Human Platelet Calmodulin-Binding Proteins: Identification and Ca^{2+}-Dependent Proteolysis upon Platelet Activation†

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ABSTRACT: Calmodulin-binding proteins have been identified in human platelets by using Western blotting techniques and ^{125}I-calmodulin. Ten distinct proteins of 245, 225, 175, 150, 90, 82 (2), 60, and 41 (2) kilodaltons (kDa) bound ^{125}I-calmodulin in a Ca^{2+}-dependent manner; the binding was blocked by ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), trifluoperazine, and nonradiolabeled calmodulin. Proteins of 225 and 90 kDa were labeled by antisera against myosin light chain kinase; 60- and 82-kDa proteins were labeled by antisera against the calmodulin-dependent phosphatase and caldesmon, respectively. The remaining calmodulin-binding proteins have not been identified. Calmodulin-binding proteins were degraded upon addition of Ca^{2+} to a platelet homogenate; the degradation could be blocked by either EGTA, leupeptin, or *N*-ethylmaleimide which suggests that the degradation was due to a Ca^{2+}-dependent protease. Activation of intact platelets by thrombin, adenosine 5′-diphosphate, and collagen under conditions which promote platelet aggregation (i.e., stirring with extracellular Ca^{2+}) also resulted in limited proteolysis of calmodulin-binding proteins including those labeled with antisera against myosin light chain kinase and the calmodulin-dependent phosphatase. Activation by the Ca^{2+} ionophores A23187 and ionomycin also promoted degradation of the calmodulin-binding proteins in the presence of extracellular Ca^{2+}; however, degradation in response to the ionophores did not require stirring of the platelet suspension to promote aggregation. Many Ca^{2+}/calmodulin-regulated enzymes are irreversibly activated in vitro by limited proteolysis. Our data indicate that limited proteolysis of Ca^{2+}/calmodulin-regulated enzymes also occurs in the intact platelet and suggest that the proteolysis is triggered by an influx of extracellular Ca^{2+} associated with platelet aggregation.

Calcium ion is an important regulator of platelet function. The elevation of intraplatelet Ca^{2+} concentration to micromolar levels triggers the platelet reaction sequence which includes shape change, aggregation, and secretion (Ardile, 1982; Feinstein et al., 1981). The Ca^{2+}-binding protein calmodulin is present in high concentrations in the platelet and is one of the primary intraplatelet receptors for the Ca^{2+} signal (Feinstein, 1982). The Ca^{2+}/calmodulin complex interacts with various calmodulin-binding proteins resulting in the propagation of the Ca^{2+} signal to enzymes and structural proteins which are involved in the platelet reaction sequence. The Ca^{2+}/calmodulin complex activates a number of platelet enzymes including myosin light chain kinase (Hathaway & Adelstein, 1979), phosphorylase kinase (Gergely et al., 1980), and a phosphoprotein phosphatase (Tallant & Wallace, 1985). In addition, under in vitro conditions, calmodulin increases the rate of Mg^{2+}-induced polymerization of platelet actin and alters the structural characteristics of the resulting actin filaments (Piazza & Wallace, 1985) which suggests that calmodulin may also be involved in the regulation of platelet microfilament structure.

Many calmodulin-regulated enzymes are irreversibly activated in a calmodulin-independent manner by limited proteolysis (Lin & Cheung, 1980; Kincaid et al., 1985; Depaoli-Roach et al., 1979; Higgli et al., 1981; Walsh et al., 1982; Manalan & Klee, 1983; Tallant & Cheung, 1984; Meijer & Guerrier, 1982; Keller et al., 1980). Calmodulin-dependent enzymes are thought to contain homologous inhibitory domains; the interaction of calmodulin with the enzyme removes inhibition in a Ca^{2+}-dependent and reversible manner while limited proteolysis degrades the inhibitory domain, resulting in irreversible activation (Lin & Cheung, 1980). Kosaki et al. (1983) have shown that human platelet calmodulin-binding proteins are readily proteolyzed under in vitro conditions by a platelet-derived Ca^{2+}-dependent protease. Fox et al. (1985) have proposed that Ca^{2+}-dependent proteolysis of actin-binding protein and protein P_{235} is important for platelet function. We have found that specific calmodulin-binding proteins also undergo limited proteolysis upon platelet activation, which suggests that Ca^{2+}-dependent proteolysis may be a physiological mechanism for irreversibly activating certain enzymes which are part of the calmodulin-mediated, Ca^{2+}-dependent regulatory pathway.

