The role of protein phosphorylation and cytoskeletal reorganization in microparticle formation from the platelet plasma membrane

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Platelets activated by various agonists produce vesicles (microparticles; MPs) from the plasma membrane. However, the mechanism of this MP formation remains to be elucidated. To investigate the possible involvement of protein phosphorylation and cytoskeletal reorganization in MP formation, the effects of various inhibitors on MP formation were investigated. Flow cytometry was employed to detect the amount of MP formation by using monoclonal antibodies against glycoprotein (GP) IIb–IIIa (NNKY 1–32) or GPIIb (Tab). The relationship between changes in cytoskeletal architecture and MP formation in the platelets activated by thrombin plus collagen was observed by scanning electron microscopy (SEM). MPs were observed in the vicinity of the terminals of pseudopods, suggesting that MPs may be released by budding of the pseudopods. Cytochalasin D (10 μM) inhibited MP formation from the activated platelets almost completely. Moreover, SEM of the cytochalasin D-treated platelets revealed the absence of shape change, pseudopod formation and MPs. These findings suggest that cytoskeletal reorganization is necessary for MP formation. Since cytoskeletal reorganization is considered to be regulated by a dynamic phosphorylation–dephosphorylation process, we investigated the effects of the protein phosphatase inhibitors, calyculin A (CLA) and okadaic acid (OA), on MP formation. Flow cytometry showed that these two inhibitors doubled MP formation in activated platelets. SEM of the platelets treated with CLA or OA demonstrated more prominent shape change and pseudopod formation in these platelets than in those without inhibitor. From these results, we conclude that cytoskeletal reorganization, which is controlled by phosphorylation, is involved in MP formation.

INTRODUCTION

Activation of platelets by a variety of agonists induces the release of small membrane vesicles named microparticles (MPs). MPs have been shown to express a high density of binding sites for assembly of the tenase and prothrombinase enzyme complexes [1]. From these observations, MPs are considered to be clinically significant in various pathological conditions such as idiopathic thrombocytopenic purpura [2,3]. Despite the recent increase in interest in MPs, information on the mechanism of MP formation is limited. Only two studies, including one of ours, suggest that the release of MPs is related to an increased cytosolic Ca^{2+} level [4,5]. We have shown that calpeptin, which is a cell-permeant calpain (Ca^{2+}-dependent protease) inhibitor, partially inhibits MP formation from platelets activated by thrombin plus collagen or A23187, and concluded that calpain is partially involved in MP formation by the proteolytic degradation of membrane-associated cytoskeletal proteins [5]. The inhibition of MP formation by calpeptin was only partial (about 30%) and was more marked in the early stage of activation. These observations suggest that MP formation is not regulated by only one mechanism, but might be regulated by other unknown pathways in which calpain is not involved. These findings prompted us to explore further the mechanisms of MP formation. In the present study we examined the effects of various inhibitors, which affect the reorganization of the cytoskeletal architecture (cytoskeletal reorganization), on the MP formation and the morphological changes of activated platelets. As an indicator of actin polymerization, cytochalasin D was used [6,7]. Okadaic acid (OA) and calyculin A (CLA), compounds isolated from sea sponges, were used as specific inhibitors of protein phosphatases type 1 and type 2A [8–12]. K-252a was also used as a non-specific protein kinase inhibitor [13]. Our results suggest that MP formation is closely related to the cytoskeletal reorganization which is mediated by protein phosphorylation.

MATERIALS AND METHODS

Materials

Prostaglandin I_2 (PGI_2) was a gift from Ono Pharmaceutical Co., Osaka, Japan, collagen was obtained from Nycomed Arzneimittel G.m.b.h. Co., München, Germany, and bovine thrombin was donated by Mochida Pharmaceutical Co., Osaka, Japan. Calibrite beads (2 μm diameter) were obtained from Becton Dickinson, San Jose, CA, U.S.A. Fluorescein 5-isothiocyanate (FITC)-labelled goat anti-mouse IgG was obtained from Organon Teknika Co., Durham, NC, U.S.A. Fura-2 AM (acetoxymethyl ester) was purchased from Dojin-Do Labo., Kumamoto, Japan. OA and CLA were obtained from Wako Pure Chemicals Co., Osaka, Japan. Cytochalasin D was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of analytical grade. Murine monoclonal antibody NNKY1-32, specific for the glycoprotein IIb–IIIa complex (GP IIb–IIIa) on either resting or activated platelets, was kindly provided by Dr.

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CLA, calyculin A; FITC, fluorescein 5-isothiocyanate; GP, glycoprotein; MP, microparticle; OA, okadaic acid; PGI_2, prostaglandin I_2; PRP, platelet-rich plasma; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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Shousaka Nomura, Kansai Medical University, Osaka, Japan [14] and murine monoclonal antibody Tab, specific for GPIIb, was generously given by Dr. Rodger P. McEver of the University of Oklahoma Health Science Center, Oklahoma, OK, U.S.A. [15,16].

