Calpain controls the balance between protein tyrosine kinase and tyrosine phosphatase activities during platelet activation

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Dedication: This paper is dedicated to the memory of Alfonso Monstero who died accidentally during the preparation of the manuscript.

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Abstract Protein phosphorylation was studied during platelet stimulation in two ranges of ionized $[Ca^{2+}]$. At ionized $[Ca^{2+}]_i \leq 1$ μM, proteins were phosphorylated. At ionized $[Ca^{2+}]_i \geq 4$ μM, phosphoproteins disappeared. Protein dephosphorylation was prevented by the combined action of calpeptin and phosphatase inhibitors. Protein tyrosine phosphatase activity was stimulated regardless of the ionized $[Ca^{2+}]_i$ level. Protein tyrosine kinase activity was stimulated at ionized $[Ca^{2+}]_i \leq 1$ μM, whereas at ionized $[Ca^{2+}]_i \geq 4$ μM, no protein tyrosine kinase activity was observed except in the presence of calpeptin. Thus, the massive tyrosine phosphoprotein disappearance observed at a high ionized $[Ca^{2+}]_i$ resulted not only in protein tyrosine phosphatase activation, but also in calpain-induced protein tyrosine kinase inactivation.

Key words: Platelet activation; Phosphorylation; Calpain; Calcium

1. Introduction

As for many different cell types, calcium is a key mediator for stimulus-response coupling in platelets. Elevation of platelet ionized cytosolic calcium ($[Ca^{2+}]_i$) is the result of a capacitative system consisting of depletion of intracellular stores and influx from the external medium [1,2]. Depending on the agonist, $[Ca^{2+}]_i$ is elevated at different levels and activates different effector enzyme(s). Phospholipase A_2, myosin light chain kinase and protein kinase C require a lower $[Ca^{2+}]_i$ [3] than do the lipid scramblase [4] or the cysteine protease calpain to be autolysed [5]. An increase in tyrosine protein phosphatase (PTP) activity was recently reported for stimulating conditions involving high $[Ca^{2+}]_i$ [6].

Calcium ionophores, which bypass receptor-coupling mechanisms and carry calcium within cells through membranes, represent useful tools to evoke changes in $[Ca^{2+}]_i$ and the impact of different $[Ca^{2+}]_i$ on platelet activation. Ionophores can evoke increases in $[Ca^{2+}]_i$, large enough to stimulate the lipid scramblase and calpain autolysis. There are, however, puzzling data concerning phosphorylation or dephosphorylation of tyrosine proteins following stimulation by ionophores [6–9]. All reports show protein phosphorylation profiles, but no measure of kinase or phosphatase activities have been performed.

In this context, we studied protein phosphorylation during platelet activation at high $[Ca^{2+}]_i$. We used ionophores in the presence of calcium and measured protein tyrosine kinase (PTK) and PTP activities in the presence of a calpain inhibitor. The results show that during platelet activation at $[Ca^{2+}]_i \geq 4$ μM, phosphorylated proteins disappear as a result of PTP activation and, above all, of simultaneous calpain-induced PTK inactivation.

2. Materials and methods

2.1. Materials

Calpeptin was from Novabiochem (Läufelfingen, Switzerland), okadaic acid (OA) from RBI (Natick, MA, USA), metrizamide and paranitrophenyl phosphate (pNPP) were from Sigma (Saint Louis, MO, USA). Electrophoresis reagents were from Bio-Rad (Hercules, CA, USA). All other chemicals were of the highest purity grade. Pervanadate was prepared by addition of a solution (1:1 v/v) of vanadate 20 mM to H_2O_2 0.07%. Since ionophores are insoluble in water and unstable, a stock solution (1 mM in Me_2SO) was kept at −20°C and freshly diluted in Me_2SO each time so that vehicle was added at less than 0.5% of the platelet volume. All results were performed in parallel with A23187 (Sigma) and ionomycin (Calbiochem, La Jolla, CA, USA). Ionomycin was twice as efficient as A23187, inducing the same effects as A23187 at 2-fold lower concentrations, in keeping with the stoichiometric properties of the two ionophores as calcium carriers [10,11].

