HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Role of Caspase in a Subset of Human Platelet Activation Responses

By Anna Shcherbina and Eileen Remold-O'Donnell

Platelets function to protect the integrity of the vascular wall. A subset of platelet activation responses that are especially important for thrombus formation include exposure of phosphatidylserine and release of microparticles, which generate procoagulant surfaces. The resemblance of these platelet activation processes to events occurring in nucleated cells undergoing apoptosis suggests a possible role for caspases. We demonstrate here the presence of caspase-3 in human platelets and its activation by physiological platelet agonists. Using cell-permeable specific inhibitors, we demonstrate a role for a caspase-3-like protease in the agonist-induced (collagen plus thrombin or Ca2+ ionophore) platelet activation events of phosphatidylserine exposure, microparticle release, and cleavage of moesin, a cytoskeletal-membrane linker protein. The role of caspase-3 in platelet activation is restricted rather than global, because other activation responses, a granule secretion, shape change, and aggregation were unaffected by caspase-3 inhibitors. Experiments with two classes of protease inhibitors show that caspase-3 function is distinct from that of calpain, which is also involved in late platelet activation events. These findings show novel functions of caspase and provide new insights for understanding of platelet activation.

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Supported by National Institutes of Health Grant No. AI39574.

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Blood, Vol 93, No 12 (June 15), 1999: pp 4222-4231
platelets. Comparative experiments with protease inhibitors showed that both caspase and calpain function in agonist-induced late events of platelet activation (PS exposure, microparticle release, and moesin cleavage) and demonstrate that the two proteases have distinct roles.

**MATERIALS AND METHODS**

**Platelet isolation.** Freshly drawn blood from normal healthy donors, who gave written consent, was collected in acid-citrate-dextrose (ACD; NIH formula A) in plastic and fractionated immediately at ambient temperature. Cells were counted using a MAX-M Blood Cell Analyzer (Coulter Corp, Hialeah, FL). The blood was centrifuged at 200g for 12 minutes to separate platelet-rich plasma (PRP). Additional ACD was added (1 part ACD per 3 parts PRP) and platelets were pelleted at 800g for 15 minutes. The platelets were resuspended in platelet buffer (10 mmol/L Tris-hydroxymethyl-methyl-2-aminoethane sulfonic acid [TES], pH 7.2, 136 mmol/L NaCl, 2.6 mmol/L KCl, 0.5 mmol/L NaH2PO4, 2 mmol/L MgCl2, 0.1% glucose, and 0.1% bovine albumin) and, after the addition of ACD (20% of final volume) and procyclin (1 µg/mL; Calbiochem, San Diego, CA), were centrifuged at 800g for 10 minutes. The isolated platelets contained no detectable erythrocytes and less than 1 leukocyte per 4,000 platelets. For activation experiments, platelets were suspended in platelet buffer and allowed to recover for 90 minutes at 37°C to ensure their resting state.

**Peptidase assay.** Platelets (5 × 10^8) in 1 mL of platelet buffer with 2 mmol/L CaCl2 and 3 µmol/L A23187 were incubated with stirring in flat-bottom polystyrene vials (14-mm diameter) at 37°C for the indicated time and were lysed by adding 1/3 vol of 4% Triton X-100, 8 mmol/L EGTA, 20 mmol/L dithiothreitol, 200 µg/mL aprotinin, 200 µg/mL benzamidine, and 200 µg/mL leupeptin. The Triton lysates (200 µL) were combined with 0.1 mmol/L of N-acetyl-Asp-Glu-Val-Asp-p-nitro anilide (DEVD-pNA; Enzyme Systems Products, Livermore, CA) or N-acetyl-Tyr-Val-Ala-Asp-p-nitroanilide (YVAD-pNA; Sigma, St Louis, MO). The reactions were incubated for 2 hours in flat-bottom 96-well plates at 37°C with monitoring of OD280. The mean ΔOD per minute was calculated from the linear range of the reaction.