Materials and Methods

Reagents. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris)[1] buffer, calcium chloride, sodium citrate, potassium chloride, nonionic detergent NP-40, adenosine 5′-diphosphate (Tris salt), collagen (type I, bovine achilles tendon), diisopropyl fluorophosphate, leupeptin, phenylmethanesulfonyl fluoride, aprotinin, pepstatin, *N*-ethylmaleimide, benzamidine, and ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid were purchased from Sigma Chemical Co. $Na^{125}I$ (13–17 Ci/μg) was from Amersham; electrophoresis reagents were obtained from Bio-Rad Laboratories; nitrocellulose sheets

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; ADP, adenosine 5′-diphosphate; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid.

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(0.45-μm pore) were from Sartorius; the calcium ionophores A23187 and ionomycin and human fibrinogen were purchsed from Calbiochem. Human thrombin was kindly provided by Dr. J. W. Fenton, II, New York Department of Health.

Calmodulin was prepared from bovine brain by affinity chromatography on columns of either fluphenazine–Sepharose (Wallace et al., 1980) or phenyl-Sepharose (Gopalakrishna & Anderson, 1982) followed by gel filtration chromatography on Sephadex G-100. Calmodulin was iodinated by the lactoperoxidase/glucose oxidase procedure (LaPorte & Storm, 1978); the ^{125}I-calmodulin was separated from the unreacted Na^{125}I and the ^{125}I-lactoperoxidase and ^{125}I-glucose oxidase, which are also produced during the iodination reaction, by affinity chromatography on a small column (0.5 × 0.5 cm) of phenyl-Sepharose (Gopalakrishna & Anderson, 1982). A typical preparation of ^{125}I-calmodulin had a specific activity of 1.2 μCi/μg.

Platelets. Blood was collected by venipuncture from healthy volunteers who denied taking aspirin during the previous 2-week period. The platelets were isolated by centrifugation and washed 2 times in Tris–citrate buffer (75 mM Tris, 12 mM citrate, and 100 mM KCl, pH 6.4) by standard procedures (Kaplan et al., 1979). The washed platelets were pelleted and resuspended in either Tyrode's buffer (138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose, 1.8 mM calcium chloride, and 0.49 mM magnesium chloride, pH 7.4) or another buffer as appropriate for the particular experiment and indicated in the figure legends. Platelets were maintained at 37 °C throughout the isolation and washing procedure. The soluble and particulate fractions were prepared by subjecting the platelets, dispersed in 50 mM Tris-HCl/3 mM EGTA, pH 7.0, to three cycles of freezing in dry ice/methanol followed by rapid thawing. The particulate fraction was collected by centrifugation (1 h, 100000*g*, 4 °C); the supernatant was used as the platelet soluble fraction. The particulate fraction was washed 1 time in 50 mM Tris-HCl/3 mM EGTA, pH 7.0, and dispersed in the same buffer.

Identification of Calmodulin-Binding Proteins. Calmodulin-binding proteins were identified by Western blotting of polyacrylamide gels onto nitrocellulose and overlays using ^{125}I-calmodulin. SDS gel electrophoresis of platelet proteins on linear gradients of 7.5–15% polyacrylamide was conducted by using the Laemmli (1970) buffer system followed by electrophoretic transfer to nitrocellulose for 5 h at 60 V according to the procedure described by Towbin (1979). ^{125}I-Calmodulin overlay of the nitrocellulose was according to a modification of our previously described procedure (Tallant & Wallace, 1985). After transfer to nitrocellulose, the remaining protein binding sites were quenched by incubating the nitrocellulose for 1 h in quench buffer [20 mM Tris-HCl, 0.15 M NaCl, 3% nonfat dry milk (Carnation), and 0.02% sodium azide, pH 7.4]. Calcium chloride (1.0 mM), nonionic detergent NP-40 (0.05%), and ^{125}I-calmodulin (10^6 cpm/mL) were then added to the solution, and incubation was continued with constant mixing for an additional 2 h. After being washed in three changes of 20 mM Tris-HCl, 0.15 M NaCl, 0.05% NP-40, 1.0 mM $CaCl_2$, and 0.02% NaN_3 (pH 7.4) over a 2-h period, the nitrocellulose was dried and autoradiographed on Kodak XAR-5 film.

