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Platelet Talin Is Phosphorylated by Calyculin A

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Abstract Calyculin A and okadaic acid, potent and cell permeable inhibitors of type 1 and type 2A protein phosphatases, inhibit platelet aggregation and secretion. However, the relationship between phosphatase inhibition and inhibition of platelet function is not well understood. We found that in unstimulated platelets, talin (P235) was phosphorylated at threonine residues by calyculin A. Furthermore, the extent of talin phosphorylation by calyculin A was closely correlated with its inhibition of thrombin-induced platelet aggregation. Since the binding of talin to platelet glycoprotein IIb/IIIa complex has been shown to be affected by its phosphorylation, these results suggest that type 1 and/or type 2A protein phosphatases may play a role in the regulation of membrane-cytoskeleton interaction through dephosphorylation of talin.

Key words: protein phosphatase, calyculin A, platelet, talin, phosphorylation, phosphoamino acid analysis

Potent and cell permeable inhibitors of type 1 and 2A protein (serine/threonine) phosphatases, i.e., okadaic acid (OA), calyculin A (CLA), and tautomycin, inhibit agonist-induced platelet aggregation and secretion [Lerea, 1991; Murata et al., 1992; Higashihara et al., 1992; Chiang, 1992]. These so-called "OA class compounds" [Sassa et al., 1989] are also demonstrated to inhibit thrombin-induced Ca^{2+} influx and exposure of fibrinogen binding sites of glycoprotein IIb/IIIa complex (GpIIb/IIIa) in platelets, which is essential for platelet aggregation [Murata et al., 1993; Sakon et al., 1993]. In accordance with the inhibitory effects on platelet reactions, various polypeptides in platelets including P50 were significantly phosphorylated by OA or CLA, but the inhibitory mechanisms are not well understood [Murata et al., 1992]. One of the phosphorylated polypeptides is a 235 KDa polypeptide (P235) considered to be talin based on its molecular weight. Talin is colocalized with GpIIb/IIIa of the platelet integrin family and has been considered to play a role in membrane-cytoskeleton interaction [Burridge and Connell, 1983; Beckerle et al., 1989]. These findings raised the possibility that phosphorylation/dephosphorylation of talin might regulate platelet function by modulating the interaction of GpIIb/IIIa and the cytoskeletal system. In the present study, therefore, attempts were made to clarify (1) whether P235 phosphorylation is correlated with the inhibition of platelet reaction by CLA, (2) whether talin (P235) is specifically phosphorylated by CLA in unstimulated platelets, and (3) which amino acid residue, i.e., serine, threonine or tyrosine, is phosphorylated by CLA treatment.

Abbreviations used: CLA, calyculin A; GpIIb/IIIa, glycoprotein IIb/IIIa complex; mAb, monoclonal antibody; OA, okadaic acid; PKC, protein kinase C; PRP, platelet rich plasma; WP, washed platelet.

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METHODS

Materials

Calyculin A (CLA) was purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Bovine thrombin was kindly provided by Mochida Pharmaceutical Co. (Osaka, Japan). Mouse monoclonal antibody (mAb) against chicken gizzard smooth muscle talin (T3287), Dowex AG1-X8, and authentic phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine) were purchased from Sigma Chemical Co. (St. Louis, MO). Calpepetin, a specific inhibitor for calpain, was synthesized in our laboratory as described [Tsujinaka et al., 1990]. [^{32}P]ortho-

phosphate was purchased from New England Nuclear (Boston, MA).

Platelet Aggregation

Venous blood from healthy volunteers was drawn into 0.1 volume of 3.8% (w/v) trisodium citrate solution. The volunteers had not taken medication affecting platelet function for at least 2 weeks before the donation of blood. Informed consent was obtained from each volunteer. Platelet rich plasma (PRP) was prepared by centrifugation at 170*g* for 15 min at room temperature. After adding EGTA and EDTA (final concentration, 2 mM), PRP was centrifuged at 850*g* for 10 min and platelets were resuspended in HEPES-glucose buffer (10 mM HEPES, 145 mM NaCl, mM KCl, 1 mM $MgSO_4$, 5 mM glucose, pH 7.4 containing 2 mM EGTA and 2 mM EDTA. Platelets were washed again with the same buffer by centrifugation at 850*g* for 5 min. Finally, washed platelets (WPs) were suspended in HEPES-glucose buffer without EDTA or EGTA.