**Immunoblotting.** Platelets (5 × 10^8/mL) were lysed by adding an equal volume of 2% sodium dodecyl sulfate (SDS), 120 mmol/L Tris-HCl, pH 6.8, 4% mercaptoethanol, 100 µg/mL leupeptin, 4 mmol/L EGTA, and 2 mmol/L diisopropyl fluorophosphate (DFP) and heating for more than 3 minutes at 100°C. The lysates were fractionated by SDS-electrophoresis on 8 or 12% polyacrylamide gels (Novex, San Diego, CA). The reactions were incubated as described above with stirring, and light transmission was measured as a function of time according to the Student's paired t-test.

**Platelet activation and flow cytometry.** For PS exposure and microparticle release experiments, 10^7 platelets in 200 µL platelet buffer with 2 mmol/L CaCl2 were placed in siliconized 7 × 45 mm glass cuvettes at 37°C in an aggregation meter (DP-247E; Sienco, Morrison, CO). A23187 (1 µmol/L), thrombin (human; 1 U/mL; Sigma), or thrombin (1 U/mL) plus collagen (20 µg/mL) was added from a diluted stock and, after an initial mixing, incubation was continued without stirring at 37°C for 1 to 20 minutes. The platelet suspensions were transferred to an approximately 22°C water bath and, after 1 minute, 50 µL was combined with fluorescein isothiocyanate-labeled annexin V (annexin V-FTTC; 1 µg/mL; PharMingen, San Diego, CA) and phycoerythrin (PE)-labeled anti-CD41 (GPIIb) MoAb (150 ng/mL; Coulter/Immunotech, Miami, FL) and incubated for 10 minutes at approximately 22°C. Samples were diluted fivefold with platelet buffer with 2 mmol/L CaCl2 for immediate analysis by flow cytometry.

Samples were acquired and analyzed using a FACS-Calibur flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA). Particles were gated for CD41^+ to distinguish platelets and platelet-derived microparticles from electronic noise. The lower limit of the platelet gate was defined on the forward scatter profile of resting platelets, and CD41^+ particles smaller than that were considered microparticles.

To measure α granule secretion, 10^7 platelets were activated as described above, and the reaction was stopped after 0, 30, or 60 seconds by adding an equal volume of 2% paraformaldehyde. Aliquots of the fixed platelets were incubated for 10 minutes with 1 µg/mL PE-labeled anti-CD62P (clone AC1.2 MoAb; Becton Dickinson, San Jose, CA) and examined by flow cytometry.

To measure aggregation in response to thrombin or A23187, 10^7 platelets were incubated as described above with stirring, and light transmission was measured as a function of time according to the manufacturer’s instruction.

**Moesin degradation assay.** After preincubation of 5 × 10^7 platelets in 1 mL platelet buffer in flat-bottom polystyrene vials, CaCl2 (2 mmol/L) and A23187 (3 µmol/L) were added and incubation was continued for 10 or 20 minutes at 37°C with stirring. The reaction was terminated by solubilizing with SDS for immunoblotting.

**TREATMENT WITH CASPASE AND CALPAIN INHIBITORS.** For inhibition experiments, carbobenzoxy-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (DEVD-fmk), carbobenzoxy-Tyr-Val-Ala-Asp(OMe)-fluoromethyl ketone (YVAD-fmk), and carbobenzoxy-Phe-Ala-fluoromethylketone (FA-fmk), or diluent was added to platelets during the final 60 minutes of preincubation, and calpeptin and E64d were added during the final 10 minutes. For moesin cleavage experiments, fluoromethylketone stocks were prepared in dimethylsulfoxide, the recommended solvent, or dimethylformamide. For PS exposure experiments, we found that preincubation of platelets with dimethylsulfoxide (0.01% to 1.0%), but not with dimethylformamide, significantly increased A23187-induced exposure of PS (data not shown). Therefore, fluoromethylketone stocks for PS exposure and all other experiments were prepared in dimethylformamide (0.2% final concentration). Using this diluent, incubation of platelets with fluoromethylketones for 1 hour did not alter resting platelet values of any of the parameters studied. Calpeptin and E64d stocks were prepared in ethanol or dimethylformamide.
minutes (Fig 1A). In parallel assays with YVAD-pNA, a caspase-1 substrate, no activity was detected (data not shown).

