Adducin in platelets: activation-induced phosphorylation by PKC and proteolysis by calpain

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Adducins are a family of cytoskeletal proteins encoded by 3 genes (alpha, beta, and gamma). Platelets express alpha and gamma adducins, in contrast to red blood cells that express alpha and beta adducins. During platelet activation with thrombin, calcium ionophore A23187, or phorbol 12-myristate 13-acetate, alpha and gamma adducins were phosphorylated by protein kinase C (PKC) as detected by an antibody specific for a phosphopeptide sequence in the highly conserved carboxy terminus. Platelet activation also led to adducin proteolysis; inhibition by calpeptin suggests that the protease was calpain. The kinase inhibitor staurosporine inhibited PKC phosphorylation of adducin and also inhibited proteolysis of adducin. Experiments with recombinant alpha adducin demonstrated that the PKC-phosphorylated form was proteolyzed at a significantly faster rate than the unphosphorylated form. The concentration of adducin in platelets was estimated at 6 μM, similar to the concentration of capping protein. Fractionation of platelets into high-speed supernatant (cytosol) and pellet (membrane and cytoskeleton) revealed a shift of PKC-phosphorylated adducin to the cytosol during platelet activation. Platelet aggregation detected turbidometrically was decreased in the presence of staurosporine and was completely inhibited by calpeptin. Thrombin-induced changes in morphology were assessed by confocal microscopy with fluorescein phalloidin and were not prevented by staurosporine or calpeptin. Our results suggest that regulation of adducin function by PKC and calpain may play a role in platelet aggregation. (Blood. 2002;99:2418-2426)

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Introduction

After stimulation with agonists, platelets undergo a complex set of events described as activation, adhesion (to endothelial cells), aggregation (to each other), and clot retraction. Activation includes secretion of granule contents by fusion with the open canalicular system, shape changes such as extension of filopodia and lamellipodia, and changes in the fibrinogen receptor, integrin αIβ3 or GPIIbIIIa, that enable it to bind fibrinogen and to create cell-cell bridges. Numerous signaling pathways are involved in the coordination of these cellular events, and regulation of cytoskeletal protein interactions is essential to normal platelet function.

One of the major cytoskeletal components of platelets is actin, which exists in the platelet as monomeric, ie, G-actin, and filamentous, ie, F-actin. In the resting platelet, most of the actin is in the monomeric state, and some exists as short actin filaments with capping proteins preventing polymerization into longer filaments. When platelets are activated, most of the G-actin polymerizes into F-actin, causing extensions of the platelet membrane into filopodia and lamellipodia. In addition to the cytoplasmic G- and F-actin in platelets, there is a membrane skeleton, a specialized structure composed of crosslinked short actin filaments with linkages to integral membrane proteins. The membrane skeleton has been most studied in red blood cells in which short actin filaments are crosslinked by spectrin tetramers. Platelets also contain spectrin in their membrane skeleton, but a more abundant component of the platelet membrane skeleton is actin-binding protein 280 (ABP-280) or filamin.

Although many components of the red cell membrane skeleton are expressed in platelets, platelets also have cytoplasmic actin filaments and microtubules contributing to their shape. The complex cytoskeleton of platelets requires careful coordination to regulate physiologic changes in these structures. We have identified another cytoskeletal component of platelets, adducin, that may have a role in the cytoskeletal changes occurring during platelet activation.

Adducin was first described in red blood cells but is found in all human cells examined and may function in the cytoplasm as well as on the membrane skeleton. Adducins (alpha, beta, and gamma) are a family of cytoskeleton proteins encoded by 3 distinct genes. The functions of adducin purified from red blood cells have been well studied by in vitro assays. Purified red cell adducin consists of alpha and beta polypeptides tightly associated into dimers or tetramers. The in vitro functions of red cell adducin (alpha/beta) include cross-linking spectrin and actin filaments, capping actin filaments, and bundling actin filaments. Adducin’s interaction with spectrin and actin is regulated via phosphorylation by several protein kinases and by calcium/calmodulin. Gamma adducin was discovered as a protein kinase C-binding protein by screening a kidney complementary DNA expression library with PKC as a probe.

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In contrast to the alpha/beta adducin complex found in red cells, the adducin complex found in platelets and most nonerythroid cells consists of alpha and gamma subunits. Platelet adducin (alpha/gamma) may have different properties from the red cell alpha/beta complex, and these properties may be crucial to adducin’s function in platelets and other nonerythroid cells. In experiments reported here, we found that alpha and gamma adducins were phosphorylated at a PKC phosphorylation site upon activation of platelets by thrombin, calcium ionophore A23187, or phorbol 12-myristate 13-acetate (PMA). PKC phosphorylation of adducin was associated with a shift of adducin from the high-speed pellet (membrane and cytoskeleton) to the high-speed supernatant (cytosol). Platelet activation also was associated with rapid proteolysis of adducin that was inhibited by the calpain-specific inhibitor calpeptin. In vitro experiments demonstrated that PKC-phosphorylated adducin was proteolyzed more rapidly than unphosphorylated adducin.