2.2. Platelet preparation and stimulation

Fresh platelet concentrates were obtained from local transfusion centers. Platelets were isolated by means of metrizamide gradients, as previously described [12], resuspended in a solution consisting of 10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM glucose, 2 mM $CaCl_2$, pH 7.4, and adjusted to 3×10^8 cells/ml. Stimulation was performed at 37°C in the cuvette of an aggregometer (Chronolog, Haverton, PA, USA) under constant stirring (1100 rpm).

2.3. $[Ca^{2+}]_i$ measurement

Washed platelets were incubated for 45 min at 37°C with 5 μM of the fluorescent intracellular calcium indicator fura-2 (acetoxymethyl ester form, Molecular Probes, Eugene, OR, USA) before being centrifuged and resuspended at a concentration of 3×10^8 cells/ml in the same solution as indicated above. Fura-2 fluorescence was monitored using a Shimadzu RF-5001 PC spectrofluorimeter (Kyoto, Japan). Excitation wavelengths were 340 and 380 nm and the emission wavelength was 495 nm. The $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [13] with a dissociation constant (K_d) of 224 nM for Ca^{2+}.

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Abbreviations: $[Ca^{2+}]_i$, ionized cytosolic calcium; OA, okadaic acid; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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2.4. Protein phosphorylation

Platelets were incubated for 60 min at 37°C with [^{32}P]phosphoric acid (NEN, Boston, MA, USA, 8500–9120 Ci/mmol, 100 μCi/ml platelet suspension) before the last wash and resuspended as above. After stimulation, platelets were solubilized in Laemmli buffer containing 2% sodium dodecyl sulfate (SDS) and 1 mM EDTA and proteins were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 13% gel. Proteins were stained with Coomassie blue and the gels were dried and autoradiographed on Hyperfilm MP (Amersham, Buckinghamshire, UK). Analysis of phosphotyrosine proteins was performed on unlabelled platelets, separated by SDS-PAGE on a 7% gel and transferred to a PDVF membrane (Millipore Corp, Bedford, MA, USA). Membranes were incubated with 4G10 antibody (UBI, Lake Placid, NY, USA) for 2 h as previously described [12] and phosphotyrosine proteins were visualized by enhanced chemiluminescence (ECL and hyperfilm-ECL, Amersham).

2.5. Assay of platelet PTP and PTK activities in platelet lysates

Platelets were lysed by incubation with 0.5 volume of 3× concentrated lysis buffer (3% NP-40, 150 mM Tris-HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM PMSF, 15 μg/ml leupeptin and 15 μg/ml aprotinin) for 30 min at 4°C. PTP activity was measured for 200 μl aliquots of platelet lysates incubated (v/v) with the reaction mixture (HEPES, 62 mM, pH 7.5) containing 8 mM pNPP as the substrate, 1 μM OA to inhibit serine/threonine phosphatases. Duplicates were performed in the presence of 1 mM pervanadate in the reaction mixture in order to be sure that the measured phosphatase activity was specifically due to tyrosine phosphatases. The absorbance change, due to conversion of pNPP to pNP, was registered for 1 h at room temperature at 405 nm using a microplate reader (Dynatech MR5000, Guernesey, IL, USA). PTK activity was measured for 250 μl aliquots of platelet lysates incubated for 30 min at 37°C in Tris buffer (50 mM, pH 7.5, containing 5 mM sodium acetate, 0.2 mM EDTA, 0.8 mM EGTA, 3 mM DTT, 30 μM vanadate, 1 mM ATP) with 1 μM biotin-labelled tyrosine containing peptide (biotin-KVEKIGEGTYGVVYK-amide) as the substrate (Pierce, Rockford, IL, USA). The reaction was quenched by piceatannol (Sigma, 600 μM to inhibit all PTKs), transfer at 4°C and centrifugation for 1 min at 12 000×*g*. The phosphorylated substrate was immobilized on a streptavidin-coated microtiter plate and the reaction mixture was washed out. The fraction of phosphorylated substrate was quantified by using a peroxidase-conjugated anti-phosphotyrosine antibody and measuring the absorbance by a dual wavelength mode (405/490 nm).

