Cytoskeletal reorganization of human platelets induced by the protein phosphatase 1/2A inhibitors okadaic acid and calyculin A

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Okadaic acid (OA) and calyculin A (CLA), which are potent and specific inhibitors of serine/threonine protein phosphatases type 1 and 2A, have been shown to induce drastic changes in platelet morphology. The aim of this study was to analyse the molecular mechanisms of OA- or CLA-induced cytoskeletal reorganization, with a specific focus on microtubules and actin filaments. Confocal fluorescence microscopy revealed that OA or CLA altered the distribution of microtubules from marginal band arrangements to homogeneous patterns, consistent with the transmission-electron-microscopic finding that microtubules were fragmented and redistributed into pseudopod-like processes. In thrombin-activated platelets, OA or CLA induced extremely long pseudopods containing an array of microtubules and actin filaments, and a condensed mass of actin filaments in the centre of platelets. OA or CLA induced the constriction of

actin filaments without an increase in filamentous (F)-actin, and also rather significantly inhibited actin polymerization in thrombin-activated platelets. Furthermore, neither OA or CLA enhanced phosphorylation of myosin light chain (MLC). By immunoprecipitation of platelet lysate with anti- α -tubulin antibody, a 90 kDa protein was co-precipitated with tubulin and was predominantly phosphorylated in the presence of OA. As the time-dependent phosphorylation of 90 kDa protein correlated well with the reorganization of microtubules, these data suggest that phosphorylation and dephosphorylation of this protein might play a role in the regulation of microtubule organization. These findings indicate that OA or CLA induces reorganization of microtubules and actin filaments via the phosphorylation of a microtubule-associated 90 kDa protein and an MLC-phosphorylation-independent mechanism.

INTRODUCTION

Platelets have been shown to change their cytoskeletal organization drastically in reaction to various physiological stimuli [1–3]. As a variety of cytoskeletal components, such as myosin, vimentin, talin and filamin, are all phosphoproteins [4–8], protein phosphorylation and dephosphorylation have been considered to play an important role in the regulation of platelet cytoskeletal structure. Therefore, in addition to protein kinases such as myosin light-chain (MLC) kinase (MLCK) or protein kinase C, protein phosphatase(s) involved in the dephosphorylation of cytoskeletal phosphoprotein may be important for the regulation of cytoskeletal organization. Okadaic acid (OA) and calyculin A (CLA), which are specific and potent inhibitors of protein phosphatases type 1 and 2A [9–13], have been used to analyse the physiological roles of these protein phosphatases in various cells [11,14–19]. We have recently found that OA or CLA induced significant morphological alterations in either resting or thrombin-stimulated platelets [14]. Similar effects of OA or CLA on cell morphology have been reported in other cells, such as fibroblasts [15,16], neurons [17] and neutrophils [18,19]. However, the regulatory mechanisms of cytoskeletal reorganization by protein phosphatase type 1 or 2A are not yet well clarified. In the present study, we investigated the effects of these inhibitors on the reorganization of microtubules and actin filaments, two major components of the platelet cytoskeleton, and demonstrated that morphological alterations by these inhibitors correlated well with the dynamic reorganization of microtubules and actin filaments. We also showed evidence of MLC-independent for-

mation of a condensed mass of actin filaments in OA- or CLA-treated platelets. In this paper, we also discuss the possible involvement of a novel microtubule-associated 90 kDa phosphoprotein in the dynamic reorganization of microtubules.

MATERIALS AND METHODS

Materials

OA and CLA were obtained from Wako Pure Chemicals Co., Osaka, Japan. Prostaglandin I_2 (PGI_2) was given by Ono Pharmaceutical Co., Osaka, Japan, and bovine thrombin by Mochida Pharmaceutical Co., Osaka, Japan. Fluorescein 5-isothiocyanate-labelled goat anti-mouse IgG was obtained from Organon Teknica Co., Durham, NC, U.S.A.; NBD (7-nitrobenz-2-oxa-1,3)-phalloidin was from Molecular Probes, Eugene, OR, U.S.A.; and anti- α -tubulin antibody was from Cedarlane Laboratories, Hornby, Ontario, Canada. Anti-phosphoserine and anti-phosphothreonine antibodies were obtained from Bio-Makor, Rehovot, Israel [20,21], and Protein A-Sepharose CL-4B was from Pharmacia LKB, Uppsala, Sweden.

Preparation of resting 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 was prepared by centrifugation at 150 *g* for 15 min and was mixed with a 0.3% volume of 5 mM PGI_2 .

Abbreviations used: OA, okadaic acid; CLA, calyculin A; F-actin, filamentous actin; MLC, myosin light chain; MLCK, MLC kinase; TEM, transmission electron microscopy; MAP, microtubule-associated protein; PGI_2 , prostaglandin I_2 .

