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Biochimica et Biophysica Acta 1190 (1994) 217–224

BBA Biochimica et Biophysica Acta

# Correlation between inhibition of cytoskeleton proteolysis and anti-vesiculation effect of calpeptin during A23187-induced activation of human platelets: are vesicles shed by filopod fragmentation?

François Bassé *, Patrick Gaffet, Alain Bienvenüe

*URA 530 CNRS, Université Montpellier II, CP 107, F-34095 Montpellier Cedex 05, France*

(Received 8 July 1993; revised manuscript received 25 October 1993)

**Abstract**

Platelets were incubated in the presence of calpeptin to inhibit calpain-mediated cytoskeleton proteolysis during further activation by $Ca^{2+}$ ionophore A23187. The appearance of filamin and myosin subfragments (93 kDa and 135 kDa, respectively) was inhibited by low calpeptin doses (1 $\mu$g/ml). Higher doses (10–20 $\mu$g/ml) were required to completely inhibit talin and filamin degradation. Vesiculation strongly depended on cytoskeleton proteolysis and was reduced by 60% when platelets were preincubated with 10 $\mu$g/ml calpeptin. Activated platelets bore longer and more filopods when pretreated with calpeptin. Filopods were straight and regular when high calpeptin doses were used, whereas they were shorter and broader with bloated surfaces when calpeptin was omitted. Some bloated areas were also found in straight filopods. These results suggest that the cytoskeleton proteolysis, and more specifically filamin proteolysis, induced bloating of filopod surfaces, thus facilitating fragmentation of filopod into vesicles.

*Key words:* Platelet; Activation; Microparticle; Calpeptin; Cytoskeleton; (Filopod)

## 1. Introduction

One of the many events occurring during platelet activation is the formation of vesicles, also called microparticles. These structures were first observed in the supernatants of platelet concentrates during blood bank conservation [1–4], and referred to as 'platelet-dust'. Microparticles were also found in the blood of patients with a cardiopulmonary-bypass [5] or autoimmune thrombocytopenia [6,7]. Vesicles were clearly observed by means of in vitro activation of platelets and further investigated [8–15].

First considered as fragments of secreted granules, analysis of the microparticle composition has clearly established that they are shed from the plasma membrane after granule secretion. Indeed, the lipid, protein, and particularly glycoprotein content of vesicles is quite close to that of the plasma membrane [8,9,11,13–17]. Glycoprotein GMP 140, localised in $\alpha$-granules in resting platelets, is found in vesicles as on the surface of remnant platelets after activation [15,18]. Moreover, the time-course of GMP 140 surface expression is shorter than the vesicle formation process [15], thus indicating that vesicle shedding is a later event than secretion during platelet activation.

The causes and molecular mechanisms involved in vesiculation are still controversial. Some authors have proposed that calpain-mediated degradation of high molecular weight cytoskeletal proteins is a prerequisite for vesicle formation [9], while others have shown that inhibition of calpain activity does not modify the amount of microparticles formed during activation [15]. Budding, blebbing, and evagination of the plasma membrane have been mentioned as potential molecular mechanism for vesicle formation, suggesting that initially vesicles look like bulges on the side of a ball. Pseudopod budding was also suggested as being the source of vesiculation [2].

In this study, we investigated the effect of calpeptin, a permeant and very potent calpain inhibitor [19,20],

* Corresponding author. Present address: M.H.L.F., 2 rue Haute, 34820 Teyran, France. Fax: +33 16144671772.


*SSDI* 0005-2736(93)E0339-C

on high molecular weight cytoskeleton protein degradation, on vesicle formation, and on shape changes in platelets when activated by the $Ca^{2+}$ ionophore A23187. The results suggest that vesicles are formed by filopod fragmentation induced by breakage of the link between the plasma membrane and cytoskeleton due to calpain-mediated proteolysis of the cytoskeleton, essentially filamin.

