Calpains are involved in phosphatidylinositol 3′,4′-*bis*phosphate synthesis dependent on the $\alpha_{IIb}\beta_3$ integrin engagement in thrombin-stimulated platelets

Nathalie Montsarrat, Claire Racaud-Sultan*, Gérard Mauco, Monique Plantavid, Bernard Payrastre, Monique Breton-Douillon, Hugues Chap

Institut Fédératif de Recherche en Immunologie Cellulaire et Moléculaire, INSERM, Unité 326, Hôpital Purpan, F31059 Toulouse Cedex, France

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Abstract In thrombin-stimulated platelets $\alpha_{IIb}\beta_3$ integrin engagement triggers both phosphatidylinositol 3′,4′-*bis*phosphate synthesis and calpain activation. We checked the possible involvement of calpains in phosphatidylinositol 3-kinase signalling pathway using a cell permeant specific inhibitor of calpains, calpeptin. In conditions where thrombin-induced platelet aggregation and secretion were not impaired, we found a dose-dependent inhibition of phosphatidylinositol 3,4-*bis*phosphate synthesis by calpeptin from 50 μg/ml. Moreover, pretreatment of platelets by both calpeptin and the peptide RGDS, an inhibitor of fibrinogen binding to activated $\alpha_{IIb}\beta_3$ integrin, did not induce additive effects on phosphatidylinositol 3,4-*bis*phosphate inhibition. Finally, the p85 regulatory subunit of phosphatidylinositol 3-kinase was still translocated to the cytoskeleton in calpeptin-treated platelets. These data indicate that calpains are involved in the regulation of $\alpha_{IIb}\beta_3$ integrin-dependent phosphatidylinositol 3-kinase signalling pathway.

Key words: $\alpha_{IIb}\beta_3$ integrin; Phosphatidylinositol 3-kinase; Calpain; Calpeptin; Platelet aggregation

1. Introduction

Calpains are Ca^{2+}-dependent cytoplasmic cysteine proteases that are widely distributed in mammalian cells [1]. In stimulated platelets, translocation to the membrane cytoskeleton and activation of calpains are detected by 10 and 30 s after thrombin addition, respectively [2]. Activation but not redistribution of calpains requires $\alpha_{IIb}\beta_3$ integrin engagement and platelet aggregation [2]. Upon platelet activation, $\alpha_{IIb}\beta_3$ integrin has been shown to promote calcium influx [3], a determining factor of calpain activation [4]. The isoforms m- and μ-calpain require 300–500 and 10–100 μM Ca^{2+} for in vitro activity, respectively. Activation of calpains results in selective hydrolysis of several platelet proteins including cytoskeletal proteins, i.e. actin-binding protein (ABP), talin, spectrin, calmodulin-binding proteins and signalling enzymes, i.e. phospholipase $C_{\beta 3}$ [5], protein kinase C [6], tyrosine kinase $pp60^{c-src}$ [7], tyrosine phosphatase PTP-1B [8]. Moreover, a calpain-dependent hydrolysis of the cytoplasmic domain of β_3 integrin has been recently described in thrombin-stimulated platelets [9]. Calpains have been involved in the shedding of procoagulant membrane vesicles and bloating of filopod surfaces in stimulated platelets [10,11], two processes which are closely related to intracellular cytoskeletal reorganization.

Thrombin activation of platelets triggers synthesis of the PtdIns 3-kinase products, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, which seems to be necessary for irreversible aggregation to occur [12]. PtdIns(3,4,5)P_3 reaches its peak value within 20 s of stimulation in a Ca^{2+}- and $\alpha_{IIb}\beta_3$ integrin-independent manner [13]. On the other hand, PtdIns(3,4)P_2 is only detectable 30 s after thrombin addition with a rapid phase before 90 s that is independent of fibrinogen/$\alpha_{IIb}\beta_3$ integrin interaction and Ca^{2+}, followed by a late phase that is associated with $\alpha_{IIb}\beta_3$ integrin engagement and Ca^{2+} [13]. Two isoforms of PtdIns 3-kinase, heterodimeric p85/p110 and monomeric p110γ, have been shown in platelets. In thrombin-stimulated platelets, we have previously shown that $\alpha_{IIb}\beta_3$ integrin regulates the translocation of the regulatory subunit p85 to the cytoskeleton, which is closely related to $\alpha_{IIb}\beta_3$ integrin-dependent PtdIns(3,4)P_2 synthesis [14]. Moreover, the redistribution of p85 allows its interaction with $p125^{FAK}$ tyrosine kinase and subsequently leads to PtdIns 3-kinase activation [14]. Because calpains and PtdIns 3-kinase are both localized in focal adhesion sites, regulated by $\alpha_{IIb}\beta_3$ integrin engagement, and involved in cytoskeleton reorganization, we have considered the possibility that the two signal transduction systems may interact during platelet activation.

