Inhibition of Platelet Aggregation by Protease Inhibitors. Possible Involvement of Proteases in Platelet Aggregation

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The possible participation of proteases in human platelet aggregation was explored using various protease inhibitors and substrates. Protease inhibitors used included naturally occurring inhibitors of serine proteases and synthetic inhibitors that modify the active site of protease. Substrates used were synthetic substrates for the trypsin type as well as for the chymotrypsin type of protease. All these inhibitors and substrates inhibited platelet aggregation and serotonin release induced by ADP, collagen, epinephrine, or thrombin. In ADP- and epinephrine-induced platelet aggregation the second phase of aggregation was most efficiently inhibited. The inhibitors suppressed the formation of malondialdehyde during platelet aggregation. Release by aggregating agents of arachidonate and its metabolites from indomethacin-treated platelets as well as nontreated platelets was also inhibited. The inhibitors appear to interact with stimulated platelets but not with unstimulated platelets. These observations suggest that the interaction of an aggregating agent with its platelet receptor activates a unique precursor serine protease that in turn activates platelet phospholipase to liberate arachidonic acid (the precursor of the potent platelet aggregating agent thromboxane A2) from platelet phospholipids.

Salzman and Chambers showed in 1964 that ADP-induced platelet aggregation was inhibited by a group of synthetic substrates for esterase. They suggested that the inhibitory reaction is specific and involves enzymes other than proteases, since some of naturally occurring inhibitors did not inhibit ADP-induced platelet aggregation. Mustard et al. reported in 1967 that the specific inhibitor of serine proteases, diisopropylphosphorofluoridate (DFP), inhibits the release of platelet serotonin and nucleotides induced by thrombin, collagen, antigen-antibody complex, or gamma globulin-coated surface.

The inhibitory effects of synthetic substrates or inhibitors of esterases on
platelet aggregation were also reported in 1968 by Kilburn and Firkin and in 1970 by Jobin et al. Recently Henson and co-workers found that a rabbit platelet aggregation and secretion caused by various immunologic stimuli as well as collagen can be inhibited by organophosphorus inhibitors of serine esterases, and they suggested that cell-associated serine proteases (esterases) are involved in rabbit platelet aggregation and secretion.

Recent studies showed that stimuli causing platelet aggregation and secretion release arachidonic acid from platelet phospholipids and induce the synthesis of potent aggregating materials from the liberated arachidonic acid.

We examined the effects of various protease inhibitors on human platelet aggregation as well as on the release of arachidonic acid and the formation of its metabolite, malondialdehyde, during platelet aggregation.

MATERIALS AND METHODS

Preparation of platelet-rich plasma (PRP). Human blood was obtained from healthy adults who had not taken any drug within the previous week. Blood was collected into 1/10 volume of 3.8% sodium citrate. PRP was prepared by centrifugation at 350 g for 6 min at room temperature. Platelet counts were determined with a Coulter counter (Coulter Electronics, Hialeah, Fla.); the counts were always in the normal range, with an average count of 300,000 platelets/μl.

Preparation of washed platelet suspensions. EDTA (0.1 M) was added to PRP to give a final concentration of 1 mM. The PRP was then centrifuged at 1700 g for 15 min at room temperature. The supernatant was discarded and the platelet pellet was resuspended in the original volume of buffered saline containing EDTA (1 mM EDTA, 5 mM D-glucose, 134 mM NaCl, 15 mM Tris-HCl, pH 7.4). The platelets were recentrifuged at 1700 g for 15 min and finally resuspended in an approximately equal volume of Tyrode's solution without calcium in order to obtain approximately 300,000 platelets/μl. Calcium chloride (0.1 M) was added to washed platelet suspension to obtain 1 mM calcium concentration immediately before each experiment.

Platelet aggregation. Aggregation of platelets was studied turbidometrically at 37°C with continuous recording of light transmission using an aggregometer (Bryston, Rexdale, Ontario). PRP or a washed platelet suspension, 0.5 ml, was mixed with 10 μl of test sample or its vehicle as the control and was incubated at 37°C with stirring for 3 min in the aggregometer before the addition of the aggregating agent. The extent of aggregation was given by the maximum change of light transmission expressed as a percentage of the control.