## 2. Materials and methods

### 2.1. Materials

Calcium ionophore A23187 was obtained from Sigma (L'Isle d'Abeau, France), and calpeptin was from Novabiochem (Switzerland). All other reagents were of the highest grade commercially available.

### 2.2. Biological

Platelets were isolated from freshly drawn blood anticoagulated with 0.15 vol. of ACD (85 mM trisodium citrate, 11 mM dextrose, 71 mM citric acid), using the previously described erythrocyte cushion procedure [21]. Finally, platelets were resuspended in Hepes buffer (136 mM NaCl, 1 mM $CaCl_2$, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mM dextrose, 10 mM Hepes, pH 7.4) at $2 \cdot 10^9$ platelets/ml. Before activation, platelets were allowed to settle for 30 min at 37°C in the presence of calpeptin when specified.

### 2.3. Activation procedure

Platelets were activated at 37°C by adding A23187 (1 $\mu$M final concentration) from a concentrated ethanolic solution without stirring of the suspension. Platelets did not aggregate, as shown by light microscopy observations. Activation efficiency was controlled by measuring dense granule secretion using [$^3$H]5-hydroxytryptamine (Amersham) as a marker for dense granules [21], and platelet lysis was evaluated by the lactate deshydrogenase activity in the platelet supernatant, as previously described [21]. Further experiments were performed 10 min after activation. Regardless of the calpeptin pretreatment, 70–80% of dense granule content was secreted after A23187 addition and platelet lysis never exceeded 3%.

### 2.4. Microparticle isolation and quantification

EGTA (5 mM) was added to the activated platelet suspension before centrifuging for 2 min at $11\,000 \times g$. The phospholipid content in supernatants was measured by spectrophotometry according to Rouser et al. [22]. No more phospholipids were recovered when the supernatants were ultracentrifuged (5 min at $160\,000 \times g$) using a Beckman Airfuge ultracentrifuge. Moreover, low-speed supernatants showed a mean 150–200 nm diameter when analysed on a sub-micron particle analyser Coulter N4S. These particles were absent from the ultracentrifuged supernatants, indicating that all vesicles sediment at high speed. Measurement of phospholipidic content of low-speed supernatants thus correctly evaluated microparticles.

### 2.5. Analysis of cytoskeletal proteins

Platelet proteins were solubilized and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described [15], using a 7.5% homogeneous gel (LKB 2050, Pharmacia, Sweden) stained with Coomassie blue. For analysis of vesicle protein patterns, low-speed supernatants of activated platelet suspensions were ultracentrifuged ($160\,000 \times g$, 5 min). The pellets were further analyzed as described above for platelets.

### 2.6. Scanning electron microscopy

Platelet suspension aliquotes were fixed by incubation for 30 min at room temperature in 2% glutaraldehyde solution brought to pH 7 with phosphate buffer, followed by a treatment with phosphate buffer containing 1% osmium tetraoxide. Fixed samples were allowed to sediment on Thermanox coverslips (Nunc, USA), then dehydrated through a graded alcohol series, and dried with carbon dioxide by the critical point method. The dried samples were sputter-coated with gold and observed at 15 kV on a Jeol JSM 6300 F microscope.

### 2.7. Evaluation of mean filopod length

Extracellular prolongations were measured on electron micrographs, and their cumulated length was divided by the number of observed platelets. Mean length was determined after examining more than 200 cells.

## 3. Results

### 3.1. Inhibition of calpain-mediated proteolysis

High molecular weight proteins were degraded when platelets were activated by adding $Ca^{2+}$ ionophore A23187 (1 $\mu$M) in the presence of 1 mM $Ca^{2+}$ (Fig. 1, lane B). Calpain-mediated proteolysis gave subfragments of filamin (190, 100 and 93 kDa), talin (190 and 48 kDa) and myosin heavy chain (135 kDa). These subfragments have been previously identified in different reports [15,23–25], and are easily observed by SDS-PAGE. The protein pattern obtained when platelets were activated in the absence of calpeptin

