Calcium mobilisation controls tyrosine protein phosphorylation independently of the activation of protein kinase C in human platelets

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Abstract

We have investigated the regulation of tyrosine proteins phosphorylation by intracellular Ca^{2+} level ($[Ca^{2+}]_i$) and protein kinase C (PKC) during platelet stimulation. We found that chelation of extracellular calcium completely prevented phosphorylation of tyrosine proteins induced by thapsigargin and phorbol 12-myristate 13-acetate (PMA), whereas, when induced by thrombin, it prevented a subset of tyrosine proteins. The selective inhibition of PKC by GF 109203X did not abolish tyrosine protein phosphorylation when induced by thrombin and thapsigargin. The results suggest that in human platelets tyrosine protein phosphorylation is dependent on $[Ca^{2+}]_i$, although direct PKC activation can also induce phosphorylation of tyrosine proteins.

Key words: Tyrosine protein phosphorylation; Ca^{2+}; Protein kinase C; Human platelets

1. Introduction

Platelet activation occurs through a rise of cytoplasmic Ca^{2+} level, and this Ca^{2+} mobilisation is accompanied by the activation of a number of kinases among which the serine/threonine protein kinase C (PKC) and tyrosine protein kinases. Tyrosine protein phosphorylations are observed during platelet activation by thrombin [1–3], ionophore A23187 and PKC activators [4], thapsigargin [5] and P256, an anti-glycoprotein IIb-IIIa (integrin $\alpha_{IIb}\beta_3$) monoclonal antibody [6]. These phosphorylations are dependent on tyrosine protein kinases such as pp60[c-src] [1–2,7], pp125[FAK] [8] and p72[syk] [9]. It is not clearly established whether tyrosine kinases depend on Ca^{2+} mobilisation [5], on PKC activation [10] or on both [4]. This prompted us to further specify the regulation of phosphorylation of tyrosine proteins. For this purpose, we compared the profiles of tyrosine protein phosphorylation induced by thrombin, a receptor-dependent agonist, phorbol 12-myristate 13-acetate (PMA), a direct activator of PKC, and thapsigargin which increases the concentration of cytoplasmic free Ca^{2+} by specific inhibition of the dense tubular system Ca^{2+}-ATPase [11–13] independently of the generation of inositol phosphates [14].

We compared the Ca^{2+} mobilisation induced by these three agonists and its relationship with PKC and tyrosine protein kinase activation in the presence of Ca^{2+} and PKC inhibitors. Our results demonstrate that phosphorylation of tyrosine proteins is dependent on Ca^{2+} mobilisation and occurs downstream of PKC only when PKC is directly activated.

2. Materials and methods

Human platelets were isolated on metrizamide gradient as described in [10]. Platelet aggregation and release were monitored using a Chrono Log (Coultronics, Margency, France) aggregometer and [14C]5-HT labelled platelets in the presence of 1 μM imipramine.

Protein phosphorylation of [32P]orthophosphate labelled platelets was detected by autoradiography after electrophoresis using a 13% polyacrylamide gel under reducing conditions. Tyrosine phosphorylation was detected by electrophoresis using a 7% polyacrylamide gel under reducing conditions and Western blotting [10]. Blots were incubated with a monoclonal anti-phosphotyrosine antibody 4G10 (1.5 μg/ml) for 1 h, with a goat anti-mouse antibody (2 μg/ml) linked to peroxydase (Amersham, Les Ulis, France) for 1 h, and developed with 4-chloro 1-naphtol.

$[Ca^{2+}]_i$ measurements were performed on indo1-labelled platelets. Indo1 fluorescence was recorded using a spectrofluorimeter (LS50, Perkin-Elmer Cetus Instruments, Norwalk, USA) with a temperature-controlled cuvette (37°C). Excitation and emission wavelengths were 331 nm and 410 nm, respectively. Intracellular free calcium ($[Ca^{2+}]_i$) was calibrated according to the derivation of Grynkiewicz et al. [15]. Briefly, $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$ where F_{min} and F_{max} are the fluorescence intensities obtained without external Ca^{2+} and at saturating Ca^{2+}, respectively, and F is the measured fluorescence intensity. K_d for indo1 at 37°C was taken to be 250 nM.

