Trypsin-Induced Phospholipase Activity in Human Platelets

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Trypsin mediates a release of arachidonic acid with resultant increase in O$_2$ consumption (a reflection of cyclo-oxygenase activity) by whole human platelets that is similar to thrombin's effect on these cells. The trypsin and thrombin effects can be differentiated in two ways: (1) at saturating concentrations the measured effects of trypsin greatly exceed those of thrombin; (2) EGTA [ethanedioxybis(ethylamine)-N,N',N'-tetra-acetate] augments the effect of thrombin but not of trypsin. Thus trypsin and thrombin probably act at different loci in the pathway that induces phospholipase activity in human platelets.

We recently showed that the thrombin-mediated burst in O$_2$ consumption by human platelets is secondary to release and subsequent oxygenation of arachidonic acid (Pickett & Cohen, 1976a). Using the same techniques, we now present evidence that trypsin induces the release of arachidonic acid and the consumption of O$_2$, thus closely mimicking the effects of thrombin. The importance of this observation is that addition of trypsin might simulate the mechanism by which thrombin brings into play the activities of phospholipase A$_2$ (EC3.1.1.4) and, by extension, cyclo-oxygenase of human platelets.

Experimental

Methods previously described were used to prepare platelet homogenates and membranes (Broekman et al., 1974), iso-osmotic (Pickett & Cohen, 1976a) and hypo-osmotic (Pickett & Cohen, 1976b) suspensions, and to measure the consumption of O$_2$ and release of fatty acids in an O$_2$-depleted reaction mixture (Pickett & Cohen, 1976a). In some studies platelets were lysed by sonication, three cycles of freeze–thawing, severe osmotic shock or exposure to detergents.

Incubation mixtures were at pH 7.4 and most often at 300 mosm, and contained, in a volume of 1.8 ml, fatty acid-poor bovine serum albumin (0.34%), Tris HCl (25 mm), KCl (116 mm) and platelet suspension. EGTA [ethanedioxybis(ethylamine)-N,N',N'-tetra-acetate] (5 mm) was added in most instances. When CaCl$_2$ was added, an osmotically equivalent amount of KCl was omitted from the medium. To decrease the osmolarity of the medium a known amount of KCl solution was replaced by water. Incubations were performed at 37°C. In studies where the release of arachidonic acid was measured, the reaction mixture was made anaerobic as described by Pickett & Cohen (1976a).

Thrombin (Ortho Pharmaceuticals, Raritan, NJ, U.S.A.) was added to the incubations, as described by Pickett & Cohen (1976a). Trypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.) unless otherwise indicated was added to give a concentration of 222 μg per incubation, the remaining proteinases (Sigma) to give concentrations of 600 μg per incubation.

Results

At the concentrations used, EGTA prevented the marked platelet-aggregating effects of thrombin. Trypsin-treated platelets did not aggregate macroscopically, or, if already spontaneously aggregated, were partially redispersed on the addition of trypsin, which at a concentration of 222 μg per incubation caused obvious platelet lysis. Spontaneous aggregation occurred only in the absence of EGTA and did not, in the absence of thrombin, affect O$_2$ consumption. In the presence of thrombin, marked aggregation, even to the point of producing a tight ball of platelets, did not affect the completeness of the extraction of lipids in general, or of fatty acids in particular.

Fig. 1 shows that the effect of trypsin on O$_2$ consumption by human platelets was similar in onset and duration to that of thrombin. At concentrations that stimulated maximal initial rates of O$_2$ consumption the effect of trypsin was twice that of thrombin (Fig. 2). In parallel experiments to those shown in Fig. 2, in the absence of EGTA four times as much unesterified arachidonic acid was recovered after adding trypsin as after adding thrombin. In the presence of EGTA, the stimulation by thrombin of arachidonic acid release was augmented 2.5-fold, whereas the trypsin-induced response was unaffected (Table 1). Addition of CaCl$_2$ did not increase the release of arachidonic acid produced by the addition of thrombin or trypsin.

The specific effect of trypsin, among proteolytic agents, was shown when additions of α-chymotrypsin, pepsin, bacterial proteinases (IV, V, VI), bromelin, ficin or papain were shown to have no effect on
Platelet suspensions or thrombin of experiment wide range make-up and platelets were similar. Consumption caused osmotic freeze-thawing, Platelet section. The in protein trypsin were in reported thrombin (Pickett acid release arachidonic Cohen, 1976a), the same. The bursts in consumption caused by thrombin and trypsin additions were similar.

Fig. 1. Polarograph tracings of O₂ consumption by human platelet suspensions after addition of (a) thrombin (6.6 units) or (b) trypsin (222 µg) in the presence of EGTA (5 mM)

Each incubation mixture contained 10 mg of platelet protein in a volume of 1.8 ml. For other details relating to make-up and conduct of incubations, see the Experimental section. The results shown are from a representative experiment of 20 with the same results. The bursts in O₂ consumption caused by thrombin and trypsin additions were similar.

Arachidonic acid release or on O₂ consumption. As we reported in studies with addition of arachidonate or thrombin (Pickett & Cohen, 1976a), the effects of trypsin were sensitive to acetylsalicylic acid but not to cyanide. Platelet homogenates or membranes, or whole platelets that were exposed to sonication, freeze–thawing, osmotic shock or detergents, over a wide range of pH and Ca²⁺ concentrations (Derksen & Cohen, 1975) were less than 10% as responsive to thrombin or to trypsin as measured by release of arachidonic acid or the stimulation of O₂ consumption.