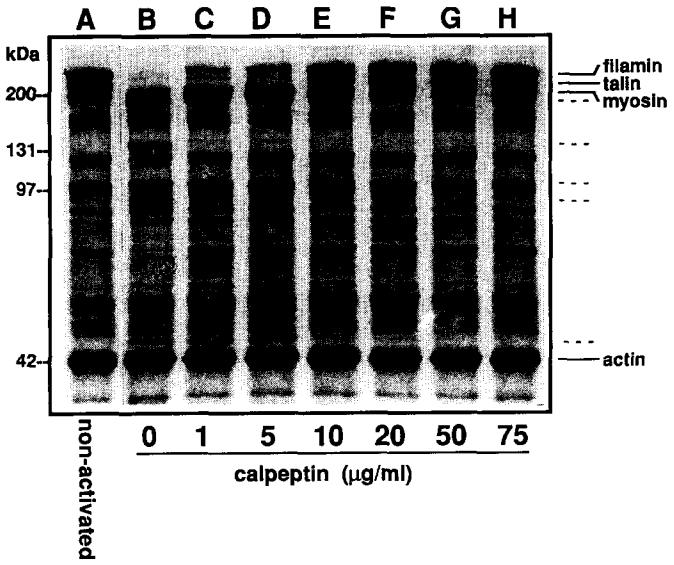


Fig. 1. Effect of calpeptin on degradation of high molecular weight cytoskeletal proteins. After previous incubation for 30 min at 37°C in the presence of various amounts of calpeptin, platelets were activated by the addition of A23187 (1 μM) in the presence of 1 mM $Ca^{2+}$. 10 min after activation, samples were denatured and analyzed by gel electrophoresis. Dashed lines indicate the major subfragments of filamin, talin and myosin proteolysis.

(lane B) showed decreased amounts of a 68 kDa protein. This peptide was earlier identified as albumin [26]. In other experiments platelets were pretreated with different amounts of calpeptin and further activated in the same way. As described in Materials and methods, granule secretion was not modified by calpeptin doses up to 100 μg/ml. Note that calpeptin did not alter platelet shape before activation, as observed by electron microscopy (not shown), nor induce any secretion or aggregation when used alone. High doses of calpeptin totally inhibited the protease activity of calpain, as shown by intact protein patterns in lanes G and H. As reported before [9,27], the ability of calpeptin to inhibit calpain activity seemed to depend on its substrate. Proteolysis of filamin and myosin was partially inhibited by calpeptin concentrations as low as 1 μg/ml, as shown by reduced presence of filamin and myosin subfragments (93 kDa and 135 kDa, respectively) in the protein pattern (lane C). Platelet spectrin consists of two polypeptides (240 and 220 kDa) present in small quantities which are partly hydrolysed by activated calpain [28,29]. According to these molecular masses and the relative proportion of spectrin and talin in platelets [28,29], the sharp 240 kDa band observed in lane B and C (Fig. 1) could be attributed to spectrin. When platelets are activated in the absence of calpeptin, the 240 kDa band corresponding to intact spectrin was not observed on SDS-PAGE (Fig. 1, lane B), in accordance to the hydrolysis of spectrin by calpain reported earlier [28]. It appeared that spectrin hydrolysis was mainly inhibited by a very low calpeptin dose (1 μg/ml) as shown by recovery of the intact 240 kDa band (Fig. 1, lane C). A higher calpeptin dose (10–20 μg/ml) was required to completely inhibit calpain activity on cytoskeleton proteins, as shown by the recovery of intact talin and filamin bands and disappearance of the talin-derived 48 kDa subfragment (lanes E and F). Longer incubation in the presence of A23187 and $Ca^{2+}$ did not modify the observed patterns. As described [25–27,30], filamin proteolysis occurs in two successive steps. The first step led to the formation of 190 kDa and 100 kDa fragments. The second allowed proteolysis of the 100 kDa fragment into a 93 kDa subfragment. Our results showed that the second step was more sensitive to calpeptin than the first. Similar conclusions can be drawn by examination of vesicle protein patterns. In fact, hydrolytic subfragments were observed simultaneously in whole suspension and vesicle protein patterns at identical calpeptin doses (not shown). Hydrolysis of the filamin-derived 190 kDa subfragment and myosin were inhibited by 1 μg/ml calpeptin. Intact talin and filamin bands were observed for calpeptin doses higher than 20 μg/ml.

