Myosin Light Chain Phosphorylation is Correlated with Cold-Induced Changes in Platelet Shape

Hayato KAWAKAMI¹, Masaaki HIGASHIHARA², Manabu OHSAKA²,

Kouji MIYAZAKI³, Mitsuo IKEBE⁴, and Hiroshi HIRANO^{1, 5}

¹*Department of Anatomy, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan*

²*Fourth Department of Internal Medicine, Kitasato University School of Medicine, Kanagawa 228-8555, Japan*

³*The First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113-0033, Japan*

⁴*Department of Physiology, Massachusetts Medical Center, Worcester MA 01655, USA*

⁵*Nittai Jusei Medical College for Judo Therapeutics, Tokyo 158-0097, Japan*

Abstract

Chilling induces shape changes in platelets from disks to spheres with abundant filopodia. Such changes were time-dependent and correlated well with the phosphorylation of 20-kDa myosin light chain (LC20). Both the shape changes and the phosphorylation were reversible. After the platelets had been chilled, myosin became incorporated into the Triton X-insoluble fraction. When the chilled platelets were immunocytochemically stained, anti-myosin antibody was localized with filamentous structures inside the filopodia. These results suggest that LC20 phosphorylation and subsequent interactions with actin filaments play a crucial role in the cold-induced changes in platelet shape and in the formation of filopodia.

Key words: platelet, chilling, shape change, myosin, phosphorylation

Introduction

The contractile proteins actin and myosin have been found in many eukaryotic cells and are thought to have important roles in cell function. In platelet cytoplasm, actin and myosin are the dominant proteins that are thought to have important roles in cellular activation (Korn, 1978). It is known that several agonists such as thrombin induce platelet responses including shape change, aggregation, secretion, and clot retraction, and it is thought that the phosphorylation of 20-kDa myosin light chain (LC20) is associated with these activations (Adelstein and Conti, 1975; Daniel *et al.*, 1981). We (Ikebe, 1989; Higashihara *et al.*, 1991) and others (Itoh *et al.*, 1992) reported that phosphorylation of platelet myosin by myosin light chain kinase (MLCK)

Correspondence to: H. Kawakami, Department of Anatomy, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan
Phone: +81-422-47-5511 Fax: +81-422-44-0866 e-mail: kaha@kyorin-u.ac.jp

increased actin-activated ATPase, and the extent of activation was dependent on the MgCl_2 concentration, and that both Ca^{2+} -ATPase and Mg^{2+} -ATPase activities were dependent on KCl concentration and markedly decreased below 0.3M KCl. These results are similar to those obtained with smooth muscle myosin (Ikebe *et al.*, 1983). Both platelet myosin and smooth muscle myosin form 10S and 6S conformation, as judged by the gel filtration, intrinsic fluorescence, and viscosity methods (Ikebe *et al.*, 1983; Higashihara *et al.*, 1991).

Since Zucker first reported a cold-induced change in platelet shape (Zucker and Borrelli, 1954), this phenomenon has become well established (Behnke, 1970; White and Krumwiede, 1973; Bennet and Lynch, 1980; White, 1982). Chilling induces platelets to undergo a change in shape from disks to spheres with filopodias. Although depolymerization of microtubules has been thought to be involved in this shape change (White, 1982), our preliminary observation revealed that microtubular rings disappeared within 15 min after chilling, whereas it took 90-120 min to accomplish the overall change in platelet shape from disk to sphere. Thus it seems to be another cytoskeletal protein that is responsible for the cold-induced shape change. Myosin (Nachmias *et al.*, 1985) and actin (Winokur and Hartwig, 1995) are possible candidates to play a crucial role in this phenomenon. In this study, by electron microscopy we observed precisely the time-dependent change in shape of chilled human platelets and also examined the effect of chilling on the phosphorylation/dephosphorylation of platelet cytoskeletal components.

Materials and Methods

Chemicals

α -Thrombin was obtained from Mochida Pharmaceutical Ltd. (Tokyo, Japan). [^{32}P] phosphoric acid was purchased from Amersham Japan (Tokyo, Japan).