Thrombin-induced platelet aggregation was performed as described previously [Murata et al., 1993]. WPs (3.0×10^8/ml) were incubated with CLA for 4 min at 37°C in the presence of mM $CaCl_2$ without stirring and for 1 min with stirring. Then, platelets were stimulated by 0.1 U/ml thrombin for 4 min and platelet aggregation was recorded by an aggregometer.

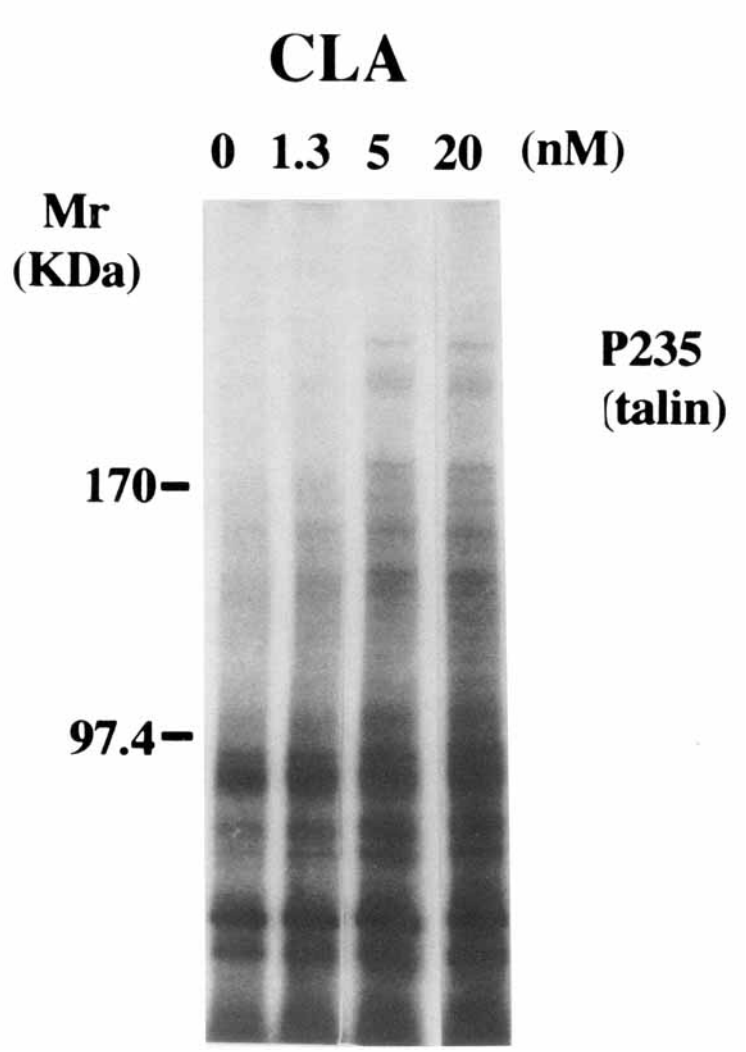

Fig. 1. Phosphorylation of P235 in CLA-treated platelets. [^{32}P]-labeled washed platelets were incubated with CLA (0–20 nM) at 37°C for 5 min and solubilized with the same volume of sample buffer containing 4% SDS and 4% 2-mercaptoethanol. Samples were then boiled and electrophoresed as described in Methods. Gels were dried and exposed to Kodak X-AR film at room temperature for 12 h. Molecular weight markers are alpha2-macroglobulin (170 KDa) and phosphorylase b (97.4 KDa).