### 3.2. Vesicle formation

Fig. 2 shows the whole cell phospholipid fraction which was found in activated platelet supernatants, indicating that vesicle formation was highly sensitive to calpeptin pretreatment. The phospholipid content in the vesicles decreased by more than 60% when platelets were preincubated with 10 μg/ml calpeptin. Higher doses of calpeptin slightly decreased the amount of phospholipid in the supernatant to a minimum of 5%

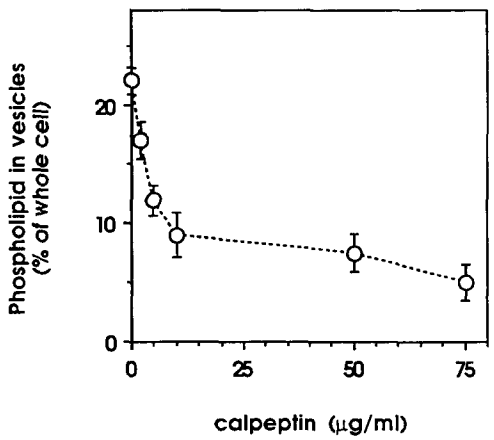


Fig. 2. Effect of calpeptin on vesicle formation. Platelets were preincubated and activated as described in Fig. 1, and then centrifuged at 12000× g for 2 min. Phospholipid contents in the supernatant were expressed as a percentage of the whole cell phospholipid content.

of whole cell phospholipid, even in the presence of large amounts of calpeptin (50 μg/ml).

### 3.3. Morphology of activated platelets

The shape of activated platelets strongly depended on previous pretreatment with calpeptin (Fig. 3). Indeed, when resting platelets were activated without calpeptin pretreatment, cell shapes were round and clenched, with very few cellular prolongations such as filopods. At higher calpeptin concentrations cellular prolongations were more numerous and longer. The mean filopod length, measured on electron micrographs, clearly depended on the calpeptin pretreatment (Fig. 4). At higher calpeptin doses, a mean length of 10 μm filopod per platelet was observed. It should be also noted that the number of protrusions per platelet increased with calpeptin doses. Activated platelets only bore single protrusions, if any, at low calpeptin doses (Fig. 3A–D). For higher calpeptin doses, protrusions were more numerous (mean of 3–4 protrusions per platelet, Fig. 3G).