In order to check the possible involvement of calpains in PtdIns(3,4)P_2 production by thrombin-stimulated platelets, we used the cell permeant specific inhibitor of calpains, calpeptin [15]. This study shows that pretreatment of platelets with calpeptin inhibits that part of PtdIns(3,4)P_2 synthesis which is dependent on $\alpha_{IIb}\beta_3$ integrin engagement upon thrombin stimulation without impairment of p85 translocation to the cytoskeleton.

2. Materials and methods

2.1. Preparation of platelets

Human platelets were isolated by centrifugation from fresh platelet concentrates (Etablissement de Transfusion Sanguine Pyrénées-Garonne, Toulouse, France) and labelled for 90 min with 0.4 mCi/ml [γ-^{32}P]phosphate (Amsterdam, Ltd., Buckinghamshire, UK) as previously described [16]. Platelets were finally resuspended in modified Tyrode's buffer (pH 7.4) containing 2.5 mM $CaCl_2$.

2.2. Calpeptin incorporation and platelet activation

Calpeptin was purchased from Novabiochem (France Biochem, Meudon, France). Calpeptin (25–75 μg/ml) in DMSO or DMSO alone

*Corresponding author. Fax: (33) 05 61 77 94 01.
E-mail: racaud@purpan.inserm.fr

Abbreviations: PtdIns(3,4)P_2, phosphatidylinositol 3′,4′-*bis*phosphate; PtdIns 3-kinase, phosphatidylinositol 3-kinase; DMSO, dimethylsulfoxide; SH, src homology; HPLC, high performance liquid chromatography

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was added to platelets for 5 min at 37°C under shaking before stimulation by thrombin. The final amount of DMSO was maximally 0.3% of the total volume. Thrombin activation of the platelets was obtained by adding 1 NIH unit of human thrombin (Sigma, St. Louis, MO, USA) per ml of suspension (3×10^8 platelets/ml). Incubation was carried on for 5 min under shaking. In some experiments, the synthetic peptide RGDS (Sigma) was added 15 s before thrombin.

Platelet release reaction was checked by [^{14}C]serotonin appearance in the supernatants after 1 min of thrombin stimulation. Aggregation was checked by turbidimetry.

2.3. Lipid extraction and analysis

Lipid extraction, which served also to quench the reactions, was a modified procedure of Bligh and Dyer [16]. Lipids were then deacylated by methylamine treatment, and separated by HPLC on a Partisphere SAX column (Whatman International Ltd., UK) using an elution gradient of 0–1 M ammonium phosphate as previously described [16]. Radioactivity was detected on line with a Berthold LB506C radioactivity monitor.

2.4. Cytoskeleton extraction and immunodetection of p85

Cytoskeleton extraction was obtained in the presence of 1% Triton X-100, which also made it possible to stop the reactions as described previously [17]. Cytoskeleton separated from Triton X-100-soluble fractions by centrifugation was then washed twice with Tris buffer containing 0.5 and 0% Triton X-100 [17]. Finally, cytoskeleton was resuspended in Tris 20 mM pH 7.4 by sonication (20 kHz for 2×10 s) using an ultrasonic cell disrupter. Proteins were solubilized, separated on 5–15% SDS-PAGE, and blotted onto nitrocellulose as described previously [14]. After nitrocellulose blockade, immunodetection of p85 was performed with a rat anti-p85 antibody from Upstate Biotechnology Inc. (New York). Antibody reaction was visualized using alkaline phosphatase-conjugated secondary antibodies.

