Journal of Cellular Biochemistry 66:54–64 (1997)

Calpain Activation in Shear-Induced Platelet Aggregation

Kazumasa Fujitani,[1]* Jun-ichi Kambayashi,[1] Hideo Ariyoshi,[1] Masato Sakon,[1] Shinobu Imajoh-Ohmi,[2] Takashi Nakamura,[1] and Morito Monden[1]

[1]Hematology Research Unit, Department of Surgery II, Osaka University Medical School, Osaka, Japan
[2]Institute of Medical Science, Tokyo University, Tokyo, Japan

Abstract Fluid shear stress has been known to activate platelet reaction such as aggregation, but the exact mechanism of shear-induced platelet aggregation (SIPA) has not been fully understood. Calpain, an intracellular calcium-activated cysteine protease, is abundant in platelets and is considered to be activated and involved in the proteolytic processes during platelet activation. A possible activation of calpain in SIPA was investigated, employing a newly developed aggregometer and specific monoclonal antibodies to detect activation of calpain. When a shear stress gradient varying between 6 and 108 dyn/cm^2 was applied to platelets, activation of μ-calpain was observed only in high-shear-stressed platelets, resulting in the proteolysis of talin. At 1 min after the onset of constant high shear stress of 108 dyn/cm^2, μ-calpain activation and proteolysis of talin were detected and increased in a time-dependent manner. Constant shear stress more than 50 dyn/cm^2, applied for 5 min, caused μ-calpain activation and proteolysis of talin, which were increased in a shear-force-dependent manner. Calpeptin, a calpain-specific peptide antagonist, caused the complete inhibition of both μ-calpain activation and proteolysis of talin, while SIPA profiles with calpeptin showed almost no change compared to those without calpeptin. These results suggest the possibility of calpain involvement in late phases of shear-induced platelet activation such as cytoskeletal reorganization. J. Cell. Biochem. 66:54–64, 1997.

Key words: calpain activation; platelet; proteolysis of talin; shear stress; shear-induced platelet aggregation (SIPA)

Platelet aggregation is essential for physiological haemostasis and contributes to the pathogenesis of arterial thrombosis. Recent evidence has suggested that shear-induced platelet aggregation (SIPA) is an important mechanism of thrombogenesis at arterial bifurcations or stenoses [Fox and Hugh, 1966; Goldsmith et al., 1976]. In stenosed atherosclerotic vessels and vasospasm, fluid shear stress may exceed the normal time average level of 20 dyn/cm^2 [Back et al., 1977; Turitto, 1982]. In vitro platelet aggregation occurs at a fluid shear stress of 30–60 dyn/cm^2 applied for 30 s [Moake et al., 1986]. The molecular mechanism of SIPA is incompletely understood. It is known that interaction of fibrinogen with glycoprotein (GP) IIb/IIIa is required at low shear stress, while interaction of von Willebrand factor (vWF) with both Gp Ib and Gp IIb/IIIa is required at high shear stress [Peterson et al., 1987; O'Brien and Salmon, 1987; Ikeda et al., 1991], leading to the transmembrane influx of Ca^{2+} [Chow et al., 1992; Ikeda et al., 1993] and the activation of protein kinase C (PKC) [Kroll et al., 1993]. Kroll et al. [1993] have recently shown that a pathological level of arterial wall shear stress (90 dyn/cm^2) induces diacylglycerol (DG)-independent PKC activation and that calcium chelators inhibit shear stress–induced p47 phosphorylation, a Mr 47,000 PKC-dependent substrate, suggesting the possibility that calcium-activated proteases play a role in platelet PKC activation associated with shear stress.

Calpains (EC 3, 4, 22, 17) are intracellular calcium-activated cysteine proteases found ubiquitously in mammalian and avian tissues and cells [Murachi, 1983a, 1989; Kawashima et al., 1988]. Their activities are regulated mainly

Abbreviations used: EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

*Correspondence to: Kazumasa Fujitani, Hematology Research Unit, Department of Surgery II, Osaka University Medical School, 2-2, Yamadaoka, Suita, Osaka 565, Japan.

Received 8 February 1996; accepted 4 March 1997

by cytosolic calcium ions and an endogenous inhibitor calpastatin [Murachi, 1983a,b]. There are two types of calpains in human platelets: μ-calpain, micromolar calcium requiring protease, and m-calpain, millimolar calcium requiring protease [Suzuki et al., 1984]. As we have reported previously, more than 90% of the total calpain activity is derived from μ-calpain in human platelets [Tsujinaka et al., 1983]. A number of endogenous calpain substrates have been reported so far: cytoskeletal proteins such as actin binding protein (ABP) [Truglia and Stracher, 1981], talin [Beckerle et al., 1986], and spectrin [Fox et al., 1987] and enzyme proteins such as PKC [Kishimoto et al., 1983, 1989], cAMP-dependent protein kinase (A-Kinase) [Beer et al., 1984], myosin light chain kinase (MLCK) [Tsujinaka et al., 1988a], phospholipase C (PLC) [Low et al., 1984], tyrosine kinase (pp60src) [Oda et al., 1993], and phosphotyrosine phosphatase 1B (PTP-1B) [Frangioni et al., 1993]. Cleavages of these endogenous substrates suggest calpain involvement in the proteolytic process during platelet activation.

In most of the previous studies on SIPA [Moake et al., 1986; Peterson et al., 1987; Kroll et al., 1993], the aggregation was measured at one time point by determining the single platelet count after shear was applied. However, Ikeda et al. [1991, 1993] have recently developed an ingenious device for direct real-time and continuous measurement of SIPA, which has enabled the detailed investigation of SIPA. Using this newly developed equipment, we investigated the effect of fluid shear stress on activation of platelet calpain and the possibility of calpain involvement in shear-induced platelet activation. In addition, we have also examined the hydrolysis of cytoskeletal protein, especially talin, which is a well-known substrate of calpain, in shear-loaded platelets.