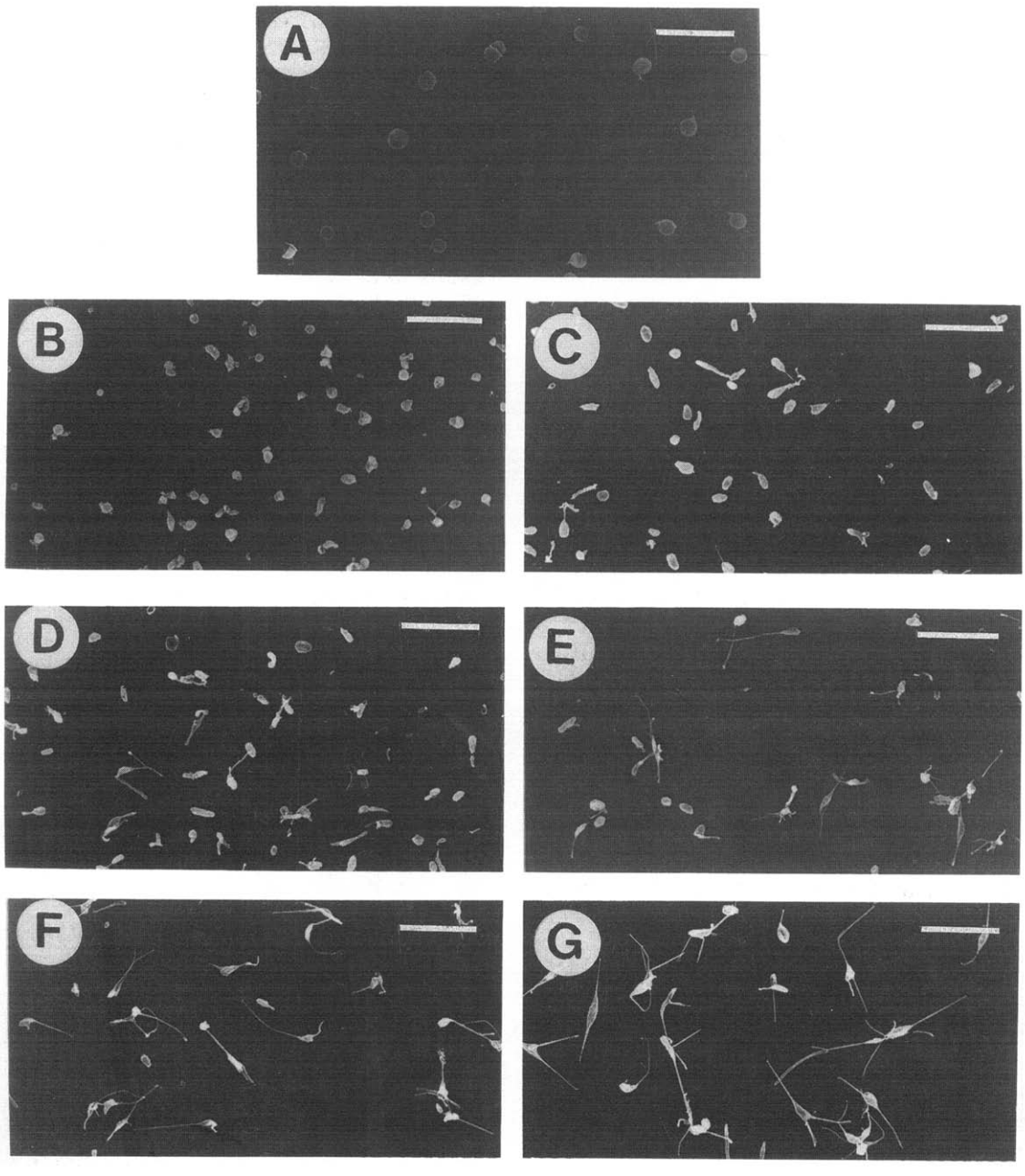


Fig. 3. Effect of calpeptin on activated platelet shape. Platelets were preincubated and activated as described in Fig. 1, and then fixed for scanning electron microscopy (see Materials and methods). A, control; B, no calpeptin; C, 2 μg/ml; D, 5 μg/ml, E, 10 μg/ml; F, 50 μg/ml; G, 75 μg/ml calpeptin. Bar, 10 μm.

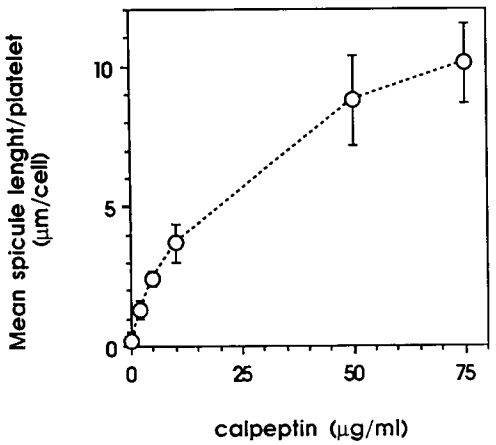


Fig. 4. Effect of calpeptin on mean filopod length per platelet. Filopods lengths were measured and the average length per platelet were determined as described in Materials and methods.