Because caspase enzymes have overlapping substrate specificities, platelets were also tested for caspase-3 antigen by immunoblotting. The specific anti–caspase-3 MoAb C31720, which recognizes an epitope in the large subunit, detected a 32-kD band in resting platelets, procaspase-3,22,23 the single-chain zymogen form of the molecule (Fig 1B, left lane). On treatment with A23187, the platelet content of procaspase-3 decreased significantly over time (Fig 1B and C; P < .02 at 10 minutes and P < .003 at 20 minutes). However, p17, the large subunit of active caspase-3, was not detected. A positive control for the ability to detect p17 was provided by mononuclear cell lysates treated with granzyme B (Fig 1B, right lane). Similar platelet immunoblot results were obtained also with caspase-3 antibodies from rabbit and with another MoAb (CPP32/p20-E8; data not shown). These findings demonstrate the presence of caspase-3 zymogen in resting platelets and its activation in A23187-treated platelets. The decrease of zymogen combined with the absence of sufficient active caspase-3 for immunoblot detection strongly suggests that the active protease is short lived in platelets.

Physiological agonists activate platelet caspase-3. Because A23187 is a potent but nonphysiological stimulus, we asked whether processing of platelet caspase-3 zymogen is induced also by physiological agonists. Thrombin, collagen, and the combination thrombin plus collagen were each found to induce processing of procaspase-3 (Table 1). The extent of procaspase-3 processing varied; the order of agonist efficiency was A23187 > thrombin + collagen > either collagen or thrombin.

Caspase inhibitor abrogates agonist-induced phosphatidylserine exposure. We next examined whether caspase is involved in the movement of negatively charged PS from the inner to the outer platelet membrane leaflet, an activation reaction synonymous with generation of the procoagulant surface. Platelets were treated with DEVD-fmk, a cell-permeant inhibitor of caspase-3–like proteases, and then stimulated with agonist. Exposed PS was measured at fixed time points by binding of annexin V-FITC.18 In response to the potent stimulant A23187, exposure of PS was rapid and extensive; 80% ± 4% (n = 4) of platelets became PS positive in 5 minutes (eg, Fig 2A). Incubation with DEVD-fmk substantially inhibited/delayed A23187-induced PS exposure (Fig 2B). At 100 µmol/L, DEVD-fmk caused 74% ± 2% inhibition of PS exposure at 3 minutes.

Table 1. Activation of Platelet Procaspase-3 by Physiological Agonists

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<tr>
<th>Agonist</th>
<th>Procaspase-3 Cleaved (%)</th>
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<tr>
<td>No additive</td>
<td>0.3 ± 1.8</td>
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<tr>
<td>Thrombin (1 U/mL)</td>
<td>14.8 ± 2.8</td>
</tr>
<tr>
<td>Collagen (10 µg/mL)</td>
<td>14.2 ± 3.9</td>
</tr>
<tr>
<td>Thrombin (1 U/mL) + collagen (10 µg/mL)</td>
<td>21.0 ± 7.2</td>
</tr>
<tr>
<td>A23187 (3 µmol/L)</td>
<td>30.2 ± 6.3</td>
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Platelets were incubated with agonist for 20 minutes. Procaspase-3 content was determined by immunoblotting with C31720 MoAb and quantitation of the 32-kD band. Results are expressed as the percentage of decrease (mean ± SEM of 3 experiments) relative to nonactivated starting platelets.

RESULTS

Detection of caspase-3 in platelets. To test for the presence of caspase, isolated platelets were lysed with Triton X-100 and peptidase activity was measured by chromogenic assay with the p-nitroanilid derivative of Asp-Glu-Val-Asp as substrate (DEVD-pNA). Substrates based on DEVD are specific for effector caspases, including caspase-3.27,28 DEVD-pNA cleaving activity was detected at low levels in lysates of resting platelets and was significantly increased on stimulation of platelets with the Ca²⁺ ionophore A23187 (P < .01), reaching maximal levels at 5