Electron Microscopy

Gel-filtered human platelets were isolated as previously described (Kawakami *et al.*, 1994). The platelets in HEPES-Tyrodé's buffer with glucose (1 mg/ml) and 1 mM CaCl_2 were preincubated for 1 hr at 37°C and then incubated for the appropriate time at 0°C (on ice) or 37°C. For morphological observations, the reaction was stopped by adding an excess volume of 2.5% glutaraldehyde-PBS (phosphate-buffered saline, pH 7.2) cooled or prewarmed at the same temperature as that for the incubation. After fixation for 1 hr, samples were prepared for scanning electron microscopy as described previously (Kawakami *et al.*, 1994). For immunoelectron microscopy, the reaction was stopped by adding 1% formaldehyde-PBS at either temperature. After fixation for 30 min, samples were re-suspended in PBS and mixed with an equal volume of saponin (0.2 mg/ml PBS) for 30 min at room temperature. After having been washed with PBS containing 1% bovine serum albumin, the samples were treated with rabbit anti-human myosin polyclonal antibody (Kawakami *et al.*, 1994) at 4°C overnight, and then with 6-nm colloidal gold-conjugated goat anti-rabbit IgG (Jackson Lab. Inc., USA) at 4°C overnight. Thereafter, they were processed by routine procedures for transmission electron microscopy. Control studies using normal rabbit IgG revealed no staining, thus confirming the specificity of the antibodies.

Classification of changes in platelet shape

Platelet shapes were classified into 4 states under scanning electron microscopy according to Nachmias *et al.* (1985): disks without filopodia, disks with filopodia, irregular disks, and irregular spheres. Over 100 platelets were photographed under each incubation condition, and the percentage of platelets in each of these 4 states was calculated.

Measurements of phosphorylation and Triton X-insoluble proteins

Platelet-rich plasma was preincubated with 1 mCi [32 P]phosphoric acid at 37°C for 1 hr (Higashihara *et al.*, 1985), and platelets were collected by filtration through Sepharose 2B equilibrated with HEPES-Tyrod's buffer containing glucose (1 mg/ml) and 1 mM CaCl₂. After incubation for the appropriate time at 0°C or 37°C, part of the samples were lysed by adding sampling buffer to cease the phosphorylation as previously described (Higashihara *et al.*, 1985), subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE), dried with filter paper and then exposed to Kodak X-Omat film. Relative intensity of autoradiogram was quantified by measuring the absorbance at 430 nm (Kawakami *et al.*, 1994). The rest of the samples were lysed by adding 0.5 volume of 4% Triton-X 100 and 0.5 volume of 4 mM phenylmethylsulphonyl fluoride according to the method of Fox *et al.* (1988). The lysate was immediately put on ice for 5 min and centrifuged at 2,000 g for 10 min. The insoluble pellets were then washed twice with HEPES-Tyrod's buffer containing 1% Triton-X 100 and 1 mM EDTA, subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. The relative amounts of proteins were determined by densitometric scanning (Fox *et al.*, 1988).

Results

Cold-induced change in platelet shape

Electron microscopy showed that chilling (0°C) induced a change in platelet shape during 0 to 120 min in an ice-water bath. Almost all platelets showed irregular spheres with many filopodia per cell after 120 min at 0°C (Fig. 1B). We classified the platelet shape into 4 types according to Nachmias *et al.* (1985): disks without filopodia, disks with filopodia, irregular disks and irregular spheres (Fig. 1A). This shape change proceeded slowly in a time-dependent fashion (Fig. 2). In the resting state, about 75% of the platelets appeared as disks without filopodia, and about 25% also were disks but had a few filopodia. After 30 min at 0°C, all 4 types were observed. After 90 min most platelets had the shape of irregular spheres, with under 5% of the platelets being irregular disks.

In contrast, thrombin (0.1 U/ml) at 37°C was earlier shown to induce rapid shape change within 1 min and most platelets became irregular spheres (Kawakami *et al.*, 1994). Platelets incubated with 1 mM EGTA instead of 1 mM CaCl₂ showed the same morphological changes after a 90-min incubation in the ice bath (data not shown). Tablin *et al.* (1996) also reported that cold-induced activation proceeded when washed platelets were stored in the presence of 10 mM EGTA.