### 3.4. Filopod morphology

Careful observation of filopod morphology allowed us to distinguish two subclasses for these structures. In fact, the few filopods observed on platelets not previously treated (or treated with small doses of calpeptin) were short, broad and bent. Their cross-sections were 200–300 nm. Moreover, their surfaces appeared irregular with some protuberances (Fig. 5A). In contrast, most of the numerous filopods observed on platelets submitted to high doses of calpeptin were straight, longer and narrower (Fig. 3G). Their surfaces were much more regular and their cross-sections smaller (100–150 nm). We also observed some bloated areas on these straight filopods, at the end and in kinked zones (Fig. 5B and C). These particular areas looked like the first above described subclass, i.e., protuberance and broadening.

## 4. Discussion

Calpain activation occurs during platelet activation induced by either physiological (thrombin + collagen) or non physiological agonists (ionophore A23187, complement protein C5b-9) [9,15,24–27,30–32]. When activation is induced by thrombin, stirring of the platelet suspension is required to activate the calpain [33]. The role of calpain activity in the general activation process was investigated using calpain inhibitors [8–10,15,27, 28,30–34]. The different structures and properties of these inhibitors [19] along with the use of different activation agonists has led to contradictory conclusions concerning the role of calpain activity in vesicle formation and in the modulation of prothrombinase activity on the platelet surface. However, it appears that calpain could play a central role in the platelet activation process and in coagulation because of its putative although yet controversial effect on vesicle formation or prothombinase activity. In this study, we examined the effect of calpeptin, a very potent membrane permeant inhibitor of calpain, on vesicle formation and platelet shape during $Ca^{2+}$ ionophore A23187 induced activation. This agonist is very powerful and induces fast (1–2 min) and total platelet activation [8,21,32,33] without aggregation, provided the cell medium is not