2.1. Materials and chemicals

Thapsigargin, PMA, imipramine and indo1 acetoxymethyl ester (indo1-AM) were purchased from Sigma (Saint Quentin Fallavier, France). Bovine thrombin was from Hoffmann La Roche (Basel, Switzerland). [14C]5-hydroxytryptamine ([14C]5-HT), and [32P]orthophosphate were from Amersham (Les Ulis, France), and metrizamide was from Nycomed AS (Oslo, Norway). The PKC inhibitor bisindolylmaleimide GF 109203X was a kind gift from Dr. Coste (Labora-

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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium level; EDTA, ethylenediaminetetraacetic acid; 5-HT, 5-hydroxytryptamine; Indo1-AM, indo1 acetoxymethyl ester; PKCI, protein kinase C inhibitor GF 109203X; PMA, phorbol 12-myristate 13-acetate; PRP, platelet-rich plasma; SDS, sodium dodecyl sulfate.

toires Glaxo, Les Ulis, France) [16], and the monoclonal anti-phosphotyrosine antibody 4G10 from Dr. Druker (Boston, USA) [17].

3. Results and discussion

3.1. Protein tyrosine phosphorylation induced by thrombin, thapsigargin and PMA

Fig. 1 shows that thrombin (0.1 U/ml) causes the increase in phosphorylation of several tyrosine proteins. Proteins with molecular masses of 56, 60, 64, 75, 80, 85, 97–105, 125 and 140 kDa have been identified by SDS/polyacrylamide gel and immunoblot analysis using the same phosphotyrosine-specific antibody as previously described [6]. With EDTA (1 mM), the aggregation-dependent 97–105 kDa doublet [10] and some other proteins were missing. Only the 140 kDa tyrosine protein phosphorylation was enhanced suggesting that a Ca^{2+}-dependent phosphatase was inhibited [18]. Bisindolylmaleimide GF 109203X (PKCI), a potent and selective inhibitor of PKC [16], did not inhibit the profile of phosphotyrosine proteins either in resting (result not shown) or in thrombin-induced platelets. Worthy of note is that the 64 kDa phosphotyrosine protein was enhanced in the presence of GF 109203X. Thapsigargin (100 nM), a platelet agonist which elevates $[Ca^{2+}]_i$ by specific inhibition of the dense tubular system Ca^{2+}-ATPase [11], also induced tyrosine protein phosphorylations. This response was independent of thromboxane A_2 production (40% aggregation with 1 mM aspirin), and of activation of phospholipase C as assessed by absence of phosphatidic acid production (result not shown), in agreement with previous results obtained in NG115-401L neuronal cells [14]. Thapsigargin induced a similar profile of phosphotyrosine proteins to that obtained with thrombin, however it was completely inhibited by EDTA. As for thrombin, GF 109203X had no inhibitory effect, and even enhanced the 64 kDa phosphotyrosine
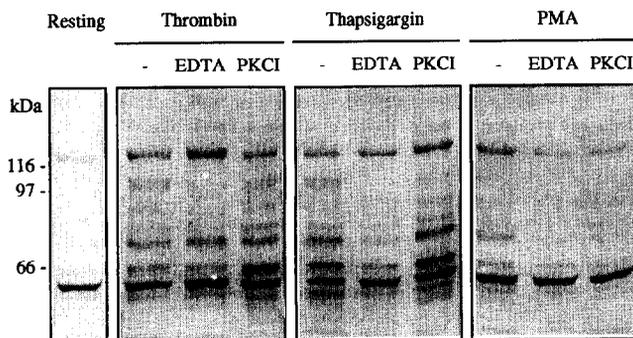

Fig. 1. Effect of EDTA and GF 109203X on trombin-, thapsigargin- and PMA-induced tyrosine protein phosphorylation in human platelets. Platelets were preincubated with $CaCl_2$ (1 mM) and stimulated for 2 min in the presence of GF 109203X (PKCI 5 μM) or EDTA (1 mM without $CaCl_2$) with thrombin (0.1 U/ml), thapsigargin (100 nM) or PMA (200 nM) as indicated. The samples were analyzed by anti-phosphotyrosine immunoblotting.
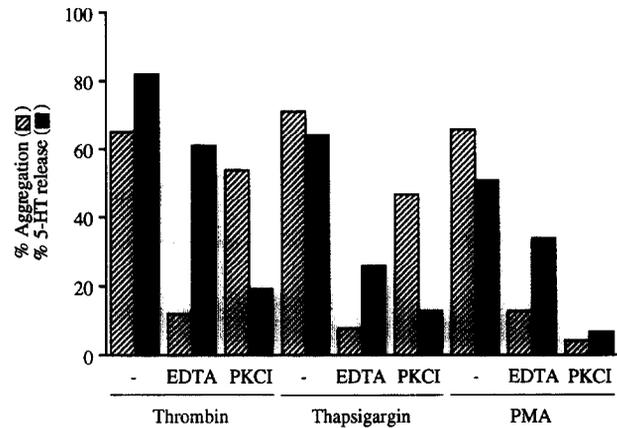