MATERIALS AND METHODS

Materials

Aspirin was purchased from NACALAI TESQUE Inc. (Kyoto, Japan). Prostaglandin I_2 (PGI_2) was a gift from Ono Pharmaceutical Co. (Osaka, Japan). Fibrinogen and mouse monoclonal antibody against talin (T-3287) were obtained from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated goat antimouse IgG was from Cappel Laboratories Inc. (Durham, NC). Alkaline phosphatase–conjugated goat antimouse IgG, alkaline phosphatase–conjugated goat antirabbit IgG, NBT (nitro blue tetrazolium) substrate, and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) substrate were purchased from SEIKAGAKU-KOGYO Inc. (Tokyo, Japan). Molecular weight standards for SDS–polyacrylamide gel electrophoresis and polyvinylidene difluoride (PVDF) membranes were obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of the highest analytical grade available. Purified multimeric vWF was kindly provided from Dr. Yoshihiro Fujimura (Department of Blood Transfusion, Nara Medical University, Nara, Japan). Mouse monoclonal IgG antibody 1A8A2, specific for both a native (80 KDa) and an activated form of μ-calpain (76 KDa) [Kasai et al., 1986], was a generous gift from Dr. Sei-ichi Kawashima (Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Rabbit monoclonal IgG antibody anti-ACTμ, specific for an activated form of μ-calpain (76 KDa), was prepared as previously reported [Kikuchi and Imajoh-Ohmi, 1995].

Preparation of Washed Platelets

Platelet-rich plasma (PRP), obtained by the centrifugation of blood from healthy donors containing one-tenth volume of 3.8% trisodium citrate, was incubated with 1 mM of aspirin for 30 min at room temperature (RT). PRP was then mixed with 0.3% volume of 5 μM PGI_2 solution and was centrifuged at 850*g* for 15 min at RT. The platelet pellet was washed twice with HEPES-buffered saline (pH 7.4, 10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 5 mM glucose) containing 1 μM of PGI_2. Platelets were suspended in HEPES-buffered saline without PGI_2 at a final concentration of 4.0×10^8/ml.

Shear-Induced Platelet Aggregation

SIPA was induced by a method described in detail by Ikeda et al. [1991, 1993]. Briefly, the modified cone-and-plate-type viscometer (Cell Function Analyzer; Medical Devices and Diagnostics Research Lab, Toray Industries Inc., Shiga, Japan) was used for continuous measurement of SIPA. Washed platelet suspension (400 μl) was applied onto the center of the polymethylmethacrylate plate in the presence of 10 μg/ml purified multimeric vWF, 100 μg/ml fibrinogen, and 1 mM $CaCl_2$ and then exposed to varying shear stress by rotating the cone located 40 μm above the plate at 24°C. Aggregation was moni-

tored continuously by recording the intensity of the light transmitted through the platelet suspension from the beginning of application of the shear forces. In this system, Lambert-Beer's law is applicable to the relation between transmitted light intensity and single platelet count [Fukuyama et al., 1989]. The extent of platelet aggregation was calculated according to Lambert-Beer's equation [Ikeda et al., 1991] and expressed as percent of light transmission, adjusting the device at 0% transmittance with initial washed platelet suspension and 100% transmittance with suspension buffer, similar to other commercial aggregometers. In selected experiments, washed platelets were preincubated for 5 min at RT with 30 μM of calpeptin (in dimethyl sulfoxide, 0.1% final, v/v), a cell-permeable, calpain-specific inhibitor [Tsujinaka et al., 1988b; Ariyoshi et al., 1991], and then exposed to varying shear stress. The remaining procedure was the same as mentioned above.

Western Blot Analysis of μ-Calpain and Talin

For Western blotting of μ-calpain and talin, SIPA was terminated at the designated time points by adding an SDS-containing buffer (2% sodium dodecyl sulfate (SDS), 2% mercaptoethanol, 10% glycerol, 30 mM Tris-HCl, 5 mM EDTA, pH 6.8, 0.001% Bromophenol Blue) followed by immediate boiling for 5 min. SDS-containing sample (20 μl) was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels or on 4–20% exponential gradient polyacrylamide gels, according to the procedure of Laemmli [1970]. Proteins were subsequently transferred from the gels onto 0.2 μm pore size PVDF membranes by the method of Towbin et al. [1979]. PVDF membranes were incubated overnight at 4°C with monoclonal antibody against μ-calpain or against talin and developed either with horseradish peroxidase–conjugated goat antimouse IgG and 3,3′-diaminobenzidine tetrahydrochloride or with alkaline phosphatase–conjugated goat antirabbit/antimouse IgG, NBT substrate, and BCIP substrate.

RESULTS

Activation of μ-Calpain and Proteolysis of Talin in Sheared Platelets

To explore the possibility of μ-calpain activation in SIPA, a shear stress gradient varying between 6 and 108 dyn/cm^2 was applied to the platelet suspension over a 6 min period (Fig. 1A). After an initial 15 s at 6 dyn/cm^2, the gradient varied between 6 and 12 dyn/cm^2 in 90 s, resulting in an increase in platelet aggregation. In the ensuing 120 s, the gradient varied between 12 and 108 dyn/cm^2, resulting initially in a decrease in aggregation and afterwards in a more pronounced second increase in aggregation. After the shear force reached 108 dyn/cm^2, it was maintained at that level for an additional 135 s, during which a continuous increase in platelet aggregation was observed. In Figure 1A, four different aggregation curves terminated at four different points are shown. Platelets obtained from the following five points, as shown in Figure 1A, were subjected to Western blotting: point 1, time 0; point 2, peak portion of low-shear-induced platelet aggregation; point 3, disaggregation portion; point 4, middle portion of high-shear-induced platelet aggregation; and point 5, end portion of high-shear-induced platelet aggregation. An activated form of μ-calpain (76 KDa) was detected at points 4 and 5 (Fig. 1B). Proteolysis of talin was observed in the same manner. Increased degradation of talin (190 KDa) was also seen at points 4 and 5 (Fig. 1C).

Time-Dependent Effect of Shear Stress on μ-Calpain Activation and on Proteolysis of Talin

In these experiments, a shear stress gradient varying between 6 and 108 dyn/cm^2 was applied to the platelet suspension (Fig. 2A). After an initial 15 s at 6 dyn/cm^2, the gradient varied between 6 and 108 dyn/cm^2 in 45 s, and the constant shear stress of 108 dyn/cm^2 was applied for various time periods. In Figure 2A, five different aggregation curves terminated at the designated time points are shown. Platelets obtained at each time point were subjected to Western blotting. An activated form of μ-calpain (76 KDa) was detected at 2 min and increased in a time-dependent manner (Fig. 2B). A corresponding time-dependent increase in the degradation of talin was also observed (Fig. 2C).

