Calpain Cleavage of Focal Adhesion Proteins Regulates the Cytoskeletal Attachment of Integrin \( \alpha_{IIb}\beta_3 \) (Platelet Glycoprotein IIb/IIIa) and the Cellular Retraction of Fibrin Clots*

(Received for publication, November 27, 1995, and in revised form, September 18, 1996)

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The intracellular thiol protease calpain catalyzes the limited proteolysis of various focal adhesion structural proteins and signaling enzymes in adherent cells. In human platelets, calpain activation is dependent on fibrinogen binding to integrin \( \alpha_{IIb}\beta_3 \) and subsequent platelet aggregation, suggesting a potential role for this protease in the regulation of postaggregation responses. In this study, we have examined the effects of calpain activation on several postaggregation events in human platelets, including the cytoskeletal attachment of integrin \( \alpha_{IIb}\beta_3 \), the tyrosine phosphorylation of cytoskeletal proteins, and the cellular retraction of fibrin clots. We demonstrate that calpain activation in either washed platelets or platelet-rich plasma is associated with a marked reduction in platelet-mediated fibrin clot retraction. This relaxation of clot retraction was observed in both thrombin and ionophore A23187-stimulated platelets. Calcium dose-response studies (extracellular calcium concentrations between 0.1 \( \mu \)M and 1 mM) revealed a strong correlation between calpain activation and relaxed clot retraction. Furthermore, pretreating platelets with the calpain inhibitors calpeptin and calpain inhibitor I prevented the calpain-mediated reduction in clot retraction. Relaxed fibrin clot retraction was associated with the cleavage of several platelet focal adhesion structural proteins and signaling enzymes, resulting in the dissociation of talin, pp60c-src, and integrin \( \alpha_{IIb}\beta_3 \) from the contractile cytoskeleton and the tyrosine dephosphorylation of multiple cytoskeletal proteins. These studies suggest an important role for calpain in the regulation of multiple postaggregation events in human platelets. The ability of calpain to inhibit clot retraction is likely to be due to the cleavage of both structural and signaling proteins involved in modulating integrin-cytoskeletal interactions.

Calpains are a family of calcium-dependent cysteine proteinases widely expressed in mammalian cells (1–3). Activation of these enzymes occurs in response to a wide range of physiological stimuli and is associated with limited proteolysis of several key cellular proteins, including the c-Fos and c-Jun transcription factors (4), the cytoskeletal proteins talin and actin-binding protein (filamin) (5), and multiple signaling enzymes, including protein kinase C, pp60c-src, and the tyrosine phosphatase PTP-1B (6–8). Although calpain-mediated proteolysis has been implicated in a broad range of pathophysiological processes, including postsischemic tissue damage and degenerative diseases (3), the precise role of these enzymes in cell function has not been established.

Calpains have been localized to points of attachment between cells and the extracellular matrix (focal adhesions) and in the cytoskeletal fraction of thrombin-stimulated platelets (9, 10). The recruitment of calpain to these sites is thought to promote its activation by membrane phospholipids and calcium and to co-localize it with target substrates (11). A growing number of these substrates have been identified in focal adhesions, in which they may participate in the assembly of cytoskeletal signaling complexes and in anchoring integrin adhesion receptors to the contractile cytoskeleton. Once anchored, integrins form a stable transmembrane linkage between extracellular matrix proteins and cytoskeletal elements, thereby allowing the extracellular transmission of cytoskeletal contractile forces necessary for fibrin clot retraction, wound healing, and tissue morphogenesis.

Studies in thrombin-stimulated platelets have demonstrated calpain-catalyzed proteolysis of multiple focal adhesion structural proteins and signaling enzymes (7, 12). Many of these proteins translocate to the cytoskeleton in an aggregation-dependent manner and participate in the formation of integrin-rich cytoskeletal signaling complexes. The formation of these complexes is thought to be critical for the tyrosine phosphorylation of multiple cytoskeletal proteins and for the stable incorporation of integrin \( \alpha_{IIb}\beta_3 \) into the contractile cytoskeleton. In this report, we have investigated the effects of calpain activation on a number of postaggregation events in human platelets. Our studies demonstrate that the calpain-catalyzed proteolysis of focal adhesion structural proteins and signaling enzymes leads to a selective defect in the ability of platelets to retract fibrin clots. This calpain-mediated relaxation of clot retraction was associated with the detachment of integrin \( \alpha_{IIb}\beta_3 \) from the contractile cytoskeleton and the dephosphorylation of multiple cytoskeletal proteins on tyrosine residues.