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solution for cytoprotection. After centrifugation at 850 *g* for 13 min, platelets were washed twice with Hepes-buffered saline (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 0.5 mM Na₂HPO₄, 5 mM glucose, pH 7.4) containing 1 μ M PGI₂, and resuspended in the same buffer without PGI₂.

Activation of platelets

Washed platelets were preincubated for 5 min at 37 °C with 2 μ M OA or 20 nM CLA dissolved in 0.1% (v/v) dimethyl sulphoxide (DMSO). In all the experiments, comparisons were made with identical matched-pair controls (platelets incubated only with 0.1% DMSO). After preincubation with inhibitors, a CaCl₂ solution was added to the platelet suspension to a final concentration of 1 mM. In some experiments, platelets were activated by the addition of 0.1 unit of thrombin/ml for various periods without stirring. Platelet reaction was terminated with fixatives as detailed below under 'Transmission electron microscopy'.

Transmission electron microscopy (TEM)

Resting and activated platelets were fixed with 2.5% glutaraldehyde (diluted in 0.1 M phosphate buffer, pH 7.5) for 15 min. In some experiments, platelet cytoskeleton was prepared by permeabilizing platelets with Triton X-100 (0.5%) containing 0.5% glutaraldehyde, as described by Hartwig and Desisto [22] and White [23]. Briefly, after 30 min fixation, platelets or platelet cytoskeletons were washed with 0.1 M-phosphate-buffered saline (PBS). They were post-fixed with 1% OsO₄ in 0.1 M PBS for 30 min and then dehydrated in a graded series of ethanols, propylene oxide, and finally embedded in Epon 812 [22,23]. 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 (Hitachi, Tokyo, Japan).

Confocal fluorescence microscopy

Resting and activated platelets (1×10^8) were fixed in 3%-phosphate-buffered paraformaldehyde containing 5 mg/ml saponin for 30 min. After fixation, platelets were allowed to settle on poly-L-lysine-coated glass slides with the aid of a Sakura AutoSmear Cytocentrifuge CF-12 (Chiyoda Manufacturing Co., Nagano, Japan). The glass slides were then gently washed with 0.1 M PBS. To study the distribution of F-actin in platelets, the platelets were stained with 3.3 μ M NBD-phalloidin for 60 min. After washing with 0.1 M PBS, the coverslips were mounted on glass slides in PermaFluor (Shandon Lipshaw, Pittsburgh, PA, U.S.A.). Samples were examined with a MultiProbe 2001 confocal laser scanning microscope (Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.). For the examination of microtubule redistribution, fixed platelets were incubated with anti- α -tubulin antibody for 30 min and then with fluorescein isothiocyanate-labelled goat anti-mouse IgG for 30 min.

Immunoblotting

Immunoprecipitated samples and the molecular-mass standards were transferred on to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Richmond, CA) (0.2 μ m pore size) with the Western-blotting technique [24], using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell apparatus (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The membranes were incubated overnight at 4 °C with a monoclonal anti-phosphoserine or anti-phosphothreonine antibody, and devel-

oped with a peroxidase-conjugated goat anti-mouse IgG and with 3,3'-diaminobenzidine tetrahydrochloride.

Measurement of F-actin (filamentous actin)

The amount of polymerized F-actin in platelets was measured after SDS/PAGE [25] with an MCID densitometer (Imaging Research Inc., St. Catharine, Ontario, Canada) as previously reported by Jennings et al. [26]. Briefly, platelets were dissolved in a lysis buffer (1% Triton X-100, 5 mM EGTA, 50 mM Tris, pH 7.4) and sedimented at 100 000 *g* for 3 h. The pellet was resuspended in SDS-sample buffer and subjected to SDS/PAGE. The amount of F-actin was also examined by using NBD-phalloidin together with flow cytometry [27].

Phosphorylation of MLC

Platelets (1×10^9 /ml) were fixed by addition of ice-cold 20% (w/v) trichloroacetic acid containing 10 mM dithiothreitol. After centrifugation at 15 000 *g* for 10 min, the platelet pellet was washed twice with ice-cold acetone containing 10 mM dithiothreitol, dried, and dissolved in 30 μ l of SDS-sample buffer containing 8 M urea. The samples were subjected to urea/glycerol gel electrophoresis (urea/PAGE) [28,29], and immunoblotting analysis was performed as described above. The membranes were incubated with anti-MLC antibody (BioMakor) for 30 min and then with biotinylated anti-mouse IgM (Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min. The membranes were finally treated with Vecstatin ABC kits (Vector Laboratories), and developed with 3,3'-diaminobenzidine tetrahydrochloride.