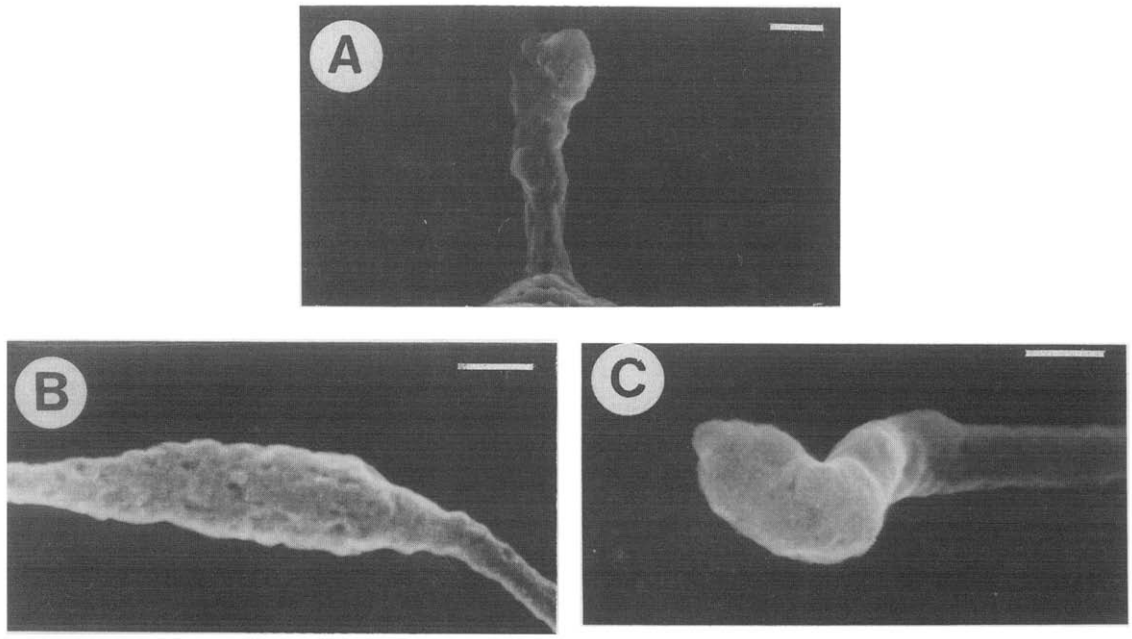


Fig. 5. Filopod morphology. A, filopod observed after A23187 activation without calpeptin pretreatment. B and C, lax and bloated surface parts found on straight filopods (50 µg/ml calpeptin). Bar, 0.2 µm.

stirred. Other stimuli also induce vesicle formation, such as complement protein C5b-9 [15], combination of thrombin and collagen [13,15,32], collagen [14] or thrombin [9] with stirring of the platelet suspension, or addition of the local anaesthetic dibucaine [9,10]. Nevertheless, ionophore A23187 is described as being the most powerful agonist for vesicle shedding [15,32], as for increasing prothrombinase activity [13,31].

We showed in this paper that calpain inhibition by calpeptin was strongly related to the inhibition of vesicle formation during A23187 induced activation. We used more concentrated platelet suspensions ($2 \cdot 10^9$ cells/ml) than in previous reports (generally $10^8$ cells/ml). The high platelet number did not modify platelet responses to a powerful agonist such as A23187, as controlled by previous experiments (not shown). Moreover, it allowed quantitative microparticle evaluation by measuring the amount of phospholipid in the activated platelet supernatants. The amount of vesicles formed during this activation procedure was reduced by 80% when platelets were previously treated with calpeptin. Moreover, comparing the effects of different calpeptin doses on cytoskeletal degradation and vesicle formation revealed that inhibition of calpain was negatively correlated with the microparticle quantity. These results agree with a previous study showing that the inhibition of calpain-mediated degradation of filamin reduces vesicle formation during platelet activation induced by a combination of thrombin and collagen [9]. Hence, we assume that hydrolysis of some cytoskeletal proteins is a prerequisite for vesicle formation. Since vesicle formation is described as being a late event in the activation process [9,15], some other events in the activation process could also act as precursors for vesicle formation, e.g., protein kinase activity [35]. In a previous study, we showed that the sudden transmembrane outflux of aminophospholipids that lead to the generation of a procoagulant platelet surface during A23187 induced activation is not counterbalanced by a reciprocal influx of choline-head phospholipids [34]. The resulting phospholipid overloading on the outer plasma membrane leaflet probably promotes, or induces the filopod formation. Indeed, platelets like erythocytes easily adopt spiculated or invaginated shapes in response to modifications in the outer/inner leaflet phospholipid balance [36,37]. Similarly, platelets like erythrocytes easily release some vesicles when high amounts of phospholipid are incorporated in the external leaflet [38,39]. We can thus assume that phospholipid transmembrane movements during activation put the membrane in a suitable state for vesicle formation, because of the resulting phospholipid overloading in the outer leaflet [34]. F-actin connects to the membrane mainly by the GPIb-IX-filamin-actin and integrin-vinculin-talin-actin complexes [40,41]. Modifying the extent of filamin or talin hydrolysis during platelet activation is predicted to influence vesicle shedding. Spectrin proteolysis was also inhibited by calpeptin and could also modulate vesicle formation.