EXPERIMENTAL PROCEDURES

Materials—Calpeptin was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Ionophore A23187 and calpain inhibitor I were from Calbiochem. All other materials were from sources we have described previously (13, 14).

Antibodies—Anti-phosphotyrosine MAb PY20 and 4G10 were supplied by Upstate Biotechnology. MAb 28–1–3 (Platelet Glycoprotein IIb/IIIa) was obtained from the National Heart Foundation Postgraduate Science Research Scholarship. To whom correspondence should be addressed. Tel.: 61-3-9895-0328; Fax: 61-3-9895-0332.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 3, Issue of January 17, pp. 1694–1702, 1997

1 The abbreviations used are: PTP, protein-tyrosine phosphatase; MAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; GP, glycoprotein; PVDF, polyvinylidene difluoride; PRP, platelet-rich plasma.
Regulation of Clot Retraction by Activated Calpain

Fig. 1. Time course of platelet aggregation and pp60<sup>src</sup> cleavage in ionophore A23187-stimulated platelets. Washed platelets (3 × 10<sup>9</sup>/ml) were pretreated with 0.3% Me<sub>2</sub>SO (DMSO) or calpeptin (100 µg/ml) for 30 min at room temperature and then activated with ionophore A23187 (1 µM) for the indicated time points while stirring. Platelet aggregation was examined in three separate experiments. Platelet aliquots were removed at the indicated time points and lysed in Laemmli reducing buffer, and total lysates were examined for pp60<sup>src</sup> proteolysis by immunoblot analysis, as described under "Experimental Procedures." The extent of proteolysis was assessed by performing densitometry on the pp60<sup>src</sup> immunoblots. These results are from one experiment, representative of three.

RESULTS

Studies in human platelets have demonstrated that several cytoskeletal-associated signaling enzymes are substrates for activated calpain. These enzymes include the Src family of nonreceptor tyrosine kinases and the nonreceptor tyrosine phosphatase PTP-1B (7, 20, 21). As these enzymes are highly expressed in platelets and translocate to the cytoskeletal fraction of aggregated platelets, they are likely to play a major role in regulating the tyrosine phosphorylation status of cytoskeletal proteins. Although the precise role of these phosphorylation events has yet to be clearly established, studies with tyrosine kinase inhibitors have suggested a potentially important role for these enzymes in regulating the cytoskeletal attachment of integrin α<sub>IIb</sub>β<sub>3</sub> (13). We therefore aimed to investigate the effects of calpain activation on the level of tyrosine phosphorylation of cytoskeletal proteins and to correlate these effects with specific platelet postaggregation events, including the cytoskeletal attachment of integrin α<sub>IIb</sub>β<sub>3</sub> and fibrin clot retraction.

Calpain-mediated pp60<sup>src</sup> Cleavage is a Postaggregation Event in Human Platelets—pp60<sup>src</sup> is the major protein tyrosine kinase identified in platelets, constituting 0.2–0.4% of total platelet protein (22). In our initial studies, we examined the time course for pp60<sup>src</sup> cleavage in ionophore A23187- and thrombin-stimulated platelets and correlated this with platelet aggregation. As demonstrated in Fig. 1, pp60<sup>src</sup> cleavage occurred well after the initiation of platelet aggregation by ionophore A23187 (1 µM), with no cleavage detected at 1 min, 20% proteolysis by 2 min, and complete proteolysis after 5 min of platelet stimulation. Consistent with a role for platelet aggregation in calpain activation and pp60<sup>src</sup> cleavage (5, 6, 12, 23), we found that not stirring ionophore A23187-stimulated plate-

lyed by ICN Biomedicals Inc. (Costa Mesa, CA). Anti-talin MAb Sd4 was purchased from Sigma. Anti-pp60<sup>src</sup> MAb 327 was a kind donation from Dr. Joan Brugge (University of Pennsylvania). Anti-GP IIB MAb SZ22 was kindly donated by Dr. Michael Berndt (Baker Medical Research Institute, Melbourne, Victoria, Australia). Both anti-calpain preautolytic and postautolytic polyclonal antibodies were kind donations from Dr. Takami C. Saijo (Tokyo Metropolitan Institute of Medical Science). Both anti-mouse and anti-rabbit peroxidase-conjugated IgG were from Silenus Laboratories (Hawthorn, Victoria, Australia).