Fig 1. Detection of caspase-3 in resting and activated platelets. (A) Caspase-3–like peptidase activity (cleavage of DEVD-pNA) of Triton X-100 lysates of resting platelets (0 time point) and platelets activated by A23187. Data shown are the mean ± SEM (n = 5). Peptidase activity was significantly increased relative to resting platelets after 5, 10, and 20 minutes with A23187 (* P < .01). In the sample 20 min + DEVD, the inhibitor DEVD-fmk was present during platelet activation and peptidase assay. (B) Caspase-3 antigen detected by immunoblot. Resting platelets and platelets activated with A23187 for 10, or 20 minutes were stained with caspase-3–specific MoAb C31720. Molecular weight marker positions are shown on the left and an arrow indicates 32-kD procaspase-3. The lanes MNC (mononuclear cell lysates treated without and with granzyme B) control for the ability to detect active caspase-3, the large subunit of which (p17) is indicated by the arrow on the right. (C) Procaspase-3 antigen quantified by immunoblots. Data shown are the mean ± SEM (n = 4). Platelet content of procaspase-3 antigen was significantly decreased relative to resting platelets after 10 (* P < .02) and 20 minutes (* P < .003) with A23187.
and 40% ± 3% at 5 minutes, and FA-fmk, a chemically similar compound lacking caspase inhibitory activity, failed to inhibit PS exposure (Fig 2B). Lower concentrations of DEVD-fmk were also inhibitory, eg, 25 µmol/L inhibited A23187-induced PS exposure by 35% ± 5% at 3 minutes and 52% ± 2% at 5 minutes (n = 3).

Platelets also expose PS when stimulated by thrombin plus collagen, in which case the response is slower and less extensive: 25% ± 4% of platelets became PS-positive by 20 minutes (Fig 2C, no inhibitor). Pretreatment of platelets with DEVD-fmk, but not with FA-fmk (not shown), significantly inhibited platelet PS exposure induced by thrombin plus collagen (Fig 2C). DEVD-fmk at 25 µmol/L caused 85% ± 6% inhibition at 5 minutes, 45% ± 8% at 10 minutes, and 55% ± 10% inhibition at 20 minutes (Fig 2C). The finding of specific inhibition by DEVD-fmk of PS exposure in response to both A23187 and to thrombin plus collagen strongly indicates that a caspase-3–like enzyme is involved in agonist-induced translocation of platelet PS to the outer membrane leaflet.

Caspase inhibitor abrogates agonist-induced microparticle release. PS exposure in activated platelets is closely linked with the release of microparticles (Zwaal and Schroit1 and Discussion). The effect of DEVD-fmk on microparticle release was examined using a flow cytometric assay to quantify microparticles (Materials and Methods). Platelet pretreatment with DEVD-fmk, but not with FA-fmk, was found to substan-
tially inhibit/delay microparticle release in response to A23187 (Fig 3A). The extent of inhibition by 100 µmol/L DEVD-fmk was 42% ± 1% at 3 minutes and 32% ± 2% at 5 minutes (Fig 3A). Lower DEVD-fmk concentrations were also inhibitory; 25 µmol/L caused 25% ± 2% inhibition at 3 minutes and 8% ± 3% inhibition at 5 minutes (n = 3). For thrombin plus collagen-treated platelets, microparticle release was also inhibited by DEVD-fmk. DEVD-fmk at 25 µmol/L caused 57% ± 3% inhibition at 5 minutes, 47% ± 4% at 10 minutes, and 58% ± 7% at 20 minutes (Fig 3B); FA-fmk had no inhibitory effect (data not shown).

Caspase inhibitor fails to prevent agonist-induced platelet aggregation and secretion of α granules. Studies were performed to determine whether DEVD-fmk inhibits other platelet activation responses, including the early responses of aggregation and α granule secretion. Aggregometer tracings showed that pretreatment with DEVD (100 µmol/L) did not alter the time course or amplitude of the aggregation response to A23187 (Fig 4A) or to thrombin (Fig 4B). To monitor α granule secretion, we measured surface expression of the α granule membrane marker CD62P, an α granule membrane protein. Pretreatment with DEVD-fmk failed to alter thrombin-induced upregulation of CD62P. Thirty seconds after thrombin addition, CD62P expression, which was negative on resting platelets, was partially upregulated in both DEVD-fmk-pretreated and control platelets and was maximally upregulated in both after 60 seconds (Fig 4C). DEVD also failed to prevent agonist-induced platelet shape change, which was detected in flow cytometry by the alteration of forward and side scatter profiles (data not shown). These findings strongly suggest that caspase-3 is not required for the early platelet activation responses of α granule secretion, shape change, and aggregation.