Isolation of platelets

Venous blood was drawn from healthy adult donors, who had not taken any medication for at least 2 weeks, and was collected into a plastic syringe containing 0.1 vol. of 3.8 % sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the anticoagulated blood at 150 *g* for 15 min. The PRP was then mixed with a 0.3 % volume of 5 μM PGI_2 solution for cytoprotection, and the mixture was centrifuged at 850 *g* for 13 min. The platelet pellet was washed twice with a 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 PGI_2. Finally, the platelets were resuspended in the same buffer without PGI_2 at a final concentration of 1×10^8/ml.

Activation of platelets with thrombin plus collagen

Washed platelets were preincubated for 5 min at 37 °C with various concentrations of cytochalasin D, OA or CLA, dissolved in a final concentration of 0.1 % (v/v) dimethyl sulphoxide. In all experiments, comparisons were made with identical matched pair controls (platelets incubated for 5 min at 37 °C with only 0.1 % dimethyl sulphoxide). After preincubation with inhibitors for 5 min at 37 °C, a $CaCl_2$ solution was added to the platelet suspension to a final concentration of 2 mM. Since our previous studies showed that a combination of thrombin plus collagen or A23187 provides optimal stimulation to evoke MP formation, the platelets were incubated at 37 °C with thrombin (0.5 unit/ml) plus collagen (10 μg/ml) without stirring for various time periods. Then 100 μl portions were fixed with 1 % paraformaldehyde.

Flow-cytometric analysis

PFA-treated platelets were first incubated with the monoclonal antibody NNKY1-32 (5 μg/ml) or Tab (5 μg/ml) at room temperature for 30 min, followed by incubation with a saturated concentration of FITC-labelled goat anti-mouse antibody (20 μg/ml) for 30 min at room temperature. The samples were then diluted with 2 ml of PBS, pH 7.5, and analysed in a FACStar or FACScan flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.). The FACStar was equipped with a 5 W argon laser and operated at 550 mW power at a wavelength of 488 nm. Fluorescein fluorescence was detected with a 530/30 band-pass filter. The instrument was calibrated daily, by using 2 μm Calibrite beads, for fluorescence and light scatter. Data were acquired with Hewlett–Packard Consort 30 Research software (Palo Alto, CA, U.S.A.) and analysed with Lysis software (Becton Dickinson). The FACScan flow cytometer was used in its standard configuration with a 15 mW 488 nm air-cooled argon laser, and the standard band-pass filter for FITC fluorescence was 530/30 nm. Data were obtained with FACScan Research software (San Jose, CA, U.S.A.). Washed platelets treated only with the second antibody were run first to set a threshold on FITC fluorescence signals to exclude background scatter. Next, only the populations that were positive for the GP IIb–IIIa complex or GP IIb were gated to distinguish the platelets and MPs from electronic noise; 10000 FITC-positive particles from each sample were analysed. To discriminate MPs from platelets, the lower limit of the platelet gate was set at the left-hand border of the forward scatter profile of the untreated platelets. The amount of MPs was expressed as a percentage of the total of all platelets and MPs.

Scanning electron microscopy (SEM)

Washed platelets were preincubated for 5 min at 37 °C with 10 μM cytochalasin D, 20 nM CLA or 2 μM OA, and then activated by thrombin (0.5 unit/ml) plus collagen (10 μg/ml). After incubation for 10 min at 37 °C, samples were fixed with 2.5 % glutaraldehyde in 0.1 M PBS, and a drop of the platelet suspension was allowed to settle on a poly-L-lysine-coated glass coverslip. Samples were post-fixed in 1 % OsO_4, dehydrated in graded alcohols and dried by the critical-point method. The dried samples were sputter-coated with platinum and observed at 10 kV in a Hitachi S-800 scanning electron microscope (Hitachi, Tokyo, Japan).

Measurement of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

PRP was incubated with 5 μM fura-2 AM at 37 °C for 45 min. The platelets were washed once with Hepes-buffered saline and suspended in the same buffer (1×10^8/ml). After the fura-2 AM-loaded platelets were preincubated at 37 °C with various concentrations of inhibitors or vehicle for 5 min, thrombin (0.5 unit/ml) plus collagen (10 μg/ml) was added in the presence of 1 mM $CaCl_2$, which was added exactly 3 min before stimulation. The fluorescence was continuously monitored at settings of 340 nm and 380 nm (excitation) and 500 nm (emission) with a fluorescence spectrophotometer. Finally $[Ca^{2+}]_i$ was determined by the method of Pollock et al. [17] and Grynkiewicz et al. [18].