Protein Phosphorylation

WPs (1.0×10^9/ml) were labeled with 200 μCi/ml [^{32}P]orthophosphate. [^{32}P]-labeled WPs were incubated with 20 nM CLA or vehicle (0.5% ethanol) for 5 min at 37°C, solubilized with SDS sample buffer [Laemmli, 1970], and electrophoresed as described elsewhere [Murata et al., 1992]. After electrophoresis, gels were dried and protein phosphorylation was evaluated by densitometric analysis following autoradiography.

Immunoprecipitation

Talin was immunoprecipitated from the Triton X-100 soluble fraction of [^{32}P]-labeled WPs by the modified procedure of Nigg et al. [1982], using anti-talin mAb (T3287). In brief, 500 μl of [^{32}P]-labeled WPs (1.0×10^9/ml) was incubated with 20 nM CLA or 0.5% ethanol as vehicle for 5 min at 37°C and lysed with 500 μl of lysis buffer (0.2% Triton X-100, 100 mM Tris-HCl, pH 7.4, 100 μM calpeptin, 10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 100 mM benzamidine, 4 mM EGTA, 4 mM EDTA) containing 20 nM CLA or vehicle. Lysates were set on ice for 30 min and centrifuged at 10,000*g* for 5 min to remove insoluble fractions. The resultant supernatants were immunoprecipitated with anti-talin mouse mAb or non-immunized mouse IgG1. The immunoprecipitant was washed four times with a mixture of glucose HEPES buffer and lysis buffer (1:1 vol/vol). Final pellets were solubilized with SDS sample buffer and boiled for 5 min. After centrifugation at 10,000*g* for 5 min, supernatants were applied on 7.5% SDS-PAGE.

Alkali Treatment of SDS-Polyacrylamide Gels

After electrophoresis, gels were treated with 1 M NaOH at 45°C for 120 min according to the method of Kohno and Pouyssegur [1986]. Following neutralization by acetic acid, the gels were dried and subjected to autoradiography.

Phosphoamino Acid Analysis

Phosphoamino acid analysis of [^{32}P]-labeled talin was performed according to the method of Cooper et al. [1983] with slight modification. The Triton X-100 soluble fraction immunoprecipitant was electrophoresed on 7.5% polyacrylamide gel. The band containing talin was excised and subjected to acid hydrolysis (6 N HCl at 110°C for 1 h). Following vacuum centrifugation, samples were neutralized by 10 mM NH_4OH and applied to Dowex AG1-X8 resin. The resin beads were washed with distilled water and amino acids were eluted by 0.1 M HCl. The eluates were concentrated and mixed with authentic phosphoserine, phosphothreonine, and phosphotyrosine. The samples were subjected to one-dimensional thin layer chromatography electrophoresed at pH 3.5 (pyridine/acetic acid/water, 1:10:189 by volume) with constant voltage of 1.3 KV for 20 min. Plates were stained with ninhydrin and [^{32}P]-labeled phosphoamino acids were visualized by autoradiography.

RESULTS

When [^{32}P]-labeled platelets were treated with CLA (0–20 nM), various polypeptides such as P235, P80, P53, P50, P20, were significantly phosphorylated within 5 min (not shown). In particular, P235 was markedly phosphorylated by CLA in a concentration-dependent manner (Fig. 1), which was correlated with the inhibition of thrombin-induced platelet aggregation (Fig. 2). The similar relationship between P235 phosphorylation and inhibition of platelet aggregation was also observed in platelets treated with OA (0–2 μM).

To confirm whether P235 is talin, the Triton X-100 soluble fraction of [^{32}P]-labeled platelets was immunoprecipitated by a monoclonal anti-