Caspase inhibitor abrogates agonist-induced cleavage of platelet moesin. The effect of DEVD-fmk was also examined on another late platelet activation response, cleavage of the cytoskeletal linker protein moesin. In the absence of inhibitor, A23187 induced cleavage of 60% ± 5% (n = 4) of moesin molecules in 20 minutes (Fig 5A, lanes 1 and 2). Cleavage of
moesin was substantially inhibited in platelets pretreated with DEVD-fmk (lane 3). The extent of inhibition of A23187-induced moesin cleavage increased over the DEVD-fmk range of 5, 25, and 50 µmol/L and was complete at 50 and 100 µmol/L (Fig 5B). Moreover, A23187 induced the conversion of procalpain to calpain in platelets preincubated with DEVD-fmk, but not in calpeptin-pretreated platelets (Fig 6, top panel, last 4 lanes). Similarly, A23187 induced the processing of procaspase-3 in platelets preincubated with calpeptin, but not in DEVD-fmk–pretreated platelets (lower panel, last 4 lanes). These findings indicate that the alteration of platelet function by DEVD-fmk does not rely on acting through calpain; similarly, the effects of calpeptin do not entail acting through caspase-3.

Effects of calpain inhibitor and caspase inhibitor on PS exposure and microparticle release. To compare the roles of calpain and caspase, platelets were pretreated with calpeptin and examined for agonist-induced PS exposure and microparticle release. Similar to previous reports, calpeptin pretreatment significantly inhibited microparticle release in response to thrombin plus collagen (Fig 7B). The extent of inhibition of microparticle release was similar for calpeptin (69% ± 6% inhibition at 20 minutes; Fig 7B) and DEVD-fmk (58% ± 7% inhibition at 20 minutes; Fig 3B), and the inclusion of DEVD-fmk together with calpeptin did not further inhibit residual microparticle release (Fig 7D).

On the other hand, calpeptin pretreatment did not inhibit PS exposure in response to thrombin plus collagen; rather, PS

![Fig 5. Inhibition by DEVD-fmk of A23187-induced cleavage of platelet moesin. (A) Platelets were preincubated for 1 hour without inhibitor or with DEVD-fmk (50 µmol/L) and treated for 20 minutes with A23187. The platelet suspensions were lysed by the addition of SDS (with protease inhibitors). Shown is an immunoblot stained with clone 38 antimoesin MoAb. (B) Quantitation of moesin immunoblots. The methods used are the same as in (A), except that platelets were preincubated with varying concentrations of FA-fmk, YVAD-fmk, and DEVD-fmk. Shown are the mean percentages of inhibition (±SEM, n = 4) of moesin cleavage relative to platelets preincubated without inhibitor.

![Fig 6. Effects of pretreatment with calpeptin or DEVD-fmk on the content of µ-procalpain/µ-calpain (top panel) and procaspase-3 (lower panel) in resting and A23187-treated platelets. Platelets were preincubated with no inhibitor or calpeptin (50 µg/mL) or DEVD-fmk (25 µg/mL) and were lysed by the addition of SDS, immediately or after stimulation with A23187 for 10 or 20 minutes. Shown are immunoblots of 1.5 x 10^7 platelets stained with B27D8 anti-µ-calpain (top panel) or C31730 anti-caspase-3 (lower panel) MoAb.]}
exposure was significantly increased (140% ± 8% increase at 20 minutes of stimulation; Fig 7A). Calpeptin pretreatment also slightly increased spontaneous PS exposure (Fig 7A, 0-minute value). Enhancement of thrombin plus collagen-induced PS exposure was observed also when platelets were pretreated with the chemically unrelated cell permeant calpain inhibitor E64d (data not shown), and enhancement by E64d was previously reported for A23187-induced PS exposure.18 Inclusion of DEVD-fmk together with calpeptin in the platelet pretreatment significantly and substantially inhibited PS exposure in response to thrombin plus collagen, including abrogation of the enhancing effect of calpeptin (Fig 7C). Together, these findings show that caspase and calpain both function in specific late events of platelet activation and that the two proteases have distinct functional roles.