Immunoprecipitation with anti- α -tubulin antibody

Thrombin-activated platelets in the absence or presence of 2 μ M OA were dissolved in ice-cold lysis buffer (25 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1 mg/ml leupeptin, 50 mM NaF, 2 mM Na₃VO₄, 1 mM phenylmethanesulphonyl fluoride, pH 7.5), mixed vigorously and incubated for 30 min at 4 °C on a rotating mixer. After centrifugation for 10 min at 15 000 *g*, the supernatant was pre-cleared by incubation with an excess of Protein A-Sepharose. The lysate was then incubated overnight at 4 °C with anti- α -tubulin antibody, followed by additional incubation with Protein A-Sepharose for 2 h. The control experiments were performed with non-immune mouse IgG₁. The immune complexes bound to Protein A-Sepharose were sedimented at 15 000 *g*, and washed with 2 $\times$ 1 ml of ice-cold washing buffer (10 mM Tris, 150 mM NaCl, 5 mM EGTA, 0.1% Nonidet P-40, pH 7.5). After the second washing, samples were resuspended in 150 μ l of SDS-containing buffer (2% SDS, 2% mercaptoethanol, 10% glycerol, 0.001% Bromophenol Blue, 30 mM Tris, pH 6.8) and boiled for 5 min. A sample of supernatant was subjected to electrophoresis on a 4–20%-acrylamide gradient gel, and immunoblotting was performed as described above.

RESULTS

Morphological changes observed by TEM

The morphological changes induced by OA or CLA treatment as studied by TEM are shown in Figure 1. Resting platelets have a discoid shape, and this lentiform appearance is supported by microtubules lying just under the plasma membrane along its greatest circumference (Figure 1a). Consistent with our previous findings with a scanning electron microscope [14], 2 μ M OA or 20 nM CLA caused membrane ruffling and induced the formation of pseudopod-like processes (Figures 1b and 1c). Dense granules

Figure 1 Effect of CLA or OA on the ultrastructure of resting and thrombin-activated platelets

(a) Resting platelet. The lentiform shape of platelets is well preserved, and circumferential bands of microtubules (MT) are clearly visible. (b, c) Platelets incubated with 20 nM CLA (b) or 2 μ M OA (c) for 5 min. These phosphatase inhibitors caused membrane ruffling and induced pseudopod-like processes. Microtubules were severed and granules were scattered throughout the cytoplasm. (d) Platelets activated by 0.1 unit/ml thrombin for 10 min. Platelets lost their discoid shape and extended pseudopods. (e, f) Thrombin-activated platelets pretreated with 20 nM CLA (e) or 20 μ M OA (f). Platelets were preincubated with these inhibitors for 5 min, and then activated by 0.1 unit/ml thrombin for 10 min. Platelets extended markedly long pseudopods in which arrays of microtubule bundles were observed in parallel with the long axis of the pseudopods. In the centre of the platelets, electron-dense amorphous masses of actin filaments were also observed. The bars represent 1.0 μ m.

and α -granules were scattered throughout the cytoplasm. Upon thrombin activation, platelets lost their discoid form and became spherical, with pseudopod formation (Figure 1d). Swollen granules after the release reaction were also observed. Pre-incubation with CLA or OA, followed by thrombin stimulation,

caused more drastic alterations in cell shape. Markedly extended pseudopods were formed, in which an array of microtubule bundles were observed in parallel with the longitudinal axis (Figures 1e and 1f). Swollen granules were also present in the periphery of the cell body. In the centre of these platelets, a mass

Figure 2 Scanning confocal images of microtubules stained with anti- α -tubulin monoclonal antibody

(a) Resting platelets. (b, c) Platelets exposed to 20 nM CLA (b) or 2 μ M OA (c) for 5 min. (d, g) Platelets activated by 0.1 unit/ml thrombin for 1 min (d) or for 10 min (g). (e, f, h, i) Platelets pretreated with 20 nM CLA (e, h) or 2 μ M OA (f, i) for 5 min and then activated by 0.1 unit/ml thrombin for 1 min (e, f), or for 10 min (h, i). In resting platelets, bright rings of microtubules were observed (a). Incubation of resting platelets with CLA or OA caused the homogeneous distribution of microtubules in the cell body and pseudopod-like processes (b, c). In thrombin-stimulated platelets, rings of microtubules became small and irregular at 1 min (d), but returned somewhat to their state at rest after 10 min (g). In contrast, in thrombin-activated platelets with prior exposure to CLA or OA, marked staining of microtubules was observed in extremely long pseudopods (e, f, h, i). The bars represent 5 μ m.

of extremely dense microfilaments was observed, more prominent than the mass of dense microfilaments in thrombin-activated platelets [30,31]. These masses of dense microfilaments were also seen within 5 min after thrombin stimulation, but at 10 min they could hardly be observed in the absence of phosphatase inhibitors (Figure 1d).