3. Results

3.1. Calpeptin inhibition of PtdIns(3,4)P_2 synthesis in thrombin-stimulated platelets

Calpeptin has been largely used as a cell permeant specific inhibitor of calpains in platelets at concentrations between 10 and 200 μg/ml [5,8,11]. Since PtdIns(3,4)P_2 synthesis is dependent on aggregation triggered by thrombin stimulation of platelets, we first checked the effects on platelet aggregation and secretion of pretreatment by calpeptin. As shown in Fig. 1, up to 75 μg/ml of calpeptin did not significantly affect platelet aggregation and secretion. The extent of platelet aggregation was reduced at 100 μg/ml of calpeptin with formation of small platelet aggregates but no reversible aggregation (Fig. 1). In conditions where platelet aggregation and secretion induced by thrombin were not affected, calpeptin treatment provoked a dose-dependent inhibition of [^{32}P]-PtdIns(3,4)P_2 already significant at 50 μg/ml (Fig. 2). With regard to preservation of platelet aggregation, maximal inhibition of [^{32}P]PtdIns(3,4)P_2 level was obtained at 75 μg/ml of calpeptin (−60% on average). These data suggest that calpains are involved in platelet PtdIns(3,4)P_2 synthesis upon thrombin stimulation.

3.2. PtdIns(3,4)P_2 synthesis inhibited by calpeptin is secondary to $\alpha_{IIb}\beta_3$ engagement

We have previously shown that $\alpha_{IIb}\beta_3$ integrin engagement was responsible for 63% ± 17 (mean ± S.D.) of the platelet PtdIns(3,4)P_2 synthesis upon thrombin stimulation [18]. We checked whether the inhibitory effect of calpeptin treatment concerned the same PtdIns(3,4)P_2 pool. Pretreatment of platelets with both the synthetic peptide RGDS in order to block $\alpha_{IIb}\beta_3$ integrin/fibrinogen interaction [18] and calpeptin did not induce significantly additive effects on [^{32}P]PtdIns(3,4)P_2 inhibition (Table 1). It should be noted that, as in the case of Glanzmann's thrombasthenic platelets lacking $\alpha_{IIb}\beta_3$ integrin [18], we never obtained a total inhibition of [^{32}P]PtdIns(3,4)P_2 in RGDS- and calpeptin-treated platelets upon thrombin stimulation. These data argue in favor of, at least, two independent pathways responsible for PtdIns(3,4)P_2 synthesis in