These results provide groundwork toward understanding a possible role of adducin in platelet function. Assays of platelet function demonstrated that platelet aggregation was decreased in the presence of staurosporine and completely inhibited by calpeptin. Thrombin-induced platelet shape change (extrusion of filopodia and lamellipodia) was not prevented by staurosporine or calpeptin. Our results suggest that regulation of adducin function by PKC and calpain may play a role in platelet aggregation.

### Materials and methods

#### Platelet preparation

After informed consent, blood was collected from healthy adult donors by a 19-gauge butterfly needle. The first 5 mL was discarded, and a second syringe was attached. Blood (30 mL) was then collected into a syringe prefilled with 5 mL acid citrate dextrose anticoagulant (85 mM sodium citrate, 111 mM dextrose, and 71 mM citric acid) and equilibrated to 37°C. The blood was centrifuged in 4 polypropylene tubes (15 mL) at 3500 × g at room temperature. The platelet-rich plasma was then transferred gently into 2 polypropylene tubes (15 mL) and proastaglandin I2 (Sigma Chemical, St Louis, MO) was added at a concentration of 0.05 μM. Platelet activation also was associated with rapid proteolysis of adducin that was inhibited by the calpain-specific inhibitor calpeptin. In vitro experiments demonstrated that PKC-phosphorylated adducin was proteolyzed more rapidly than unphosphorylated adducin.

#### Recombinant alpha adducin

Full-length human alpha adducin was expressed in BL21(DE3) pLysS Escherichia coli by using the pCR7/NT-TOPO expression vector (Invitrogen, Carlsbad, CA). The vector was constructed so that the amino terminus of alpha adducin is synthesized in frame with a 6-histidine tag. The 6-histidine tag permitted purification of the expressed polypeptide by using immobilized metal affinity chromatography. For purification, the cobalt-containing Talon Superflow Resin (Clontech, Palo Alto, CA) was used. Extraction and elution buffers were prepared according to the user manual for isolation under native conditions. Recombinant adducin was further concentrated by using centrifugal filters (Millipore, Bedford, MA).

#### Quantitation of adducin in platelets

Adducin levels in platelet samples were determined by using optical density measurements from Western blots and reference to known adducin levels in samples of recombinant human alpha adducin. The concentration of recombinant alpha adducin was determined by using the Biorad protein assay kit with BSA as standard. Several dilutions of recombinant alpha adducin and platelet samples from 2 individuals were run on a 4% to 15% gradient gels (Biorad) at 100 V. The same sample volume was loaded for the corresponding Coomassie blue-stained gels and the gels that were used for the Western blots. Gels were stained for 15 minutes in Coomassie Brilliant Blue R-250 (0.1% Coomassie blue, 50% methanol, 10% acetic acid), followed by destaining (10% methanol, 7% acetic acid). Samples run on duplicate gels were transferred to nitrocellulose electrophotoorphically and hybridized with antibodies. Nitrocellulose blots were incubated overnight at 4°C in buffer containing phosphate-buffered saline (PBS), 4% bovine serum albumin (BSA; Fraction V; Sigma), 0.1% Triton X-100, and primary antibody. Blots were washed 3 times at room temperature in the same buffer without BSA, then incubated with protein A conjugated to horseradish peroxidase (Biorad) in buffer with BSA for 60 minutes at room temperature. Blots were washed again 4 times, then incubated with chemiluminescence substrate (Amersham, Piscataway, NJ) and exposed to film. The antibodies used were either antiphosphorylated adducin (rabbit immunoaffinity-purified immunoglobulin G; Upstate Biotechnology, Lake Placid, NY), or a general antiadducin antibody made in our laboratory. The antiadducin antibody was generated in rabbits by using several synthetic adducin peptides conjugated with glutaraldehyde to rabbit serum albumin (Sigma). The rabbit serum was affinity purified on a column of recombinant human alpha adducin, yet it reacts with alpha, beta, and gamma adducins.