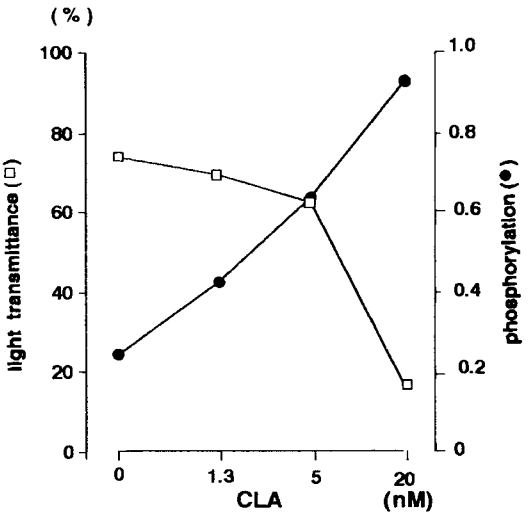

Fig. 2. Correlation between P235 phosphorylation and thrombin-induced platelet aggregation in CLA-treated platelets. Thrombin-induced platelet aggregation was monitored with an aggregometer as described in Methods. Light transmittance at 4 min after thrombin stimulation was plotted (□). The extent of phosphorylation of P235 was densitometrically quantified and expressed (●) by the ratio against a phosphoprotein (MW = 65 KDa) whose level of phosphorylation did not change in response to thrombin or CLA.

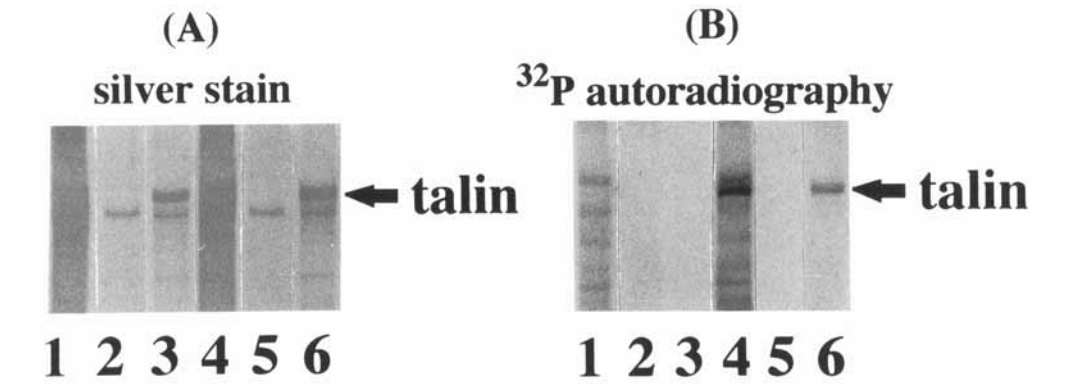

Fig. 3. Immunoprecipitation of talin from the lysate of CLA-treated platelets. [^{32}P]-labeled platelets were incubated with vehicle (lanes 1–3) or 20 nM CLA (lanes 4–6) and then solubilized with 0.1% Triton X-100. Talin was immunoprecipitated from the Triton X-100 soluble fraction by using anti-talin mAb or mouse IgG1. Triton X-100 soluble fraction of [^{32}P]-labeled platelets treated with vehicle (lane 1) or 20 nM CLA (lane 4) and immunoprecipitate from the same sample (vehicle: lane 3, CLA 20 nM: lane 6) were electrophoresed as described in Methods. Negative controls with mouse IgG1 were also performed from the same sample (vehicle: lane 2; CLA 20 nM: lane 5). The results of silver staining (**A**) and autoradiogram (**B**) are shown as the representative data of three independent experiments with different blood donors.

body against talin (T3287). The amount of precipitable talin was identical between CLA-treated and nontreated platelets (Fig. 3A). Talin was not immunoprecipitated by nonimmune IgG1, but some peptides were similarly precipitated (Fig. 3A, lanes 2, 5). By autoradiography, a single [^{32}P]-labeled polypeptide of the same molecular weight (235 KDa) was demonstrated in CLA-treated platelets (Fig. 3B, lane 6) but not in nontreated platelets (Fig. 3B, lane 3). No radioactivity was detected when nonimmune IgG1 was used (Fig. 3B, lane 5). These results indicate that talin is phosphorylated by CLA in unstimulated platelets.