Immuno-electron microscopy of platelets and MPs

After the washed platelets had been incubated with thrombin plus collagen for 15 min at 37 °C, the platelets and MPs were fixed with 4 % PFA and 0.1 % glutaraldehyde in 0.1 M PBS (pH 7.5). The sample was centrifuged at 10000 *g* for 5 min. As over 99 % of the platelets were sedimented under this condition, the supernatant was used as samples containing only MPs. These MPs were embedded in 1.5 % agarose and cut into 2 mm^3 cubes. After overnight incubation of the blocks at 4 °C with anti-(GP IIb–IIIa) antibody (NNKY1-32) (10 μg/ml) they were processed for immunostaining with the avidin–biotin–peroxidase complex (ABC) method [19] by using a kit from Vector Laboratories (Burlingame, CA, U.S.A.). The blocks were then processed for transmission electron microscopy (TEM), post-fixed for 1 h in 1 % OsO_4 in 0.1 M PBS, dehydrated in a graded series of ethanols and propylene oxide, and embedded in Epon 812. Thin sections were cut on a Reichert–Jung Ultracut ultramicrotome (Leica A.G., Vienna, Austria), stained with 2 % uranyl acetate and lead citrate and examined with a Hitachi H-7000 electron microscope operated at 75 kV.

RESULTS

Detection of platelet-derived MPs by flow cytometry

Figures 1(a)–1(c) show the typical scattering patterns of platelets and MPs. Although only a small percentage (8 %) of MPs was detected in the resting platelets, a significant amount of MPs was generated when the platelets were activated by thrombin (0.5 unit/ml) plus collagen (10 μg/ml) or Ca^{2+} ionophore A23187

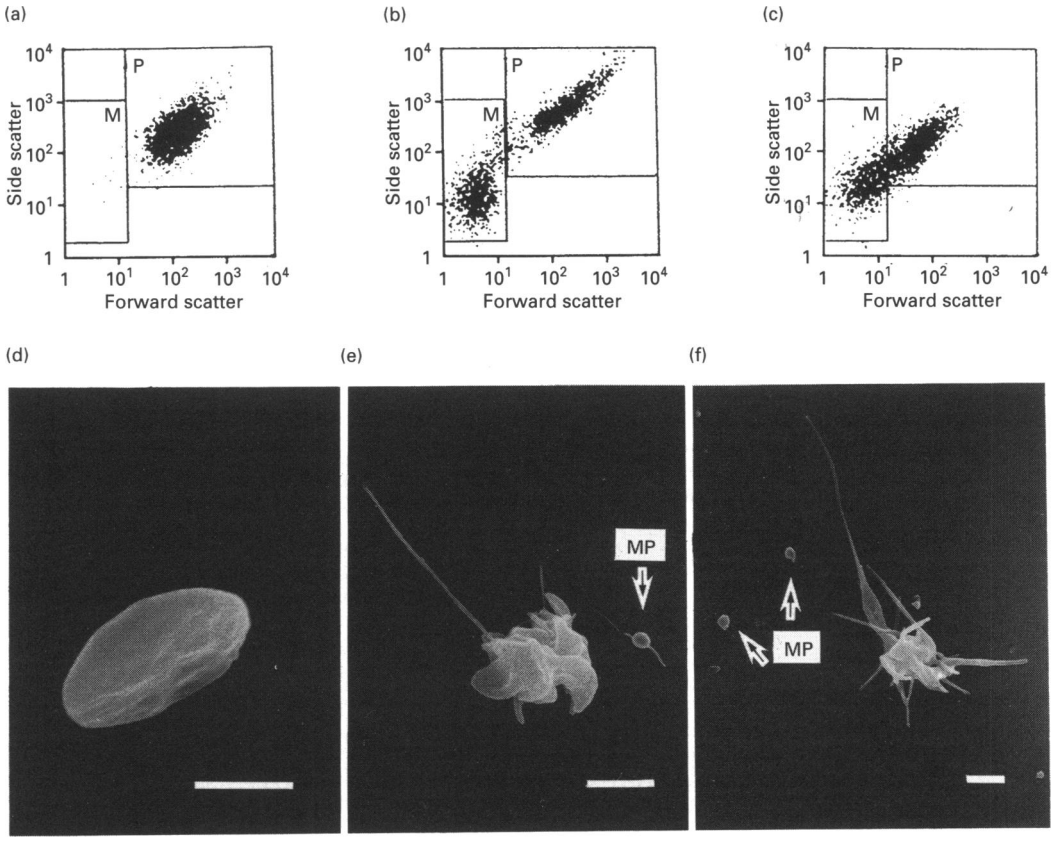

Figure 1 Scattering patterns of platelets and MPs by flow cytometry (a–c) and scanning electron micrographs of platelets and MPs (d–f)