Immunological Procedures. Polyclonal rabbit antibodies specific for the 60000-dalton subunit (subunit A) of the bovine brain calmodulin-dependent phosphatase (calcineurin) were prepared in rabbits by using phosphatase subunit protein excised from an SDS–polyacrylamide gel as an antigen; the antiserum was purified by affinity chromatography on a column of phosphatase–Sepharose (Tallant & Wallace, 1985). Affinity-purified IgG from rabbit antiserum against chicken gizzard myosin light chain kinase (Guerriero et al., 1981) was provided by Dr. Vince Guerriero, Jr., Department of Cell Biology, Baylor College of Medicine; affinity-purified IgG from polyclonal antiserum against turkey gizzard myosin light chain kinase (de Lanerolle et al., 1981) was provided by Dr. Primal de Lanerolle, Department of Physiology and Biophysics, University of Illinois at Chicago. The purified IgG fraction of polyclonal rabbit antiserum against chicken gizzard caldesmon (Ngai & Walsh, 1985) was provided by Dr. Michael P. Walsh, Department of Medical Biochemistry, The University of Calgary. These antisera were used to correlate the electrophoretic mobility of specific calmodulin-binding proteins with the platelet calmodulin-dependent phosphoprotein phosphatase, myosin light chain kinase, and caldesmon using immunoblotting procedures previously described (Tallant & Wallace, 1985). ^{125}I-Labeled protein A, iodinated by the chloramine T procedure (Hunter & Greenwood, 1962), was used to visualize the immunoreactive protein bands by autoradiography.

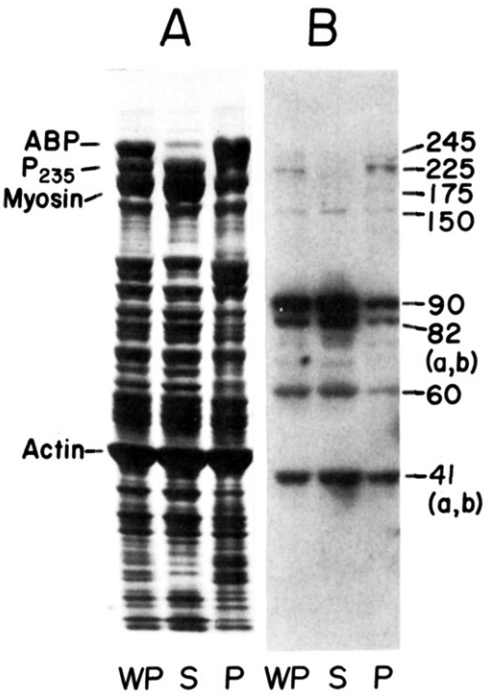

FIGURE 1: Identification of human platelet calmodulin-binding proteins. Whole platelet proteins (lanes WP) as well as the soluble (lanes S) and particulate (lanes P) platelet protein fractions were resolved by SDS gel electrophoresis and either stained for total protein with Coomassie brilliant blue (panel A) or Western blotted with ^{125}I-calmodulin in the presence of 1 mM $CaCl_2$ (panel B) and autoradiographed to identify calmodulin-binding proteins. An equal amount of protein (150 μg) was applied to each lane. The numbered arrows refer to the molecular weight (×10^{-3}) of each calmodulin-binding protein. Several of the major protein bands in the platelet are identified including actin binding protein (ABP), a 235-kDa protein (P_{235}), myosin, and actin.

Protein Determination. Protein was determined according to Lowry et al. (1951) after the proteins were precipitated with 10 volumes of 10% perchloric acid/1% phosphotungstic acid. Bovine serum albumin (Bio-Rad Laboratories) was used as a standard.