To examine which amino acid residue of talin, i.e., serine, threonine, or tyrosine, is phosphorylated, SDS-polyacrylamide gels were alkali-treated according to the method of Kohno and Pouyssegur [1986]. As shown in Figure 4, 20 nM CLA-induced phosphorylation of P235 was not reversed by alkali treatment, indicating that threonine and/or tyrosine, but not serine residues, were phosphorylated in CLA-treated platelets. Phosphoamino acid analysis of [^{32}P]-labeled peptides immunoprecipitated by anti-talin antibody revealed that threonine residues were predominantly phosphorylated following CLA treatment (Fig. 5).

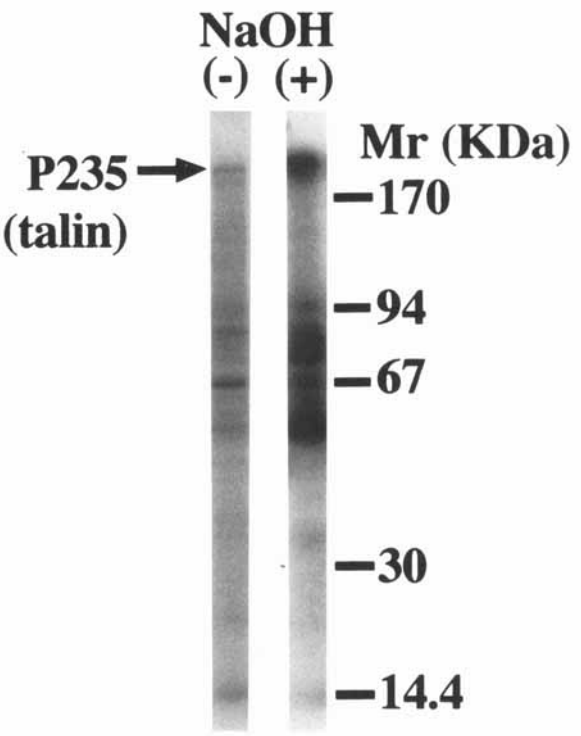

Fig. 4. Alkali-resistant phosphoproteins of CLA-treated platelets. [^{32}P]-labeled washed platelets were incubated with 20 nM CLA at 37°C for 5 min. Platelets were then solubilized and electrophoresed on 4–20% gradient polyacrylamide gels as described in Figure 1. Gels were incubated with 1 M NaOH at 45°C for 120 min and neutralized by acetic acid. Alkali- or non-alkali-treated gels were exposed to Kodak X-AR film at room temperature for 36 and 12 h, respectively. Molecular weight markers were alpha2-macroglobulin (170 KDa), phosphorylase b (97.4 KDa), albumin (67 kDa), carbonic anhydrase (30 KDa), and alpha-lactalbumin (14.4 KDa).

DISCUSSION

In the present report, we have demonstrated that talin (P235) is phosphorylated by CLA, a potent and specific inhibitor of type 1 and 2A protein phosphatases [Ishihara et al., 1989], and