Preparation of Washed Platelets and Platelet Aggregation Studies—Human platelets were obtained from healthy volunteers who had not taken antiplatelet medication in the preceding 2 weeks and washed as described previously (14). Washed platelets were finally resuspended in modified Tyrode's buffer (10 mM Hepes, 12 mM NaHCO<sub>3</sub>, pH 7.5, 137 mM NaCl, 2.7 mM KCl, and 5 mM glucose), and preincubated with either calpeptin (2–100 µg/ml), calpain inhibitor I (2–10 µm), or vehicle (0.3% Me<sub>2</sub>SO) for 30 min at room temperature. Washed platelets (3 × 10<sup>9</sup>/ml) were stimulated with 0.01–1 µM ionophore A23187 and/or thrombin (1 unit/ml) at room temperature in the presence or absence of the indicated concentration of CaCl<sub>2</sub>. Fibrinogen (0.3 mg/ml) was included in the platelet suspensions when ionophore A23187 was used as a single agonist. All aggregations were initiated by stirring the platelet suspensions at 950 rpm for 10 min at 37 °C in a four-channel automated platelet analyzer (KyoTo Daiichi, Japan). The extent of platelet aggregation was defined arbitrarily as the percentage of change in optical density as measured by the automated platelet analyzer.

Platelet Subcellular Fractionation—Washed platelets were lysed with 1 volume of 10 mM Trition X-100 lysis buffer (200 mM Tris- HCl, pH 7.4, 10% Triton X-100, 10 mM EGTA, 20 mM EDTA, 2 mM sodium vanadate, 250 µM phenylmethylsulfonyl fluoride, and 50 µM calpain inhibitor I) to 9 volume of platelets, then gently agitated for 60 min at 4 °C. Triton X-100-soluble and -insoluble (cytoskeletal) fractions were prepared by centrifugation for 4 min at 15,000 × g.

Miscellaneous Methods—SDS-PAGE was performed according to the method of Laemmli (18). Fibrinogen was purified from fresh frozen plasma as described previously by Jakobsen and Koerulf (19). Protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin as a standard.
Regulation of Platelet-mediated Fibrin Clot Retraction by Activated Calpain—Our previous studies have suggested that the phosphorylation of cytoskeletal proteins on tyrosine residues by nonreceptor tyrosine kinases may be important for regulating the cytoskeletal attachment of integrin \( \alpha_{\text{IIb}}\beta_3 \) and the cellular retraction of fibrin clots (13). The ability of calpain to regulate the phosphorylation status of cytoskeletal proteins...
and to cleave focal adhesion structural proteins involved in anchoring integrins to the cytoskeleton has suggested a potential role for this protease in the regulation of clot retraction. We therefore performed a series of experiments in PRP and washed platelets to correlate calpain activation with changes in clot retraction. Previous studies examining the role of activated calpain in the regulation of clot retraction have reported no differences in the rate and extent of clot retraction in the presence or absence of calpeptin (23). However, it is unlikely that calpain was substantially activated in these studies, as the platelets were not aggregated during the clot retraction assay. In preliminary studies, we examined clot retraction in a PRP assay system, as PRP is the normal physiological medium used for platelet aggregation and clot retraction studies. The stimulation of platelets with thrombin (5 units/ml) alone (Fig. 3A, tube 1) or thrombin (5 units/ml) in the presence of calcium (10 mM), without stirring (i.e. no aggregation; Fig. 3A, I, tube 3), resulted in 88 ± 2% (Fig. 3A, II, lane 1) and 84 ± 6% (Fig. 3A, II, lane 3) (n = 5) retraction of fibrin clots, respectively. However, when platelets were stimulated with thrombin (5 units/ml) and calcium (10 mM) while stirring for 45 s (i.e. conditions that promote platelet aggregation and calpain activation; Fig. 3A, I, tube 4), the extent of clot retraction was markedly reduced to 32 ± 11% (n = 5) (Fig. 3A, II, lane 4). It was unlikely that this defect in clot retraction was purely a technical artifact related to the formation of large platelet aggregates, as an 83 ± 5% (n = 5) (Fig. 3A, II, lane 2) retraction of fibrin clots was observed in thrombin-aggregated platelets when calcium was omitted from the reaction mixture (Fig. 3A, I, tube 2).

To confirm that these inhibitory effects on platelet-mediated clot retraction were due to calpain activation, we examined clot retraction in a washed platelet assay system. With this assay it was much easier to examine the effects of pharmacological inhibitors of calpain, as these platelet suspensions contained no plasma components, such as albumin or plasma lipoproteins, which may bind these lipophilic compounds and sequester them from platelets. Furthermore, to exclude the possibility that the reduction in clot retraction observed in aggregated platelets was a technical artifact due to uneven platelet dispersion throughout the fibrin clot, we activated calpain in the absence of platelet stirring and aggregation by stimulating platelets with ionophore A23187 (1 μM). This agonist was particularly useful in these studies, as it has the unique ability to activate calpain in the absence of platelet aggregation (27). As demonstrated in Fig. 3B, platelet stimulation by thrombin (1 unit/ml) alone (Fig. 3B, lane 1) or thrombin with calpeptin (100 μg/ml) (Fig. 3B, lane 2) resulted in 90 ± 7% and 90 ± 9% (n = 5) retraction of fibrin clots, respectively. However, in the presence of ionophore A23187, thrombin-stimulated clot retraction was reduced to 49 ± 9% (n = 5) (Fig. 3B, lane 3). This reduction in clot retraction was prevented by pretreating platelets with 100 μg/ml calpeptin (83 ± 5%) (Fig. 3B, lane 4).