Aggregating agents. Collagen. Bovine tendon collagen (Sigma Chemical, St. Louis, Mo.) (100 mg) was minced and homogenized with 30 ml of 0.85% NaCl at 0°C. The coarse materials were removed by centrifugation, and the supernatant fluid was stored at 4°C before use.

Epinephrine and ADP. Epinephrine (Sankyo Pharmaceutical, Tokyo, Japan) or ADP (Sigma) was dissolved with 0.85% NaCl to various concentrations immediately before use and kept ice cold. The epinephrine solution was protected from light during experiments.

Thrombin. Thrombin (bovine, Parke Davis, Detroit, Mich.) was further purified by the method of Lundblad and stored as a 5000-U/ml solution in 50% glycerol at −20°C until its use. Thrombin was diluted in 0.85% NaCl to a desired concentration immediately before use.

Protease inhibitors. Natural protease inhibitors tested were soybean trypsin inhibitor (SBTI) (Worthington Biochemical, N.J.), aprotinin (Trasylo1, Bayer Chemical, Osaka), and leupeptin (kindly supplied by Dr. T. Aoyagi, Institute of Microbial Chemistry, Tokyo). These were dissolved in Tris-buffered saline (Tris 0.05 M-HCl, NaCl 0.15 M, pH 7.4).

Synthetic protease inhibitors tested were disopropylphosphorofluoridate (DFP), phenylmethylanesulfonyl fluoride (PMSF), N-tosyl-L-lysylchloromethylketone (TLCK), N-tosyl-L-phenylalanlylcholoromethylketone (TPCK), and 4-(2-carboxyethyl)-phenyl-trans-4-aminomethylcyclohexane carboxylate (AMCHA-CEP1). DFP and PMSF were dissolved in isopropyl alcohol. TLCK and TPCK were dissolved in water and ethanol, respectively. AMCHA-CEP1 was dissolved in Tris-buffered saline. AMCHA-CEP1 was donated by Daiichi Pharmaceutical, Tokyo; all of the other chemicals were purchased from Sigma Chemical.

Synthetic substrates. Synthetic substrates for protease tested were acetylglycinelysine methyl
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...suspensions were extracted, separated chromatographically and then assayed for radioactivity as described. PRP or the washed platelet suspension (0.5 ml) was mixed with 10 μl of a protease inhibitor or its vehicle and incubated at 37°C with stirring for 3 min before the addition of an aggregating agent. Stirring was continued for 5 min after the addition of aggregating agent, and 0.2 ml of 100% (w/v) trichloroacetic acid (TCA) in 1 N HCl was added and mixed to stop the reaction. The mixture was stored at 4°C for 30 min and then centrifuged at 2000 g for 20 min. The supernatant (0.4 ml) was mixed with 0.1 ml 1% sodium thiobarbiturate (Tokyo Kasei Chemical, Tokyo), and the mixture, in a closed vessel, was placed in a boiling water bath for 30 min. After cooling, the mixture was centrifuged at 2000 g for 20 min. The absorbance of the supernatant was read at 532 nm and the value obtained was converted to MDA using the reference curve constructed with the known amount of MDA (>99% pure, Tokyo Kasei). The controls were run substituting aggregating agents with their respective vehicles. MDA formation during platelet stimulation by an aggregating agent was determined from the difference between the value obtained with an aggregating agent and its control.

**Assay of malondialdehyde (MDA) formation.** MDA formed in PRP or washed platelet suspensions during platelet stimulation by an aggregating agent in the presence and absence of a protease inhibitor was assayed according to a method modified slightly from one previously described. PRP or the washed platelet suspension (0.5 ml) was mixed with 10 μl of a protease inhibitor or its vehicle and incubated at 37°C with stirring for 3 min before the addition of aggregating agent. Stirring was continued for 5 min after the addition of aggregating agent, and 0.2 ml of 100% (w/v) trichloroacetic acid (TCA) in 1 N HCl was added and mixed to stop the reaction. The mixture was stored at 4°C for 30 min and then centrifuged at 2000 g for 20 min. The supernatant (0.4 ml) was mixed with 0.1 ml 1% sodium thiobarbiturate (Tokyo Kasei Chemical, Tokyo), and the mixture, in a closed vessel, was placed in a boiling water bath for 30 min. After cooling, the mixture was centrifuged at 2000 g for 20 min. The absorbance of the supernatant was read at 532 nm and the value obtained was converted to MDA using the reference curve constructed with the known amount of MDA (>99% pure, Tokyo Kasei). The controls were run substituting aggregating agents with their respective vehicles. MDA formation during platelet stimulation by an aggregating agent was determined from the difference between the value obtained with an aggregating agent and its control.