Fig. 1. Cold-induced changes in platelet shape. Scanning electron micrographs of human platelets after a 30-min (A) or 120-min (B) incubation in an ice-water bath. Shape changes were classified into four states according to Nachmias *et al.* (1985); disks without filopodia (a), disks with filopodia (b), irregular disks (c) and irregular spheres (d). Almost all platelets showed irregular spheres with many filopodia per cell after 120 min at 0°C.

Cold-induced myosin phosphorylation

Figure 3 shows the time course of myosin phosphorylation of human platelets. Myosin phosphorylation increased gradually and reached a plateau by 90 min. The extent of the phosphorylation was almost the same as that obtained with thrombin (0.1 U/ml, 1 min at 37°C). Myosin phosphorylated by chilling was almost completely dephosphorylated within 5 min of rewarming. Pleckstrin (47-kDa protein) was also phosphorylated (see the inset of Fig. 3), and

Fig. 2. Percentage of platelets in each of the 4 shape categories as a function of chilling time. Disks without filopodia (a), disks with filopodia (b), irregular disks (c), and irregular spheres (d).

the extent of this phosphorylation paralleled that of LC20 phosphorylation (data not shown).

Cold-induced rearrangement of cytoskeletal proteins

Figure 4A shows the cold-induced rearrangement of cytoskeletal proteins. Not only myosin but also actin and actin-binding protein (ABP) increased in the Triton-insoluble fractions of both chilled and thrombin-treated platelets. The relative increase in the protein content of myosin was larger than that of actin or ABP. Figure 4B shows the change in the relative amount of these three proteins after chilling. The relative amount of myosin was the same as that in the thrombin (0.1 U/ml)-induced change after 1 min at 37°C.

Myosin association with filopodia

Figure 5 shows the localization of myosin in the filopodia of chilled human platelet as revealed by immuno-gold labeling. Filamentous structures, probably composed of actin bundles, were also seen in the filopodia. Microtubules disappeared in both peripheral area and filopodia within 15 min of chilling.

Discussion

The goal of this study was to clarify the role of myosin phosphorylation in cold-induced changes in platelet shape. Our morphological data confirmed the previous report of Nachmias *et al.* (1985). Four types of shape change were observed, and the number of irregular spheres increased in a time-dependent fashion. Two interesting findings are that these shape changes were correlated with the extent of phosphorylation of LC20 and these changes were reversible.

Fig. 3. Time course of cold-induced phosphorylation of myosin light chain (LC20). The extent of phosphorylation was plotted as a function of time of chilling (●) or of incubation with thrombin (0.1 U/ml) at 37°C (▲). Inset shows the autoradiogram of phosphorylation effected by chilling. Lane 1: 0 min; lane 2: 30 min; lane 3: 60 min; lane 4: 90 min; lane 5: 120 min; lane 6: thrombin (0.1 U/ml, 1 min at 37°C); lane 7: chilling for 120 min, then 37°C for 5 min.

The contribution of myosin phosphorylation to the shape changes was further confirmed by 2 findings: First, the incorporation of myosin into the Triton X-insoluble fraction proceeded in a time-dependent fashion (Fig. 4). Secondly, immuno-electron microscopic observation showed that myosin was associated with bundles of actin fibers in the filopodia (Fig. 5).

Why is myosin phosphorylated by chilling? We propose 2 mechanisms, one involving an increase in $[Ca^{2+}]_i$ by chilling and the other, inactivation of myosin phosphatase by chilling. A slight increase in $[Ca^{2+}]_i$ of 100–200 nM in chilled human platelets has been reported (Winokur and Hartwig, 1995; Oliver *et al.*, 1999). There was very little difference in the $[Ca^{2+}]_i$ increase in the presence or absence of extracellular Ca^{2+} (Oliver *et al.*, 1999). The increase in $[Ca^{2+}]_i$ during chilling is likely to be due to more than a single mechanism, e.g., some release of internal calcium stores from the dense tubule system, or cold-induced decrease in the activity of calcium pump to extrude calcium (Nakamura, 1989; Schuttlesworth and Thompson, 1991; Vostal *et al.*,