Dose-Dependent Effect of Shear Stress on μ-Calpain Activation and on Proteolysis of Talin

In these experiments, after an initial 15 s at 6 dyn/cm^2, the shear stress gradient varied between 6 and the designated degree of force in 45 s, and then the designated constant shear stress ranging from 12–108 dyn/cm^2 was applied to

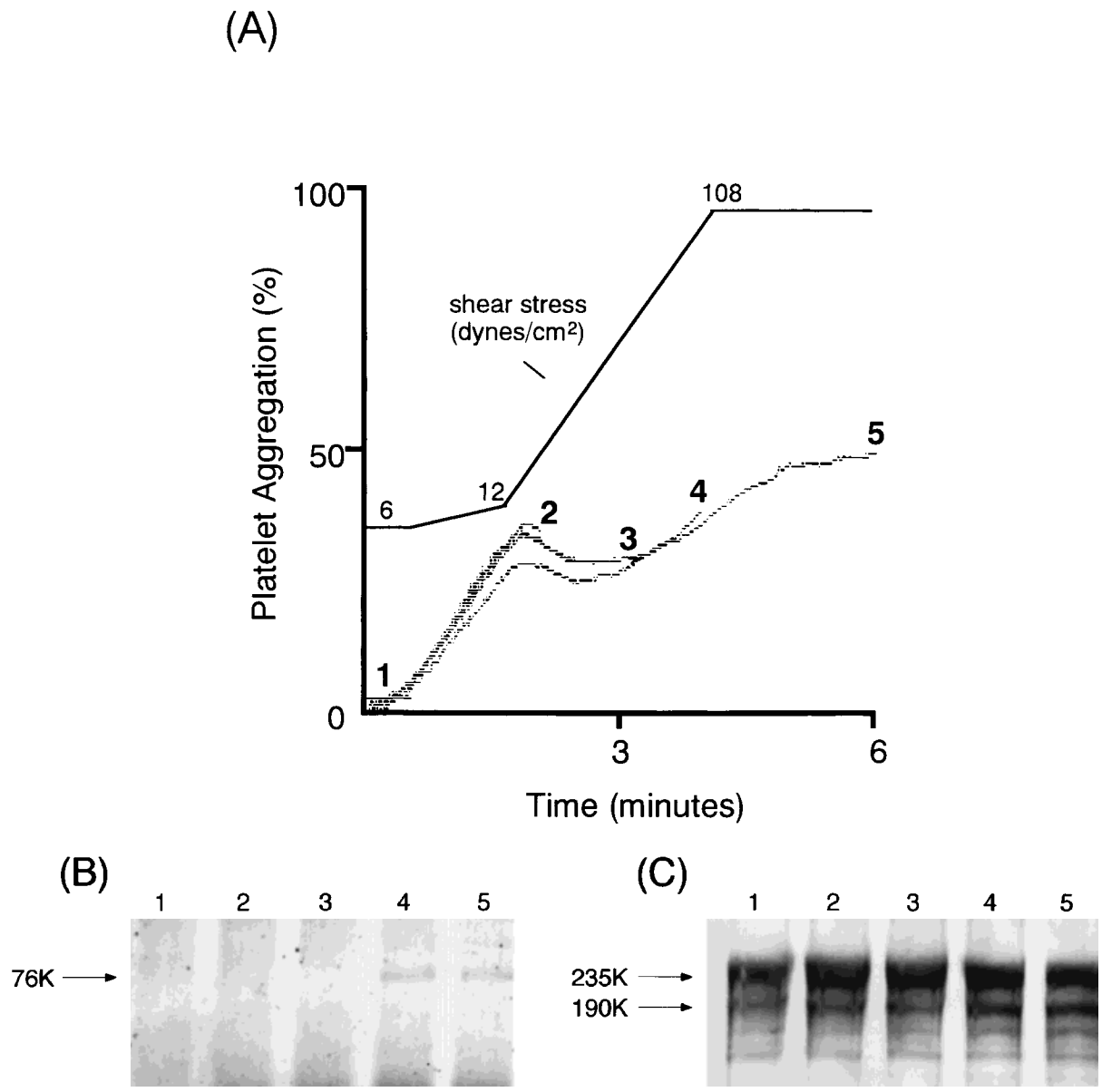

Fig. 1. Activation of μ-calpain and proteolysis of talin in SIPA. A shear stress gradient as shown was applied to the platelet suspension. Four different SIPA profiles terminated at four different points are shown (**A**). Platelets obtained from the designated five points were subjected to SDS-PAGE and electroblotted onto PVDF membranes. The membranes were then immunostained with monoclonal antibody specific for an activated form of μ-calpain (**B**) or against talin (**C**) as described in Materials and Methods. These data are representative of three separate experiments.

the platelet suspension for 5 min (Fig. 3A). Five different aggregation curves induced by the designated degree of constant shear stress are shown. Constant shear stress of 108 and 80 dyn/cm^2 induced the same extent of aggregation, and that of 50, 18, and 12 dyn/cm^2 induced another lower degree of aggregation. Platelets aggregated by each constant shear stress were subjected to Western blotting. An activated form of μ-calpain (76 KDa) was detected at shear stress of more than 50 dyn/cm^2 and increased in a shear force–dependent manner (Fig. 3B). Degradation product of talin (190 KDa) was also increased in the same manner as μ-calpain (Fig. 3C).

Effect of Calpeptin on SIPA, Shear-Induced μ-Calpain Activation, and Proteolysis of Talin

In order to further clarify whether μ-calpain activation is essential in SIPA and whether