**Serotonin release.** Serotonin release was measured by the following procedure. One hundred parts of PRP were incubated for 45 min at 37°C with one part of serotonin-14C-creatinine sulfate (55 mCi/m mole; Radiochemical Centre, Amersham, England) diluted in buffered saline (10 μCi/ml). PRP, 0.5 ml, thus treated was mixed with 10 μl of test sample or its vehicle and was incubated at 37°C with stirring for 3 min in the aggregometer tube; then 5 μl of aggregating agent (or its vehicle for the control sample) were added. Stirring was continued for an additional 5 min, and 0.2 ml of 0.1% glutaraldehyde was subsequently added and mixed to stop the reaction. Samples were then centrifuged at 2000 g for 20 min. The radioactivity of the supernatant (0.4 ml) was determined by liquid scintillation counting. The control was run in a similar way without addition of an aggregating agent.

**Release of arachidonic acid or its metabolites.** [1-14C]-arachidonic acid (14C-AA) (55 Ci/m mole, Radiochemical Centre) in a concentration of 5 μCi/ml in ethanol was added to PRP at a ratio of 1 μl 14C-AA to 1 ml PRP. The PRP was then incubated at 37°C for 1 hr. From PRP thus treated, washed platelet suspensions were made according to the method described above. Uptake of radioactivity by platelets varied between 20% and 45%. Greater than 90% of the incorporated radioactivity was found in the platelet phospholipids when the lipids from the platelet suspensions were extracted, separated chromatographically and then assayed for radioactivity as described by Bills et al. A platelet membrane fraction prepared from the platelet suspensions according to a method described previously was found to contain approximately 95% of radioactivity incorporated into platelets.

The 14C-AA-incorporated platelet suspensions thus prepared were used for the study on the release of arachidonic acid and its metabolites from platelets during platelet stimulation by an aggregating agent in the presence or absence of a protease inhibitor or indomethacin (Merck-Banyu, Tokyo). The 14C-AA-incorporated washed platelet suspension, 0.4 ml, was mixed with 10 μl of a protease inhibitor, indomethacin, or their respective vehicles and incubated at 37°C with stirring for 3 min before the addition of an aggregating agent or its vehicle as the control. Stirring was continued for 5 min after the addition of an aggregating agent, and 0.2 ml of ice-cold 0.1 M EDTA was subsequently added and mixed to stop the reaction. The tube was immediately placed in ice to be cooled and then centrifuged at 2000 g for 15 min at 4°C. The radioactivity of the supernatant (0.4 ml) was determined by liquid scintillation counting.

**Scintillation counting procedures.** Each sample was mixed with 1 ml soluene 350 (Packard Instrument, Downer's Grove, Ill.) in a counting vial and left at room temperature overnight. The sample was subsequently mixed with 5 ml scintillator (5 g PPO and 0.5 g POPOP/liter toluene), and the radioactivity was estimated with a Packard 3330 liquid scintillation counter.

**Amidolytic assay of thrombin.** Thrombin activity was measured spectrophotometrically using tripeptide-N-benzoyl-l-phenylalanyl-l-valyl-l-arginine-p-nitroanilide (S-2160) (Kabi Diagnostica,
Stockholm) as a substrate. The enzyme solution was mixed with 0.1 mM S-2160 in Tris-imidazole-buffered saline (0.05 M Tris, 0.05 M imidazole, 0.135 M NaCl, pH 8.3) and incubated at 37°C. The initial rate of thrombin-catalyzed hydrolysis of the substrate at 37°C was measured by recording the increase of the absorbance at 405 nm with a Hitachi 333 spectrophotometer equipped with a thermostated cuvette holder and connected with a Hitachi 056 recorder (Hitachi, Tokyo).

Statistical analysis was performed by Student’s one-tailed t test for paired samples. A p value >0.05 was considered to represent a statistically nonsignificant change.