Reorganization of microtubules studied by confocal fluorescence microscopy

The spatial rearrangement of microtubules induced by CLA or OA was studied by confocal fluorescence microscopy, using an anti- α -tubulin antibody. In resting platelets, bright rings of microtubules characteristic of discoid platelets were observed (Figure 2a). When incubated with OA or CLA for 5 min, the

staining of α -tubulin became more widespread and homogeneous in the cytoplasm, concomitantly with morphological changes of the platelets (Figures 2b and 2c). Furthermore, pseudopod-like processes projecting from the centre of the platelets were also stained. At 1 min after thrombin activation, the diameter of the microtubules decreased, indicating centralization of the microtubules (Figure 2d). Microtubules were also identified in the pseudopods. When preincubated with OA or CLA, thrombin-activated platelets showed more marked staining of α -tubulin in the long pseudopods (Figures 2e and 2f). At 10 min, an almost identical dense staining of α -tubulin in the long pseudopods was observed (Figures 2h and 2i), whereas microtubules in the thrombin-activated platelets without phosphatase inhibitors nearly reverted to the ring shape, as could be seen in resting platelets (Figure 2g).

Figure 3 Scanning confocal images of F-actin stained with NBD-phalloidin

(a) Resting platelets. (b, c) Platelets exposed to 20 nM CLA (b) or 2 μ M OA (c) for 5 min. (d) Platelets activated by 0.1 unit/ml thrombin for 1 min (d) or for 10 min (g). (e, f, h, i) Platelets preincubated with 20 nM CLA (e, h) or 2 μ M OA (f, i) for 5 min and then activated by 0.1 unit/ml thrombin for 1 min (e, f) or for 10 min (h, i). In resting platelets, homogeneous distribution of F-actin was observed (a). Incubation with CLA or OA caused the formation of F-actin not only in the cell body, but also in pseudopod-like processes (b, c). In thrombin-activated platelets, homogeneous staining of F-actin was observed in both the cell body and pseudopods (d, g). In thrombin-activated platelets with prior exposure to CLA or OA, the formation of F-actin could be seen in the long pseudopods. F-actin was concentrated in the centre of the cell body, forming a condensed mass of actin filaments (h, i). The bars represent 5 μ m.

Reorganization of actin filaments studied by confocal fluorescence microscopy

Confocal-fluorescence-microscopic examination with NBD-phalloidin revealed a homogeneous distribution of F-actin in unstimulated platelets (Figure 3a). After treatment with 20 nM CLA or 2 μ M OA for 5 min, actin filaments could be observed not only in the cell body but also in the pseudopod-like processes (Figures 3b and 3c). Thrombin-activated platelets showed homogeneous staining of F-actin in the cell body, although F-actin was also identified in the pseudopods (Figures 3d and 3g). Both OA and CLA induced drastic reorganization of actin filaments in thrombin-stimulated platelets (Figures 3e, 3f, 3h and 3i). F-actin was observed in the long pseudopods. Actin filaments appeared

to be more concentrated in the central area of the cell bodies, compared with those in platelets treated with thrombin alone.

Cytoskeletal reorganization observed by TEM

In resting platelets, circumferential bundles of microtubules appeared at the periphery of the detergent-resistant cytoskeleton (Figure 4a). However, when platelets were treated with OA or CLA for 5 min, the circumferential bands of microtubules were disrupted and many short fragments of microtubules were dispersed randomly throughout the cytoplasm. Moreover, microtubules were found to have entered into several pseudopod-like processes (Figures 4b and 4c). When platelets were stimulated with thrombin, the microtubules became irregular in the peri-

Figure 4 Effect of CLA or OA on the cytoskeletal structure of resting and thrombin-activated platelets

(a–c) The cytoskeleton of platelets in the presence of vehicle (a), CLA (b) or OA (c). (d–f) Platelets activated by 0.1 unit/ml thrombin for 10 min in the presence of vehicle (d), CLA (e) or OA (f). After incubation, platelets were treated with 0.5% Triton X-100 containing 0.5% glutaraldehyde. In resting platelets, a circumferential bundle of microtubules (MT) is evident underneath the membrane (a). Platelet cytoskeleton from a sample treated with 20 nM CLA (b) or 2 μM OA for 5 min (c) showed that the circumferential microtubules were severed into short fragments and that some of the fragments were dispersed to the pseudopod-like processes. In thrombin-activated platelets at 10 min, the peripheral layer became irregular, but the circumferential microtubules remained under the cell surface even though they became irregular (d). In thrombin-activated platelets with prior exposure to CLA (e) or OA (f), arrays of microtubules were observed in parallel with the long axis of the pseudopods. A mass of constricted microfilaments was also observed in the central region of the cell (e, f). The bars represent 1.0 μm.