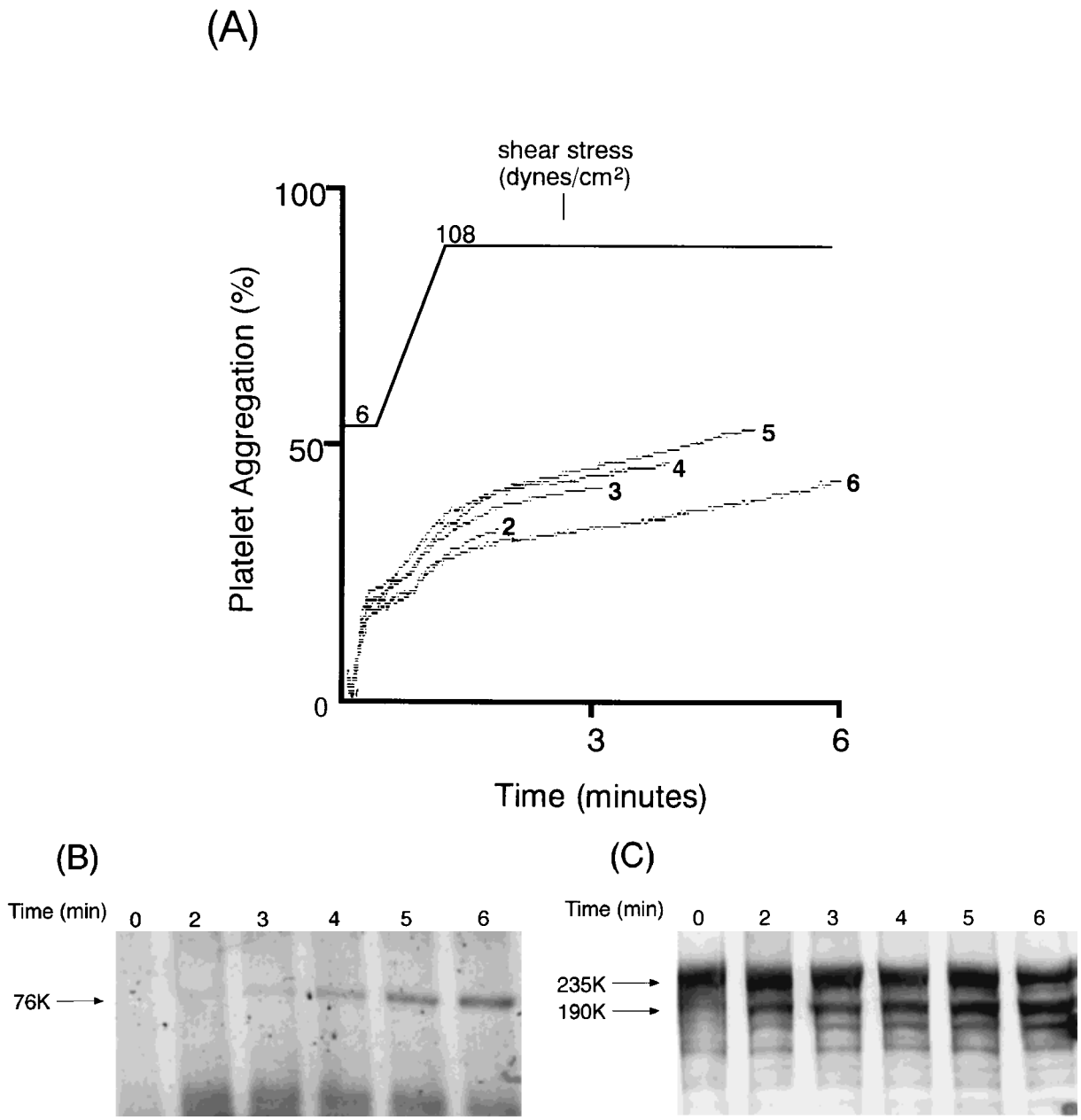

Fig. 2. Time-dependent effect of shear stress on μ-calpain activation and on proteolysis of talin. A shear stress gradient as shown was applied to the platelet suspension. After the shear stress reached 108 dyn/cm^2 at 1 min, constant shear stress of 108 dyn/cm^2 was applied for various time periods. Five different SIPA profiles terminated at the designated time points (min) are shown (**A**). Platelets obtained at each time point were subjected to Western blotting for an activated form of μ-calpain (**B**) or for talin (**C**) as described in Fig. 1. These data are representative of three separate experiments.

such an activation is involved in the proteolysis of talin, we preincubated washed platelets with 30 μM of calpeptin, a calpain-specific inhibitor, for 5 min at RT and then exposed them to varying shear stress. The same shear stress gradient as mentioned in Figure 1A was applied. 4 different aggregation curves terminated at 4 different points are shown in Figure 4A. Platelets obtained from the same five points as described in Figure 1A were subjected to Western blotting. An activated form of μ-calpain (76 KDa) was not detected at any point (Fig. 4B). Only a native form of μ-calpain (80 KDa) was observed (Fig. 4C). Proteolysis of talin was also not observed (Fig. 4D). Baseline talin degradation product (190K), which was