Resting platelets (**a**), or platelets incubated with thrombin plus collagen (**b**) or with A23187 (**c**) without stirring for 10 min were fixed with 1% paraformaldehyde and stained with NNKY 1-32 (**a–c**) followed by FITC-labelled goat anti-mouse IgG. 'P' indicates platelet gate and 'M' indicates MP gate. For SEM, washed platelets at rest (**d**), or platelets activated by thrombin plus collagen (**e**) or by A23187 (**f**) were fixed with 2.5% glutaraldehyde for 30 min and then treated as described in the Materials and methods section. Resting platelets were a discoid shape (**d**) and few MPs were observed (**a**, **d**). Platelets activated by thrombin plus collagen extended pseudopods, and MPs seemed to be released from the end of the pseudopods (**e**). Platelets activated by A23187 extended pseudopods, and numerous MPs were observed (**f**). Bar = 1 μm.

(1 μM). Figures 1(d)–1(f) show representative SEM pictures of platelets at rest, activated by thrombin (0.5 unit/ml) plus collagen (10 μg/ml) and activated by A23187 (1 μM). Most of the resting platelets maintained a discoid form (Figure 1d). By contrast, after the platelets were activated by thrombin plus collagen, many pseudopods were formed, and MPs appeared to be released by the budding of the pseudopods (Figure 1e). The platelets activated by A23187 also drastically extended longer pseudopods and liberated many MPs (Figure 1f). In view of the possibility that MPs might be released from pseudopods (Figure 1e), we examined the relationship between cytoskeletal reorganization and MP formation.

Effect of cytochalasin D on MP formation

Cytochalasin D, a fungal metabolite that inhibits actin polymerization and induces depolymerization of formed actin filaments [6,7], inhibited MP formation from platelets activated by thrombin plus collagen, as detected by flow cytometry (Figure 2). This inhibition by cytochalasin D was dose-dependent. After incubation for 15 min cytochalasin D decreased MP formation by 50% at a concentration of 10 μM, which decreased actin polymerization almost completely in thrombin-stimulated platelets [6,7]. However, 10 μM cytochalasin D had no effect on the release reaction or on the elevation of $[Ca^{2+}]_i$ (results not shown).

Morphological studies were also performed by SEM to compare the morphology of platelets with MP formation. Cytochalasin D did not alter the discoid shape of resting platelets, and inhibited shape change and pseudopod formation of the platelets activated by thrombin plus collagen. Moreover, MP formation could hardly be observed when morphological changes and pseudopod formation were inhibited by cytochalasin D (results not shown). These findings were consistent with those obtained by flow cytometry, as shown in Figure 2.

Effect of protein phosphatase inhibitors on MP formation

Since cytoskeletal reorganization is considered to be regulated by a dynamic phosphorylation–dephosphorylation process [20–24], we examined the effects of protein phosphorylation on MP formation by using CLA or OA, which inhibit protein phosphatase types 1 and 2A. Figure 3 shows the effect of CLA or OA on the amount of MP formation from platelets activated by thrombin plus collagen. After incubation for 5 min with thrombin plus collagen at 37 °C, CLA (20 nM) or OA (2 μM) enhanced MP formation approx. 2.5–3-fold, as calculated from flow-cytometry findings. Since intracellular Ca^{2+} has been shown to play an important role in MP formation [4,5], the effect of CLA on the increase in $[Ca^{2+}]_i$ in platelets activated by thrombin plus collagen was investigated (Figure 4). CLA at concentrations of

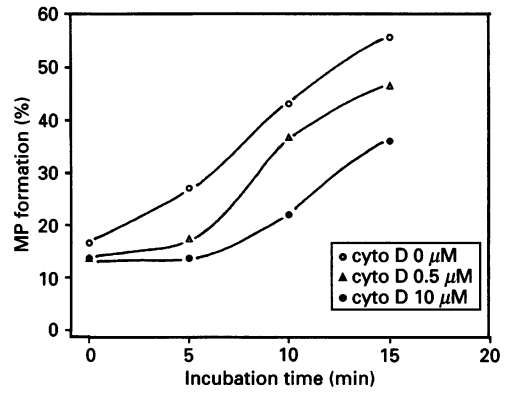

Figure 2 Effect of cytochalasin D on MP formation

Cytochalasin D (cyto D) inhibited MP formation dose-dependently. The amount of MPs was expressed as described in the Materials and methods section.