RESULTS

Inhibition of Platelet Aggregation by Protease Inhibitors

Platelet aggregation induced by collagen, epinephrine, ADP, arachidonic acid, or thrombin was inhibited by the presence of protease inhibitors. Typical results obtained with SBTI are shown in Fig. 1. As seen in ADP- or epinephrine-induced platelet aggregation, SBTI inhibited most efficiently the second-phase aggregation, and the inhibition of the second-phase aggregation was more remarkable than that of collagen-induced aggregation when the degrees of inhibition achieved with the same concentration of an inhibitor were compared. The first-phase aggregation was also inhibited when the concentration of the inhibitor was increased. All the protease inhibitors tested inhibited platelet aggregation in a similar way. Serotonin release associated with platelet aggregation was also inhibited by the inhibitors, and the extent of the inhibition of the release was proportional to that of the inhibition of aggregation (Table 1).

Inhibition was dose dependent, and approximately 50% of inhibition of collagen (20 μg/ml)-induced aggregation of platelets in plasma as well as of washed platelets was achieved by the following ranges of concentrations of inhibitors: SBTI 1–5 μM; aprotinin 1–6 μM; leupeptin 0.2–0.5 mM; DFP 0.7–2 mM; PMSF 0.2–1 mM; TLCK 0.02–0.2 mM; TPCK 0.05–0.2 mM; and AMCHA-CEP, 0.02–0.1 mM. These ranges of effective concentrations were obtained from experiments on five different platelet preparations. Platelet aggregation induced by stimulants other than thrombin was similarly inhibited by the same range of concentrations of the inhibitors. Slightly higher concentrations of SBTI were needed for the inhibition of thrombin-induced platelet aggregation; SBTI 15 μM gave 63%, 65%, and 68% inhibition of aggregation induced by...
**Table 1. Effect of Protease Inhibitors on Platelet Aggregation and Serotonin Release**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Collagen (20 μg/ml)</th>
<th>ADP (2 μM)</th>
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<tr>
<td></td>
<td>Aggregation (%)</td>
<td>Release (%)</td>
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<tr>
<td>SBTI</td>
<td>5 µM</td>
<td>47</td>
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<tr>
<td></td>
<td>10 µM</td>
<td>19</td>
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<tr>
<td>Aprotinin</td>
<td>1 µM</td>
<td>51</td>
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<td></td>
<td>2 µM</td>
<td>36</td>
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<tr>
<td></td>
<td>4 µM</td>
<td>0</td>
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<tr>
<td>DFP</td>
<td>2 mM</td>
<td>17</td>
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<tr>
<td>PMSF</td>
<td>2 mM</td>
<td>10</td>
</tr>
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*The control values of aggregation and release were obtained without addition of inhibitors and are expressed as 100%.
†Primary aggregation only.

0.06 U/ml thrombin on three separate experiments, whereas the same concentration of SBTI was usually high enough to exert complete inhibition of collagen-induced aggregation. (Experiments to demonstrate inhibition of thrombin-induced aggregation by protease inhibitors other than SBTI are not meaningful because inhibitors other than SBTI can directly inhibit thrombin activity.)

Inhibition was instantaneous and did not require the preincubation of platelets with inhibitors before platelet stimulation. Figure 2 shows that SBTI inhibited the aggregation even when SBTI was added at the same time as the stimulant or added during the “induction time of aggregation.” Furthermore, SBTI was still able to inhibit platelet aggregation to some extent when added after the aggregation process was already started (Fig. 2). This type of immediate action was also seen with aprotinin and DFP.

![Fig. 2. Inhibition of collagen-induced platelet aggregation in plasma by addition of SBTI at various times. Experimental conditions as in Fig. 1. (A) Control without addition of SBTI; (B) aggregation profiles with addition of SBTI at various times indicated by small arrows.](image-url)
Inhibition of Platelet Aggregation by Synthetic Substrates for Serine Proteases

Platelet aggregation and the release reaction induced by the stimulants were also inhibited by synthetic substrates for serine proteases, including substrates for proteases of trypsin type (AGLMe, TAMe, TLMe, BAN!, BANA) and of chymotrypsin type (ATEE, APNE). Inhibition was dose dependent, and about 50% inhibition of collagen (20 μg/ml)-induced aggregation of platelets in PRP was achieved by the following concentrations of substrates: AGLMe 1 mM; ATEE 0.7 mM; TAMe 0.5 mM; BANA 0.4 mM; BAN! 0.2 mM; APNE 0.2 mM; and TLMe 0.1 mM. These values were obtained from the experiments done on the same platelet preparation.