3. Results

3.1. $[Ca^{2+}]_i$ increase induced by ionophores

$[Ca^{2+}]_i$ was measured on fura-2-loaded platelets, in the presence of 2 mM external calcium (Fig. 1). The basal $[Ca^{2+}]_i$ was 60–100 nM. Stimulation by 0.05 μM ionomycin (left tracing) increased $[Ca^{2+}]_i$ progressively in the first few seconds. It reached 300 nM at 1 min and a maximum level of 350–700 nM depending on the donor. Using 0.5 μM ionomycin (right tracing), the increase was extremely rapid and $[Ca^{2+}]_i$ reached 1 μM at 10 s, 2 μM at 15 s and over 4 μM at 30 s. Similar results were obtained with fluo-3-loaded platelets stimulated by A23187 (unshown results), i.e. the basal $[Ca^{2+}]_i$ could be increased 5–10 times or up to 50–100 times with 0.1 and 1 μM A23187, respectively.

3.2. Platelet protein phosphorylation

The impact of the two different $[Ca^{2+}]_i$ levels on protein phosphorylation was studied on ^{32}P-labelled proteins and on phosphotyrosine proteins (Fig. 2). Two different profiles were obtained. The higher the $[Ca^{2+}]_i$, the less phosphorylated proteins were observed. At $[Ca^{2+}]_i \leq 1$ μM (left panels), two proteins were phosphorylated (Fig. 2A), i.e. p47 and p20 corresponding to pleckstrin and myosin light chain, respectively [14], and tyrosine proteins were phosphorylated (Fig. 2B). When $[Ca^{2+}]_i$ was over 4 μM (right panels), ^{32}P-labelled proteins were initially phosphorylated in the first 20 s and progressively dephosphorylated thereafter (Fig. 2A). Most phosphotyrosine proteins disappeared, including the prominent kinase Src [15] (Fig. 2B). We confirmed that high $[Ca^{2+}]_i$ causes cytoskeletal proteolysis [16]. On Coomassie blue-