peral area and followed the contour of the pseudopods (Figure 4d). Microfilaments became more prominent in the submembranous region compared with those in resting platelets, and were also found to extend into the pseudopods. Microtubules were still present at the cell periphery, but most of them were irregular. Prior exposure to 20 nM CLA (Figure 4e) or 2 μM OA (Figure 4f) drastically altered the pattern of cytoskeletal

reorganization in the thrombin-stimulated platelets. Microtubules were reorganized, extending radially to the periphery of the platelets, and were also found to be orientated in the direction of the longitudinal axis of the pseudopods (Figures 4e and 4f). A mass of concentrated microfilaments was observed in the central area of the platelets, consistent with the results shown in Figures 1(e), 1(f), 3(h) and 3(i). In contrast, microfilaments were observed

Table 1 Effect of OA or CLA on the amount of F-actin

Washed platelets were incubated for 5 min with DMSO (final concn. 0.1%), 20 nM CLA, or 2 μ M OA. For further experiments, after preincubation with 20 nM CLA or 2 μ M OA, platelets were activated by 0.1 unit/ml thrombin for 1 min or 10 min. The platelets were then treated with lysis buffer containing 1% Triton X-100 and sedimented at 100 000 *g* for 3 h. The amount of F-actin was determined by SDS/PAGE and densitometry as described in the Materials and methods section. Results are means $\pm$ S.D. of three independent experiments: **P* < 0.05 as compared with control, as determined by Student's *t* test.

Condition	F-actin content (% of total actin)
Unstimulated	
Control	61.6 $\pm$ 1.1
20 nM CLA	65.4 $\pm$ 2.8
1 μ M OA	64.4 $\pm$ 1.9
1 min after thrombin activation	
Control	71.3 $\pm$ 2.5
20 nM CLA	59.3 $\pm$ 2.4
1 μ M OA	60.6 $\pm$ 2.3
10 min after thrombin activation	
Control	70.6 $\pm$ 2.2
20 nM CLA	58.6 $\pm$ 1.4
1 μ M OA	47.4 $\pm$ 1.0

Figure 5 Time course of MLC phosphorylation

Platelets were activated by 0.1 unit/ml thrombin, and then the reaction was terminated by adding 20% ice-cold trichloroacetic acid containing 10 mM dithiothreitol. The percentage of MLC phosphorylation (mono- or di-phosphorylation) was calculated by using a laser-densitometric scan of transblots with anti-MLC-20 antibody (see the Materials and methods section). OA or CLA did not increase MLC mono-phosphorylation in resting or thrombin-activated platelets. They did not enhance di-phosphorylation of MLC in either resting or thrombin-activated platelets (results not shown). Representative data obtained from three separate experiments with identical results are shown.

scattered loosely in the area between the submembranous region and the central mass of concentrated microfilaments.

Measurement of F-actin

The effects of OA or CLA on actin polymerization in either thrombin-stimulated or non-stimulated platelets were studied by SDS/PAGE of Triton X-100-resistant cytoskeleton (Table 1).

Figure 6 Serine/threonine phosphorylation of 90 kDa protein by OA

(a) Effect of 2 μ M OA on proteins immunoprecipitated by anti- α -tubulin antibody. Washed platelets, preincubated with 2 μ M OA or vehicle (DMSO) before thrombin activation (0.1 units/ml), were solubilized in the lysis buffer as described in the Materials and methods section. The platelet lysate was immunoprecipitated with anti- α -tubulin monoclonal antibody, subjected to SDS/PAGE and stained with Coomassie Blue. OA did not affect the pattern of proteins immunoprecipitated by anti- α -tubulin antibody (a). (b, c) Effect of 2 μ M OA on phosphorylation of co-migrating proteins with α -tubulin. Immunoprecipitates generated by anti- α -tubulin antibody from the lysate of thrombin-activated platelets were further examined by immunoblotting with the anti-phosphoserine (b) and anti-phosphothreonine (c) antibodies. Washed platelets preincubated with 2 μ M OA or vehicle for 5 min before thrombin activation are shown in the lanes at 0 min (first two lanes on the left-hand side of the Figure). Although various proteins were co-precipitated with α -tubulin (a), OA remarkably enhanced the phosphorylation of serine and threonine residues in 90 kDa protein (b, c).

Thrombin stimulation significantly increased the amount of actin in the platelet cytoskeleton. Exposure of platelets to 2 μ M OA or 20 nM CLA for 5 min appeared to increase the actin content of the cytoskeleton, but the difference was not statistically significant. Pretreatment of platelets with OA or CLA significantly inhibited thrombin-induced actin polymerization, which is consistent with their inhibitory effects on the increase in intracellular Ca^{2+} of thrombin-activated platelets [32]. Similar results (not shown) were obtained by using flow-cytometric analysis with NBD-phalloidin.