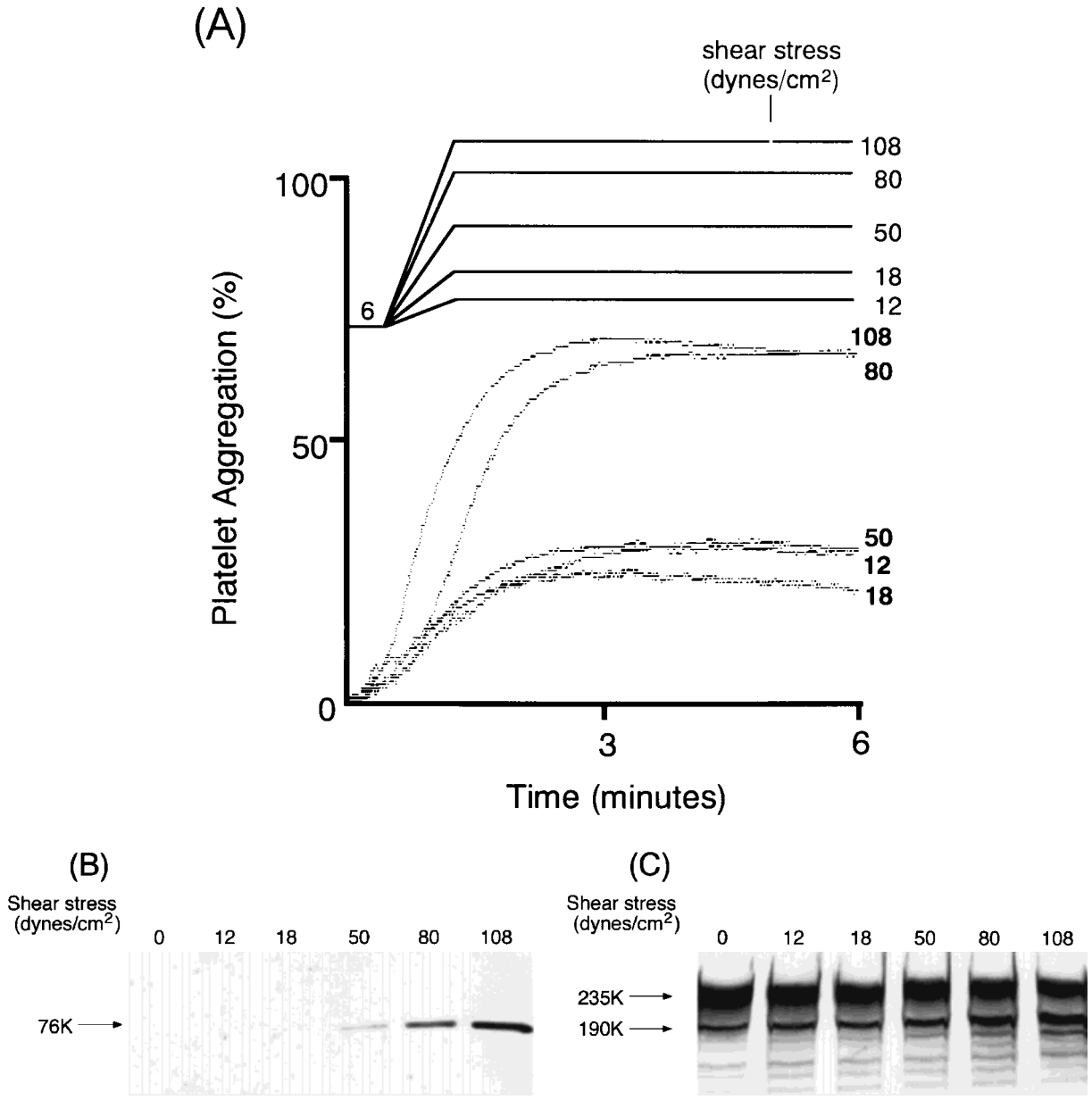

Fig. 3. Dose-dependent effect of shear stress on μ-calpain activation and on proteolysis of talin. Five different shear stress gradients as shown were applied to the platelet suspension. After the shear stress reached the designated degree of force at 1 min, constant shear stress ranging from 12–108 dyn/cm^2 was applied for 5 min. Five different SIPA profiles induced by various degrees of constant shear stress are shown (**A**). Platelets aggregated by each constant shear stress were subjected to Western blotting for an activated form of μ-calpain (**B**) or for talin (**C**) as described in Fig. 1. These data are representative of three separate experiments.

observed at time 0 in Figures 1C, 2C, and 4D, was due to preparation maneuvers of washed platelets. Though there appears to be a difference in the amount of baseline talin degradation product among figures, each degradation product (190K) was densitometrically quantitated to be 20.1–26.4% of whole talin molecule (190K + 235K). In spite of the complete inhibition of μ-calpain activation by calpeptin, these SIPA profiles in Figure 4A showed almost no change compared to those without calpeptin in Figure 1A.

DISCUSSION

SIPA at high shear stress is irreversible and reflects the interaction between vWF and its

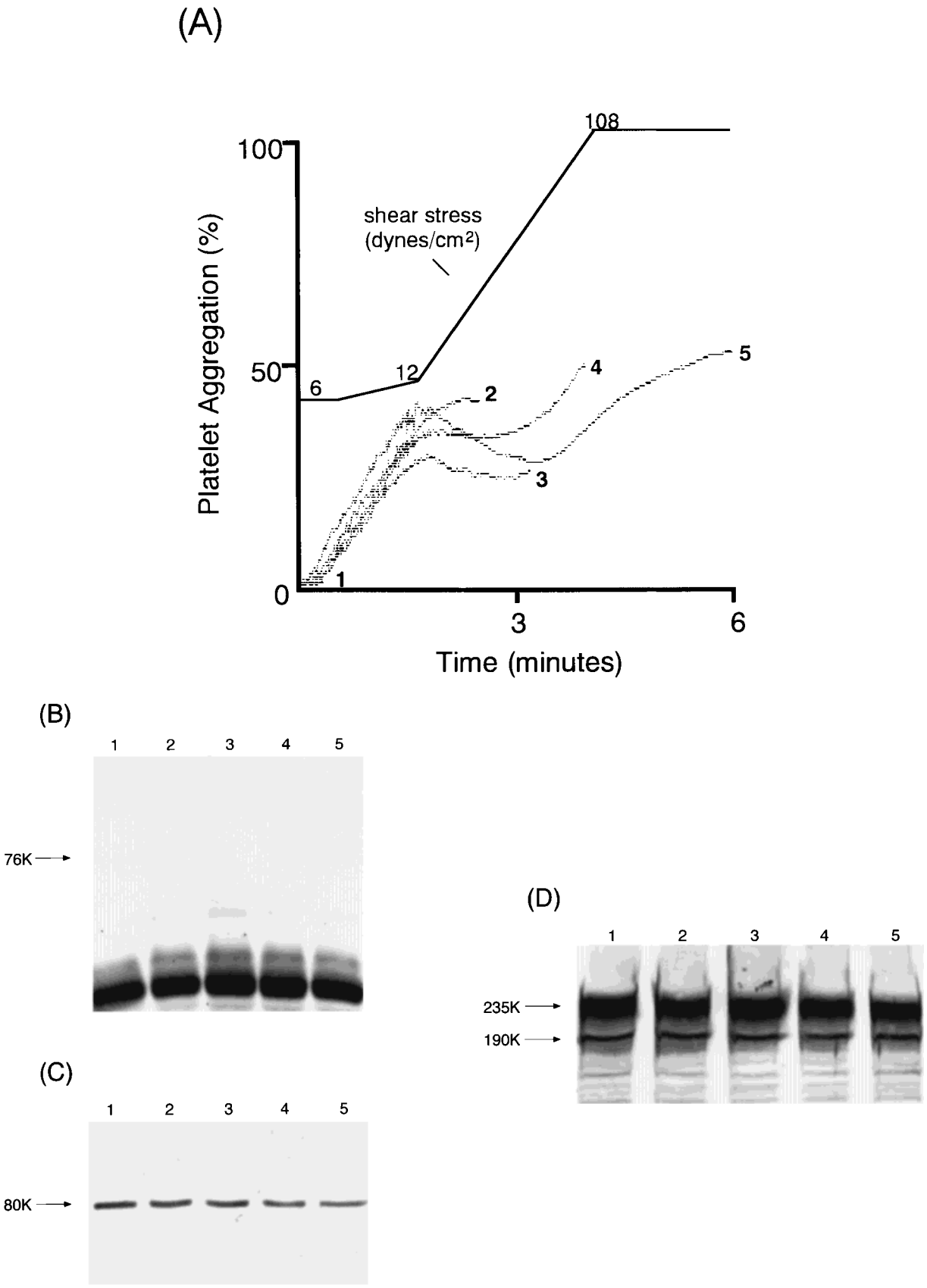

Fig. 4. Effect of calpeptin on SIPA, shear-induced μ-calpain activation, and proteolysis of talin. Washed platelets were preincubated with 30 μM of calpeptin for 5 min at RT and then exposed to a shear stress gradient as shown. 4 different SIPA profiles terminated at 4 different points are shown (**A**). Platelets obtained from the designated five points were subjected to SDS-PAGE and electroblotted onto PVDF membranes. The membranes were then immunostained with monoclonal antibody specific for an activated form of μ-calpain (**B**), specific for both native and activated forms of μ-calpain (**C**) or against talin (**D**) as described in Materials and Methods. These data are representative of three separate experiments.