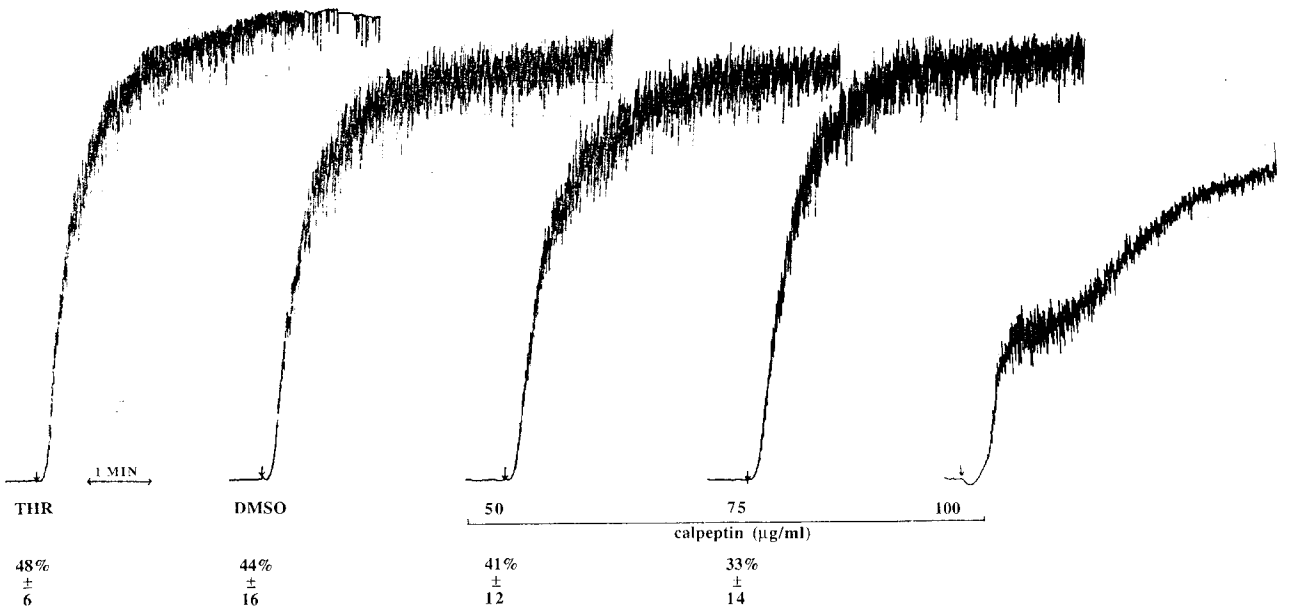

Fig. 1. Effect of calpeptin treatment on thrombin-induced platelet aggregation and secretion. Washed platelets were pretreated in the absence or presence of calpeptin (50–100 μg/ml) or DMSO (vehicle; 0.3%) for 5 min and were stimulated by thrombin (1 NIH U/ml). [^{14}C]Serotonin secretion and aggregation were measured as described in Section 2. Platelet aggregation data are from one representative of three independent experiments with similar results. Serotonin release data are means ± S.D. of four independent experiments and are represented below aggregation curves.

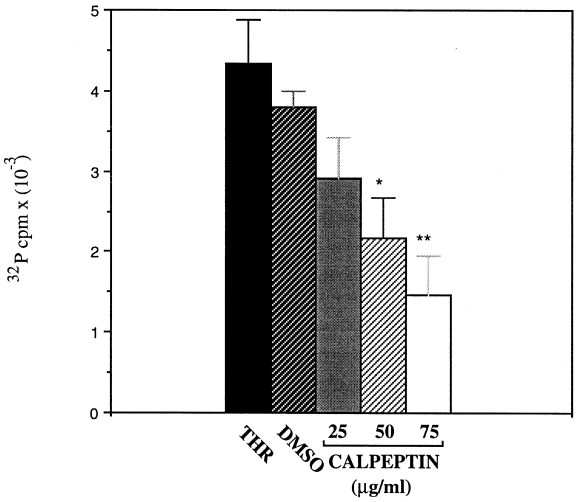

Fig. 2. Dose-dependent inhibition of PtdIns(3,4)P_2 production in thrombin-stimulated platelets by calpeptin. Washed platelets were pretreated in the absence (THR) or presence of increased concentrations of calpeptin (25–75 μg/ml) or 0.3% DMSO for 5 min and were stimulated by thrombin (1 NIH U/ml). Lipids were extracted and separated as described in Section 2. The [^{32}P]PtdIns(3,4)P_2 level (from 9×10^8 platelets) is expressed in counts/min, and data are means ± S.E. from four independent experiments. Radioactivity of [^{32}P]PtdIns(3,4)P_2 was undetectable in samples of resting platelets and is not presented. Student's *t*-test (unpaired) gave probabilities of the observed means to be identical as indicated: $^*P<0.05$, $^{**}P<0.01$.

thrombin-stimulated platelets. Only $\alpha_{IIb}\beta_3$ integrin-dependent pathway of PtdIns(3,4)P_2 synthesis seems to be inhibited by calpeptin treatment.

3.3. *Translocation to the cytoskeleton of the p85 regulatory subunit of PtdIns 3-kinase is not inhibited upon calpeptin treatment*

Our previous data have demonstrated that translocation of the p85 regulatory subunit of PtdIns 3-kinase to the cytoskeleton was closely related to PtdIns(3,4)P_2 synthesis in thrombin-stimulated platelets [14]. Since calpains have been involved in cytoskeleton reorganization upon platelet stimulation, we asked for a possible modification of p85 localization upon calpeptin treatment. As shown in Fig. 3, this was not the case since p85 translocation to the cytoskeleton was not impaired by calpeptin until 75 μg/ml.