To further strengthen our hypothesis that calpain is the responsible protease mediating relaxed fibrin clot retraction, we performed ionophore A23187 dose-response studies. We monitored calpain activation in these studies using antibodies against the inactive large subunit of calpain (80 kDa) and the autoproteolytic activated form (76 kDa). Previous studies in human platelets have demonstrated calcium-dependent autoproteolytic conversion of the 80-kDa subunit of calpain to its 76-kDa active form (3). The antibodies used in these studies have been raised against synthetic peptides corresponding to the N-terminal sequence of the large subunit of both forms of calpain (28). Immunoblot analysis of whole cell lysates with these antibodies represents a sensitive, specific, and direct

![Figure 3](https://example.com/figure3.png)

**FIG. 3. Effect of calpain activation on platelet-mediated fibrin clot retraction.** A. I. Thrombin (5 units/ml) was added to platelet-rich plasma (3 × 10^8/ml) in the presence or absence of CaCl_2 (10 mM) as indicated. The platelet suspensions were either left unstirred or stirred for 45 s to induce the formation of platelet aggregates. Tube I, thrombin (5 units/ml) without stirring; tube 2, thrombin (5 units/ml) with stirring; tube 3, thrombin (5 units/ml) and calcium (10 mM) without stirring; tube 4, thrombin (5 units/ml) and calcium (10 mM) with stirring. II. Clot retraction was quantitated by measuring the residual volume of serum after removal of the clot, as described under “Experimental Procedures.” These results represent the mean ± S.E. (bars) of five separate experiments. B. Washed platelets (3 × 10^8/ml) were pretreated with 0.3% Me_2SO or calpeptin (100 μg/ml) in the presence of CaCl_2 (1 mM) for 30 min at room temperature. Platelets were activated with thrombin (5 units/ml) in the presence or absence of ionophore A23187 (1 μM) without stirring. Clot retraction was quantitated as described above. These results represent the mean ± S.E. (bars) of five separate experiments.
monitored by either calpain autolysis or calpain substrate pro-
calcium. Higher concentrations of extracellular calcium were
the presence of low micromolar concentrations of extracellular
ionophore A23187 and thrombin-stimulated platelets required
extracellular calcium by the addition of 1 mM EGTA and 2 mM
MgCl₂ (2 mM) without stirring. Platelets were lysed in Laemmli reduc-
ing buffer, and the whole cell lysates were examined for calpain autol-
ysis and pp60c-src cleavage, resulted in 60 kDa form of talin and the largest 190-kDa talin fragment,
reached rapid proteolysis of talin in ionophore A23187-stimu-
lated platelets (Fig. 6A). Cleavage of talin from its 230-kDa
native form to the 190-kDa fragment was observed within 15 s
of platelet stimulation and was complete by 3 min. In agree-
ment with previous studies (23), we observed that calpeptin
(100 μg/ml) pretreatment of platelets dramatically slowed the
rate of talin cleavage but did not consistently prevent cleavage
altogether. The effect of this cleavage on the cytoskeletal associ-
ation of talin was investigated by fractionation platelets into
Triton X-100-soluble and -insoluble (cytoskeletal) extracts. Ionophore A23187 stimulation of platelets was associated with
a progressive increase in the cytoskeletal content of talin
throughout the first 60 s of platelet activation (Fig. 6B). The
association of both the 230- and 190-kDa forms of talin with
the cytoskeleton was transient, however, with complete disso-
ciation of the 190-kDa fragment observed after 4 min of platelet
stimulation. This cytoskeletal dissociation was not due to fur-
ther proteolysis of the fragment, as the total cell levels of the
190-kDa fragment remained constant throughout the period of
examination (Fig. 6A). An important role for calpain in regu-
late the cytoskeletal attachment of talin was confirmed in
calpeptin-treated platelets, in which reduced proteolysis of
talin prevented its dissociation from the cytoskeleton (Fig. 6B).
We correlated the effect of talin cleavage on the cytoskeletal
attachment of integrin αIIbβ3. Time course experiments re-
vealed a close correlation between the cytoskeletal dissociation of
talin with that of integrin αIIbβ3 (GP IIb) (Fig. 6B, B–D). As
with talin, pretreating platelets with calpeptin resulted in both
higher and more sustained level of integrin expression incorpo-
ration into the cytoskeleton. Previous reports have suggested
that the dissociation of integrin αIIbβ3 from the membrane
cytoskeleton does not correlate with talin cleavage (23). Our
studies are consistent with these findings, as we observed
dramatic reduction of talin within the first 30 s of platelet
stimulation, yet the bulk of integrin αIIbβ3 (GP IIb) did not
associate from the cytoskeleton until 2–3 min after platelet
activation (Fig. 6, compare A and B with C). To exclude the
possibility that the dramatic reduction in the cytoskeletal con-
tent of talin and integrin αIIbβ3 was due to a global reduction in
total cytoskeletal protein, the talin and integrin αIIbβ3 immu-
noblots were stained with Coomassie Brilliant Blue. The total
amount of filamentous actin (Fig. 6D) and a range of other
cytoskeletal proteins (not shown) increased approximately 2–3-
fold following ionophore A23187 stimulation of platelets and
was largely maintained throughout the period of examination.
Hence it is unlikely that the calpain-mediated reduction in the
cytoskeletal content of integrin αIIbβ3 and talin is attributable
to gross changes in the total amount of cytoskeletal protein.