RESULTS

Ten calmodulin-binding proteins were detected when a nitrocellulose blot was incubated with ^{125}I-calmodulin in the

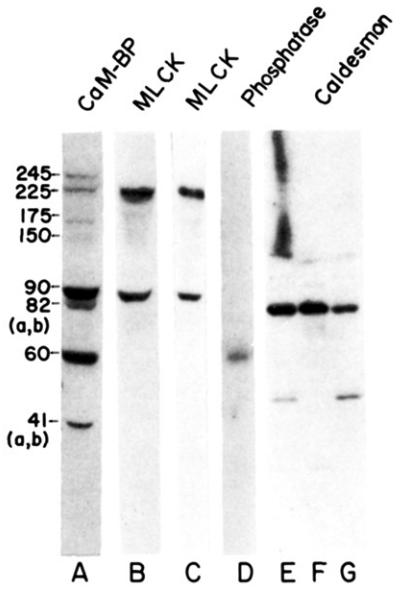

FIGURE 2: Correlation of electrophoretic mobilities of specific calmodulin-binding proteins with platelet myosin light chain kinase, calmodulin-dependent phosphatase, and caldesmon. Either whole platelet proteins (lanes A–E) or heat-stable (lane F) and heat-denatured (lane G) platelet protein fractions were resolved by SDS–polyacrylamide gel electrophoresis, Western blotted with either ^{125}I-calmodulin to identify calmodulin-binding proteins (CaM-BP) (lane A), affinity-purified IgG against chicken gizzard myosin light chain kinase (MLCK) (lane B), turkey gizzard myosin light chain kinase (lane C), subunit A of the phosphoprotein phosphatase (lane D), or the IgG fraction of chicken gizzard caldesmon antiserum (lanes E–G), and autoradiographed. Heat-treated fractions were prepared by incubating a platelet homogenate dispersed in 20 mM Tris-HCl/3 mM EGTA (pH 7.5) for 5 min in a boiling water bath and centrifugation (10 min, 10000g) to collect the heat-denatured proteins; the soluble fraction was considered heat stable. Lanes A–D, 300 μg of protein; lane E, 150 μg of protein; lane F, 30 μg of protein; lane G, 140 μg of protein. Lanes F and G contain 3 times and an equal amount, respectively, of the heat-stable and heat-denatured proteins derived from the amount of protein in lane E. The molecular weight ($\times 10^{-3}$) is indicated for each calmodulin-binding protein.

presence of Ca^{2+} (Figure 1, panel B). None of these proteins bound ^{125}I-calmodulin when EGTA and EDTA or an excess of nonradiolabeled calmodulin was included in the buffer. The calmodulin antagonist trifluoperazine also blocked the binding of ^{125}I-calmodulin (data not shown). ^{125}I-Calmodulin bound predominantly to proteins of 90, 82, 60, and 41 kDa; lower amounts of ^{125}I-calmodulin were bound to proteins of 245, 225, 175, and 150 kDa. The 82- and 41-kDa regions consist of two distinct calmodulin-binding proteins (see below); they are distinguished in Figure 1 as 82a,b and 41a,b. Autoradiographs of much longer exposures of the ^{125}I-calmodulin overlays confirmed that the 245-, 225-, and 175-kDa calmodulin-binding proteins were present only in the particulate fraction (data not shown); the other calmodulin-binding proteins were found in both the soluble and particulate fractions but were predominantly soluble.

Two different polyclonal antibody preparations for myosin light chain kinase bound to platelet proteins corresponding in electrophoretic mobility to the 225- and 90-kDa calmodulin-binding proteins (Figure 2). The possibility that the 225-kDa protein is a dimer of the 90-kDa protein held together by disulfide bonds was considered; however, reduction of a platelet homogenate with dithiothreitol followed by alkylation of sulfhydryl groups with *N*-ethylmaleimide according to procedures utilized for platelet proteins with disulfide bonds which are difficult to reduce (Phillips & Agin, 1977) had no effect on the pattern of immunoreactive proteins or calmodulin-binding proteins (data not shown). Both the 225-kDa protein which bound calmodulin (Figure 1) and the 225-kDa protein which was labeled by the antibody (data not shown) were found only in the particulate fraction of the platelet. The 90-kDa calmodulin-binding protein was found predominantly in the soluble fraction with a similar amount also present in the particulate fraction (Figure 1); a similar distribution was found for the 90-kDa protein labeled with the antibody (data not shown). A polyclonal antibody specific for the 60-kDa subunit of the calmodulin-dependent phosphatase[2] bound to a platelet protein with a mobility identical with that for the 60-kDa calmodulin-binding protein (Figure 2, lane D).