Table 1
Effect of tetrapeptide RGDS on calpeptin-induced inhibition of PtdIns(3,4)P_2 synthesis

Inhibitor	Inhibition of PtdIns(3,4)P_2 synthesis (%)
Calpeptin	54 ± 14
RGDS	73 ± 16
Calpeptin+RGDS	83 ± 12

Platelet preparation and activation by thrombin (1 NIH U/ml) were as described under Section 2. Calpeptin (75 μg/ml) and RGDS (100 μM) were added to the platelet suspension 5 min and 15 s before thrombin, respectively. Results represent percentages of inhibition of [^{32}P]PtdIns(3,4)P_2 labeling compared to control values (platelets stimulated with thrombin in the absence of inhibitor). Values represent means ± S.D. ($n=4$).

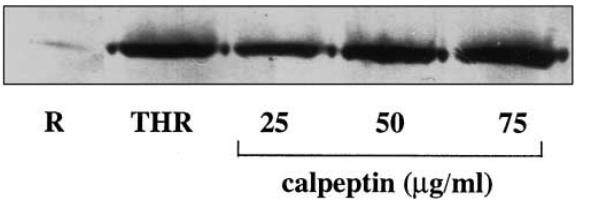

Fig. 3. Effect of calpeptin on cytoskeletal relocalization of p85 upon thrombin stimulation. After processing as described in Section 2, cytoskeletal proteins from 3×10^8 platelets in resting state (R) or pretreated in the absence (THR) or presence of calpeptin (25–75 μg/ml) and stimulated by thrombin (1 NIH U/ml) were probed with anti-p85.

4. Discussion

The aim of this study was to check for the possible involvement of calpains in the regulation of $\alpha_{IIb}\beta_3$ integrin-dependent PtdIns 3-kinase activity in platelets. For this purpose, we used a cell permeant specific inhibitor of calpains, calpeptin, and followed ^{32}P incorporation into the only PtdIns 3-kinase product, PtdIns(3,4)P_2, whose synthesis is partially controlled by $\alpha_{IIb}\beta_3$ integrin engagement.

In conditions where $\alpha_{IIb}\beta_3$ integrin engagement was preserved (normal platelet secretion and aggregation), we observed a dose-dependent inhibition of PtdIns(3,4)P_2 synthesis upon calpeptin treatment. Interestingly, in these conditions, the maximal calpeptin inhibition value of PtdIns(3,4)P_2 level (−60%) was close to that obtained with RGDS alone, which is a competitive inhibitor of fibrinogen/$\alpha_{IIb}\beta_3$ integrin interaction. Moreover, we did not observe additive inhibitory effects when platelets were pretreated with both inhibitors. These data indicate that calpains are involved in the regulation of $\alpha_{IIb}\beta_3$ integrin-dependent PtdIns(3,4)P_2 synthesis.

Calpains selectively hydrolyse cytoskeletal proteins such as talin or filamin [11]. Since we have previously shown that the $\alpha_{IIb}\beta_3$ integrin-dependent PtdIns(3,4)P_2 synthesis was correlated to the translocation of the p85 regulatory subunit to the cytoskeleton in thrombin-stimulated platelets [14], we checked for the localization of p85 upon calpeptin treatment. In conditions where calpeptin induced 60% inhibition of PtdIns(3,4)P_2, p85 remained fully translocated to the cytoskeleton. Thus, calpains could influence either the translocation of the p110 catalytic subunit of PtdIns 3-kinase, or its regulation, or the degradation of PtdIns(3,4)P_2.