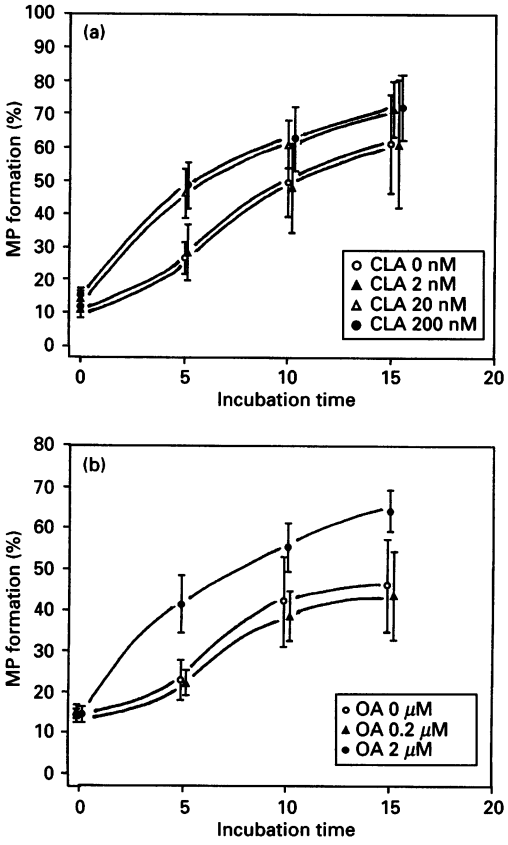

Figure 3 Effect of CLA and OA on MP formation

Washed platelets were preincubated for 5 min at 37 °C with various concentration of CLA (**a**) or OA (**b**) and then activated by thrombin plus collagen. MP formation was detected by using flow cytometry and calculated as described in the Materials and methods section. CLA and OA enhanced thrombin-plus-collagen-induced MP formation by approx. 2.5–3-fold. Results are expressed as means ± S.D. ($n = 4$). The amount of MPs was expressed as described in the Materials and methods section.

more than 20 nM, which potentiated MP formation, was found to inhibit the increase in $[Ca^{2+}]_i$ by approx. 70%, suggesting that CLA does not enhance MP formation through Ca^{2+}-dependent

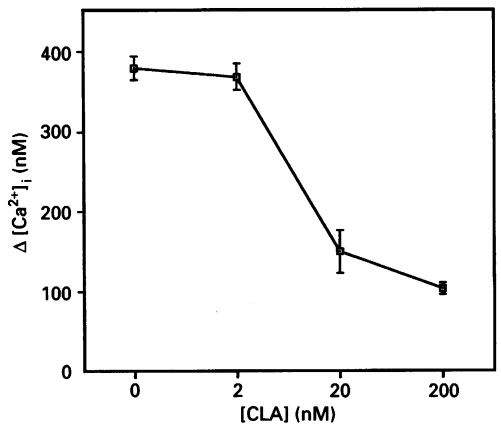

Figure 4 Effect of CLA on $[Ca^{2+}]_i$ in platelets activated by thrombin plus collagen

Fura-2 AM-loaded platelets were preincubated with various concentration of CLA and activated by thrombin plus collagen. CLA at a concentration of over 20 nM inhibited the increase in $[Ca^{2+}]_i$ by approx. 70%. Results are expressed as means ± S.D. ($n = 4$).

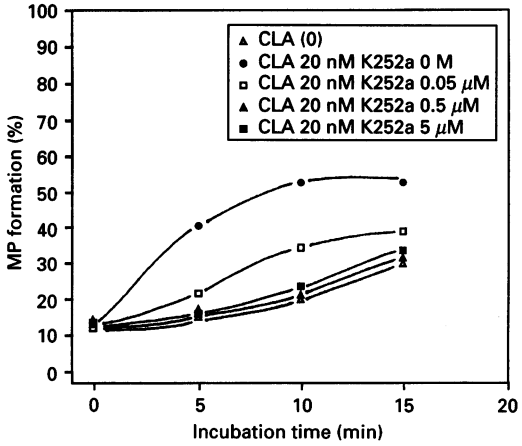

Figure 5 Inhibitory effect of protein kinase inhibitor K-252a on CLA-induced increase in MP formation

Washed platelets were preincubated with K-252a and CLA (20 nM) at 37 °C for 5 min in the absence of external Ca^{2+} (with 2 mM EDTA), then activated by thrombin plus collagen. Samples were fixed with 1% PFA and stained with monoclonal antibody Tab (anti-GPIIb antibody). K-252a inhibited the CLA-induced increase in MP formation dose-dependently. The amount of MPs was expressed as described in the Materials and methods section.

mechanisms. As these two inhibitors elevate the state of phosphorylation of platelet proteins [8–12], our present data suggest that protein phosphorylation plays an important role in MP formation. Furthermore, as shown in Figure 5, addition of the non-selective protein kinase inhibitor K-252a inhibited the CLA-induced increase in MP formation dose-dependently (Figure 5).