Fig. 4. Cold-induced rearrangement of cytoskeletal proteins. (A) SDS-polyacrylamide electrophoresis of Triton-insoluble fraction of platelets. Lanes 1–4: 12.5% polyacrylamide; lanes 5–8: 7.5% polyacrylamide. Lane 1: molecular weight standard; lanes 2 and 6: untreated platelets; lanes 3 and 7: platelets chilled for 60 min; lanes 4 and 8: thrombin-treated (0.1 U/ml, 1 min at 37°C) platelets; lane 5: purified platelet myosin. ABP: actin binding protein. MHC: myosin heavy chain. (B) Relative protein contents. Protein contents plotted as a function of time of chilling, were calculated by the method of Fox *et al.* (1988).

1991). If the increase in $[Ca^{2+}]_i$ is the case, it could explain a part of our present data showing the phosphorylation of both myosin LC20 and pleckstrin, a 47-kDa protein that is the target protein of Ca^{2+} /phospholipid-dependent protein kinase C (Imaoka *et al.*, 1983; Fig. 3). Although

Fig. 5. Electron microscopic distribution of anti-myosin antibody in human platelet after chilling for 90 min. Colloidal gold particles representing anti-myosin binding sites are seen in the filopodia. Arrows: actin-like filament. Bar: 0.1 μm .

Winokur and Hartwig (1995) and Hoffmeister *et al.* (2001) insisted the association of actin with cold-induced shape changes, they made no comments about myosin phosphorylation. Second possibility is that myosin may have phosphorylated by an imbalance between MLCK (myosin light chain kinase) and phosphatase activities caused by chilling. We previously reported the purification of myosin-associated protein phosphatase from chicken gizzard smooth muscle (Mitsui *et al.*, 1992), and Q_{10} (the factor by which the rate constant increases by raising the temperature by 10°C) value of this phosphatase to be very high, i.e. about 5.2 in contrast to that of MLCK, which is about 2.0 (Mitsui *et al.*, 1994). Thus the suppression of this phosphatase activity might be much greater than that of MLCK during the change in temperature from 37°C to 0–4°C. This imbalance could induce the phosphorylation of LC20 even if $[\text{Ca}^{2+}]_i$ is not increased by chilling. Further studies are needed to determine if myosin phosphatase with the same properties as the enzyme in gizzard smooth muscle (Mitsui *et al.*, 1994) exists in human platelets.

Acknowledgment

The authors wish to thank to Mr. M. Fukuda and Ms. C. Okada (Laboratory for Electron Microscopy, Kyorin University School of Medicine) for their technical assistances. This work was supported in part by a grant-in aid for scientific research from the Japan Society for the Promotion of Science.