**Relationship Between Calpain Activation, the Cytoskeletal**
**Attachment of Integrin αIIbβ3, and Clot Retraction**—To exam-
in more detail the relationship between calpain activation
and the cytoskeletal attachment of integrin αIIbβ3, we per-

![Fig. 4. Ionophore dose response for calpain activation and relaxed clot retraction.](image-url)

means of monitoring calpain activation within the cell (29). As
shown in Fig. 4, concentrations of ionophore A23187 that activ-
ate platelets without activating calpain (0.01 and 0.05 μM), as
monitored by calpain autolysis and pp60c-src cleavage, resulted
in 72.7 ± 3.5 and 70 ± 5.4% retraction of fibrin clots, respecti-
vely. Higher concentrations of ionophore A23187 (0.25 and 1
μM) resulted in calpain activation and pp60c-src cleavage and
were associated with a substantial reduction in clot retraction
(46 ± 2.8 and 37.4 ± 5.2%, respectively). The chelation of
extracellular calcium by the addition of 1 mM EGTA and 2 mM
MgCl₂ to the platelet reaction mixtures abolished calpain activ-
lation by 1 μM ionophore A23187 and restored effective clot
retraction (72.3 ± 2.9%).

Further evidence supporting a role for calpain in the regu-
lation of clot retraction stemmed from calcium dose-response
studies. Previous reports have demonstrated an absolute re-
quirement for extracellular calcium for calpain activation in-
duced by both pharmacological and physiological agonists (2).
As demonstrated in Fig. 5, A and B, calpain activation in both
ionophore A23187 and thrombin-stimulated platelets required
the presence of low micromolar concentrations of extracellular
calcium. Higher concentrations of extracellular calcium were
associated with a progressive increase in calpain activation, as
monitored by either calpain autolysis or calpain substrate pro-
teolysis, with maximal activation observed with 1.0 mM cal-
cium (Fig. 5, A and B). In each of these experiments, there was a
strong correlation between the extent of calpain activation
and the reduction in clot retraction. Furthermore, in all of the
studies reported here the inhibitory effects of calpain were
limited to the clot retraction process, with normal platelet
aggregation and [14C]serotonin release in response to thrombin
or ionophore A23187 (data not shown).