platelet receptors, GP Ib-IX and Gp IIb/IIIa, whereas SIPA at low shear stress is reversible and assumed to reflect the binding of fibrinogen to GP IIb/IIIa [Peterson et al., 1987; O'Brien and Salmon, 1987; Ikeda et al., 1991]. Platelet aggregation induced by high shear stress is known to occur at 81 ± 20.1 dyn/cm^2 in normal citrated PRP [Murata et al., 1993]. As shown in Figure 1, our SIPA profiles in washed platelets showed the close similarity to those in PRP, and μ-calpain activation and proteolysis of talin were observed only in the irreversible phase of platelet aggregation at high shear stress. In accordance with these findings, Frangioni et al. [1993] reported that PTP-1B cleavage catalyzed by calpain occurred in irreversible platelet aggregation, not in reversible platelet aggregation, induced by ADP (adenosine 5′-diphosphates). Other calpain substrates such as ABP [Fox et al., 1993] and pp60src [Oda et al., 1993] are also cleaved in an aggregation-dependent manner. Fox et al. [1993] demonstrated that the binding of the extracellular domain of GpIIb/IIIa to its adhesive ligand was required for calpain activation. In SIPA, binding of vWF to GPIIb/IIIa under a high shear stress may trigger the activation of calpain, although the detailed mechanism by which integrin engagement leads to calpain activation is unclear.

As shown in Figure 2, μ-calpain activation and proteolysis of talin were detected at 1 min (the earliest time point studied) after the onset of high shear stress of 108 dyn/cm^2 and increased in a time-dependent manner. Consistent with these findings, calpain activation is detected at 30 or 60 s after the addition of thrombin to a platelet suspension [Fox et al., 1993; Ariyoshi et al., 1993a]. In addition, many calpain substrates, like ABP, talin, pp60src, and PTP-1B, are also shown to be cleaved in a time-dependent manner in washed platelets treated with A23187 or thrombin [Oda et al., 1993; Frangioni et al., 1993].

Activation of μ-calpain and proteolysis of talin were detected at shear stress of more than 50 dyn/cm^2 and increased in a shear force–dependent manner in Figure 3. Consistent with these findings, Chow et al. [1992] reported an increase of $[Ca^{2+}]_i$ (cytoplasmic Ca^{2+} concentration) induced by the binding of vWF to GpIb under the shear stress was proportional to the shear force. Comparing the results at 108 dyn/cm^2 with those at 80 dyn/cm^2, we see that the degree of μ-calpain activation was not proportional to the extent of platelet aggregation. Cleavages of ABP and PTP-1B catalyzed by calpain were shown to be proportional to the extent of platelet aggregation in washed platelets stimulated with thrombin [Fox et al., 1993; Frangioni et al., 1993]. Transmembrane signal, which activates intracellular calpain, initiated by the binding of vWF to GpIIb/IIIa under a high shear stress might not reflect directly the extent of platelet aggregation, although the exact nature of the transmembrane signal is not known. Constant shear stress of 50 dyn/cm^2 induced a lower degree of aggregation, accompanied by μ-calpain activation. Comparing this finding with the results shown in Figure 1, we see that even low shear stress, if it is lasting, may be able to cause μ-calpain activation.

Although calpeptin completely inhibited μ-calpain activation and proteolysis of talin in SIPA, SIPA profiles with or without calpeptin were closely similar (comparing those in Fig. 4A with those in Fig. 1A). These findings suggest that μ-calpain activation, leading to the proteolysis of talin, is not essential in SIPA. We previously showed that calpeptin does not inhibit platelet aggregation, the rise of $[Ca^{2+}]_i$, and inositol 1,4,5-trisphosphate (IP_3) formation induced by physiological agonists, even at doses which completely block cleavage of ABP and talin by calpain [Ariyoshi et al., 1991]. Calpain may not be involved in the early phases of platelet activation such as shape change, secretion, and aggregation induced not only by physiological agonists but also by physical shear stress. We have recently demonstrated the possibility of calpain involvement in the late phases of platelet activation such as cytoskeletal reorganization, leading to microparticle formation [Yano et al., 1993], and Ca^{2+} uptake [Ariyoshi et al., 1993b, 1995]. When platelets are exposed to a constant high shear stress of 108 dyn/cm^2, $[Ca^{2+}]_i$ has been shown to increase slowly in parallel with the aggregation [Ikeda et al., 1993], resulting in a pattern distinct from that generated by physiological agonists. In this study, we have shown that μ-calpain activation was detected at 1 min after the onset of a high shear stress of 108 dyn/cm^2 and increased in a time-dependent manner (Fig. 2). In addition, calpain is also considered to be involved in cytoplasmic Ca^{2+} restoration through the activation of dense tubular system Ca^{2+}-ATPase by limited proteolysis following platelet activation

[Ariyoshi et al., 1993b]. These findings suggest that calpain might play a role in delaying the $[Ca^{2+}]_i$ rise in the late phases of shear-induced platelet activation. From the physiological viewpoint, μ-calpain activation in SIPA might contribute to the pathogenesis of arterial thrombosis through microparticle formation, which is rich in coagulation factor-V and -VIII and thought to have important roles in the coagulation system [Gilbert et al., 1991], from aggregating platelets.