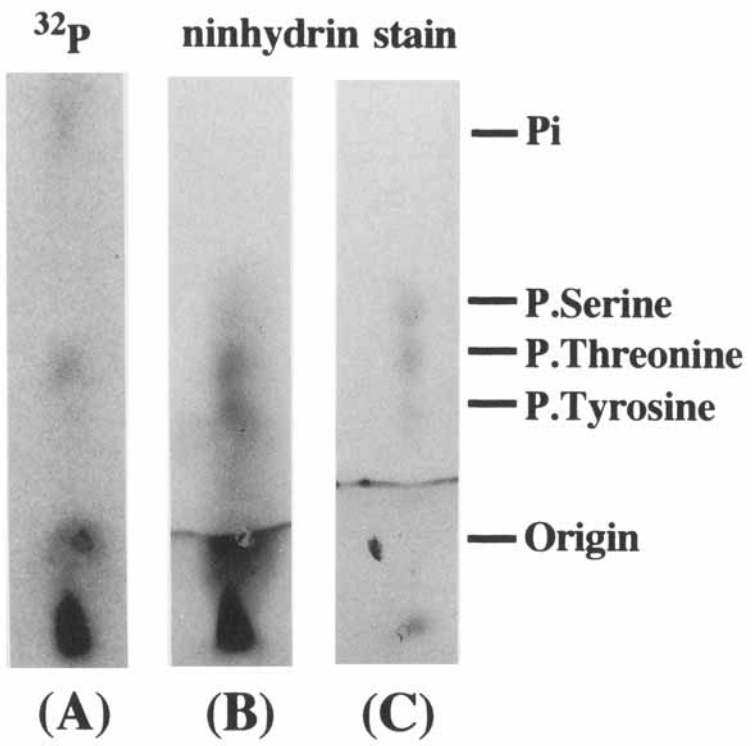

Fig. 5. Phosphoamino acid analysis of phosphorylated talin. Protein immunoprecipitated by anti-talin mAb from Triton X-100 soluble fraction of CLA-treated [^{32}P]-labeled platelets was electrophoresed as described in Methods. The gels were dried and stained with Coommasie brilliant blue. The band containing talin was excised and hydrolyzed with 6 M HCl. The radiolabeled phosphoamino acids were mixed with authentic phosphoserine (P. Serine), phosphothreonine (P. Threonine), and phosphotyrosine (P. Tyrosine). The sample was then subjected to one-dimensional thin-layer chromatography. The plate was exposed to Kodak X-AR film at −80°C for 7 days with enhancing screens (**A**) and stained with ninhydrin (**B**). The mixture of authentic phosphoamino acids was also electrophoresed and stained with ninhydrin (**C**).

that its phosphorylation is closely correlated with the inhibition of agonist-induced platelet aggregation. This inhibitory effect of CLA on platelet reaction does not appear to result from nonspecific effects on plasma membrane because 200 nM CLA (10-fold higher concentration than was used in this study) did not cause any conformational changes of plasma membrane when examined by ANS (1-anilino-8-naphthalene sulphate) or DPH (1.6-diphenyl-1,3,5-hexatriene) [Sakon et al., 1994]. Furthermore, the removal of extracellular CLA by gel filtration restored thrombin induced platelet aggregation (Murata, unpublished work). These results suggest that CLA specifically and reversibly inhibits type 1 and/or type 2A phosphatases in platelets and that the phosphorylation of talin may result from their inhibition by CLA, although the stoichiometry of this phosphorylation was not determined.

The presence of both type 1 and 2A serine/threonine protein phosphatases has been identified in platelets [Lerea, 1991; Walker and Watson, 1992]. They do not require divalent cations for their activities, unlike type 2B and 2C serine/threonine phosphatases, whose activation requires Ca^{2+} and Mg^{2+}, respectively. Therefore, type 1 and 2A protein phosphatases are supposed to be active, and to be involved in the dephosphorylation of talin in unstimulated platelets, although type 1 protein phosphatase activity is also shown to be regulated by endogenous inhibitor 2 [Shenolikar and Nairn, 1991].

It remains unknown which protein kinase(s) are involved in the phosphorylation of talin in resting platelets. When the lysate of [^{32}P]-labeled platelets was incubated with CLA, the effect of CLA on talin phosphorylation was not significant (data not shown). Since the lysis buffer contains 2 mM EGTA and EDTA, these results suggest that the kinase(s) involved in talin phosphorylation requires divalent cations like Ca^{2+} or Mg^{2+}. One of the possible enzymes is protein kinase C (PKC), which has been shown to phosphorylate talin [Litchfield and Ball, 1986, 1990], but it remains unknown whether PKC is

active in CLA treated platelets. Recently, Bertagnolli et al. [1993] reported that talin is phosphorylated by 1.0 U/ml thrombin and 10 μM phorbol-12-myristate-13-acetate in platelets. We did not find phosphorylation of talin in 0.1 U/ml thrombin-stimulated platelets (data not shown), suggesting that phosphatases may be active even in stimulated platelets.