#### Western blotting and Coomassie blue-stained gels

Samples were separated by SDS polyacrylamide gel electrophoresis on 4% to 15% gradient gels (Biorad) at 100 V. The same sample volume was loaded for the corresponding Coomassie blue-stained gels and the gels that were used for the Western blots. Gels were stained for 15 minutes in Coomassie Brilliant Blue R-250 (0.1% Coomassie blue, 50% methanol, 10% acetic acid), followed by destaining (10% methanol, 7% acetic acid). Samples run on duplicate gels were transferred to nitrocellulose electrophotoorphically and hybridized with antibodies. Nitrocellulose blots were incubated overnight at 4°C in buffer containing phosphate-buffered saline (PBS), 4% bovine serum albumin (BSA; Fraction V; Sigma), 0.1% Triton X-100, and primary antibody. Blots were washed 3 times at room temperature in the same buffer without BSA, then incubated with protein A conjugated to horseradish peroxidase (Biorad) in buffer with BSA for 60 minutes at room temperature. Blots were washed again 4 times, then incubated with chemiluminescence substrate (Amersham, Piscataway, NJ) and exposed to film. The antibodies used were either antiphosphorylated adducin (rabbit immunoaffinity-purified immunoglobulin G; Upstate Biotechnology, Lake Placid, NY), or a general antiadducin antibody made in our laboratory. The antiadducin antibody was generated in rabbits by using several synthetic adducin peptides conjugated with glutaraldehyde to rabbit serum albumin (Sigma). The rabbit serum was affinity purified on a column of recombinant human alpha adducin, yet it reacts with alpha, beta, and gamma adducins.

#### Platelet activation

Platelet activation involved the addition of human thrombin (Sigma), calcium ionophore A23187 (Sigma), or PMA (Sigma). Platelet activators and inhibitors, except for thrombin, were dissolved in dimethyl sulfoxide (DMSO). Thrombin was dissolved in a solution containing 150 mM NaCl and 10 mM Tris-HCl, pH 7.4. Calcium chloride was added to all platelet samples just before activation for a final concentration of 2.5 mM. Platelets were incubated with the kinase inhibitor staurosporine (Calbiochem, La Jolla, CA) or the calpain inhibitor calpeptin (Novabiochem, La Jolla, CA) for 10 to 30 minutes before activation with thrombin. For each experiment, platelets were divided into 1 mL microfuge tubes and kept at 37°C in a water bath. After platelet activation by thrombin, calcium ionophore, or PMA, the samples were inverted 6 times and incubated at 37°C for different lengths of time depending on the experiment. To end the experiment, a volume of Laemmli sample buffer equal to 25% of the platelet volume was added, and the sample was boiled for 10 minutes. Laemmli sample buffer (Biorad) contained 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 25% glycerol, 5% beta-mercaptoethanol, and 0.01% Bromphenol Blue.

#### Adducin levels in platelets

Adducin levels in platelet samples were determined by using optical density measurements from Western blots and reference to known adducin levels in samples of recombinant alpha adducin. The concentration of recombinant alpha adducin was determined by using the Biorad protein assay kit with BSA as standard. Several dilutions of recombinant alpha adducin and platelet samples from 2 individuals were run on a 4% to 15% polyacrylamide gel and transferred to nitrocellulose. Western blotting with the antiadducin antibody was performed as described earlier. Adducin levels in platelet samples were determined by using optical density measurements from scanned gel images analyzed by using the public domain National Institutes of Health (NIH) Image program. The amount of alpha adducin in each platelet sample was calculated from the standard curve. The number of platelets was determined by a resistive-particle counter after column purification, and the concentration of alpha adducin per platelet was calculated by dividing the amount of adducin from Western blot by the number of platelets loaded on the gel.

#### Calpain proteolysis of recombinant adducin

Recombinant alpha adducin (240 ng/μL) was phosphorylated by incubation in a reaction-containing adenosine triphosphate 1.6 mM, CaCl2, 4.2 mM, and inhibitors, except for thrombin, were dissolved in dimethyl sulfoxide (DMSO). Thrombin was dissolved in a solution containing 150 mM NaCl and 10 mM Tris-HCl, pH 7.4. Calcium chloride was added to all platelet samples just before activation for a final concentration of 2.5 mM. Platelets were incubated with the kinase inhibitor staurosporine (Calbiochem, La Jolla, CA) or the calpain inhibitor calpeptin (Novabiochem, La Jolla, CA) for 10 to 30 minutes before activation with thrombin. For each experiment, platelets were divided into 1 mL microfuge tubes and kept at 37°C in a water bath. After platelet activation by thrombin, calcium ionophore, or PMA, the samples were inverted 6 times and incubated at 37°C for different lengths of time depending on the experiment. To end the experiment, a volume of Laemmli sample buffer equal to 25% of the platelet volume was added, and the sample was boiled for 10 minutes. Laemmli sample buffer (Biorad) contained 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 25% glycerol, 5% beta-mercaptoethanol, and 0.01% Bromphenol Blue.
Platelet fractionation