Recent studies indicate that shear-induced changes of platelet $[Ca^{2+}]_i$ are due entirely to the transmembranous influx of calcium, suggesting that PLC-mediated production of IP_3 is not involved in platelets responding to shear stress [Chow et al., 1992; Ikeda et al., 1993]. Kroll et al. [1993] have observed that a pathological level of arterial wall shear stress (90 dyn/cm^2) does not stimulate platelet PLC-mediated sn-1,2-diacylglycerol (DG) production or hydrolysis of the phosphatidylinositol 4,5-bisphosphate (PIP_2) but induces PKC activation, and in their report calcium chelators inhibited shear stress–induced PKC activation. Calpain might be involved in platelet PKC activation associated with shear stress. Cleavage of pp60src by calpain reduces its kinase activity 7.6-fold [Oda et al., 1993] and that of PTP-1B by calpain increases its enzymatic activity twofold [Frangioni et al., 1993]. These findings suggest the possibility of calpain involvement in altering tyrosyl phosphorylation. Razdan et al. [1994] observed a shear loading time– and shear stress-dependent tyrosine phosphorylation of various substrates. Shear stress may affect the balance between platelet tyrosine kinase and tyrosine phosphatase activity through μ-calpain activation.

In the present study, we investigated SIPA at 24°C. This temperature is not physiologic. However, SIPA at room temperature (22–25°C) in the cone-and-plate viscometer has been established and investigated in many previous studies [Chow et al., 1992; Goto et al., 1992, 1995; Alevriadou et al., 1993; Kroll et al., 1993; Razdan et al., 1994; Kamat et al., 1995; Oda et al., 1995, 1996]. SIPA at room temperature is known to enhance the detectability of the changes associated with SIPA, which would proceed in a more subtle manner at 37°C. In addition, processes of platelet activation in SIPA at room temperature are known to be accompanied by many physiologic changes, such as elevation of intracellular ionized calcium concentration [Chow et al., 1992; Ikeda et al., 1993], activation of protein kinase C [Kroll et al., 1993], and protein tyrosine phosphorylation [Razdan et al., 1994; Oda et al., 1995]. Thus, the present study performed at 24°C is believed to reflect the changes at the physiologic condition.

In conclusion, the present study indicates that activation of μ-calpain is observed in shear-stressed human platelets in a shear loading time– and shear strength–dependent manner, resulting in the proteolysis of specific endeogenous substrates such as talin. The activation is not a cause of SIPA but may have a significant role in cytoskeletal reorganization, signal transduction, and Ca^{2+} equilibrium in the late phases of shear-induced platelet activation by limited proteolysis of cytoskeletal proteins, PKC, and Ca^{2+}-ATPase.

ACKNOWLEDGMENTS

We thank Dr. Yoshihiro Fujimura (Department of Blood Transfusion, Nara Medical University) for the kind offer of purified multimeric vWF and Dr. Sei-ichi Kawashima (Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science) for supplying anti-μ-calpain antibody. We are grateful to Dr. Yasuo Ikeda (Department of Internal Medicine, Keio University School of Medicine) for providing the cone-and-plate viscometer for the measurement of SIPA and to Dr. Narendra Tandon for linguistic revision.

REFERENCES

Alevriadou BR, Moake JL, Turner NA, Ruggeri ZM, Folie BJ, Phillips MD, Schreiber AB, Hrinda ME, McIntire LV (1993): Real-time analysis of shear-dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. Blood 81:1263–1276.

Ariyoshi H, Shiba E, Kambayashi J, Sakon M, Tsujinaka T, Uemura Y, Mori T (1991): Characteristics of various synthetic peptide calpain inhibitors and their application for the analysis of platelet reaction. Biochem Int 23:1019–1033.

Ariyoshi H, Shiba E, Sakon M, Kambayashi J, Yoshida K, Kawashima S, Mori T (1993a): Translocation of human platelet calpain-I. Biochem Mol Biol Int 30:63–72.

Ariyoshi H, Shiba E, Kambayashi J, Sakon M, Kawasaki T, Yoshida K, Mori T (1993b): Stimulation of human platelet Ca^{2+}-ATPase and Ca^{2+} restoration by calpain. Cell Calcium 14:455–463.

Ariyoshi H, Kambayashi J, Sakon M (1995): Calpains and calpastatin in human blood platelets. Platelets 6:1–5.

Back LD, Radbill JR, Crawford DW (1977): Analysis of pulsatile, viscous blood flow through diseased coronary arteries of man. J Biomech 10:339–353.

Beckerle MC, Halloran TO, Burridge K (1986): Demonstration of a relationship between talin and P235, a major substrate of the calcium dependent protease in platelets. J Cell Biochem 30:259–270.

Beer DG, Butley MS, Malkinson AM (1984): Developmental changes in the endogenous Ca^{2+}-stimulated proteolysis of mouse lung cAMP-dependent protein kinases. Arch Biochem Biophys 228:207–219.

Chow TW, Hellums JD, Moake JL, Kroll MH (1992): Shear stress induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. Blood 80:113–120.

Fox JA, Hugh AE (1966): Localization of atheroma. A theory based on boundary layer separation. Br Heart J 28:388–399.

Fox JEB, Reynolds CC, Morrow JS, Phillips DR (1987): Spectrin is associated with membrane-bound actin filaments in platelets and is hydrolyzed by the Ca^{2+}-dependent protease during platelet activation. Blood 69: 537–545.

Fox JEB, Taylor RG, Taffarel M, Boyles JK, Goll DE (1993): Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin, glycoprotein IIb-IIIa. J Cell Biol 120:1501–1507.

Frangioni JV, Oda A, Smith M, Salzman EW, Neel BG (1993): Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. EMBO J 12:4843–4856.

Fukuyama M, Sakai K, Itagaki I, Kawano K, Murata M, Kawai Y, Watanabe K, Handa M, Ikeda Y (1989): Continuous measurement of shear-induced platelet aggregation. Thromb Res 54:253–260.

Gilbert GE, Sims PJ, Wiedmer T, Furie B, Furie BC, Shattil SJ (1991): Platelet-derived microparticles express high affinity receptors for factor VIII. J Biol Chem 266:17261–17268.

Goldsmith HL, Marlow JC, Yu SK (1976): The effect of oscillatory flow on the release reaction and aggregation of human platelets. Microvasc Res 11:335–359.

Goto S, Ikeda Y, Murata M, Handa M, Takahashi E, Yoshioka A, Fujimura Y, Fukuyama M, Handa S, Ogawa S (1992): Epinephrine augments von Willebrand factor–dependent shear-induced platelet aggregation. Circulation 86:1859–1863.