SEM

To study the effects of these two inhibitors on platelet morphology, SEM studies were performed. Platelets activated by thrombin plus collagen altered their shape from discoid to echinocytic and then extended many pseudopods (Figures 6a and 6b). Treatment with CLA (20 nM) or OA (2 μM) caused most of

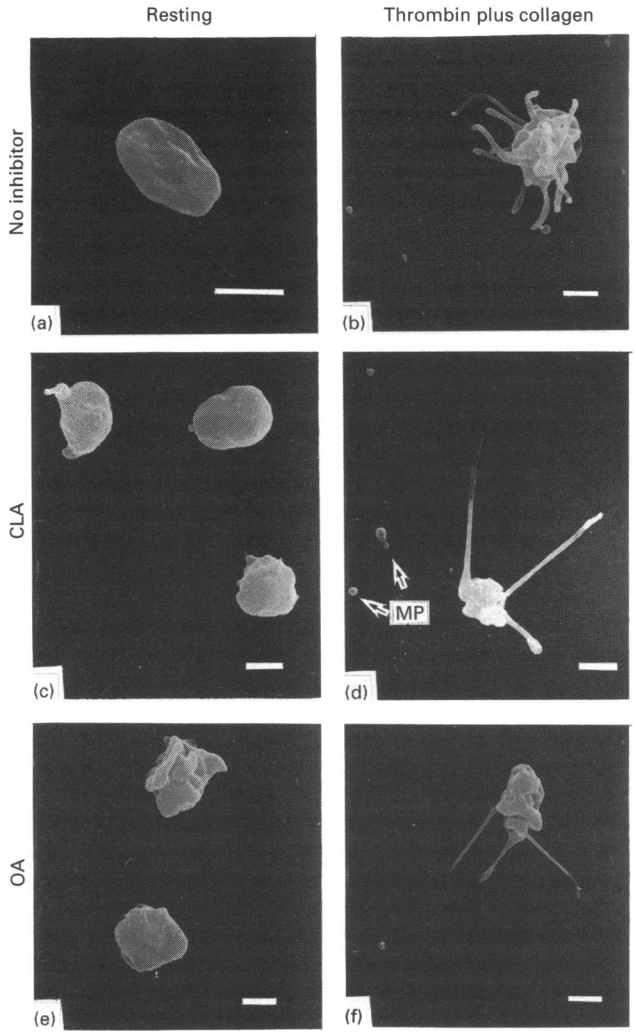

Figure 6 Effect of CLA or OA on platelet morphology

Washed platelets preincubated with CLA (20 nM) or OA (2 μM) were activated by thrombin plus collagen for 15 min. They were fixed with 2.5% glutaraldehyde and treated for SEM as described in the Materials and methods section. (**a**) Resting platelet; (**b**) platelet activated by thrombin plus collagen; (**c**) platelets incubated with 20 nM CLA for 5 min; (**d**) platelet preincubated with 20 nM CLA for 5 min and then activated by thrombin plus collagen for 15 min; (**e**) platelets incubated with 2 μM OA for 5 min; (**f**) platelet preincubated with 2 μM OA for 5 min and then activated by thrombin plus collagen for 15 min. The treatment with CLA (20 nM) or OA (2 μM) induced platelet shape changes and pseudopod formation. In platelets activated by thrombin plus collagen, these two inhibitors caused more prominent shape change and longer pseudopod formation compared with those without inhibitors. Bar = 1 μm.

the platelets to exhibit ruffling on the membrane and to change to echinocyte-like shape (Figures 6c and 6e). When the platelets were activated by thrombin plus collagen, these two inhibitors caused more prominent shape change and longer pseudopod formation compared with the platelets without inhibitors (Figures 6d and 6f) and many MPs were seen, as in Figure 6(d).

TEM

TEM was performed to observe the ultrastructure of MPs. Figures 7(a)–7(c) show immunolabelling of MPs with the anti-(GP IIb–IIIa) monoclonal antibody. As is clearly visible in these

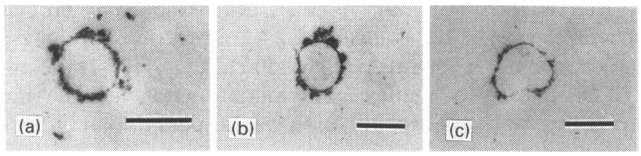

Figure 7 Immuno-electron micrographs of platelets and MPs

(**a**–**c**) MPs stained with NNKY 1-32 were treated for TEM as described in the Materials and methods section. Bar = 1 μm.

micrographs, peroxidase reaction product darkened the surface of the MPs, including an abundant distribution of GPIIb–IIIa on MPs. The size of most of the MPs was less than 0.5 μm, and they did not contain any mitochondria or α-granules but only a few filamentous structures, which were presumably part of the cytoskeleton.