Preincubation in the presence of calpeptin doses up to 100 $\mu$g/ml modified neither granule secretion, nor transmembrane movements of phospholipids which led to generation of a procoagulant platelet surface during A23187 induced activation [34]. However, A23187-activated platelets exhibited very different shapes depending on the calpeptin pretreatment. The major effect of calpeptin was that activated platelets bore longer and more numerous filopods when cytoskeletal proteins were not proteolyzed, while untreated activated platelets only bore rare broader and shorter filopods (Figs. 3 and 4). Indeed, the mean filopod length positively correlated with the disappearance of vesicles from platelet supernatants. This suggests that the appearance of vesicles in the supernatant and disappearance of long filopods were related phenomena, and that vesicles were released by fragmentation of the filopods. According to this proposal, filopod shapes were shown to depend on calpeptin pretreatment (Figs. 3 and 5). Long and straight filopods were found after high calpeptin pretreatment doses, whereas they were shorter and broader with lower doses. The filopod surface was very irregular, showing protuberances in this latter instance. Hence, the presence of broad filopods and bloated surfaces positively correlated with high vesicle formation and cytoskeleton proteolysis. Since calpain-mediated degradation of filamin induced breakdown of bonds between the cytoskeleton and the plasma membrane [9,40–42], it is likely that this proteolysis can specifically increase membrane flexibility and give it an irregular surface. It is well documented that talin hydrolysis strengthens the connection between integrins and the cytoskeleton, thus enabling the contractile force of the actomyosin complex to be transmitted to the extracellular fibrin network via the talin-integrin complex [40,41]. Thus, talin proteolysis does not release the membrane from the constraints of the cytoskeleton. Elsewhere, spectrin is present in low quantities in platelets, and no connection with the membrane has been evidenced. Moreover, the spectrin meshwork is described as a passive structure, held in platelets by the GPIb-IX-filamin-actin complex in a compressed form, and may only account for the toughness of circulating resting platelets [29]. So filamin cleavage could be singled out as being mainly responsible for vesicle formation, in addition to the external leaflet overloading resulting from the transmembrane redistribution of phospholipid during activation [21,34]. We thus consider that broad filopods with bloated surfaces were due to cytoskeleton degradation, and most likely to filamin proteolysis, and that perturbed areas were sites of microparticle shedding. Some irregular and bloated local areas were also ob-

served on some parts of straight filopods, suggesting that cytoskeleton degradation and the related perturbation of the filopod membrane could occur very locally. Accordingly pseudopodias terminated by 'a balloon-like' structure were described in a previous study on collagen-activated platelets [43]. Interestingly, basal shedding of vesicles was observed in the presence of high calpeptin doses, although the proteolytic activity of calpain was inhibited. Moreover, the few vesicles shed under these conditions exhibited intact high molecular weight proteins, as reported for microparticles resulting from C5b-9-induced platelet activation in the presence of another calpain inhibitor, leupeptin [15]. This suggests that filopods could be divided into fragments containing unproteolysed filamin and talin, because of some fractures in these relatively fragile structures, or the appearance of a bloated local area along this filopod. In fact, a single area is sufficient to induce shedding of the terminal part of the filopod, thus involving vesicles with intact filamin and talin. We also noted that the amount of shed vesicles did not further decrease when filopods continued to elongate in the presence of higher calpeptin doses (50–75 $\mu$g/ml). This suggests that high calpeptin doses could favourably act on the formation of long filopods in addition to its effect on calpain-mediated proteolysis.

Platelet shapes without any filopods, as observed when platelets were activated without calpain inhibition (Fig. 3), are not typical of physiologically activated platelets. Vesicle formation and cytoskeletal protein degradation were more pronounced and occurred faster when efficient agonists such as ionophore A23187 and complement protein C5b-9 were used instead of milder conditions. It can be assumed that the dendritic shape of platelets activated with physiological agonists reflects a less drastic calpain action, and thus reduced fragmentation of filopods.

A direct implication of our proposal is that platelet shape change during A23187-mediated activation occurs in two steps. First, an intracellular $Ca^{2+}$ increase induces transmembrane redistribution of phospholipids and modification of actin organisation, both leading to formation of long filopods. Secondly $Ca^{2+}$-activated calpain progressively proteolyzes filamin among other substrates thus enabling the formation of some potential sites of cracking on filopods from which vesicles could shed.

## 5. Acknowledgements

This work was supported by grants from the Centre National de la Recherche Scientifique (URA 530) and from the University of Montpellier II.

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