Fig. 2. Effect of EDTA and GF 109203X on trombin-, thapsigargin- and PMA-induced aggregation and [^{14}C]5-HT release. [^{14}C]5-HT-labelled platelets were preincubated for 5 min in the presence of imipramine (1 μM) before addition of $CaCl_2$ (1 mM), and of thrombin (0.1 U/ml), thapsigargin (100 nM) or PMA (200 nM) for 2 min. GF 109203X (PKCI 5 μM) or EDTA (1 mM without $CaCl_2$) was added 1 min before activation as indicated. Values are the mean of 3 to 6 experiments. Detailed conditions are described in section 2.

protein. PMA (200 nM), a direct activator of PKC, induced the same profile of tyrosine protein phosphorylations as did thrombin and thapsigargin [10]. However, these phosphorylations were completely inhibited in the presence of both EDTA and GF 109203X. Thus, with all three agonists, we found that external Ca^{2+} is essential for tyrosine protein phosphorylation, suggesting that intracellular Ca^{2+} is predominant over PKC for tyrosine kinase activity in human platelets.

3.2. Aggregation and secretion induced by thrombin, thapsigargin and PMA

Thrombin, thapsigargin and PMA induced platelet aggregation and 5-HT release from dense granules as measured by release of [^{14}C]5-HT (Fig. 2). With all three agonists, GF 109203X (5 μM) prevented [^{14}C]5-HT release. This confirmed that activation of PKC is necessary for platelet granule secretion [19]. Addition of EDTA (1 mM) prevented aggregation and reduced basal $[Ca^{2+}]_i$ and had only a limited effect on thrombin-induced release. It prevented thapsigargin-induced aggregation and [^{14}C]5-HT release. In this case, Ca^{2+} entry is essential for thapsigargin-induced PKC activation, and thus chelation of external Ca^{2+} prevented secretion, RGDS peptide, a specific inhibitor of platelet aggregation did not modify [^{14}C]5-HT release (result not shown).

3.3. Effects of EDTA and GF 109203X on thrombin-induced activation

Thrombin (0.1 U/ml) led to the phosphorylation of the 47 kDa protein called pleckstrin (P47), used as a marker for PKC activation, and the 20 kDa protein recognized as the myosin light chain (P20), phosphorylated by the

Ca^{2+}-calmodulin dependent myosin light chain kinase [20] (Fig. 3A). GF 109203X (5 μM), inhibited thrombin-induced pleckstrin phosphorylation, whereas EDTA (1 mM) had no effect on pleckstrin phosphorylation. Using indo1-labelled platelets, thrombin (0.1 U/ml) induced an increase of $[Ca^{2+}]_i$ from 116 ± 6 nM to a maximum of 434 ± 26 nM (mean ± S.E.M., $n = 12$) (Fig. 3B). Removal of extracellular calcium by addition of EDTA (1 mM) significantly reduced the basal $[Ca^{2+}]_i$ to 55 ± 3 nM, as previously described [21], and also the thrombin-induced $[Ca^{2+}]_i$ rise to 155 ± 13 nM (mean ± S.E.M., $n = 10$). In agreement with a previous report [22], Ca^{2+} mobilisation was not decreased by the selective inhibition of PKC.

3.4. Effects of EDTA and GF 109203X on thapsigargin-induced activation

Thapsigargin (100 nM) led to the phosphorylation of both pleckstrin and myosin light chain [23]. These phosphorylations were independent of thromboxane, since, in the presence of aspirin, phosphorylation of proteins was not detectable before 45 s of thapsigargin activation, compared to 15 s in the absence of aspirin. Thapsigargin also induced an increase of the $[Ca^{2+}]_i$ up to 398 ± 32 nM (mean ± S.E.M., $n = 11$), although slower than with thrombin (Fig. 4). EDTA inhibited thapsigargin-induced $[Ca^{2+}]_i$ rise which was of only 89 ± 10 nM (mean ± S.E.M., $n = 10$) after 3 min. EDTA also inhibited thapsigargin-induced pleckstrin and myosin light chain phosphorylation, suggesting that in this case a threshold $[Ca^{2+}]_i$ was determinant for both myosin light chain kinase and PKC activation. Among the three different isoforms of PKC described in platelets, PKC-δ isozyme is not Ca^{2+}-sensitive, and the classical isozymes PKC-α and -β are dependent on Ca^{2+} and

diacylglycerol synergistically [24–25]. In the case of thapsigargin there was no production of diacylglycerol, PKC activity was thus stimulated by the only Ca^{2+} mobilisation. As a result, in the presence of EDTA, it was inactive. Moreover, as for thrombin, thapsigargin-induced Ca^{2+} mobilisation was not decreased by the selective inhibition of PKC.