Column-purified platelets were divided into 1.5-mL Beckman microfuge tubes at 37°C (900 μL per tube). Thrombin (0.1 U/mL or 1 U/mL) and CaCl2 (2.5 mM) were added, and samples were inverted 6 times and kept at 37°C. Some samples were preincubated with calpeptin (200 μg/mL) for 30 minutes. At indicated time points after activation, 100 μL concentrated lysis buffer was added (final concentration 1% Triton X-100, 2.5 mM EGTA, 4 mM Pefabloc [Roche, Indianapolis, IN], 0.01 mg/mL Aprotinin [Sigma], 10 mM sodium orthovanadate [Calbiochem], 0.01 mg/mL Leupeptin [Sigma], 1 mM phenylmethylsulfonyl fluoride [Sigma]). Samples were vortexed and put on ice for 20 minutes. Samples were then centrifuged in a Beckman TLA 100.4 ultracentrifuge at 55 000 rpm (126 203 × g) for 3 hours. Gel samples were made by adding 400 μL Laemmli sample buffer to 800 μL supernatant and resuspending the pellet with 750 μL Laemmli sample buffer. Samples were boiled for 10 minutes and loaded proportionately (45 μL supernatant to 22.5 μL pellet) onto 4% to 15% gradient polyacrylamide gels as described earlier. Western blotting was performed as described above, and relative optical density was determined for adducin and PKC-phosphorylated adducin by using NIH Image software.

Platelet aggregation assay

Column-purified platelets were assayed by using a 4-channel platelet aggregation chronogenic kinetic system (PACKS-4; Helena Laboratories, Beaumont, TX) with constant stirring (1000 rpm) at 37°C. Platelets were incubated with 0.3 μM staurosporine, 200 μg/mL calpeptin, or 0.8% DMSO (vehicle control) for 30 minutes before activation. After 30 minutes, samples were added to siliconized glass tubes with a stir bar and activated with 1 U/mL thrombin in the presence of 2.5 mM CaCl2. Maximum aggregation within 2 minutes was determined by the aggregometer and averaged for 3 experiments. P was determined by Tukey studentized range test.

Fluorescence microscopy

Platelets were prepared by column purification as described above. Duplicate samples were made for Western blotting and for microscopy. After incubation with the inhibitor staurosporine (0.3 μM) or calpeptin (200 μg/mL) for 30 minutes or none, platelets were activated with thrombin for 60 seconds. Activation was stopped by addition of glutaraldehyde (final concentration 2%). AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR) was added to each sample (final concentration 0.002 U/μL phalloidin), and samples were kept at 4°C overnight or longer. On the day of microscopy, samples were pelleted in a microfuge at 10 000 rpm (6720g) for 5 minutes. After the supernatant was removed, the pellet was resuspended with 1 mL PBS, vortexed, and spun again. Pellets were resuspended in 200 μL PBS, and aliquots were placed on glass slides with a coverslip for examination with a Leica confocal microscope.
resting platelets (lane 0), there was a low level of PKC phosphorylation of adducin, but, within 10 seconds of addition of thrombin, there was a large increase in the level of PKC phosphorylation of adducin. The upper band, migrating at approximately 125 kDa, is alpha adducin, and the 2 lower bands are alternatively spliced isoforms of gamma adducin (80 and 90 kDa) (for review see Suriyapperuma et al15). Beta adducin is not expressed in platelets.14 The antibody used in panel C recognizes alpha and gamma adducins independent of their state of phosphorylation. This antibody was used as a control to demonstrate that adducin was present (but relatively unphosphorylated) in the resting platelets, lane 0. In addition, the general adducin antibody demonstrated that the amount of adducin decreased with time of exposure to thrombin and started as quickly as 10 seconds. The intensity of the thrombin-induced PKC-phosphorylated bands appeared constant from 10 seconds to 5 minutes (panel B), yet the decreased amount of total adducin from 10 seconds to 5 minutes (panel C) would suggest that there is an increased proportion of phosphorylated adducin with time of exposure to thrombin. From this experiment it is not possible to determine whether the adducin being proteolyzed is phosphorylated or not; the PKC-phosphorylated adducin could be preferentially proteolyzed while PKC phosphorylation of unphosphorylated adducin would continue, giving a net result that the level of phosphorylated adducin would appear unchanged. It should be noted that if platelets were prepared without prostacyclin in the column buffers, then the adducin was already PKC phosphorylated before addition of thrombin, and a lower yield of platelets was obtained (data not shown).