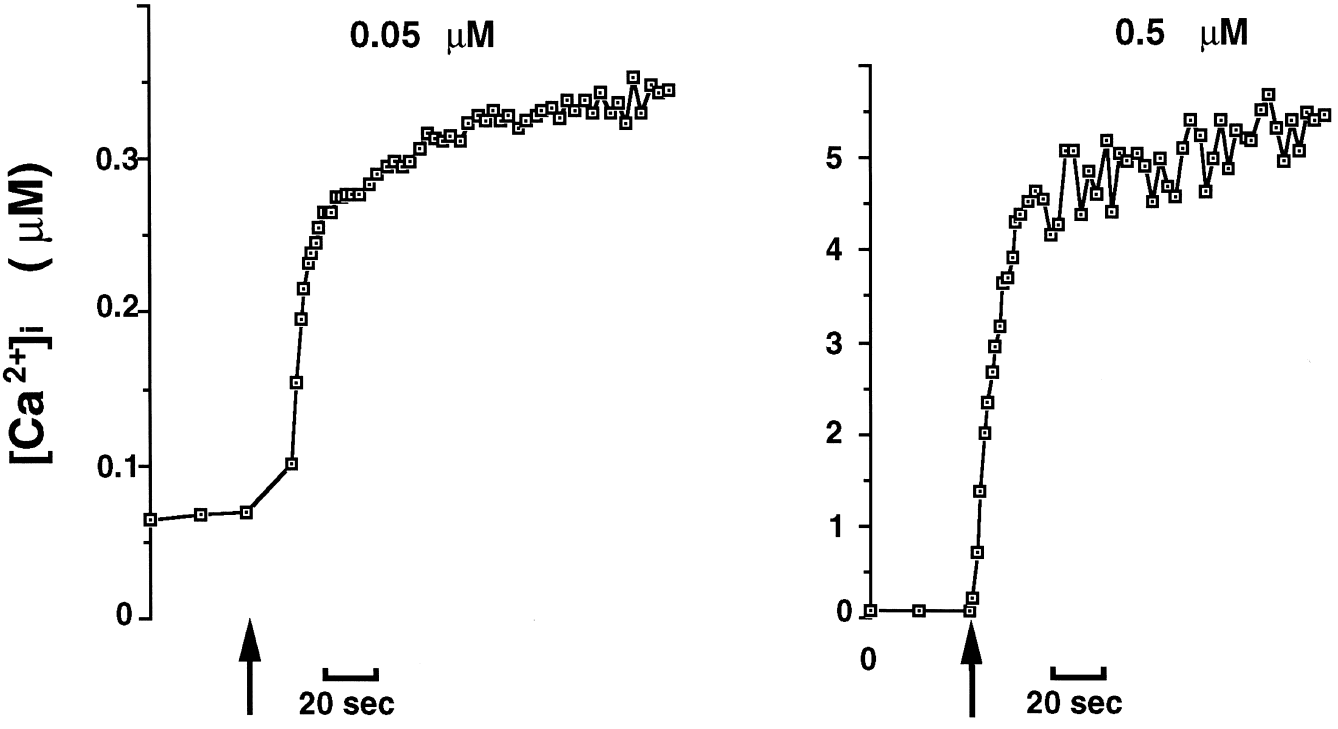

Fig. 1. $[Ca^{2+}]_i$ during ionophore-induced platelet activation. Fura-2-loaded platelets were stimulated (arrow) by 0.05 μM (left) or 0.5 μM (right) ionomycin and $[Ca^{2+}]_i$ was registered. Results are expressed in μM of $[Ca^{2+}]_i$ within platelets and are representative of 5–7 experiments.