DISCUSSION

Recently accumulated data suggest that agonist-induced MP formation is closely related to elevation of $[Ca^{2+}]_i$ [4,5]. Our previous study also showed that calpeptin (a calpain-specific inhibitor) inhibits MP formation dose-dependently, and the time course of the degradation of actin-binding protein, a well-known calpain substrate, correlated well with MP formation [5]. However, the inhibition of MP formation by calpeptin is only partial, suggesting that more than one mechanism is involved in MP formation.

The findings by SEM suggested the possibility that MPs are released from near the tip of pseudopods (Figure 1e). Various investigators reported a similar phenomenon in the formation of platelets from megakaryocytes. Using TEM, Radley and Haller [25] reported that megakaryocytes extend processes into sinusoids and that these processes undergo attenuation and constriction at their distal end to form platelets. In spite of intensive investigation on platelet formation from megakaryocytes, the exact mechanism underlying the release of MPs from platelets has not yet been clarified. Sims and Wiedmer [26] reported that SEM observation of platelets treated with complement components C_{5b-9} revealed irect vesiculation or budding of the platelet wall. On the other hand, Crawford [27] observed terminal swellings on the platelet pseudopods in a number of human and animal platelets and offered the possibility that MPs were the result of the budding of pseudopods. Our observation by SEM supports the findings of Crawford [27] rather than those of Sims and Haller [26]. Regardless of these differences, however, it can be generally agreed that MP formation is closely related to unique morphological changes in activated platelets, especially pseudopod formation, which might be the result of intracellular cytoskeletal reorganization. As we expected, cytochalasin D, which inhibits actin polymerization, inhibited not only the morphological change, but also MP formation to a large extent. This finding suggests an essential role of cytoskeletal reorganization in MP formation.

Since it has been reported by many investigators that cytoskeletal reorganization in various cells is regulated by a dynamic phosphorylation–dephosphorylation process [20–24], the role of protein phosphorylation on MP formation was investigated by using CLA or OA, which specifically inhibit protein phosphatases types 1 and 2A [8–12]. Protein phosphatases type 1 and 2A are two of the four major serine and threonine protein phosphatases in mammalian cells [28–30]. The major difference between CLA

and OA is their inhibitory potency with respect to protein phosphatase 1. The IC_{50} values of CLA for protein phosphatase 1 are about 2 nM, compared with 60–500 nM OA [12]. Therefore it is mandatory to employ both inhibitors to elucidate the involvement of the phosphorylation–dephosphorylation process in cellular function.

Interestingly, treatment of resting platelets with CLA or OA induced a morphological change (Figures 6c and 6e), and when the platelets were activated by thrombin plus collagen, CLA or OA induced longer pseudopods compared with those without the inhibitors (Figures 6d and 6f). Considering that a variety of cytoskeletal components which perform major roles in cytoskeletal reorganization are phosphoproteins, and that the level of phosphorylation of these proteins correlates closely with the degree of change in platelet shape [20–24], CLA- or OA-induced morphological changes and pseudopod formation are likely to be the result of increased levels of protein phosphorylation of cytoskeletal proteins. Moreover, CLA (20 nM) or OA (2 μM) had no significant effect on the membrane fluidity of the platelets, as determined by the use of 1-anilino naphthalene-8-sulphonate (N. Iwamoto, K. Murata, M. Sakon, J. Kambayashi, Y. Yano and T. Mori, unpublished work). These data support and reinforce the active involvement of protein phosphorylation in MP formation. We show for the first time that CLA or OA induced shape change of platelets, although other investigators have reported a similar effect of OA or CLA on fibroblasts [31], neurons [32] and neutrophils [33].

As shown in Figure 3, CLA or OA greatly enhanced MP formation from platelets activated by thrombin plus collagen. As intracellular Ca^{2+} concentration is considered to be closely related to MP formation [4,5], the effects of these inhibitors on intracellular Ca^{2+} elevation in activated platelets was investigated. Figure 4 shows the inhibitory effect of CLA on the elevation of intracellular Ca^{2+} in activated platelets. A similar effect was also seen with OA (results not shown). Therefore, it is unlikely that the principal mechanism for the increase in MP formation by CLA or OA is due to an enhanced level of intracellular Ca^{2+}. As CLA and OA have been shown to inhibit protein phosphatase types 1 and 2A specifically [8–12], our data strongly suggest that protein phosphorylation is deeply involved in MP formation. This possibility is also supported by the evidence that the CLA-induced increase in MP formation was inhibited by a non-specific kinase inhibitor, K-252a (Figure 5), although the identification of unknown kinases involved is under investigation.

In conclusion, it appears that there are at least two mechanisms involved in MP formation from activated platelets. One is a Ca^{2+}-dependent pathway, in which calpain might be involved, and the other is a phosphorylation-related pathway, which causes cytoskeletal reorganization.

