

DISCUSSION: The interaction of thrombin with the platelet surface induces a membrane potential change, a shape alteration, secretion of granule contents, and eventually aggregation. This stimulation of platelet response requires active thrombin (23, 24) and involves binding either to two species of binding sites (19) or to one type controlled by negative cooperativity (17) as measured by equilibrium binding studies with

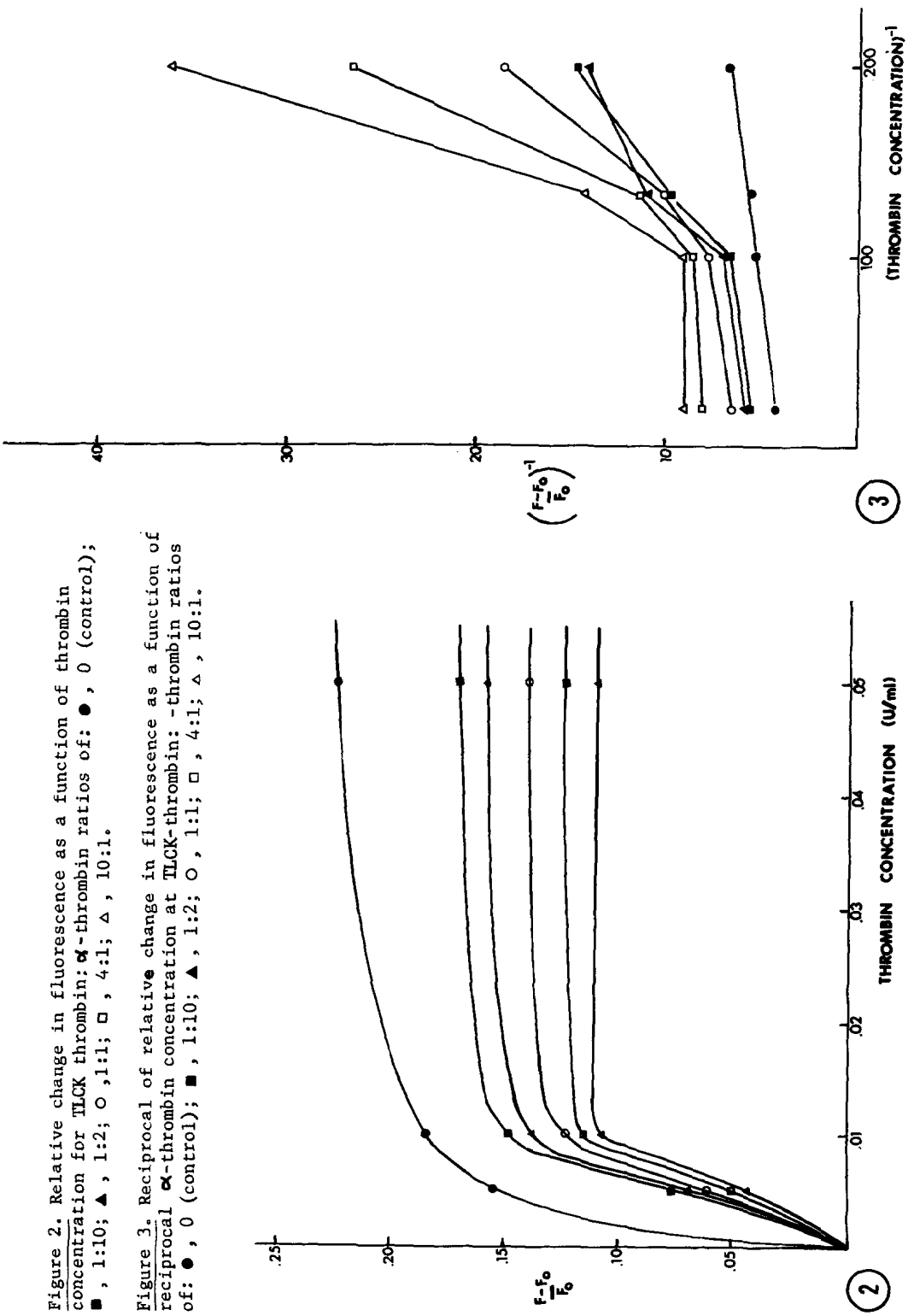

Figure 2. Relative change in fluorescence as a function of thrombin concentration for TLCK thrombin: α-thrombin ratios of: ● , 0 (control); ■ , 1:10; ▲ , 1:2; ○ ,1:1; □ , 4:1; △ , 10:1.