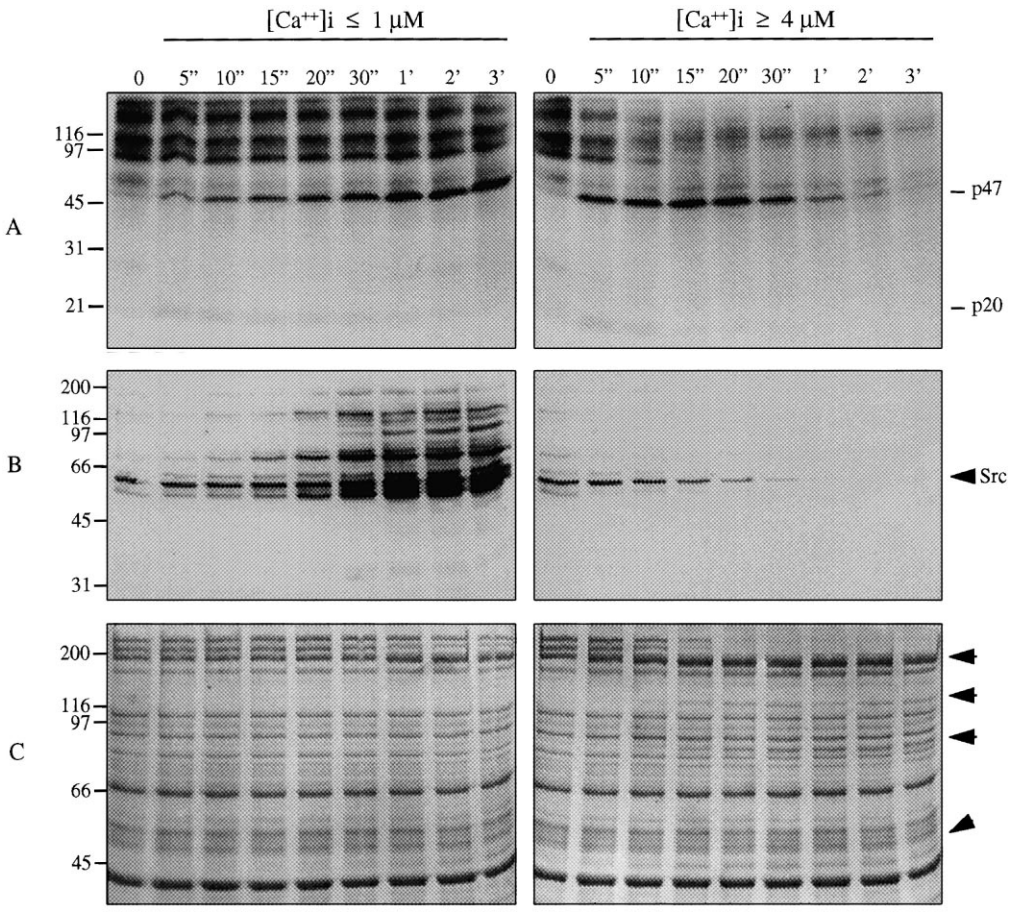

Fig. 2. Platelet protein phosphorylation and $[Ca^{2+}]_i$ relationship. Platelets were stimulated by A23187 0.1 μM (left panels) or 1 μM (right panels) for the indicated times. (A) Autoradiography of a gel from ^{32}P-labelled platelet proteins resolved by SDS-PAGE on a 13% gel. Positions of pleckstrin (p47) and myosin light chain (p20) are indicated. (B) Western blots of phosphotyrosine proteins from unlabelled platelets. After SDS-PAGE on a 7% gel, transfer to a PDVF membrane and an immunoblot with 4G10, phosphotyrosine proteins were revealed with the ECL system. (C) Coomassie blue of platelet proteins separated on a 10% gel. Arrows indicate new bands generated. Results are representative of 5–7 experiments.

stained gel, bands of 200 kDa and above disappeared and new bands were visualized (Fig. 2C, right panel).

3.3. The effect of inhibitors on protein phosphorylation

The protein dephosphorylation observed at high $[Ca^{2+}]_i$ suggested that phosphatases were activated. We therefore used the phosphatase inhibitors OA and pervanadate to inhibit serine/threonine phosphatases and tyrosine phosphatases, respectively. Because of the degradation of high molecular weight proteins occurring at high $[Ca^{2+}]_i$, we also used calpeptin, the membrane permeant calpain inhibitor.

Pre-incubation with OA increased the basal protein phosphorylation state (Fig. 3A) and prevented high $[Ca^{2+}]_i$-induced dephosphorylation of p47 and p20, but not that of high molecular weight proteins. Calpeptin prevented the disappearance of high molecular weight ^{32}P-labelled proteins, but had no effect on p47 and p20 dephosphorylation. Thus, the phosphorylation of high molecular weight proteins and the phosphorylation of p47 and p20 required that OA acted in concert with calpeptin.

The addition of pervanadate to platelets before stimulation at high $[Ca^{2+}]_i$ partly prevented dephosphorylation of tyrosine proteins (Fig. 3B, compare to Fig. 2B, right panel). Calpeptin had only a minimal effect except for some recovery of Src phosphorylation. The combination of pervanadate and calpeptin not only prevented dephosphorylation, but also allowed some tyrosine phosphorylation. The latter result was almost similar to that observed following stimulation by most other agonists [17] (Fig. 2B, left panel), except for the absence of the doublet of 97–110 kDa proteins, known to be strictly dependent on platelet aggregation [18].

3.4. Tyrosine phosphatase and tyrosine kinase activities

Since the massive protein dephosphorylation concerned more drastically tyrosine rather than serine/threonine-phosphorylated proteins, we studied the relationship between gross platelet PTP and PTK activities and the $[Ca^{2+}]_i$ level. The activities were also measured in the presence of calpeptin.