Received 18 August 1993/19 November 1993; accepted 6 December 1993

We are extremely grateful to Dr. Rodger P. McEver for supplying antibody Tab. Thanks are also due to Dr. Shosaku Nomura and Dr Kojiro Yasunaga for kindly providing monoclonal antibody NNKY1-32.

REFERENCES

1 Sims, P. J., Faioni, E. M., Weidmer, T. and Shattil, S. J. (1988) J. Biol. Chem. **263**, 18205–18212
2 Wenche, J. Y., Horstman, L. L., Arce, M. and Ahn, Y. S. (1992) J. Lab. Clin. Med. **119**, 334–345
3 Nomura, S., Yanabu, M., Kido, H., Fukori, T., Yamaguchi, K., Soga, T., Nagata, H., Kokawa, T. and Yasunaga, K. (1991) Ann. Hematol. **62**, 103–107
4 Wiedmer, T., Shattil, S. J., Cunningham, M. and Sims, P. J. (1990) Biochemistry **29**, 623–632
5 Yano, Y., Shiba, E., Kambayashi, J., Sakon, M., Kawasaki, T., Fujitani, K., Kang, J. and Mori, T. (1993) Thromb. Res. **71**, 385–396
6 Fox, J. E. B. and Phillips, D. R. (1981) Nature (London) **292**, 650–652
7 Casella, J. F., Flanagan, M. D. and Lin, S. (1981) Nature (London) **293**, 302–305
8 Tachibana, K., Scheuer, P. J. and Tukitani, Y. (1981) J. Am. Chem. Soc. **103**, 2469–2471
9 Bialojan, C. and Takai, A. (1988) Biochem. J. **256**, 283–290
10 Takai, A. and Bialojan, C. (1987) FEBS Lett. **217**, 81–84
11 Kato, Y., Fusetani, N. and Matunaga, S. (1986) J. Am. Chem. Soc. **108**, 2780–2781
12 Ishihara, H., Martin, B. L. and Brautigan, D. L. (1989) Biochem. Biophys. Res. Commun. **159**, 871–877
13 Kase, H., Iwagashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Muradata, C., Sato, A. and Kaneko, M. (1987) Biochem. Biophys. Res. Commun. **142**, 436–440
14 Nomura, S. and Kokawa, T. (1990) in Automation and New Technology in Clinical Laboratory (Okuda, L., ed.), pp. 287–294, Blackwell Scientific Publications, Oxford
15 McEver, R. P., Beneet, E. M. and Martin, M. N. (1983) J. Biol. Chem. **258**, 5269–5275
16 McEver, R. P., Baenziger, N. L. and Magerus, P. W. (1980) J. Clin. Invest. **66**, 1311–1318
17 Pollock, W. K., Rink, T. J. and Irvine, R. F. (1986) Biochem. J. **235**, 869–877
18 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
19 Hsu, S., Raine, L. and Fanger, H. (1981) J. Histochem. Cytochem. **29**, 577–580
20 Zhuang, Q. Q., Rosenberg, S. and Lawrence, J. (1984) Biochem. Biophys. Res. Commun. **118**, 508–511
21 Maurer, D. R., Majercik, M. H. and Bourguignon, L. Y. W. (1988) Cell Biol. Int. Rep. **12**, 271–288
22 Pasquale, E. B., Mather, P. A. and Singer, S. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5507–5511
23 Chou, Y., Rosevear, E. and Goldman, R. D. (1988) Proc. Natl. Acad. Sci. U.S.A. **86**, 1885–1889
24 Chou, Y., Bischoff, J. R., Beach, D. and Goldman, R. D. (1990) Cell **62**, 1063–1071
25 Radley, J. M. and Haller, J. (1982) Blood **60**, 213–219
26 Sims, P. J. and Wiedmer, T. (1986) Blood **68**, 556–561
27 Crawford, N. (1971) Br. J. Haematol. **21**, 53–69
28 Cohen, P. (1989) Annu. Rev. Biochem. **58**, 453–508
29 Cohen, P. and Cohen, P. T. W. (1989) J. Biol. Chem. **264**, 21435–21438
30 Sakon, M., Kambayashi, J., Kajiwara, Y., Uemura, Y., Shiba, E., Kawasaki, T. and Mori, T. (1990) Biochem. Int. **22**, 149–161
31 Chartier, L., Rankin, L. L. and Allen, R. E. (1991) Cell Motil. Cytoskeleton **18**, 26–40
32 Sacher, M. G., Athlan, E. S., Mushynski, W E. (1992) Biochem. Biophys. Res. Commun. **186**, 524–530
33 Kreienbühl, P., Keller, H., and Niggli, V. (1992) Blood **80**, 2911–2919