Activation of PKC is a well-studied component of thrombin-induced platelet activation.26-30 To verify that the phosphorylation of adducin detected with the antiphosphoadducin antibody was due to the action of PKC, platelets were stimulated with PMA that directly activates PKC. Figure 2 shows a comparison of thrombin activation (1 U/mL) versus activation with PMA (100 nmol/L). In panel B, the phosphospecific antibody demonstrated very low levels of adducin phosphorylation in resting platelets (lane 1) and increased adducin phosphorylation with exposure to thrombin (lanes 2 and 3) or PMA (lanes 4-6). In panel C, the general adducin antibody again demonstrated a large amount of adducin in resting platelets (lane 1) and decreased adducin in both thrombin and PMA-activated platelets (lanes 2-6). These results confirm that the thrombin-induced phosphorylation of adducin detected with this antibody was due to the action of PKC.

To characterize the calcium dependence of the adducin phosphorylation and proteolysis, platelets were activated with the calcium ionophore A23187. Figure 3 shows platelet activation by using increasing concentrations of calcium ionophore A23187 (0.1 to 4 μM for 2 minutes). Panel A shows that extensive proteolysis of ABP-280 and talin occurred at higher concentrations of A23187 (80 and 90 kDa) (for review see Suriyapperuma et al15). Beta adducin is not expressed in platelets.14 The antibody used in panel C recognizes alpha and gamma adducins independent of their state of phosphorylation. This antibody was used as a control to demonstrate that adducin was present (but relatively unphosphorylated) in the resting platelets, lane 0. In addition, the general adducin antibody demonstrated that the amount of adducin decreased with time of exposure to thrombin and started as quickly as 10 seconds. The intensity of the thrombin-induced PKC-phosphorylated bands appeared constant from 10 seconds to 5 minutes (panel B), yet the decreased amount of total adducin from 10 seconds to 5 minutes (panel C) would suggest that there is an increased proportion of phosphorylated adducin with time of exposure to thrombin. From this experiment it is not possible to determine whether the adducin being proteolyzed is phosphorylated or not; the PKC-phosphorylated adducin could be preferentially proteolyzed while PKC phosphorylation of unphosphorylated adducin would continue, giving a net result that the level of phosphorylated adducin would appear unchanged. It should be noted that if platelets were prepared without prostacyclin in the column buffers, then the adducin was already PKC phosphorylated before addition of thrombin, and a lower yield of platelets was obtained (data not shown).

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In panel B, phosphorylation of adducin was seen at lower concentrations of A23187 (0.1 and 1 μM), but at higher concentrations (2 and 4 μM) less phosphorylation was seen because the adducin was already proteolyzed (panel C). At 0.1 μM A23187, adducin was phosphorylated by PKC but not yet proteolyzed, suggesting that PKC phosphorylation precedes proteolysis of adducin in platelets. At lower concentrations of A23187 (1-50 nmol/L), PKC phosphorylation of adducin was not detected (data not shown). EGTA blocked both the phosphorylation and the proteolysis of adducin (data not shown). These results suggest that the phosphorylation of adducin is calcium dependent, which confirms the results above, suggesting that PKC phosphorylated adducin during platelet activation.

Platelets contain a calcium-dependent protease, calpain, that is known to proteolyze the cytoskeletal proteins ABP-280, talin, spectrin, and cortactin10,27,31 as well as the integrin β332,33 during platelet activation. To test whether calpain was responsible for the proteolysis of adducin during platelet activation, platelets were activated with thrombin in the presence or absence of calpeptin, a membrane-permeable calpain inhibitor.24 Panel A of Figure 4 shows thrombin-induced proteolysis of ABP-280 and talin in lanes 3 to 5. This proteolysis was blocked by preincubation of platelets with 100 μg/mL calpeptin before the addition of thrombin, shown in lanes 6 to 8. Panel B (lanes 1 and 2) shows lack of PKC phosphorylation of adducin in resting platelets and platelets incubated with calpeptin alone (200 μg/mL). Activation of platelets with thrombin caused PKC phosphorylation of adducin (lanes 3-8). The intensity of the PKC phosphorylated bands was much stronger in the samples pretreated with calpeptin (lanes 6-8) because proteolysis of adducin was blocked. Panel C demonstrates this inhibition of proteolysis using the general antiadducin antibody.
Lanes 3 to 5 show a decreased amount of adducin in thrombin-activated platelets compared with the controls (lanes 1 and 2). The amount of adducin in lanes 6 to 8 was the same as the control, demonstrating that calpeptin blocked proteolysis of adducin.