Activation increased the basal platelet PTP activity whatever the $[Ca^{2+}]_i$ level (Fig. 4). By contrast, the basal PTK activity was increased at $[Ca^{2+}]_i \leq 1$ μM, but not at $[Ca^{2+}]_i \geq 4$ μM. In the latter conditions, pre-incubation with calpeptin had no significant effect on the PTP activity, but increased the PTK activity to a level comparable to that observed at low $[Ca^{2+}]_i$. The results suggest that at $[Ca^{2+}]_i \geq 4$ μM, PTKs were inactivated by calpain.

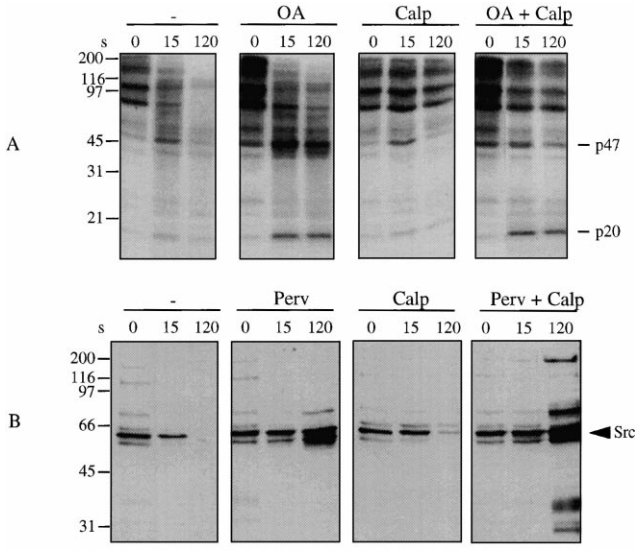

Fig. 3. Effects of phosphatase and calpain inhibitors on protein phosphorylation. (A) Autoradiography of a gel from ^{32}P-labelled platelet proteins as in Fig. 2. Positions of plekstrin (p47) and myosin light chain (p20) are indicated. Before stimulation for the indicated times by 0.5 μM ionomycin, calpeptin (Calp, 100 μg/ml) and OA (0.5 μM) were incubated for 30 and 5 min, respectively. (B) Western blot of phosphotyrosine proteins as in Fig. 2. The position of the tyrosine kinase Src is indicated. Pervanadate (Perv, 100 μM) and calpeptin (Calp, 100 μg/ml) were pre-incubated for 1 and 30 min, respectively. Results are representative of 3–5 experiments.

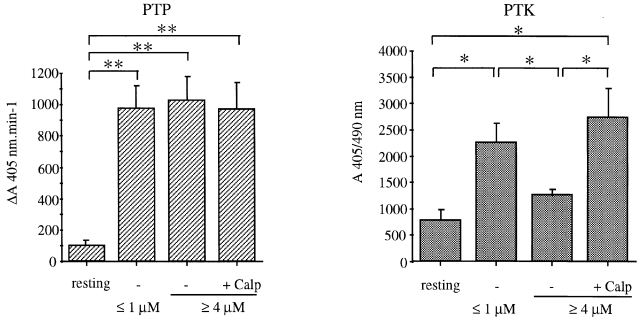

Fig. 4. Platelet PTP and PTK activities. Platelets were either unstimulated (resting) or stimulated by 0.1 μM A23187 ($Ca^{2+} \leq 1$ μM) or by 1 μM A23187 ($Ca^{2+} \geq 4$ μM) for 2 min in the presence of 2 mM $CaCl_2$ at 37°C with constant stirring. Calpeptin (100 μg/ml) or vehicle (Me_2SO) was pre-incubated for 30 min. PTP activity (left) was measured by the generation of *p*-nitrophenolate from pNPP and measured by the absorbance at 405 nm and registered for 30 min. PTK activity (right) was measured by the phosphorylation of a biotin-labelled tyrosine containing peptide and is expressed as the absorbance *A* at 405/490 nm. Results are mean ± S.E.M. of seven experiments (*t*-test: * = $P \leq 0.05$, ** = $P \leq 0.005$).