Figure 3. Reciprocal of relative change in fluorescence as a function of reciprocal α-thrombin concentration at TLCK-thrombin: -thrombin ratios of: ● , 0 (control); ■ , 1:10; ▲ , 1:2; ○ , 1:1; □ , 4:1; △ , 10:1.

labeled thrombin. If there are two populations, they comprise approximately 450 to 1050 of high and 45,000 to 95,000 of low affinity for active thrombin, as evaluated by binding studies with radioisotope-labeled enzyme.

The blocking of the active site by TLCK does not affect the platelet equilibrium binding characteristics of thrombin (21), while modification of the macromolecular substrate (fibrinogen) binding site with tetranitromethane (TNM) modifies the binding, and decreases, but does not abolish, the ability of the TNM-modified thrombin to induce platelet secretion and aggregation (20, 21). Only an unblocked thrombin active site, rather than both an unblocked active site and a binding site, is hence required for platelet stimulation. Studies such as these can give information on equilibrium binding, and are valid for thrombin because it has been shown (1) that the enzyme does not turn over. Equilibrium measurements do not, however, yield information on kinetic processes, such as rates of reaction in the presence or absence of inhibitors.

In contrast with equilibrium binding studies, the data shown here do provide kinetic information on the rate of reaction of α-thrombin with its receptor in the presence, by preincubation, of the competitively binding TLCK-thrombin. This rate, determined as a rate of stimulation of platelet receptor response, depends upon the relative ratio of TLCK-thrombin to active thrombin. However, even at very high thrombin concentrations, the TLCK-thrombin is not fully displaced, the extent to which it remains being a function of the relative TLCK-thrombin:thrombin ratio (cf. Figure 2 at $1/[T] > 100$).

Our data do not allow calculation of the relative affinity constants for blocked and native α-thrombin since we are dealing with <u>rates</u> of stimulation, not with eventually attained equilibrium concentrations. Both Figure 2 and Figure 3 demonstrate that a maximal fluorescence is always attained with approximately 0.01-0.012 U/ml of thrombin but that the magnitude of that maximal response is dependent on the TLCK-thrombin:throm-

bin ratio. The response is lower, but still present, even when the ratio is 100:1 (data not shown). Thus some, but not all, of the stimulatable sites exhibit a more rapid reaction with free than with blocked enzyme.

These kinetic observations fit the hormone-receptor model proposed by Detwiler (16), provided one can assume that the formation of a complex between the blocked inactive enzyme and some specific receptor moiety (e.g. Tr_o in 16) is sufficiently favored so that a 100 fold increase in the relative amount of TLCK-thrombin at constant thrombin concentration reduces the response by a factor of 2 to 2.5 (Figure 3).

The most likely explanation for the discontinuity in the 1/v vs. 1/[E] curves (Figure 3) at saturation is the presence of more than one species of binding, as previously proposed (1, 2, 16). The curves are not very compatible with the negative cooperativity model which has also been suggested (3, 19). Unlike the ATP release (16), the platelet membrane responses show that there is competition for stimulation as well as for binding, but the saturation effect above 0.01 to 0.012 thrombin U/ml implies that this holds largely for one species of binding site, presumably the one exhibiting higher affinity for thrombin; therefore both species of binding sites are involved in stimulation of platelets although they exhibit different thrombin affinities.

In terms of platelet-thrombin interaction models (1, 2, 16, 27), these results are all compatible with the suggestion that thrombin activitates platelets by more than one mechanism, if the separate mechanisms involve separate receptors of different thrombin affinity. The receptor with highest affinity would interact with the lowest thrombin concentrations and would be competitively inhibited by active site-blocked thrombin. Although no definitive answer to the platelet thrombin interaction mechanism can be drawn from these studies, all of these results strongly support the presence of two types of thrombin receptor sites on the platelet membrane.

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