Antibodies against chicken gizzard caldesmon, a calmodulin- and actin-binding protein (Sobue et al., 1981), react with a platelet protein corresponding to the two 82-kDa calmodulin-binding proteins (Figure 2, lane E). The antibodies also labeled several other platelet proteins, but the reaction was much less than that obtained with the 82-kDa protein. This may be due to nonspecific interaction of the IgG with platelet proteins or be due to contaminants in the caldesmon used to prepare the antibody. Caldesmon is stable to heat treatment, remaining in solution following incubation for several minutes in boiling water (Bretscher, 1984). In an attempt to provide further evidence that the 82-kDa calmodulin-binding protein is caldesmon, we prepared a heat-stable and heat-denatured platelet fraction and determined if both an 82-kDa calmodulin-binding protein and also the immunoreactive protein remained in solution. A substantial portion of the 82-kDa immunoreactive protein was present in the heat-stable fraction (Figure 2, lane F); however, calmodulin-binding proteins were found only in the heat-denatured fraction (data not shown). These results provide evidence for the identity of an 82-kDa immunoreactive protein as caldesmon but raise questions concerning the inability of the heat-stable immunoreactive protein to bind ^{125}I-calmodulin.

Addition of Ca^{2+} to a platelet homogenate caused a time-dependent change in the composition of the calmodulin-binding proteins (Figure 3). The 245-, 225-, 175-, 90-, 82-, and 60-kDa proteins either disappeared or were reduced during the 1-h incubation with Ca^{2+}. Faint calmodulin-binding proteins at 150, 115, 74, 68, 55, and 37 kDa became more pronounced during the incubation period; the 74-, 68-, and 55-kDa proteins were prominent sites for ^{125}I-calmodulin binding after 1 h of incubation. When excess EGTA was present, the composition of calmodulin-binding proteins did not change. Leupeptin was also effective in blocking the change in composition of calmodulin-binding proteins, and *N*-ethylmaleimide was partially effective (Figure 4). None of the other protease inhibitors tested including DFP, PMSF, aprotinin, pepstatin, and benzamidine was effective.

The data shown in Figures 3 and 4 also provide evidence that the 82- and 41-kDa regions are composed of two distinct calmodulin-binding proteins. In Figure 3, the 41-kDa region is resolved into two bands with very similar mobilities. The insert to Figure 3 shows that these two bands were best resolved in an autoradiograph in which the exposure time was less; however, the exposure time which was optimal for bands 41a and 41b was not sufficient to show the high molecular weight calmodulin-binding proteins. In Figure 4, the lanes containing leupeptin and EGTA-treated platelets show the 82-kDa region as a broad faintly exposed area (82a) superimposed upon a tighter, more intensely exposed band (82b). In the lane containing the NEM-treated platelets, only the

[2] E. A. Tallant and R. W. Wallace, manuscript in preparation.