Phosphorylation of MLC

To study the involvement of MLC phosphorylation in the formation of the condensed mass of actin filaments, the effects of OA or CLA on MLC phosphorylation were investigated. Figure 5 shows the time course of MLC phosphorylation in thrombin-activated platelets, with the peak at 1–3 min after addition of 0.1 unit/ml thrombin. The result shows that neither OA or CLA enhanced the phosphorylation of MLC, nor did they enhance the dephosphorylation of MLC.

Immunoprecipitation by anti- α -tubulin antibody

Figure 6(a) shows the Coomassie-Blue-stained pattern of proteins co-migrating with α -tubulin from the lysate of platelets preincubated with or without 2 μ M OA. OA did not influence the patterns of proteins immunoprecipitated by anti- α -tubulin antibody. The phosphorylation of proteins co-precipitated with α -tubulin was further examined by immunoblotting with anti-phosphoserine or with anti-phosphothreonine monoclonal antibody. It was found that 5 min preincubation with OA significantly induced the phosphorylation of 90 kDa protein (at 0 min in Figures 6b and 6c). After thrombin stimulation, the 90 kDa protein was significantly phosphorylated on serine and threonine residues at 1 min, but 10 min later it was also completely dephosphorylated. In the presence of OA, 90 kDa protein was slightly dephosphorylated until 3 min after thrombin stimulation, but the dephosphorylation of 90 kDa protein was completely inhibited at 10 min.

DISCUSSION

Recently we found that OA and CLA, specific inhibitors of protein phosphatase type 1 and 2A [9,13], induced morphological changes in both resting and thrombin-activated platelets [14]. The present study was designed to elucidate the role of protein phosphatase type 1 or 2A in the regulation of platelet cytoskeletal structure. Figure 7 schematically summarizes the effects of these phosphatase inhibitors on cytoskeletal reorganization. In resting platelets, circumferential bundles of microtubules are lying underneath the plasma membrane, whereas actin filaments are distributed homogeneously throughout the cytoplasm [1,24,30] (Figure 7a). At 1 min after thrombin stimulation, microtubules are significantly centralized, and organelles are concentrated in the cell centre in conjunction with rings of microtubules and actin filaments [1,24,30] (Figure 7c). At 10 min after activation, most pseudopods have disappeared, and microtubule bundles are forced back to the cell periphery, although they are irregular (Figure 7d). Treatment with OA or CLA causes disruption of microtubule bundles into short fragments. Most of them are randomly distributed throughout the cell body, and some are reorganized into short pseudopod-like processes along with actin filaments (Figure 7e). Pretreatment with OA or CLA and subsequent stimulation by thrombin cause the reorganization of

microtubules radially to the periphery, and the formation of long pseudopods consisting of parallel arrays of long microtubules (Figure 7f). Actin filaments are concentrated in the cell body. After 10 min activation, the cytoskeletal structure of microtubules and actin filaments remains the same as observed at 1 min, and long pseudopods are maintained (Figure 7g).

Kreienbühl et al. [18] reported that OA-induced changes in the shape of neutrophils were associated with a remarkable reorganization of microfilaments, although they were not accompanied by an increase in F-actin formation. It has also been shown that OA or CLA completely prevents the formation of F-actin in neutrophils induced by phorbol 12-myristate 13-acetate, *N*-formylmethionyl-leucyl-phenylalanyl-lysine (fNLPNTL) [18], leukotriene B₄ and platelet-activating factor [19]. We also showed that preincubation of platelets with 2 μ M OA or 20 nM CLA significantly inhibited the increase in F-actin induced by thrombin (Table 1). Since, in our study, platelets were activated by 0.1 unit/ml thrombin without stirring, to avoid platelet aggregation, the total increase in F-actin by thrombin was less than that reported in other studies in which platelets were activated by agonists with stirring [33]. However, the inhibition of the thrombin-induced increase in F-actin by OA or CLA was significant ($P < 0.05$), suggesting that OA or CLA induced the reorganization of actin filaments into pseudopods (Figures 3 and 4), without any increase in F-actin (Table 1). One possible explanation for this finding is that these phosphatase inhibitors significantly inhibit the thrombin-induced increase in intracellular Ca^{2+} concentration [32], since Ca^{2+} plays a central role in actin polymerization [34]. Thus morphological changes in platelets are not always accompanied by actin polymerization.