DISCUSSION

Our studies with specific reagents demonstrate the presence of at least one caspase family protease in platelets. Peptidase activity corresponding to the effector protease caspase-3 was detected in lysates of resting platelets and was significantly increased in A23187-activated platelets. Specific antibodies identified the zymogen procaspase-3 in resting platelets, and stimulation with agonist induced processing of procaspase-3. Active platelet caspase-3 appears to be short-lived, because it did not accumulate at levels adequate for detection by immunoblot.

Processing/activation of platelet procaspase-3 was induced by several characterized platelet agonists. The order of agonist effectiveness in activating procaspase-3, A23187 > thrombin > collagen > either thrombin or collagen (Table 1), is the same as their order of effectiveness in inducing PS exposure,25,26 microparticle release,25,26 and moesin cleavage.16a

Three agonist-induced platelet activation responses were found to be significantly inhibited by the specific caspase inhibitor DEVD-fmk, which has the greatest specificity for the effector proteases caspase-3 and caspase-7.7,8 DEVD-fmk inhibited A23187-induced cleavage of the cytoskeletal protein moesin in a dose-dependent manner and inhibition was complete at 50 µmol/L. DEVD-fmk inhibited exposure of platelet PS and release of microparticles in response to A23187 and to thrombin plus collagen. The extent of DEVD-fmk inhibition of PS exposure was dose-dependent. The finding of inhibition of these three platelet responses by DEVD-fmk strongly indicates that caspase-3, or a caspase with specificity similar to caspase-3, is required for a subset of late-occurring platelet activation events.
moein cleavage, PS exposure, and microparticle release. In contrast, treatment with DEVD-fmk had no effect on other (earlier) events of platelet activation, including α-granule secretion, shape change, and aggregation. Thus, the caspase-3 inhibitor is not a global inhibitor of platelet responses, but rather is directed to a subset of events, including those that are important for the generation of the procoagulant platelet and microparticle surfaces.

Another protease, calpain, was previously shown to be involved in a subset of platelet activation events, not including aggregation, shape change, or secretion, and we, therefore, performed parallel testing of calpain inhibitors and caspase inhibitors. These experiments showed that the calpain inhibitor calpeptin and the caspase-3 inhibitor DEVD-fmk are comparably effective in preventing microparticle release in thrombin plus collagen-treated platelets. On the other hand, calpeptin pretreatment substantially increases PS exposure in response to thrombin plus collagen, whereas DEVD-fmk inhibits agonist-induced PS exposure, including abrogating the increase effected by calpeptin.

Whereas DEVD-fmk completely inhibited agonist-induced moein cleavage, inhibition of PS exposure was only partial. Possibly, low levels of caspase that escape inhibition may suffice to support partial PS exposure, but not moein cleavage. Alternatively, caspase may be absolutely required for moein cleavage, but platelets may have caspase-dependent and caspase-independent pathways for activating PS exposure. Support for the latter possibility is provided by the finding that PS exposure in nucleated cells undergoing apoptosis is also abrogated by inhibitors of caspase-3, including DEVD-fmk. However, in Jurkat T cells, PS exposure is caspase-dependent when apoptosis is induced by Fas ligand, but caspase-independent when the inducing agent is cytolytic granules.