The nonsubstrate compounds Tos-Gly and Tos-Arg-amide, which possess tosyl residues and are structurally close to synthetic substrates such as TAMe and TLMe, were tested for their inhibitory activity on platelet aggregation. The necessary concentrations of those compounds for approximately 50% inhibition on platelet aggregation in PRP ranged from 1 to 5 mM, values approximately 10 times higher than those needed for TAMe and TLMe.

Restoration of Platelet Aggregability by Removal of Inhibitors

Platelets were not able to respond to the aggregation stimulants in the presence of 2 mM DFP. However, the platelet aggregability was restored when

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**Fig. 3. Restoration of aggregability of DFP-treated platelets by washing procedure.** PRP was preincubated with 2 mM DFP for 3 min. These DFP-treated platelets in plasma did not aggregate with addition of collagen (not shown in figure). Washed platelets prepared from DFP-treated PRP, however, did aggregate with an addition of collagen (B) but failed to aggregate when the washed platelets were pretreated again (C). Aggregation of washed platelets prepared from the same but nontreated PRP shown as control (A). Collagen (10 μg) used as stimulant for each 0.5 ml PRP or washed platelets.
DFP was removed from the milieu by a simple washing procedure (Fig. 3). The same phenomenon was also seen in SBTI-mediated inhibition of platelet aggregation, in which removal of SBTI restored the aggregability of platelets.

**Inhibition of MDA Production by Protease Inhibitors**

The amount of MDA generated in PRP and also in washed platelet suspensions under stirring by the addition of an aggregating agent was reduced by the presence of a protease inhibitor. Representative results are shown in Fig. 4. The inhibition was seen with every inhibitor tested (SBTI, aprotinin, DFP, and AMCHA-CEP,) regardless of the type of aggregating agents used. The range of concentrations of each inhibitor required to inhibit MDA formation was the same as that required for the inhibition of aggregation.

**Inhibition of Release of Arachidonic Acid and its Metabolites by Protease Inhibitors**

Release of arachidonic acid and its metabolites from platelets during platelet stimulation by collagen or thrombin was inhibited by the presence of protease inhibitors.
inhibitor. Representative results are shown in Fig. 5. The inhibition was seen with every inhibitor tested (SBTI, aprotinin, and DFP). The range of concentrations of each inhibitor required to inhibit the release was the same as that required for the inhibition of aggregation. Approximately 50% inhibition (average) of the collagen-induced release was achieved with 5 μM SBTI, 5 μM aprotinin, or 2 mM DFP (Fig. 5). Thrombin-induced release was reduced to 32% (average) on the control by the presence of 20 μM SBTI (Fig. 5).

When 14C-AA-labeled platelets preincubated with 20 μM indomethacin were stimulated by collagen (20 μg/ml PRP), there was no or less than 20% aggregation elicited and no formation of MDA. The release of radioactivity, however, was still observed, although the amount of radioactivity release was decreased to 59% (average) of the control without indomethacin (Fig. 5). The release of radioactivity from these indomethacin-treated platelets by collagen was further diminished by the presence of protease inhibitor (Fig. 5), 24% and 16% release (average) when compared to the control in the presence of 5 μM SBTI or 2 mM DFP, respectively (Fig. 5).

When 14C-AA-labeled platelets were preincubated with 20 μM indomethacin before stimulation by thrombin (0.08 U/ml), there was approximately 20% inhibition of aggregation by indomethacin, and no formation or less than 30% of control formation of MDA was observed. The release of radioactivity was decreased to 57% (average) of the control without indomethacin (Fig. 5). The release of radioactivity from these indomethacin-treated platelets by thrombin was further diminished by the presence of 20 μM SBTI to 17% (average) as compared to the control (Fig. 5).