Several earlier studies have described a dense mass of microfilaments separated from the membrane in agonist-stimulated platelets [31,35]. In our study, pretreatment with 2 μ M OA or 20 nM CLA induced a greater concentration of the mass of microfilaments in the centre of thrombin-activated platelets (Figures 3 and 4), which remained unchanged even at 10 min after activation. Since these inhibitors did not enhance F-actin formation (Table 1), actin polymerization does not seem to account for the formation of this concentrated mass of microfilaments. Stark et al. [35] suggested the possible involvement of MLC phosphorylation in the formation of the concentrated mass of actin filaments. As OA or CLA inhibits MLC phosphatase [11], these phosphatase inhibitors may enhance the phosphorylation of MLC, which has been shown to strengthen actin-myosin interaction [37]. Thus the hypothesis of the involvement of enhanced MLC phosphorylation in the formation of a dense mass of actin filaments is feasible. However, in our study, OA or CLA did not enhance the phosphorylation level of MLC in thrombin-stimulated platelets when examined by Western blotting using anti-MLC20 monoclonal antibody (Figure 5). These results are consistent with the findings by Lerea [38], but differ from those reported by Chartier et al. [15] and Stark et al. [35]. Lerea [38] reported that there was no difference in thrombin-induced MLC phosphorylation between thrombin-stimulated platelets with and without OA treatment, when examined by two-dimensional PAGE of ^{32}P -labelled platelets. Chartier et al. [15] showed that 15 min incubation with CLA increased phosphorylation of MLC in 3T3 fibroblasts, but they used 100 nM CLA, a concentration 5 times that used in this study. Stark et al. [35] also demonstrated that 10 μ M OA induced an increase in MLC phosphorylation in permeabilized platelets. As intracellular Ca^{2+} is determined by extracellular Ca^{2+} (10 μ M) in permeabilized platelets, MLCK is considered to be fully activated under the conditions used by these investigators. In contrast, OA or CLA has been shown to attenuate a thrombin-induced increase

Figure 7 Diagram summarizing the effect of OA or CLA on the reorganization of microtubules and actin filaments in resting and thrombin-activated platelets

(a) In resting platelets, circumferential bundles of microtubules are lying underneath the plasma membrane. Actin filaments are distributed throughout the whole cell, but are observed densely under the plasma membrane (membrane skeleton). (c) In platelets activated by thrombin for 1 min, centralization of microtubules occurs and organelles are concentrated in the cell centre with tight rings of constricted microtubules and actin filaments. Some microtubules extend from the circumferential microtubule bundles into pseudopods along with actin filaments. (d) At 10 min after thrombin activation, microtubule bundles are irregular, but have returned to the cell periphery. (e) Treatment of resting platelets with OA or CLA (5 min) induces severing of microtubules into short fragments and their redistribution into short pseudopod-like processes along with actin filaments. (f) Pretreatment of thrombin-activated platelets (5 min) with OA or CLA caused extremely long pseudopods, in which parallel arrays of long microtubules were observed. Actin filaments were concentrated in the cell body. (g) No further reorganization of microtubules or actin filaments occurs at 10 min after thrombin stimulation.

in intracellular Ca^{2+} in intact platelets by inhibiting the Ca^{2+} influx [32]. Thus these different results may be accounted for by different experimental conditions, such as concentration of phosphatase inhibitor, assay method for MLC phosphorylation and preparation of platelets. Therefore these findings suggest that other mechanisms may be involved in the formation of the concentrated mass of actin filaments. Our findings that CLA or OA induced the formation of a constricted mass of actin filaments without any increase in MLC phosphorylation may be explained by so called 'latch state' in smooth muscle, which was originally proposed by Murphy and colleagues [39,40]. The latch state is characterized by sustained smooth-muscle constriction without any increase in MLC phosphorylation or intracellular Ca^{2+} [39,40]. Other regulatory proteins, such as caldesmon and calponin, may be implicated in this state, because their regulatory function on smooth muscle is also considered to be regulated by phosphorylation and dephosphorylation. In fact, Winder and Walsh [41] and Nakamura et al. [42] demonstrated that calponin is phosphorylated by protein kinase C and Ca^{2+} /calmodulin-dependent protein kinase II, and that it is dephosphorylated by protein phosphatase type 1 and 2A. They showed that phosphorylation of calponin by either protein kinase C or Ca^{2+} /calmodulin-dependent protein kinase II abolishes inhibition of actomyosin ATPase activity by calponin [41,42]. Caldesmon is also phosphorylated by protein kinase C, Ca^{2+} /calmodulin-dependent protein kinase II and casein kinase II, and is dephosphorylated by a protein phosphatase [43,44]. Thus the down-regulation of these proteins by phosphorylation enhances the actomyosin ATPase activity, which may result in the dense mass of actin filaments.

TEM and confocal fluorescence microscopy showed that OA or CLA induced dramatic reorganization not only of actin filaments but also of microtubules (Figures 2, 3 and 4). Since circumferential microtubule bundles are considered to support the discoid form of platelets [1,45,46], OA or CLA is also thought to induce platelet morphological changes through the reorganization of microtubules. In thrombin-activated platelets, OA or CLA significantly affected the reorganization of microtubules. Microtubules were no longer organized in bundles, but were distributed radially in the cell body, extending into extremely long pseudopods even at 10 min after activation. Although the involvement of microtubules in pseudopod formation has not been exactly clarified yet, the following are considered to be possible explanations [30,47]: in platelets activated by agonists, microtubule bundles are decreased in size in the equatorial plane, and the free ends of the compressed bundles are elongated and enter into pseudopods. Another possibility may be that general disruption of microtubule bundles occurs upon platelet activation, and then the resulting free ends move into pseudopods. So far as platelets treated with OA or CLA are concerned, the latter hypothesis may be more feasible, because the centralization of microtubule bundles could hardly be observed in OA- or CLA-treated platelets.