References

- Adelstein, R.S., Conti, M.A. (1975). Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity. *Nature* **256**: 597–598.
- Behnke, O. (1970). Microtubules in disk-shaped blood cells. *Int. Rev. Exp. Pathol.* **9**: 1–92.
- Bennett, W.F., Lynch, G. (1980). Low-temperature induction of calcium-dependent protein phosphorylation in blood platelets. *J. Cell Biol.* **86**: 280–285.
- Daniel, J.L., Molish, I.R., Molman, H. (1981). Myosin phosphorylation in intact platelets. *J. Biol. Chem.* **256**: 7510–7514.
- Fox, J.E.B., Boyles, J.K., Berndt, M.C., Steffen, P.K., Anderson, L.K. (1988). Identification of a membrane skeleton in platelets. *J. Cell Biol.* **106**: 1525–1538.
- Higashihara, M., Maeda, H., Yatomi, Y., Takahata, K., Oka, H., Kume, S. (1985). The platelet protein phosphorylation induced by a monoclonal antibody against human platelets (TP82). *Biochem. Biophys. Res. Commun.* **133**: 306–313.
- Higashihara, M., Takahata, K., Kurokawa, K. (1991). Effect of phosphorylation of myosin light chain by myosin light chain kinase and protein kinase C on conformational change and ATPase activities of human platelet myosin. *Blood* **78**: 3224–3231.
- Hoffmeister, K.M., Falet, H., Toker, A., Barkalow, K.L., Stossel, T.P., Hartwig, J.H. (2001). Mechanisms of cold-induced platelet actin assembly. *J. Biol. Chem.* **276**: 24751–24759.
- Ikebe, M., Hinkins, S., Hartshorne, D.J. (1983). Correlation of enzymatic properties and conformation of smooth muscle myosin. *Biochemistry* **22**: 4580–4587.
- Ikebe, M. (1989). Phosphorylation of a second site for myosin light chain kinase on platelet myosin. *Biochemistry* **28**: 8750–8755.
- Imaoka, T., Lynham, J.A., Haslam, R.J. (1983). Purification and characterization of the 47,000-dalton protein phosphorylated during degranulation of human platelets. *J. Biol. Chem.* **258**: 11404–11414.
- Itoh, K., Hara, T., Shibata, N. (1992). Diphosphorylation of platelet myosin by myosin light chain kinase. *Biochim. Biophys. Acta* **1133**: 286–292.
- Kawakami, H., Higashihara, M., Song, X.-H., Kurokawa, K., Ikebe, M., Hirano, H. (1994). Okadaic acid induces marked shape change of human platelets. *J. Smooth Muscle Res.* **30**: 57–64.
- Korn, E.D. (1978). Biochemistry of actomyosin-dependent cell motility (a review). *Proc. Natl. Acad. Sci. USA* **75**: 588–599.
- Mitsui, T., Inagaki, M., Ikebe, M. (1992). Purification and characterization of smooth muscle myosin-associated phosphatase from chicken gizzards. *J. Biol. Chem.* **267**: 16727–16735.
- Mitsui, T., Kitazawa, T., Ikebe, M. (1994). Correlation between high temperature dependence of smooth muscle myosin light chain phosphatase activity and muscle relaxation rate. *J. Biol. Chem.* **269**: 5842–5848.
- Nachmias, V.T., Kavalier, J., Jacobowitz, S. (1985). Reversible association of myosin with the platelet cytoskeleton. *Nature* **313**: 70–72.
- Nakamura, J. (1989). pH and temperature resolve the kinetics of two pools of calcium bound to the sarcoplasmic reticulum Ca^{2+} -ATPase. *J. Biol. Chem.* **264**: 17029–17031.
- Oliver, A.E., Tablin, F., Walker, N.J., Crowe, J.H. (1999). The internal calcium concentration of human platelets increases during chilling. *Biochim. Biophys. Acta* **1416**: 349–360.
- Shuttleworth, T.J., Thompson, J.L. (1991). Effect of temperature on receptor-activated changes in $[\text{Ca}^{2+}]_i$ and their determination using fluorescent probes. *J. Biol. Chem.* **266**: 1410–1414.
- Tablin, F., Oliver, A.E., Walker, N.J., Crowe, L.M., Crowe, J.H. (1996). Membrane phase transition of intact human platelets: Correlation with cold-induced activation. *J. Cell. Physiol.* **168**: 305–313.
- Vostal, J.G., Jackson, W.L., Shulman, N.R. (1991). Cytosolic and stored calcium antagonistically control tyrosine phosphorylation of specific platelet proteins. *J. Biol. Chem.* **266**: 16911–16916.
- White, J.G., Krumwiede, M. (1973). Influence of cytochalasin B on the shape change induced in platelets

- by cold. *Blood* **41**: 823–832.
- White, J.G. (1982). Influence of taxol on the response of platelets to chilling. *Am. J. Pathol.* **108**: 184–195.
- Winokur, R. Hartwig, J.H. (1995). Mechanism of shape change in chilled human platelets. *Blood* **85**: 1796–1804.
- Zucker, M.B., Borrelli, J. (1954). Reversible alterations in platelet morphology produced by anticoagulants and by cold. *Blood* **9**: 602–608.

(Received October 20, 2001: Accepted December 22, 2001)