Discussion

Phospholipase activity of pig pancreas is augmented by storage, even at 4°C. de Haas et al. (1968) were the first to show that tryptic digestion converts pancreatic pro-phospholipase into the active phospholipase. Duchesne et al. (1972) found in studies with rats that the phospholipase activity of platelet-poor plasma is unmasked by crude trypsin as well as by clotting. This led Duchesne et al. (1972) to suggest that a substance in platelets, themselves weak in phospholipase activity, has a proteolytic action that mimics the effect of trypsin. The present results suggest that the phospholipase activity induced by trypsin in suspensions of human platelets would, by analogy with the studies on pancreas (de Haas et al., 1968), appear to result from tryptic modification of a pro-phospholipase in the platelets themselves. The locus of such a pro-phospholipase is likely to be in the membranes, which represent the subcellular fraction with the greatest concentration of both substrate and active enzyme (Derksen & Cohen, 1975). However, compared with intact platelets, disrupted platelets showed a markedly smaller response to added trypsin (or to thrombin). Yet homogenates or membranes can oxygenate added arachidonic acid, and platelets in 80 mM media contain mitochondria that show tightly coupled respiration (Pickett & Cohen, 1976b). Thus it would appear that the structural integrity of the platelet or the co-operation of a non-membranous component, or both, are indispensable to the abilities of trypsin or thrombin to induce the phospholipase activity that furnishes unesterified arachidonic acid. Except for EGTA, all chemical and physical agents that were tried either abolished or did not affect the actions of trypsin and thrombin that we have studied. That EGTA was able to augment the effect of thrombin but not that of trypsin suggests that each of these compounds influences a different point in the pathway that initiates phospholipase activity.

In earlier studies we found that phospholipase A₂ activity of human platelet membrane preparations, in keeping with that from other sources, has an absolute requirement for Ca²⁺ (Derksen & Cohen, 1975). Subsequently, we showed that thrombin induced phospholipase activity in intact platelets that had been washed and resuspended in Ca²⁺-free buffer and then exposed to 5 mM-EGTA (Pickett & Cohen, 1976a). In the present work with the intact platelet, EGTA was apparently able to enhance the effect of thrombin on phospholipase A₂ activity without enhancing its own access to the Ca²⁺ which is an essential cofactor for the phospholipase. This suggests that EGTA, by chelating surface calcium, alters
Fig. 2. Initial rates of O₂ consumption by human platelets in response to addition of various amounts of (a) thrombin and (b) trypsin in the presence of EGTA (5 mM)

Each incubation mixture contained 5 mg of platelet protein in a volume of 1.8 ml. For other details, see the Experimental section. Each point is the mean for three experiments, each in duplicate. At the concentrations used to obtain the results shown in Fig. 1, both compounds were maximally active and the stimulatory effect of trypsin was twice that of thrombin.

Table 1. Effect of EGTA on trypsin- and thrombin-mediated release of arachidonic acid

<table>
<thead>
<tr>
<th>Arachidonic acid released (nmol)</th>
<th>+EGTA</th>
<th>Control</th>
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<tbody>
<tr>
<td>Thrombin</td>
<td>22.5±2.3</td>
<td>9.0±0.5</td>
</tr>
<tr>
<td>Trypsin</td>
<td>35.5±2.5</td>
<td>34.7±4.9</td>
</tr>
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Platelet shape (Zucker & Borelli, 1954) or volume (Bull & Zucker, 1965), and in so doing facilitates binding of thrombin to the platelet surface (Tollefsen et al., 1973). Such an effect, in turn, could have enhanced the ability of thrombin to induce proteolysis or the release reaction (Tollefsen et al., 1973), either of which could affect phospholipase activity in a way that is at present unknown. Attention is drawn to a facilitating role for an intracellular component, which is perhaps mobilized by the release reaction, since thrombin and trypsin were much more effective in stimulating the release of arachidonic acid in whole platelets than in membranes.

Trypsin, which is unlike thrombin in that it has no specific receptor sites on human platelets (Tollefsen et al., 1973), seemed able to activate phospholipase A₂ activity equally well in the presence or absence of EGTA. Our data are insufficient to say whether the trypsin effect was specific at concentrations that caused the maximum release of arachidonic acid. The proteolytic attack by trypsin was severe enough to cause platelet lysis, which, in a non-specific manner, could have activated phospholipase A₂. However, disruption of platelets by sonication, freeze-thawing, detergents or osmotic shock failed to produce the same effect. Thus a specific effect of trypsin on the phospholipase A₂ complex cannot be ruled out. Such an effect could derive from cleavage of a pro-phospholipase or removal of barrier molecules that normally isolate the phospholipase from activating influences elsewhere in the cell.

We conclude that, compared with thrombin, trypsin is a more powerful stimulator of human platelet phospholipase activity. The effect of thrombin on platelet phospholipase activity may be mediated by the release of a trypsin-like intracellular proteinase that leads to activation of a membrane-bound pro-phospholipase.
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References