The present study demonstrates for the first time that CLA or OA induces the disruption and reorganization of microtubules in platelets, although other investigators have reported a similar effect of OA on microtubules in interphase pig kidney cells in culture [48]. Furthermore, Neal et al. [49] reported that addition of OA to the extracts of interphase sea-urchin eggs

induced rapid conversion into short dynamic microtubules typical of mitosis. In spite of these recent findings, the mechanism of microtubule reorganization induced by CLA or OA has not yet been clarified. The plastic features of microtubules are mainly a consequence of the inherent dynamic properties of tubulin polymers. In addition, microtubule-associated proteins (MAPs) have been shown to regulate the stability of microtubules. In brain, MAPs such as tau, MAP-2 and MAP-4 are known to promote tubulin nucleation and assembly and also to stabilize polymerized microtubules [50]. Furthermore, phosphorylation of the microtubule-binding domain in these MAPs causes a marked decrease in tubulin-polymerizing ability, suggesting that phosphorylation and dephosphorylation of MAPs may play an important role in the reorganization of microtubules [51]. Recently, two MAPs, i.e. 210 K MAP, which cross-reacts with anti-210 K MAP antibody in HeLa cells [52], and an IEF (isoelectric focusing)-51K protein [53], have been identified in human platelets. To investigate whether the phosphorylation of MAPs is involved in CLA- or OA-induced microtubule reorganization, the phosphorylation of proteins co-precipitated with tubulin was studied. Although a number of proteins were co-precipitated by anti- α -tubulin antibody, 90 kDa protein was predominantly phosphorylated on serine and threonine residues by CLA or OA treatment (Figure 6). The other proteins were not significantly phosphorylated, which is consistent with the findings that phosphorylation of MAP-1A, MAP-1B and MAP-4 in fibroblasts is not affected by CLA [54]. In unstimulated platelets, 90 kDa protein was phosphorylated by OA in conjunction with microtubule reorganization. This result suggests that the phosphatase responsible for dephosphorylation of the 90 kDa protein is active in the resting state, and that OA inhibits this phosphatase activity. The 90 kDa protein was also phosphorylated by thrombin stimulation and dephosphorylated at 10 min in the absence of OA. Our finding that 90 kDa-protein phosphorylation is closely related to microtubule reorganization (Figures 2, 3 and 6) suggests that dephosphorylation of 90 kDa protein by protein phosphatase 1 or 2A may play a role in the stabilization of microtubules. Although OA inhibited the dephosphorylation of 90 kDa protein in thrombin-stimulated platelets, this inhibition was not complete (Figures 6b and 6c). In fact, phosphorylation of the 90 kDa protein decreased at 1 and 3 min after thrombin stimulation even in the presence of OA, but then it reverted to the pre-treatment level at 10 min. These results raise another possibility, that one or more unidentified protein phosphatase(s) is activated after thrombin stimulation and that this phosphatase(s) may be responsible for the temporary dephosphorylation of 90 kDa phosphoprotein.

Electron micrographs of platelets treated with CLA or OA revealed shorter fragments of microtubules (Figures 4b and 4c), suggesting that shorter microtubules are generated by severing rather than by depolymerization from the microtubule ends. Several investigators have suggested that unidentified microtubule-severing proteins may play an important role in microtubule reorganization at the onset of mitosis by protein phosphorylation and dephosphorylation. Verde et al. [55] and Vale [56] showed that microtubule length decreased in accordance with the increase in p34^{cdc2} kinase activities in *Xenopus* egg extracts, and suggested that microtubule-severing activity is regulated by the balance between p34^{cdc2} kinase and protein phosphatase type 1 activities. As p34^{cdc2} kinase was identified in sheep and human platelets by Samiei et al. [57] and Lerea [58], it is possible that the phosphorylation of 90 kDa proteins by p34^{cdc2} kinase is involved in severing and reorganizing microtubules in OA- or CLA-treated platelets, although further study is necessary to determine whether p34^{cdc2} kinase is re-

sponsible for the phosphorylation of this microtubule-associated protein.

In this study, we demonstrated that protein phosphatase type 1 or 2A is important for maintaining the organization of microtubules and actin filaments in both resting and agonist-stimulated platelets. We also suggested that an unknown microtubule-associated 90 kDa protein is involved in microtubule reorganization, and that actin filaments are reorganized and concentrated in the centre of platelets through a mechanism independent of MLC phosphorylation.

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