**Effect of SBTI on Thrombin Activity**

Purified thrombin and SBTI in Tris-buffered saline, pH 7.4, were mixed and incubated at 37° C. The control was run by replacing SBTI with the buffered saline. The concentrations of thrombin and SBTI in the incubation mixture were 12.5 U/ml and 50 or 500 μM, respectively. After 5, 10, and 20 min of incubation, 0.2 ml of the incubation mixture was withdrawn and the remaining thrombin activity was measured by amidolytic assay. No inhibition of thrombin activity by SBTI was observed when compared to the control.

**DISCUSSION**

Stimuli such as collagen, thrombin, antiplatelet antibody, C3 bound to zymosan, and platelet-activating factor derived from antigen-stimulated, IgE-sensitized rabbit basophils can cause secretion of serotonin of rabbit platelets. Henson and co-workers found that the secretion was inhibited by the organophosphorus inhibitors that specifically and irreversibly inhibit serine protease (esterases) by phosphorylating the serine group in the active site of the enzyme. Noninhibitory organophosphorus compounds, which resemble structurally the phosphorylating agents but which cannot phosphorylate the active serine, were unable to inhibit the secretion. Those workers consequently suggested that inhibitory organophosphorus inhibits platelet aggregation and secretion by phosphorylating the active serine of stimulus-activatable serine proteases involved in platelet aggregation and secretion. Their observations and
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suggestions are in accordance with the earlier reports that platelet aggregation was inhibited by protease inhibitors or synthetic substrates for proteases.\(^1\)\(^4\)

In the present study various other serine protease inhibitors in addition to organophosphorus were demonstrated to be inhibitory on platelet aggregation and serotonin release. These included active-site serine phosphorylating agent DFP; active-site serine sulfonylating agent PMSF; active-site histidine alkylating agents TLCK and TPCK; a synthetic competitive inhibitor, AMCHA-CEP;\(^1\)\(^2\) naturally occurring small peptide inhibitor leupeptin; and naturally occurring macromolecular inhibitors aprotinin and SBTI. The last two macromolecular inhibitors were found to be most effective when considered on a molar basis. This finding, together with the immediate inhibition observed, suggests that proteases involved in platelet aggregation may be on the outer surface of platelet plasma membranes, which are accessible to these macromolecular inhibitors.

Another support for the hypothesis that proteases are involved in platelet aggregation came from the observation that synthetic substrates for proteases can inhibit platelet aggregation. The inhibition was considered to be specific because some compounds that are not substrates for proteases but that are structurally similar to the substrates were not inhibitory. Hydrolysis of substrates also resulted in a loss of inhibitory activity on platelet aggregation. These findings may suggest that the substrates for proteases inhibit platelet aggregation by competing with natural substrates for proteases that are involved in platelet aggregation.

DFP- or SBTI-treated platelets, which were unresponsive to aggregating agents, restored their aggregability after the inhibitors were removed from the milieu by a simple washing procedure. This fact may indicate, as suggested by Henson et al.,\(^6\) that the putative proteases exist as precursor forms (zymogens) in platelets and can be activated only after platelets were stimulated by aggregating agents, since the inhibitors interact only with activated proteases and form tightly bound complexes with enzymes that cannot be dissociated by a simple washing procedure.

Recent studies indicate that aggregating agents release arachidonic acid from phospholipids in platelets and induce the synthesis of potent aggregation-inducing substances from the liberated arachidonic acid.\(^7\)\(^8\) These potent substances are endoperoxides and thromboxanes,\(^1\)\(^3\) both of which are intermediate products of the cyclooxygenase pathway of arachidonic acid metabolism. The other pathway of arachidonic acid metabolism is the lipoxygenase pathway, which yields nonaggregating compounds. In the present study the effects of protease inhibitors on the liberation of arachidonic acid and on the formation of MDA were explored. MDA is one of the endproducts of the cyclooxygenase pathway of arachidonic acid metabolism, and the measurement of MDA formation may be used as an indication of the production of the metabolites through the cyclooxygenase pathway.\(^1\)\(^4\)