**Calpain-mediated Cleavage of Talin and the Dissociation of
Talin and Integrin αIIbβ3 from the Contractile Cytoskeleton**—
The ability of cells to transmit cytoskeletal contractile forces to
extracellular matrices is dependent on the anchorage of inte-
grins to the cytoskeleton. The stable association of integrins
with actin filaments requires a number of intermediary pro-
teins, including talin, α-actinin, and vinculin (30). We investi-
gated whether the calpain-catalyzed cleavage of talin was asso-
ciated with the dissociation of either talin or integrin αIIbβ3
from the contractile cytoskeleton. Immunoblot analysis of total
cell lysates, with an antibody that recognizes the native 230-
kDa form of talin and the largest 190-kDa talin fragment,
revealed rapid proteolysis of talin in ionophore A23187-stimu-
lated platelets (Fig. 6A). Cleavage of talin from its 230-kDa
native form to the 190-kDa fragment was observed within 15 s
of platelet stimulation and was complete by 3 min. In agree-
ment with previous studies (23), we observed that calpeptin
(100 μg/ml) pretreatment of platelets dramatically slowed the
rate of talin cleavage but did not consistently prevent cleavage
altogether. The effect of this cleavage on the cytoskeletal associ-
ation of talin was investigated by fractionation platelets into
Triton X-100-soluble and -insoluble (cytoskeletal) extracts.
Ionophore A23187 stimulation of platelets was associated with
a progressive increase in the cytoskeletal content of talin
throughout the first 60 s of platelet activation (Fig. 6B). The
association of both the 230- and 190-kDa forms of talin with
the cytoskeleton was transient, however, with complete disso-
ciation of the 190-kDa fragment observed after 4 min of platelet
stimulation. This cytoskeletal dissociation was not due to fur-
ther proteolysis of the fragment, as the total cell levels of the
190-kDa fragment remained constant throughout the period of
examination (Fig. 6A). An important role for calpain in regu-
late the cytoskeletal attachment of talin was confirmed in
calpeptin-treated platelets, in which reduced proteolysis of
talin prevented its dissociation from the cytoskeleton (Fig. 6B).

We correlated the effect of talin cleavage on the cytoskeletal
attachment of integrin αIIbβ3. Time course experiments re-
vealed a close correlation between the cytoskeletal dissociation of
talin with that of integrin αIIbβ3 (GP IIb) (Fig. 6, B–D). As
with talin, pretreating platelets with calpeptin resulted in both
higher and more sustained level of integrin αIIbβ3 incorpora-
tion into the cytoskeleton. Previous reports have suggested
that the dissociation of integrin αIIbβ3 from the membrane
cytoskeleton does not correlate with talin cleavage (23). Our
studies are consistent with these findings, as we observed
extensive proteolysis of talin within the first 30 s of platelet
stimulation, yet the bulk of integrin αIIbβ3 (GP IIb) did not
dissociate from the cytoskeleton until 2–3 min after platelet
activation (Fig. 6, compare A and B with C). To exclude the
possibility that the dramatic reduction in the cytoskeletal con-
tent of talin and integrin αIIbβ3 was due to a global reduction in
total cytoskeletal protein, the talin and integrin αIIbβ3 immu-
noblots were stained with Coomassie Brilliant Blue. The total
amount of filamentous actin (Fig. 6D) and a range of other
cytoskeletal proteins (not shown) increased approximately 2–3-
fold following ionophore A23187 stimulation of platelets and
was largely maintained throughout the period of examination.
Hence it is unlikely that the calpain-mediated reduction in the
cytoskeletal content of integrin αIIbβ3 and talin is attributable
to gross changes in the total amount of cytoskeletal protein.
formed experiments on ionophore A23187-stimulated platelets that had been resuspended in either calcium-free or calcium-containing buffers. Platelet stimulation with 1 mM ionophore A23187 for 5 min in the presence of 1 mM CaCl₂ resulted in complete conversion of inactive calpain to its activated form. Under these assay conditions, integrin αIIbβ₃ was no longer detectable within the cytoskeleton by immunoblot analysis (Fig. 7A). In contrast, the resuspension of platelets in buffers containing 1 mM EGTA and 2 mM MgCl₂, prior to ionophore A23187 stimulation, prevented calpain activation and restored the association of integrin αIIbβ₃ with the contractile cytoskeleton.

**FIG. 5. Calcium dose response for calpain activation and relaxed clot retraction.** A, washed platelets (3 × 10⁸/ml) were activated with ionophore A23187 (1 μM) for 30 min, in the presence of EGTA (1 mM) and MgCl₂ (2 mM) or the indicated concentrations of CaCl₂, without stirring. Platelets were lysed in Laemmli reducing buffer, and the whole cell lysates were examined for pp60⁰-src proteolysis (upper panel) or calpain activation (C) by immunoblot analysis. % Inactive Calpain, amount of the intact form of calpain (80 kDa) in whole cell lysates, as determined by densitometric measurements of calpain immunoblots. These results are from one experiment, representative of three. In parallel experiments, platelets were activated with ionophore A23187 (1 μM) in the presence of atroxin (0.1 μg/ml) and exogenous fibrinogen (1 mg/ml). The extent of clot retraction was quantitated after 60 min (histogram), as described in Fig. 2. These results represent the mean ± S.D. (bars) of four separate experiments. B, washed platelets (3 × 10⁸/ml) were activated with ionophore A23187 (1 μM) in the presence of thrombin (1.0 units/ml) and the indicated concentrations of CaCl₂ and MgCl₂, prior to ionophore A23187 stimulation, prevented calpain activation and restored the association of integrin αIIbβ₃ with the contractile cytoskeleton.