The observation of rapid PKC phosphorylation and calpain proteolysis of adducin during platelet activation suggested that PKC phosphorylation might promote the proteolysis of adducin. To test this possibility, we preincubated platelets with a kinase inhibitor to determine whether blocking phosphorylation of adducin would also block proteolysis of adducin. Figure 5 shows the results of preincubating platelets with staurosporine (0.1 and 0.3 μM) before activation with thrombin. Panel B shows the typical PKC phosphorylation of adducin with addition of thrombin (lanes 2 and 3) but no PKC phosphorylation of adducin in platelets preincubated with staurosporine before activation with thrombin (lanes 5-8). Lane 1 shows resting platelets, and lane 4 shows platelets preincubated with staurosporine and not activated with thrombin. Panel C, using the general adducin antibody, shows the typical proteolysis of adducin in thrombin-activated platelets (lanes 2 and 3) but no proteolysis in the platelets pretreated with staurosporine before thrombin activation (lanes 5-8). These results suggest that proteolysis of adducin could be dependent on PKC phosphorylation of adducin. It is also possible that staurosporine inhibited proteolysis of adducin by indirectly inhibiting the activation of calpain.

To compare adducin with other actin-binding proteins in platelets and to better understand its possible role in platelet activation, we quantitated the amount of adducin in platelets. Recombinant alpha adducin was used to generate a standard curve for analysis of the concentration of adducin in platelets by Western blot. Figure 6A shows a Western blot with whole platelet extracts from 2 individuals (lanes marked X) and 5 dilutions of recombinant alpha adducin (lanes 1-5). Panel B shows the standard curve derived from this Western blot and the positions of the 2 platelet samples (triangles) on the curve. The amount of adducin per platelet based on these 2 samples was determined as 3.5 × 10⁻⁶ ng and 4.3 × 10⁻⁶ ng (platelet samples contained 7,939,850 and 8,320,000 platelets). Given a mean platelet volume of 8 fL, a mean adducin value of 3.9 × 10⁻⁶ ng/platelet, and a molecular weight of 81 kDa (based on the amino acid sequence rather than mobility), the concentration of alpha adducin in a platelet was calculated as 6 μM. This concentration corresponds to approximately 28,800 molecules of alpha adducin per platelet. It is not known whether alpha and gamma adducins exist as a complex in platelets, although evidence from other cells suggests that they are tightly associated. If the complex consists of 2 alpha subunits and 2 gamma subunits, then the concentration of the adducin tetramer would be 3 μM, and there would be approximately 14,424 molecules per platelet.

To test our hypothesis that PKC phosphorylation of adducin promotes proteolysis by calpain, we examined calpain proteolysis of recombinant alpha adducin with and without phosphorylation by PKC. Figure 7 shows the results of a typical experiment in panels A (anti-adducin Western blot) and B (anti-phosphoadducin Western blot). PKC-phosphorylated adducin decreased to a level of 12% after 60 seconds of incubation with calpain I, whereas unphosphorylated adducin decreased to a level of 58% after 60 seconds of incubation with calpain I (average of 3 experiments quantitated in panel C). On very long exposures, there was a band corresponding...
to an intermediate product of proteolysis at about 55 kDa, but this band was also proteolyzed over time, confirming our results with platelets that stable intermediate products of proteolysis are not seen after platelet activation. On Coomassie blue–stained gels, the disappearance of the 125 kDa alpha adducin band confirmed that adducin was proteolyzed by calpain and not just undergoing a change in conformation, making it no longer recognizable by antibodies. Quantitation of calpain proteolysis shown in panel C demonstrated that the rate of proteolysis of PKC-phosphorylated adducin was faster than the rate of proteolysis of unphosphorylated adducin. The difference between the slopes of the 2 curves was statistically significant at \( P < .001 \).

PKC phosphorylation of adducin has been shown to be associated with a shift of adducin from the membrane skeleton to the cytosol in MDCK cells treated with PMA.\(^{35}\) To determine whether the PKC phosphorylation of adducin in platelets was associated with a shift in subcellular location, we fractionated platelets into high-speed (126 000 \( g \times 3 \) hours) supernatant and pellet fractions at time points after addition of dilute thrombin (0.1 U/mL), Western blots were performed on supernatant and pellet fractions, and the optical density of adducin and PKC-phosphoadducin bands were quantitated with NIH Image. Figure 8A shows increased adducin in the supernatant and decreased adducin in the pellet versus time of exposure to thrombin. Although the amount of adducin in the pellet continued to decrease with time, the amount in the supernatant remained constant, so that total adducin decreased over time, consistent with proteolysis. Figure 8B shows the amount of PKC-phosphorylated adducin in supernatant versus pellet fractions (thrombin 0.1 U/mL, \( n = 2 \)). There was a rapid increase of PKC-phosphorylated adducin in the supernatant fraction (10 seconds), followed by a decrease consistent with proteolysis.

To examine the effect of PKC phosphorylation of adducin without proteolysis, platelets were preincubated with calpeptin (200 \( \mu \)g/mL) before thrombin activation (1 U/mL, \( n = 2 \)). Figure 8C shows that PKC-phosphorylated adducin in the supernatant continued to increase over time in contrast to the decrease seen without calpeptin (Figure 8B). PKC-phosphorylated adducin in the pellet also increased up to the 30-second time point and then decreased, consistent with movement of phosphoadducin to the supernatant. Together, these data suggest an association between PKC phosphorylation of adducin and movement to the cytosol during thrombin activation of platelets.