The data presented here suggest that calpains could represent an early step in $\alpha_{IIb}\beta_3$ integrin-mediated signal transduction that is necessary for PtdIns(3,4)P_2 accumulation. In our conditions, platelet aggregation was not significantly modified when 60% of PtdIns(3,4)P_2 production was inhibited. This result suggests that the PtdIns(3,4)P_2 pool inhibited by calpeptin treatment could be not involved in the irreversible character of platelet aggregation as suggested for platelets stimulated with thrombin-receptor-activating peptide (TRAP) [12]. Alternatively, thrombin stimulation might trigger additional signalling pathways making it possible to encompass PtdIns 3-kinase inhibition, in contrast with TRAP-induced platelet aggregation. On the other hand, calpain-dependent PtdIns(3,4)P_2 production could be involved in calpain-dependent platelet vesiculation since the *Saccharomyces cerevisiae* protein VPS34 is a PtdIns 3-kinase implicated in vacuole morphogenesis [19], and Glanzmann's thrombas-

thenic platelets seem to be not able to vesiculate in response to thrombin [20]. More widely, our demonstration of PtdIns 3-kinase regulation by calpains could be of importance with regard to the involvement of calpains in differentiation and cell cycle regulation.

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References

[1] Croall, D.E. and Demartino, G.N. (1991) Physiol. Rev. 71, 813–847.
[2] Fox, J.E.B., Taylor, R.G., Tafferel, M., Boyles, J.K. and Goll, D.E. (1993) J. Cell Biol. 120, 1501–1507.
[3] Yamaguchi, A., Yamamoto, N., Kitagawa, H., Tanoue, K. and Yamazaki, H. (1987) FEBS Lett. 225, 228–232.
[4] Pasquet, J.M., Dachary-Prigent, J. and Nurden, A. (1996) Eur. J. Biochem. 239, 647–654.
[5] Banno, Y., Nakashima, S., Hachiya, T. and Nozawa, Y. (1995) J. Biol. Chem. 270, 4318–4324.
[6] Kishimoto, A., Kajikawa, N., Shiota, M. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 1156–1164.
[7] Oda, A., Druker, B.J., Ariyoshi, H., Smith, M. and Salzman, E.W. (1993) J. Biol. Chem. 268, 12603–12608.
[8] Frangioni, J.V., Oda, A., Smith, M., Salzmann, E.W. and Neel, B.G. (1993) EMBO J. 12, 4843–4856.
[9] Du, X., Saido, T.C., Tsubuki, S., Indig, F.E., Williams, M.J. and Ginsberg, M.H. (1995) J. Biol. Chem. 270, 26146–26151.
[10] Fox, J.E.B., Austin, C.D., Reynolds, C.C. and Steffen, P.K. (1991) J. Biol. Chem. 266, 13289–13295.
[11] Bassé, F., Gaffet, P. and Bienvenüe, A. (1994) Biochim. Biophys. Acta 1190, 217–224.
[12] Kovacsovics, T.J., Bachelot, C., Toker, A., Vlahos, C.J., Duckworth, B., Cantley, L.C. and Hartwig, J.H. (1995) J. Biol. Chem. 270, 11358–11366.
[13] Sorisky, A., King, W.G. and Rittenhouse, S.E. (1992) Biochem. J. 286, 581–584.
[14] Guinebault, C., Payrastre, B., Racaud-Sultan, C., Mazarguil, H., Breton, M., Mauco, G., Plantavid, M. and Chap, H. (1995) J. Cell Biol. 129, 831–842.
[15] Tsujinaka, T., Kajiwara, Y., Kambayashi, J., Sakon, M., Higuchi, N., Tanaka, T. and Mori, T. (1988) Biochem. Biophys. Res. Commun. 153, 1201–1208.
[16] Sultan, C., Breton, M., Mauco, G., Grondin, P., Plantavid, M. and Chap, H. (1990) Biochem. J. 269, 831–834.
[17] Grondin, P., Plantavid, M., Sultan, C., Breton, M., Mauco, G. and Chap, H. (1991) J. Biol. Chem. 266, 15705–15709.
[18] Sultan, C., Plantavid, M., Bachelot, C., Grondin, P., Breton, M., Mauco, G., Lévy-Toledano, S., Caen, J.P. and Chap, H. (1991) J. Biol. Chem. 266, 23554–23557.
[19] Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D. and Emr, S.D. (1993) Science 260, 88–91.
[20] Gemmell, C.H., Sefton, M.V. and Yeo, E.L. (1993) J. Biol. Chem. 268, 14586–14589.