For rapid exposure of PS, platelets rely on a Ca2+-dependent mechanism, likely involving the recently cloned lipid scramblase, a transmembrane protein with a Ca2+ binding motif that rapidly moves phospholipids between membrane leaflets. Other requirements for activation of this mechanism are not known. Induction of platelet PS exposure and microparticle release generally occur as linked processes. For example, thrombin, a potent stimulator of aggregation, is a weak inducer of both PS exposure and microparticle release, which are best stimulated by A23187 or thrombin + collagen or collagen in that order of effectiveness. In Scott syndrome, an inherited bleeding disorder characterized by impaired lipid scramblase activity, ie, failure of agonist-induced PS exposure, patient platelets also fail to release microparticles. By strictly regulating intracellular Ca2+ levels or through the use of calpain inhibitors, PS exposure can be induced without microparticle release (eg, Fig 7), but agonist-induced microparticle release has not been observed in the absence of PS exposure. Such instances of apparent linkage have led several investigators to hypothesize that PS exposure (loss of lipid asymmetry) is required for microparticle release.

Thus, of the agonist-induced activation events examined in this study, aggregation, shape change, and granule secretion, previously found to be unaffected by calpain inhibition, are also unaffected by caspase-3 inhibition (Table 2). PS exposure was inhibited by caspase-3 inhibitor and not by calpain inhibitor; rather, (agonist-induced) PS exposure was substantially enhanced in calpeptin-pretreated platelets. In a recent study, another platelet activation event, filopod extension in response to A23187, was also enhanced in calpeptin-pretreated platelets. Finally, moein cleavage and microparticle release are prevented by either calpain inhibition or caspase-3 inhibition (Table 2).

Together, these findings strongly indicate that caspase-3 functions in agonist-induced activation of the PS exposing mechanism (Table 3), possibly by cleavage/activation of a component of the PS exposing enzyme complex or by cleavage/destruction of an inhibitory/regulatory protein. Based on the findings that PS exposure is potentiated by calpeptin and E64d, we hypothesize that calpain normally terminates the action of the PS exposing system (Table 3). Calpain may act also to terminate filopod extension. Whether caspase-3 acts directly at the level of micro particle release cannot be concluded from the present data, because inhibition of PS exposure by DEVD-fmk may suffice to prevent microparticle release. Likewise, the apparent requirement for both calpain and caspase-3 to cleave moein requires further study. These cumulative findings confirm a direct role for calpain in microparticle release and demonstrate a distinct role for caspase-3 in activating PS exposure in response to agonist.

Although best known as effectors of apoptosis, caspases function also in maturation of the cytokines, interleukin-1 by caspase 137,38 and interleukin-16 by caspase 3.39 Also, caspase-3 activity was found in nonapoptotic mitogen-activated T cells, and a caspase-3–like protease functions in terminal differentiation of lens epithelial cell. The present platelet findings

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<th>Hypothesized Direct Action of</th>
<th>Caspase-3</th>
<th>Calpain</th>
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<tbody>
<tr>
<td>α-Granule release</td>
<td>–</td>
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<tr>
<td>Shape change</td>
<td>–</td>
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<td>Aggregation</td>
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</tr>
<tr>
<td>Moein cleavage</td>
<td>(+)*</td>
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*(+)* indicates uncertain.
represent an additional example of caspase function in a nonapoptotic setting.

Recent studies have identified naturally occurring human caspase inhibitors, the IAP (inhibitors-of-apoptosis) family of protease inhibitors and the related tumor cell protein survivin. These discoveries suggest pharmacological therapies to induce apoptosis, eg, of tumor cells, or to prevent apoptosis, eg, in neurodegenerative disease. These efforts have relevance to platelet function in coagulation and thrombosis in that agents that inhibit caspases may be useful to prevent pathological thrombotic events. From another perspective, it would be prudent that clinical trials to alter apoptosis by targeting caspases be designed bearing in mind the possible effect of the therapy on platelet function in coagulation and thrombosis.

Altogether, these findings provide a basis for in-depth studies of the molecular mechanisms of caspase family protease action in platelet activation and programmed generation of procoagulant platelets.

ACKNOWLEDGMENT

The authors thank Drs Zhinan Xia and Judy Lieberman for providing granzyme B, Dr Anthony Bretscher for advice, Dr Dianne Kenney for advice and the use of equipment, and Drs John Hartwig and Andrey Prodeus for critical reading of the manuscript. We are grateful to the blood donors for their cooperation.

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