3.5. Effects of EDTA and GF109203X on PMA-induced activation

PMA (200 nM) induced a strong phosphorylation of pleckstrin, and to a smaller extent phosphorylation of the myosin light chain, without causing any $[Ca^{2+}]_i$ rise (Fig. 5). EDTA did not affect PMA induced pleckstrin phosphorylation, but slightly inhibited that of the myosin light chain. GF 109203X inhibited PMA-induced pleckstrin and myosin light chain phosphorylation. Indeed, the phosphorylation of myosin light chain is also dependent on PKC [26].

3.6. Conclusion

Thus, the addition of EDTA reduced protein tyrosine phosphorylation, induced by all three agonists. These results suggest that platelet tyrosine kinase activity is controlled by intracellular Ca^{2+}. Also when PKC was selectively inhibited, tyrosine protein phosphorylation was not abolished in the case of thrombin and thapsigargin-induced stimulation, suggesting that only Ca^{2+} entry was responsible for tyrosine protein phosphorylation, independently of PKC activation. In cultured human epidermal keratinocytes, a rapid Ca^{2+} mobilisation was proposed to stimulate a phosphatase activity resulting in pp60$^{c\text{-}src}$ kinase activity [27]. Indeed, this phosphatase dephosphorylates Tyr-527 the carboxy-terminal phosphotyrosine residue reported to be responsi-
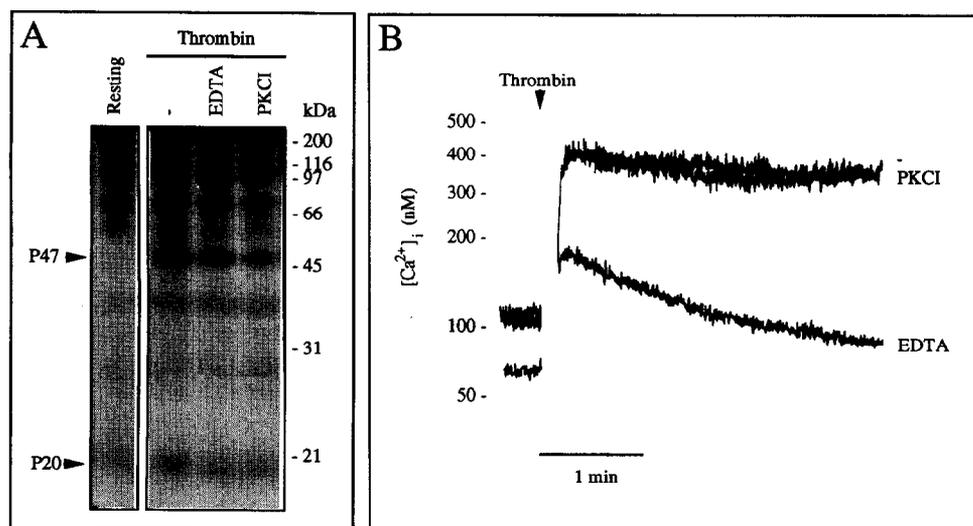