To determine how adducin phosphorylation by PKC and proteolysis by calpain may be involved in platelet function, platelet aggregation was assayed by using a 4-channel aggregometer. Four samples were tested simultaneously: untreated control, staurosporine-treated, calpeptin-treated, or DMSO-treated control. Thrombin (1 U/mL) was used to stimulate aggregation in the presence of 2.5 mM CaCl\(_2\). Results of a typical experiment are shown in Figure 9. Untreated platelets (A) and DMSO-treated control platelets (B) showed no difference and aggregated within 2 minutes to a maximum of 77\% \( \pm \) 0.8\% and 76.8\% \( \pm \) 1.7\% (mean of 3 experiments). Aggregation of staurosporine-treated platelets (C) was inhibited to a maximum of 64.1\% \( \pm \) 0.5\%; the difference from control was statistically significant (\( P = .003 \)). Aggregation of calpeptin-treated platelets (D) was completely inhibited with a maximum of 3.8\% \( \pm \) 1.9\%; the difference from control was statistically significant (\( P = .003 \)). These results suggest that PKC phosphorylation is not absolutely required for platelet aggregation but, if inhibited, leads to a delay in platelet aggregation. These results also show that calpain function is essential for platelet aggregation under our conditions of platelet preparation and

![Figure 5. Staurosporine blocked thrombin-induced PKC phosphorylation and proteolysis of adducin. Preincubation of platelets with the kinase inhibitor staurosporine (10 minutes) completely blocked thrombin-induced PKC phosphorylation of adducin (B) and proteolysis (C). Thrombin concentration, 1 U/mL; CaCl\(_2\), 2.5 mM.](image)

![Figure 6. Quantitation of adducin in platelets. (A) Western blotting was performed with known amounts of recombinant alpha adducin (lanes 1-5) for comparison to platelet samples from 2 individuals (lanes marked X). (B) A standard curve was generated by using NIH Image software to quantitate bands from (A) and convert to relative optical density. Circles correspond to known amounts of adducin; triangles correspond to platelet samples.](image)
activation. Inhibition of adducin phosphorylation by PKC and proteolysis by calpain could have a role in the observed inhibition of platelet aggregation, although PKC and calpain have a large number of substrates other than adducin.

Aggregation of platelets is a complex process comprising several steps: secretion of granules, extension of filopodia and lamellipodia by F-actin polymerization, and modulation of the integrin αIIbβ3 to permit binding of fibrinogen and generation of cell-cell linkages. We have demonstrated that adducin is present in sufficient quantity to cap all barbed ends of actin filaments in resting platelets and that adducin moves from skeleton to cytosol during platelet activation. It is possible that phosphorylation by PKC causes adducin to be released from actin-capping sites, thereby allowing polymerization of short actin filaments into long actin filaments. Thrombin-stimulated platelets were examined by confocal microscopy under conditions shown above that inhibit adducin phosphorylation by PKC and/or proteolysis by calpain. Fixed platelets were labeled with AlexaFluor 488 phalloidin to stain F-actin, and results are shown in Figure 10. Panel A shows control platelets that maintained the characteristic disc morphology of unactivated platelets. Panel B shows platelets after thrombin stimulation with extension of filopodia and lamellipodia and F-actin polymerization. Platelets in panels C and D were incubated with staurosporine (C) or calpeptin (D) before addition of thrombin and showed similar extension of filopodia and lamellipodia with F-actin polymerization. The results shown are typical of 3 independent experiments on different days using different platelet donors. These results demonstrated that PKC phosphorylation and calpain proteolysis of adducin were not necessary for the changes in morphology observed by confocal microscopy with phalloidin staining.

Figure 8. Movement of PKC-phosphorylated adducin to cytosol with thrombin activation. Column-purified platelets were activated with dilute thrombin (0.1 U/mL) for increasing times and fractionated by high-speed centrifugation (126,000g for 3 hours) into supernatant and pellet fractions. Samples were run on SDS-polyacrylamide gel electrophoresis and Western blotted; relative optical density of bands was quantitated by using NIH Image software. (A) Typical experiment showing increasing adducin in supernatant versus decreasing adducin in pellet. (B) Distribution of PKC-phosphorylated adducin between supernatant and pellet fractions over time (n = 2). (C) Platelets were preincubated with calpeptin (200 μg/mL for 30 minutes), stimulated with thrombin (1 U/mL), then fractionated as above. PKC-phosphorylated adducin continued to increase in the supernatant while decreasing in the pellet fraction.
Discussion