Goto S, Salomon DR, Ikeda Y, Ruggeri ZM (1995): Characterization of the unique mechanism mediating the shear-dependent binding of soluble von Willebrand factor to platelets. J Biol Chem 270:23352–23361.

Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, Anbo H, Kawai Y, Watanabe K, Itagaki I, Sakai K, Ruggeri ZM (1991): The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. J Clin Invest 87:1234–1240.

Ikeda Y, Handa M, Kamata T, Kawano K, Kawai Y, Watanabe K, Kawakami K, Sakai K, Fukuyama M, Itagaki I, Yoshioka A, Ruggeri ZM (1993): Transmembrane calcium influx associated with von Willebrand factor binding to Gp Ib in the initiation of shear-induced platelet aggregation. Thromb Haemost 69:496–502.

Kamat SG, Michelson AD, Benoit SE, Moake JL, Rajasekhar D, Hellums JD, Kroll MH, Schafer AI (1995): Fibrinolysis inhibits shear stress–induced platelet aggregation. Circulation 92:1399–1407.

Kasai Y, Inomata M, Hayashi M, Imahori K, Kawashima S (1986): Isolation and characterization of monoclonal antibodies against calcium activated neutral protease with low calcium sensitivity. J Biochem 100:183–190.

Kawashima S, Hayashi M, Saito Y, Kasai Y, Imahori K (1988): Tissue distribution of calcium-activated neutral proteinases in rat. Biochim Biophys Acta 965:130–135.

Kikuchi H, Imajoh-Ohmi S (1995): Activation and possible involvement of calpain, a calcium-activated cysteine protease, in down-regulation of apoptosis of human monoblast U937 cells. Cell Death Differ 2:195–199.

Kishimoto A, Kajikawa N, Shiota M, Nishizuka Y (1983): Proteolytic activation of calcium-activated, phospholipid-dependent protein kinase by calcium-dependent neutral protease. J Biol Chem 258:1156–1164.

Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominaga M, Kuroda T, Nishizuka Y (1989): Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem 264: 4088–4092.

Kroll MH, Hellums JD, Guo Z, Durante W, Razdan K, Hrbolich JK, Schafer AI (1993): Protein kinase C is activated in platelets subjected to pathological shear stress. J Biol Chem 268:3520–3524.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Low MG, Carroll RC, Weglicki WB (1984): Multiple forms of phosphoinositide-specific phospholipase C of different relative molecular masses in animal tissues. Biochem J 221:813–820.

Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellums JD (1986): Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress induced platelet aggregation. J Clin Invest 78:1456–1461.

Murachi T (1983a): Calpain and calpastatin. Trends Biochem Sci 8:167–169.

Murachi T (1983b): Intracellular Ca^{2+} protease and its inhibitor protein: Calpain and calpastatin. In Cheung WY (ed): "Calcium and Cell Function." New York: Academic Press, pp 377–410.

Murachi T (1989): Intracellular regulatory system involving calpain and calpastatin. Biochem Int 18:263–294.

Murata M, Fukuyama M, Satoh K, Fujimura Y, Yoshioka A, Takahashi H, Handa M, Kawai Y, Watanabe K, Ikeda Y (1993): Low shear stress can initiate von Willebrand factor–dependent platelet aggregation in patients with type IIB and platelet-type von Willebrand disease. J Clin Invest 92:1555–1558.

O'Brien JR, Salmon GP (1987): Shear stress activation of platelet glycoprotein IIb/IIIa plus von Willebrand factor causes aggregation: Filter blockage and the long bleeding time in von Willebrand's disease. Blood 70:1354–1361.

Oda A, Druker BJ, Ariyoshi H, Smith M, Salzman EW (1993): pp60src is an endogenous substrate for calpain in human blood platelets. J Biol Chem 268:12603–12608.

Oda A, Yokoyama K, Murata M, Tokuhira M, Nakamura K, Handa M, Watanabe K, Ikeda Y (1995): Protein tyrosine phosphorylation in human platelets during shear stress–induced platelet aggregation (SIPA) is regulated by glycoprotein (GP) Ib/IX as well as GP IIb/IIIa and requires intact cytoskeleton and endogenous ADP. Thromb Haemost 74:736–742.

Oda A, Miyakawa Y, Druker BJ, Ozaki K, Yabusaki K, Shirasawa Y, Handa M, Kato T, Miyazaki H, Shimosaka A, Ikeda Y (1996): Thrombopoietin primes human platelet aggregation induced by shear stress and by multiple agonists. Blood 87:4664–4670.

Peterson DM, Stathopoulos NA, Giorgio TD, Hellums JD, Moake JL (1987): Shear-induced platelet aggregation requires von Willebrand factor and platelet membrane glycoprotein Ib and IIb-IIIa. Blood 69:625–628.

Razdan K, Hellums JD, Kroll MH (1994): Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). Biochem J 302:681–686.

Suzuki K, Kawashima S, Imahori K (1984): Structure and function of Ca^{2+}-activated protease. In Ebashi S, Endo M, Imahori K, Kakiuchi S, Nishizuka Y (eds): "Calcium Regulation in Biological Systems." New York: Academic Press, pp 213–226.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Truglia JA, Stracher A (1981): Purification and characterization of a calcium dependent sulfhydryl protease from human platelets. Biochem Biophys Res Commun 100: 814–822.

Tsujinaka T, Shiba E, Kambayashi J, Kosaki G (1983): Purification and characterization of a low calcium requiring form of Ca^{2+}-activated neutral protease from human platelets. Biochem Int 6:71–80.

Tsujinaka T, Kajiwara Y, Kambayashi J, Sakon M, Mori T (1988a): Studies on myosin light chain phosphorylation in intact platelets, utilizing a cell penetrating thiol protease inhibitor. Thromb Res 51:365–372.

Tsujinaka T, Kajiwara Y, Kambayashi J, Sakon M, Higuchi N, Tanaka T, Mori T (1988b): Synthesis of a new cell penetrating calpain inhibitor (calpeptin). Biochem Biophys Res Commun 153:1201–1208.

Turitto VT (1982): Blood viscosity, mass transport, and thrombogenesis. Prog Hemost Thromb 6:139–177.

Yano Y, Shiba E, Kambayashi J, Sakon M, Kawasaki T, Fujitani K, Kang J, Mori T (1993): The effects of calpeptin (a calpain specific inhibitor) on agonist induced microparticle formation from the platelet plasma membrane. Thromb Res 71:385–396.