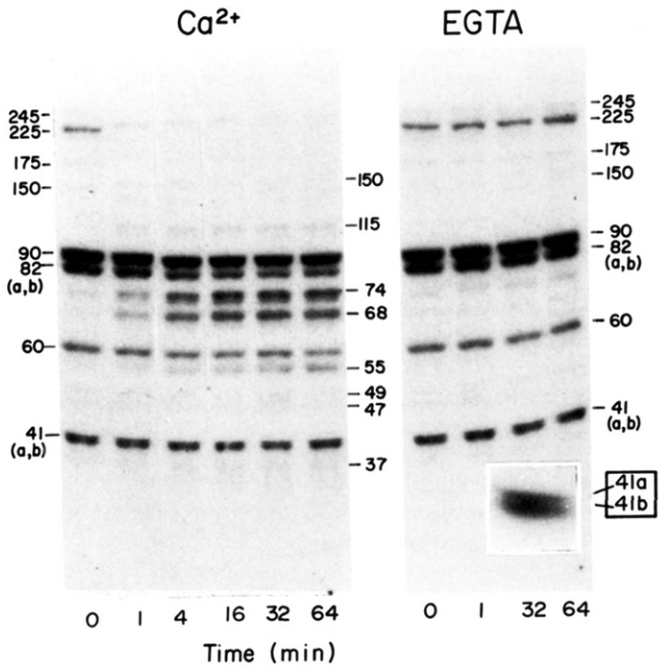

FIGURE 3: Ca^{2+}-dependent proteolysis of human platelet calmodulin-binding proteins. Human platelets (10^9/mL) in Ca^{2+}- and Mg^{2+}-free Tyrodes buffer containing 2 mM EGTA were subjected to three cycles of freezing in dry ice/methanol. The platelet homogenate was divided into two fractions, one of which was made 2.1 mM in $CaCl_2$ to give a concentration of 100 μM free Ca^{2+}. The platelets were incubated at 37 °C, and at the times indicated at the bottom of the figure, 100-μL aliquots were withdrawn and transferred to a tube in a boiling water bath containing EGTA and SDS (5 mM and 1% final concentrations) where they were incubated for an additional 2 min. The platelet calmodulin-binding proteins were identified after SDS gel electrophoresis by Western blotting and autoradiography as described under Materials and Methods. The numbered arrows refer to the molecular weight ($\times 10^{-3}$) of the calmodulin-binding proteins (left and right) or the calmodulin-binding proteolytic fragments (middle). The insert at the bottom right shows an enlargement of a shorter exposure of the 41-kDa calmodulin-binding region of the autoradiograph; the 41a and 41b bands are labeled.

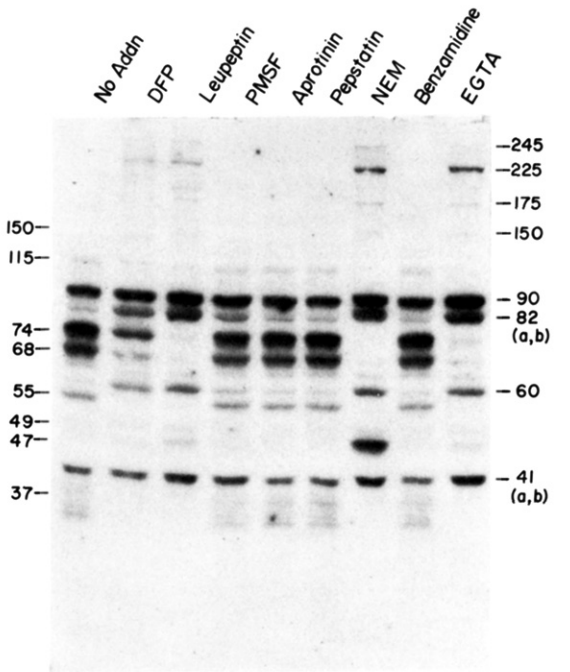

FIGURE 4: Effect of various protease inhibitors on the proteolysis of platelet calmodulin-binding proteins. Human platelets (10^9/mL) in Ca^{2+}- and Mg^{2+}-free Tyrodes buffer were aliquoted into tubes containing either no addition or the protease inhibitors DFP (1 mM), leupeptin (120 μM), PMSF (100 μM), aprotinin (1.0 thrombin inhibitory unit/mL), pepstatin (75 μM), NEM (1 mM), benzamidine hydrochloride (2 mM), or EGTA (2 mM). The platelets were made 0.1 mM in $CaCl_2$, subjected to three cycles of freezing in dry ice/methanol, and incubated at 37 °C for 10 min. The samples were then made 1% in SDS and incubated for an additional 2 min in boiling water. Aliquots (100 μL) of the platelet proteins were resolved by SDS gel electrophoresis and the calmodulin-binding proteins identified by Western blotting with ^{125}I-calmodulin. The numbered arrows refer to the kilodaltons of the calmodulin-binding proteins (right) and the calmodulin-binding proteolytic fragments (left).