4. Discussion

We investigated the relationship between $[Ca^{2+}]_i$, protein phosphorylation and the balance between PTP and PTK activities. We showed that during stimulation at high $[Ca^{2+}]_i$, up to 50–100 times the basal level, phosphoproteins disappear by calpain-induced inactivation of PTKs rather than by stimulation of PTPs, whose activity is increased whatever the elevation of $[Ca^{2+}]_i$.

During platelet stimulation at $[Ca^{2+}]_i \leq 1$ μM, several kinases are activated, protein kinase C, the calcium/calmodulin-dependent kinase that phosphorylates the myosin light chain and tyrosine kinases [12,17]. By contrast, at $[Ca^{2+}]_i \geq 4$ μM, all proteins including phospho-serine and -threonine proteins became less phosphorylated than in unstimulated platelets. Pleckstrin was initially phosphorylated, but could not be maintained in the phosphorylated form. Dephosphorylation of subsets of tyrosine proteins [8] or of most tyrosine proteins [6] has been reported during A23187-induced stimulation. Here, we show that dephosphorylation also involved phospho-serine and -threonine proteins.

Activation of one or more phosphatase(s) by high $[Ca^{2+}]_i$ could be responsible for the tipping of the balance between kinases and phosphatases towards the disappearance of phosphoproteins. OA prevented pleckstrin (p47) and myosin light chain (p20) dephosphorylation, but failed to restore the presence of high molecular weight proteins, which are phosphorylated [19] because these proteins were degraded. Indeed, the kinetics of high molecular weight (200 kDa and over) protein dephosphorylation followed their disappearance. As a result, pre-incubation with calpeptin restored the presence of high molecular weight phosphorylated proteins. A role for calpain to down-regulate protein kinase C has been proposed [20]. Calpain alters by limited proteolysis protein kinase C [21] and calcium/calmodulin-regulated enzymes [22,23]. Taken together, the results suggest that calpain contributes to the control of serine/threonine protein phosphorylation during platelet activation.

The massive protein dephosphorylation involved more drastically and more rapidly tyrosine-phosphorylated proteins, rather than phospho-serine and -threonine proteins. Platelet PTP activity is indeed stimulated during ionophore-induced stimulation [6,24,25]. We show that the gross PTP activity was increased regardless of the $[Ca^{2+}]_i$. The addition of pervanadate, or phenylarsine oxide (not shown), did not restore the phosphorylation. Platelet PTP activity thus cannot explain the phosphotyrosine protein dephosphorylation. By contrast, the PTK activity was increased at $[Ca^{2+}]_i \leq 1$ μM, but not at high $[Ca^{2+}]_i$. Moreover, the addition of calpeptin had no effect on the PTP activity, but restored the PTK activity. Thus, a high $[Ca^{2+}]_i$ hindered PTK activity, as a result of calpain activation. This is consistent with the observation that calpain participates in tyrosine protein phosphorylation and dephosphorylation [26] and inactivates the prominent platelet tyrosine kinase Src [27]. The absence of phosphotyrosine proteins was thus due to prevention of the PTK activity, which, at a reduced level, could not compensate for the PTP activity to allow some tyrosine phosphorylation of proteins.

In conclusion, during stimulation by ionophores in the presence of calcium leading to high $[Ca^{2+}]_i$, gross PTK activity was not induced. Pasquet et al. [6] reported that following stimulation by a mixture of thrombin plus collagen, only a subpopulation of platelets showed extensive dephosphorylation of tyrosine proteins. This subpopulation corresponded to platelets that had shed microparticles. PTK inactivation would thus occur only in a platelet subpopulation and resulting tyrosine phosphoprotein profiles of all platelets would show phosphorylated proteins. Ionophores are much stronger agonists than thrombin plus collagen and stimulate almost every platelet to shed microparticles [28], inducing tyrosine phosphoproteins to disappear. Calpain-dependent protein dephosphorylation might represent an irreversible deleterious

process of proteolysis of signalling enzymes, including kinases, resembling the apoptosis of nucleated cells [29].

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