Fig. 3. Effect of EDTA and GF 109203X on thrombin-induced protein phosphorylation and $[Ca^{2+}]_i$ rises. [^{32}P]orthophosphate- (panel A) or indo1-AM-labelled platelets (panel B) were preincubated with $CaCl_2$ (1 mM) and stimulated for 2 min with thrombin (0.1 U/ml) in the presence of GF 109203X (PKCI 5 μM) or EDTA (1 mM without $CaCl_2$). Autoradiograms are representative of 4 experiments.
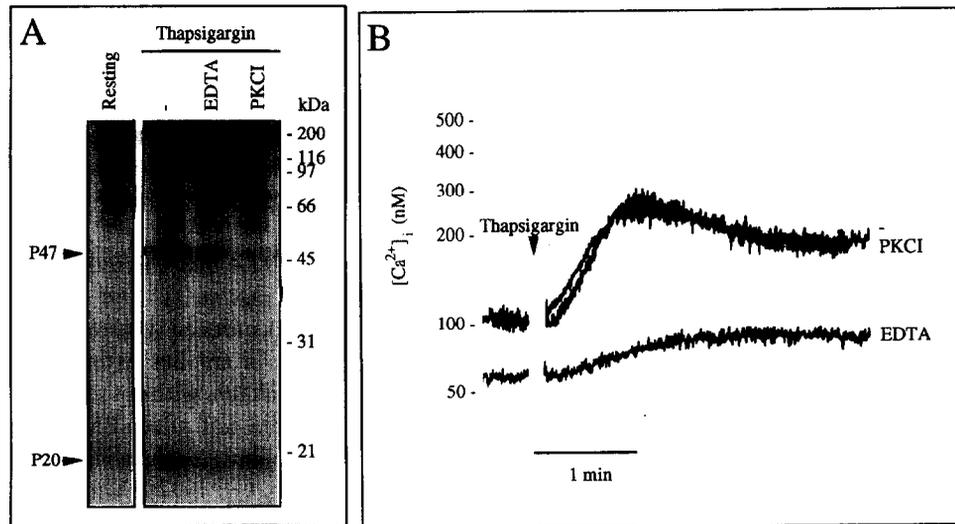
Fig. 4. Effect of EDTA and GF 109203X on thapsigargin-induced protein phosphorylation and $[Ca^{2+}]_i$ rises. [^{32}P]orthophosphate- (panel A) or indol-AM-labelled platelets (panel B) were preincubated with $CaCl_2$ (1 mM) and stimulated for 2 min with thapsigargin in the presence of GF 109203X (PKCI 5 μM) or EDTA (1 mM without $CaCl_2$) (100 nM). Autoradiograms are representative of 4 experiments.

ble for the down-regulation of pp60^{c-src} protein tyrosine kinase activity [28]. Since pp60^{c-src} is the most representative protein tyrosine kinase in platelets [29], a similar regulation could occur in human platelets. However PMA could also induce tyrosine protein phosphorylation [10]. In this case, the phosphorylation of the Ser-12 of the pp60^{c-src} protein tyrosine kinase could be implied, which would elevate the affinity of the kinase for its substrates [30].

In conclusion, we have demonstrated that tyrosine protein phosphorylation was abolished when $[Ca^{2+}]_i$ was reduced below 100 nM. Furthermore, direct activation of PKC can also induce protein tyrosine phosphoryla-

tion. Ca^{2+} mobilisation is, however, essential for tyrosine protein kinase activation in human platelets.

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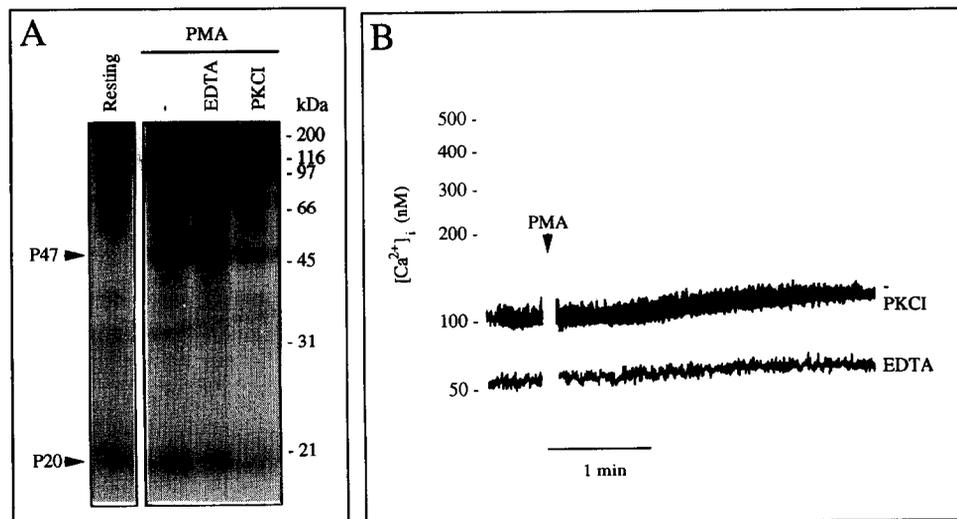[2] Golden, A. and Brugge, J.S. (1989) Proc. Natl. Acad. Sci. USA 86, 901–905.

Fig. 5. Effect of EDTA and GF 109203X on PMA-induced protein phosphorylation and $[Ca^{2+}]_i$ rises. [^{32}P]orthophosphate- (panel A) or indol-AM-labelled platelets (panel B) were preincubated with $CaCl_2$ (1 mM) and stimulated for 2 min with PMA (200 nM) in the presence of GF 109203X (PKCI 5 μM) or EDTA (1 mM without $CaCl_2$). Autoradiograms are representative of 3 experiments.

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