**FIG. 6. Time course for talin cleavage and the dissociation of talin and integrin αIIbβ₃ (GP IIb) from the contractile cytoskeleton.** Washed platelets (3 × 10⁸/ml) were pretreated with 0.3% Me₂SO or calpeptin (100 μg/ml) for 10 min at room temperature and then stimulated with ionophore A23187 (1 μM) for the indicated time points while stirring. Platelets were lysed and fractionated into Triton X-100-soluble or cytoskeletal extracts, as described under “Experimental Procedures.” Whole cell lysates or cytoskeletal extracts were subjected to immunoblot analysis using monoclonal antibodies against talin or GP IIb. Cytoskeletal actin was quantitated by densitometry after staining the PVDF membranes with Coomassie Brilliant Blue. Results are from one experiment, representative of three.
The studies presented in this article define an important role for calpain in the regulation of multiple postaggregation events in human platelets. We have demonstrated under a variety of different experimental conditions that the calpain-catalyzed cleavage of several focal adhesion structural proteins and signaling enzymes in human platelets leads to a selective defect in the ability of platelets to retract fibrin clots. This reduction in clot retraction was associated with reduced incorporation of integrin αIIbβ3 into the contractile cytoskeleton and the dephosphorylation of multiple cytoskeletal proteins on tyrosine residues.

Although there are a number of cysteine and serine proteases in human cells, we have provided several lines of evidence suggesting that calpain is likely to be the responsible protease regulating fibrin clot retraction. First, relaxed fibrin clot retraction was only observed under experimental conditions that promoted calpain activation. These conditions include the requirement for extracellular calcium in both thrombin- and ionophore A23187-stimulated platelets and the necessity for platelet aggregation when thrombin was used as a single agonist. Second, dose-response studies in ionophore A23187-stimulated platelets and the necessity for platelet aggregation when thrombin was used as a single agonist. Second, dose-response studies in ionophore A23187-stimulated platelets revealed a close correlation between calpain activation and relaxed clot retraction. Third, pretreatment of platelets with two different inhibitors of calpain restored clot retraction in a dose-dependent manner (Fig. 7B) and correlated with its ability to inhibit calpain activation (data not shown).

**FIG. 7.** Correlation between calpain activation, the cytoskeletal association of integrin αIIbβ3, and clot retraction. Washed platelets (3 x 10^7/ml) were pretreated with either 1 mM EGTA and 2 mM MgCl2, 0.3% Me2SO, or the indicated concentrations of calpeptin for 30 min at room temperature in the presence of fibrinogen (1.0 mg/ml). All assays, except those containing EGTA (1 mM) and MgCl2 (2 mM), were performed in the presence of CaCl2 (1 mM). A, platelets were activated with ionophore A23187 (1 μM) in the presence of atroxin (0.1 μg/ml) without stirring. Clot retraction was quantitated after 60 min of platelet stimulation. These results represent the mean ± S.D. (bars) of four separate experiments. In parallel experiments, ionophore A23187-stimulated platelets (30 min) were lysed and fractionated into Triton X-100-soluble or cytoskeletal extracts. Whole cell lysates were subjected to immunoblot analysis using an antibody against the 80-kDa preautolytic form of calpain. Immunoblot analysis was performed on cytoskeletal extracts using a monoclonal antibody against GP IIb. B, washed platelets were activated with thrombin (1 unit/ml) while stirring for 45–60 s to induce platelet aggregation. Clot retraction was quantitated after 60 min of platelet stimulation. These results represent the mean ± S.D. (bars) of four separate experiments.
as the release of procoagulant-rich microparticles from the platelet membrane, a role for calpain in the regulation of fibrin clot retraction has not been established (23, 31). One of the major technical obstacles in examining the role of calpain in clot retraction is the need to induce platelet aggregation to activate the protease. The formation of platelet aggregates may lead to uneven platelet dispersion throughout the fibrin clot, resulting in an artificial decrease in clot retraction. To overcome this technical problem we have used washed platelets treated with the pharmacological agonist ionophore A23187. This agonist has well characterized effects on platelet function and has the advantage of activating calpain in the absence of platelet aggregation (27). Several lines of evidence suggest that the results we have obtained in ionophore A23187-stimulated platelets are not unique to this agonist and are likely to be physiologically relevant. First, the inhibitory effects of calpain activation on clot retraction were not limited to ionophore A23187-stimulated platelets, as we observed a similar functional defect in thrombin-stimulated platelets under assay conditions that favored calpain activation. Second, calpain-mediated proteolysis of Src family kinases, PTP-1B and talin is also observed in platelets activated by physiological agonists, such as thrombin and collagen, and is associated with the dissociation of integrin \(\alpha_{IIb}\beta_3\) from the membrane cytoskeleton (31). Third, the ability of calpain to regulate the cytoskeletal attachment and signaling function of pp60
superscript
src
subscript
and PTP-1B is a feature of both ionophore A23187- and thrombin-stimulated platelets (6, 7, 21). Finally, the calpain-induced dephosphorylation of multiple platelet proteins that we have observed in ionophore A23187-stimulated platelets has recently been reported in thrombin-aggregated platelets (24).