CLA has been shown to be a specific and potent inhibitor of type 1 and type 2A serine/threonine protein phosphatases, but it may increase tyrosine phosphorylation because type 2A protein phosphatase has been shown to dephosphorylate not only serine/threonine residues but also tyrosine residue [Goris et al., 1988]. However, results from alkali treatment of SDS gels and phosphoamino acid analysis revealed that talin was phosphorylated on threonine residue in CLA-treated platelets.

Talin has been demonstrated to play a role in the linkage between cytoskeletal system and integrins in various eukaryotic cells [Horwitz et al., 1986; Beckerle and Yeh, 1990; Luna, 1991; Luna and Hitt, 1992]. In platelets, talin is a major cytosolic protein consisting of 3% of total platelet protein, but it also has been demonstrated to function as an anchoring protein between actin filament and GpIIb/IIIa complex (integrin alphaIIb/beta3) [Collier and Wang, 1982]. Since the phosphorylation of talin is shown to affect its affinity to integrins [Burn et al., 1988; Pasquale et al., 1986; Turner et al., 1989; Qwarnstrom et al., 1991], phosphorylation (or dephosphorylation) of talin may modulate the activation of GpIIb/IIIa or induce the reorganization of cytoskeleton by influencing its affinity to GpIIb/IIIa and cytoskeleton. In support of this hypothesis, it was recently found that the same treatment by CLA (20 nM, 37°C, 5 min) as in this study, caused specific morphological changes in platelets in accordance with reorganization of actin filaments [Yano et al., 1994]. Furthermore, CLA suppresses various platelet reactions by inhibiting Ca^{2+} influx [Murata et al., 1993]. Therefore, CLA inhibitable protein phosphatases, most likely type 1 and 2A protein phosphatases, might be involved in maintaining the normal membrane-cytoskeleton relationship by dephosphorylating talin.

In conclusion, talin is dephosphorylated on threonine residues by type 1 and/or 2A protein phosphatases and this dephosphorylation in unstimulated platelets may play a role in the regulation of platelet function.

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REFERENCES

Beckerle MC, Yeh RK (1990): Talin: Role at sites of cell-substratum adhesion. Cell Motil Cytoskeleton 16:7–13.

Beckerle MC, Miller DE, Bertagnolli ME, Locke SJ (1989): Activation-dependent redistribution of the adhesion plaque protein, talin, in intact human platelets. J Cell Biol 109: 3333–3346.

Bertagnolli ME, Locke SJ, Hensler ME, Bray PF, Beckerle MC (1993): Talin distribution and phosphorylation in thrombin-activated platelets. J Cell Sci 106:1189–1199.

Burn P, Kupfer A, Singer SJ (1988): Dynamic membrane-cytoskeletal interactions: Specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. Proc Natl Acad Sci USA 85:497–501.

Burridge K, Connell L (1983): A new protein of adhesion plaques and ruffling membranes. J Cell Biol 97:359–367.

Chiang TM (1992): Okadaic acid and vanadate inhibit collagen-induced platelet aggregation; The functional relation of phosphatase on platelet aggregation. Thromb Res 67: 345–354.

Collier NC, Wang K (1982): Purification and properties of human platelet P235. J Biol Chem 257:6937–6943.

Cooper JA, Sefton BM, Hunter T (1983): Detection and quantification of phosphotyrosine in proteins. Methods Enzymol 99:387–402.

Goris J, Pallen CJ, Parker PJ, Hermann J, Waterfield MD, Merlevede W (1988): Conversion of a phosphoseryl/threonyl phosphatase into a phosphotyrowyl phosphatase. Biochem J 256:1029–1034.

Higashihara M, Takahata K, Kurokawa K, Ikebe M (1992): The inhibitory effects of okadaic acid on platelet function. FEBS Lett 307:206–210.

Horwitz A, Duggan K, Buck C, Beckerle MC, Burridge K (1986): Interaction of plasma membrane fibronectin receptor with talin: A transmembrane linkage. Nature (London) 320:531–533.

Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, Hartshorne DJ (1989): Calyculin A and okadaic acid: Inhibitors of protein phosphatase activity. Biochem Biophys Res Commun 159:871–877.

Kohno M, Pouyssegur J (1986): alpha-Thrombin-induced tyrosine phosphorylation of 43000- and 41000-Mr proteins is independent of cytoplasmic alkalinization in quiescent fibroblasts. Biochem J 238:451–457.

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

Lerea KM (1991): Thrombin-induced effects are selectively inhibited following treatment of intact human platelets with okadaic acid. Biochemistry 30:6819–6824.

Litchfield DW, Ball EB (1986): Phosphorylation of the cytoskeletal protein talin by protein kinase C. Biochem Biophys Res Commun 134:1276–1283.

Litchfield DW, Ball EH (1990): Phosphorylation of high molecular weight proteins in platelets treated with 12-o-tetradecanoylphorbol-13-acetate. Biochem Int 20:615–621.

Luna EJ (1991): Molecular links between the cytoskeleton and membranes. Curr Opin Cell Biol 3:120–126.

Luna EJ, Hitt AL (1992): Cytoskeleton-plasma membrane interactions. Science 258:955–964.

Murata K, Sakon M, Kambayashi J, Yukawa M, Ariyoshi H, Shiba E, Kawasaki T, Kang J-H, Mori T (1992): The effects of okadaic acid and calyculin A on thrombin induced platelet reaction. Biochem Int 26:327–334.

Murata K, Sakon M, Kambayashi J, Yukawa M, Yano Y, Fujitani K, Kawasaki T, Shiba E, Mori T (1993): The possible involvement of protein phosphatase 1 in thrombin-induced Ca^{2+} influx of human platelets. J Cell Biochem 51:442–445.

Nigg EA, Sefton BM, Hunter T, Walter G, Singer SJ (1982): Immunofluorescent localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic src peptide. Proc Natl Acad Sci USA 79:5322–5326.

Pasquale EB, Maher PA, Singer SJ (1986): Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. Proc Natl Acad Sci USA 83:5507–5511.

Qwarnstrom EE, MacFarlane SA, Page RC, Dower SK (1991): Interleukin 1 beta induces rapid phosphorylation and redistribution of talin: A possible mechanism for modulation and of fibroblast focal adhesion. Proc Natl Acad Sci USA 88:1232–1236.

Sakon M, Murata K, Fujitani K, Yano Y, Kambayashi J, Uemura Y, Kawasaki T, Shiba E, Mori T (1993): Caluculin A inhibits the exposure of fibriogen receptor in thrombin-stimulated platelets. Biochem Biophys Res Commun 195: 139–143.

Sakon M, Murata K, Kambayashi J (1994): The involvement of protein phosphatases in platelet activation. Platelets 5:130–134.

Sassa T, Richter WW, Uda N, Suganuma M, Suguri H, Yoshizawa S, Hirota M, Fujiki H (1989): Apparent "activation" of protein kinases by okadaic acid class tumor promoters. Biochem Biophys Res Commun 159:939–944.

Shenolikar S, Nairn AC (1991): Protein phosphatases: Recent progress. Adv Second Messenger Phosphorylation Res 23:1–121.

Tsujinaka T, Ariyoshi H, Uemura Y, Sakon M, Kambayashi J, Mori T (1990): Potential participation of calpain in platelet activation studied by use of a cell penetrating calpain inhibitor (calpeptin). Life Sci 46:1059–1066.

Turner CE, Pavalko FM, Burridge K (1989): The role of phosphoarylation and linited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity. J Biol Chem 264:11938–11944.

Walker TR, Watson SP (1992): Okadaic acid inhibits activation of phospholipase C in human platelets by mimicking the actions of protein kinases A and C. Br J Pharmacol 105:627–631.

Yano Y, Kambayashi J, Shiba E, Sakon M, Oiki E, Fukuda K, Kawasaki T, Mori T (1994): The role of protein phosphorylation and cytoskeletal reorganization in microparticla formation from the platelet plasma membrane. Biochem J 299:303–308.