MDA formation during platelet aggregation was strongly suppressed by the addition of protease inhibitor (Fig. 4). The question then arose of whether this reduction of MDA formation could be attributed to the decrease of release of arachidonic acid or to the decrease of cyclooxygenase activity. To answer this question, release of arachidonic acid and its metabolites from platelets was
examined using $^{14}$C-arachidonic acid–incorporated platelets. Release of radioactivity into the milieu during platelet aggregation was strongly reduced by the addition of protease inhibitor (Fig. 5). Released radioactivity was most likely a mixture of free arachidonic acid and metabolites of cyclooxygenase and lipooxygenase pathways, which was shown by Rittenhouse-Simmons and Deykin.\textsuperscript{15} When the cyclooxygenase pathway of platelets was completely blocked by treatment with indomethacin, the platelets released less radioactivity upon stimulation by aggregating agents. Since indomethacin does not affect the release of arachidonic acid from platelets,\textsuperscript{7,8} the reduction of release of radioactivity was most likely caused by the absence of production of cyclooxygenase metabolites. The absence of production was actually indicated by lack of formation of MDA.

When indomethacin-treated platelets were stimulated by an aggregating agent in the presence of protease inhibitor, the release of radioactivity was further diminished (Fig. 5). This can be explained by the two alternative mechanisms: protease inhibitors either suppress the release of arachidonic acid by preventing activation of phospholipase or prevent the transformation of arachidonic acid to metabolites via lipoxygenase pathway. The latter mechanism is less likely, since the lipoxygenase metabolites have not been shown to be involved in platelet aggregation. The former mechanism seems to be more likely, since the concentrations of the inhibitors necessary to inhibit the release of radioactivity are the same as those needed for inhibition of platelet aggregation. It is therefore suggested that the interaction of an aggregating agent with its platelet receptor directly or indirectly activated a unique precursor serine protease localized on the platelet’s outer membrane. The activated enzyme can then activate phospholipase A$_2$, which is also localized on the outer membrane,\textsuperscript{16} to liberate arachidonic acid.

Recently, Pickett et al. showed that trypsin can activate phospholipase A$_2$ activity in platelet membranes to release arachidonic acid\textsuperscript{17} by analogy with tryptic activation of pancreas pro phospholipase.\textsuperscript{18} This observation is in accordance with our hypothesis that proteases are involved in platelet aggregation. They also suggested from the comparison with thrombin that the effect of thrombin on platelet phospholipase activity may be indirect and mediated by the release or activation of a trypsinlike protease that leads to activation of a membrane-bound pro phospholipase.\textsuperscript{17} In this connection, it is interesting to note that SBTI, which is not inhibitory on thrombin activity, as shown by previous studies\textsuperscript{19,20} as well as the present one, can inhibit thrombin-induced platelet aggregation, MDA formation, and $^{14}$C- AA release (Figs. 4 and 5).

Both the trypsin inhibitor TLCK and the chymotrypsin inhibitor TPCK were almost equally effective in inhibition of platelet aggregation. This is partly explained by the fact that the selectivity of these inhibitors with respect to the types of enzyme (trypsin or chymotrypsin) is not absolute but is only a quantitative difference.\textsuperscript{21} However, almost equal inhibition of platelet aggregation by both inhibitors suggests that there may be other mechanisms operating in these particular situations in addition to active-site–directed inhibition of proteases. Additional mechanisms may be related to the common structural characteristics of inhibitors such as chloromethyl ketone.

Although the inhibitors inhibited primarily the second phase of aggregation,
they also inhibited the primary phase of aggregation when their concentrations were increased. This cannot be readily explained by the proposed hypothesis that protease inhibitors inhibit platelet aggregation by blocking platelet proteases involved in the activation of phospholipase in platelet membranes, since the primary phase is not considered to involve activation of prostaglandin synthesis.22,23 It may be possible that an additional mechanism is involved in the inhibition of platelet aggregation.

It was recently suggested that the intracellular concentration of cyclic adenosine 3′, 5′-monophosphate (cAMP) may regulate the thrombin-induced stimulation of phospholipase activity.24,25 The question as to whether or not protease inhibitors have any effect on platelet cAMP levels before and after stimulation of platelets with aggregating agents is unanswered at present.

Finally, the possibility that serine protease inhibitors are able to inhibit directly the particular phospholipase A2 activity present in platelets cannot be ruled out at the present time, although phospholipases in general are not inhibited by the inhibitors of serine proteases.26

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