Previous studies in thrombin- and collagen-stimulated platelets have suggested that calpain-mediated cleavage of cytoskeletal proteins is responsible for the dissociation of as much as 16% of total platelet integrin \(\alpha_{IIb}\beta_3\) from the membrane cytoskeleton of aggregated platelets (31). Although talin is considered to play a critical role in anchoring integrins to the contractile cytoskeleton, these studies have highlighted that the calpain-mediated cleavage of talin does not appear to correlate with the release of integrin \(\alpha_{IIb}\beta_3\) from the cell surface. Our time course experiments in ionophore A23187-stimulated platelets are consistent with this possibility, as they clearly demonstrate that the cytoskeletal dissociation of integrin \(\alpha_{IIb}\beta_3\) lags well behind the initial cleavage of talin. These observations suggest a role for additional calpain-mediated proteolytic events in dissociating integrin-cytoskeletal contacts. A recent report has demonstrated that the cytoplasmic domain of \(\beta_3\) integrins is cleaved at multiple sites by calpain in vitro, raising the distinct possibility that direct proteolysis of integrins can regulate their association with the cytoskeleton (32).

In previous studies we have demonstrated that tyrosine phosphorylation events in human platelets play a key role in regulating the cytoskeletal attachment of integrin \(\alpha_{IIb}\beta_3\) (13). The studies reported in this article are consistent with these findings, as they demonstrate a close correlation between cytoskeletal protein dephosphorylation and the cytoskeletal dissociation of integrin \(\alpha_{IIb}\beta_3\). Furthermore, studies in human fibroblasts have demonstrated that the tyrosine phosphorylation of cytoskeletal proteins, following the ligation and aggregation of integrins on the cell surface, is essential for stable interaction between filamentous actin and integrins (33). Although these observations highlight the importance of tyrosine phosphorylation events in regulating cytoskeletal-integrin contacts, they provide limited insight into the molecular events modulating this interaction. For example, the level of tyrosine phosphorylation of talin, \(\beta\)-integrin, and vinculin is low in normal adherent cells, suggesting that direct phosphorylation of these proteins is an unlikely mechanism by which integrins become anchored to the cytoskeleton. In contrast, the vinculin-binding proteins pallxin and tensin are prominent tyrosine-phosphorylated proteins in focal adhesions (34). The phosphorylation of pallxin may also be important for regulating its association with other cytoskeletal proteins, such as vinculin and talin. An attractive hypothesis is that the phosphorylated forms of pallxin and/or tensin bind to vinculin and unmask its talin and actin binding sites. This “active conformation” of vinculin may in turn stabilize the interaction between integrins and the underlying cytoskeleton (35). It is likely that the calpain-mediated cleavage of tyrosine kinases and phosphatases leads to a reduction in the level of phosphorylation of cytoskeletal proteins, such as pallxin and tensin. The dephosphorylation of these proteins may undermine the stable association of vinculin with actin filaments and talin, ultimately leading to the disassembly of integrin-cytoskeletal contacts.

The studies reported here on human platelets clearly have important implications for adhesion processes in other cells. Calpain is a ubiquitous protease, which has been localized to focal adhesions in a variety of adherent cells (9). The ability of calpain to cleave a growing number of focal adhesion proteins suggests a potentially important role for this enzyme in the regulation of these cellular structures. Our studies indicate that one of the functions of these protease is to regulate the transmission of cytoskeletal contractile forces to extracellular matrices. Furthermore, our studies suggest that calpain may also have an important signal-terminating role within the cell. The ability of integrins to promote calpain activation, leading to the proteolysis and down-regulation of cytoskeletal-associated signaling enzymes, suggests a potentially novel means by which these adhesion receptors can limit their own signaling function. Whether these proteolytic events have flow-on effects to other signaling pathways linked to cell adhesion will be an important area for future investigation.

Acknowledgment—We thank Dr. T. C. Saido for his generous donation of anti-calpain antibodies.

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