We have shown that adducin was rapidly phosphorylated by PKC in platelets activated with thrombin, PMA, or calcium ionophore A23187, as defined by the use of a PKC site-specific phosphopeptide antibody. There are several possibilities for how PKC phosphorylation may regulate the function of adducin. Studies on the interaction of gamma adducin with PKC suggested that unphosphorylated gamma adducin can bind PKC, and, when PKC phosphorylates gamma adducin, it is released from its association. Further studies are needed to determine whether PKC and unphosphorylated adducin exhibit this interaction in platelets. Phosphorylation of recombinant alpha and beta adducin by PKC has been shown to decrease their interactions with spectrin and actin in vitro, and treatment of MDCK cells with PMA caused a redistribution of adducin from membrane to cytosol as seen by light microscopy. We have shown that adducin moves from high-speed pellet (membrane and cytoskeleton fraction) to supernatant (cytosolic fraction) during platelet activation and levels of PKC-phosphorylated adducin are much higher in the cytosol versus the pellet. These observations suggest that activation-induced PKC phosphorylation of adducin releases it from binding sites in the membrane skeleton or cytoskeleton.

Erythrocyte adducin (alpha/beta) has been shown to cap actin filaments at the barbed end, and PKC phosphorylation reduces the F-actin capping activity of adducin. Platelets contain 0.5 μM F-actin barbed ends, based on measurements of average filament length and amount of polymerized actin per platelet. We have demonstrated that sufficient adducin is present (3-6 μM) to cap all actin filaments in the resting platelet. The concentration of adducin in platelets is similar to the 2 to 5 μM concentration of capping protein. However, the dissociation constant for binding to barbed ends is only 100 nmol/L for adducin versus 1 to 2 nmol/L for capping protein. In addition, it has not yet been demonstrated that platelet adducin (alpha/gamma) has actin-capping activity.

Adducin is also a substrate for rho kinase, and phosphorylation by rho kinase, in contrast to phosphorylation by PKC, increases adducin’s interaction with F-actin. Rho kinase is also activated during platelet activation, so it is not yet known how the combination of phosphorylation by PKC and rho kinase will affect adducin function in platelets.

One important observation from our studies is that adducin was proteolyzed during platelet activation. Inhibition of the proteolysis by calpeptin suggests that calpain is likely to be the protease involved. Calpain is an important component of platelet activation, causing highly specific calcium-induced proteolysis of several cytoskeleton proteins, including ABP-280, talin, spectrin, and cortactin. In addition, calpain proteolyses the β3 subunit of the αIIbβ3 integrin, the fibrinogen receptor, during platelet activation. This is the first report of adducin as a substrate for calpain proteolysis and may represent an important pathway for regulation of adducin function in platelets as well as other cells. Results of in vitro experiments with recombinant alpha adducin demonstrated that PKC-phosphorylated adducin was proteolyzed by calpain more quickly than unphosphorylated adducin. Demonstrating the influence of PKC phosphorylation on adducin proteolysis in the absence of binding proteins such as spectrin or actin suggests a mechanism whereby PKC phosphorylation of adducin directly causes a conformational change of adducin, permitting rapid proteolysis by calpain. A similar result has recently been shown in which the PKC-phosphorylated form of adducin was...
preferentially proteolyzed by caspase-3 in cisplatin-treated renal epithelial cells.40 Phosphorylation of adducin by PKC may be a common mechanism for regulating adducin proteolysis by several proteases.

Others have shown that inhibition of calpain blocks platelet granule secretion, aggregation, and spreading on glass.41 Our results inhibiting proteolysis of adducin and platelet aggregation with calpeptin are consistent with adducin having a role in platelet aggregation. Targeted deletion of the μ-calpain gene in mice demonstrated that μ-calpain is crucial to normal platelet aggregation and clot retraction.42 The proteolysis of ABP-280, talin, and β3 integrin was not inhibited in platelets from the μ-calpain null mice, suggesting that proteolysis of the cytoskeleton is not required for platelet aggregation or clot retraction. However, platelets from the μ-calpain null mice still contain m-calpain, so it is not possible to conclude definitively about the role of the cytoskeleton in platelet aggregation from the μ-calpain null mice.

We have shown that adducin is crucial to the normal architecture of red blood cells.43 Targeted disruption of the beta adducin gene in mice caused a phenotype similar to the human disease hereditary spherocytosis. Platelets were unaffected in these mice because platelets do not express beta adducin. Future studies with targeted disruption of alpha and gamma adducin genes are likely to provide answers about the specific role of adducin in platelets.

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References

Adducin in platelets: activation-induced phosphorylation by PKC and proteolysis by calpain

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