82b-kDa band is missing, and an additional 47-kDa calmodulin-binding protein is present; NEM is apparently capable of protecting all of the calmodulin-binding proteins from proteolytic attack with the exception of the 82b protein which was degraded to produce the 47-kDa calmodulin-binding protein. Platelets with no addition or platelets treated with DFP, PMSF, aprotinin, pepstatin, or benzamidine retained small amounts of the 82b-kDa protein while the 82a-kDa protein was almost completely degraded. Due to variability in the resolving power of the polyacrylamide gels, it was not always apparent that the 82- and 41-kDa regions consist of two distinct calmodulin-binding proteins; however, the two calmodulin-binding proteins in these regions were resolved numerous times during the course of these experiments.

Proteolysis of platelet calmodulin-binding proteins also occurred upon activation of intact platelets with agents known to elevate intraplatelet Ca^{2+} concentration. Figure 5 shows that in the presence of extracellular Ca^{2+}, activation of platelets by thrombin, ADP plus fibrinogen, collagen, and the Ca^{2+} ionophores A23187 and ionomycin caused a decrease in the 90-, 82-, and 60-kDa calmodulin-binding proteins. Concomitant with the decrease in these proteins was an increase in the 74-, 68-, 55-, and 37-kDa calmodulin-binding proteins which are most likely proteolytic products of the calmodulin-binding proteins which decreased in quantity. In addition, the 245-, 225-, and 150-kDa calmodulin-binding proteins disappeared upon activation with the Ca^{2+} ionophores. The degree of proteolysis of the different proteins was variable depending upon the activating agent used. Activation with thrombin and ADP plus fibrinogen was associated with the least amount of proteolytic degradation; the Ca^{2+} ionophores were the most effective, while collagen was intermediate in promoting proteolysis of the binding proteins. Extracellular Ca^{2+} was essential for proteolysis; when EGTA was substituted for Ca^{2+}, no proteolysis of the calmodulin-binding proteins was observed. Stirring the platelets during activation to promote aggregation was also an important factor; when platelets were activated by thrombin, ADP plus fibrinogen, and collagen without stirring, little proteolysis of the calmodulin-binding proteins occurred even when Ca^{2+} was present in the buffer. However, the Ca^{2+} ionophores were equally effective as inducers of Ca^{2+}-dependent proteolysis with or without stirring during platelet activation (data not shown).

Degradation of the calmodulin-binding proteins appeared to be highly specific. A Coomassie brilliant blue stained gel corresponding to the gel in Figure 5 showed that, with the exception of the degradation of actin-binding proteins and P_{235} as previously described (Fox et al., 1985), there was no difference in the total protein pattern before and after platelet activation in the presence of extracellular Ca^{2+} (data not shown). Apparently, the 90-, 82-, and 60-kDa calmodulin-binding proteins are minor constituents of the total composition of platelet proteins since their degradation is observed only when they are labeled with ^{125}I-calmodulin.

Because our in vitro experiments indicated that lysis of platelets in the presence of extracellular Ca^{2+} resulted in

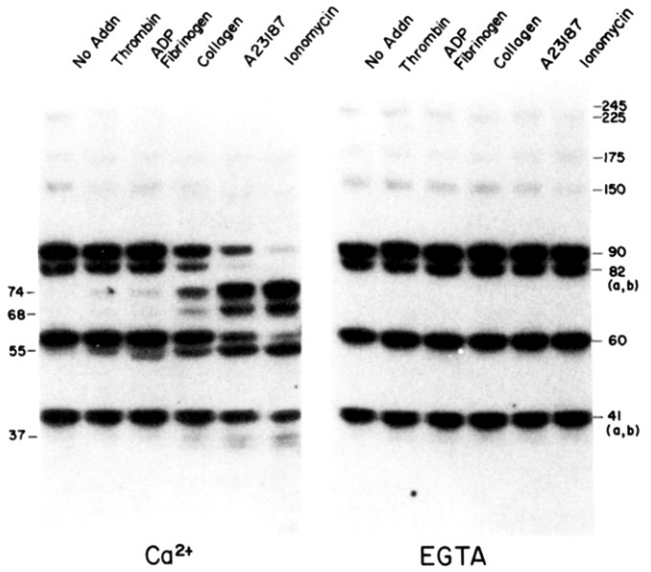

FIGURE 5: Proteolysis of calmodulin-binding proteins upon platelet activation. Human platelets (10^9/mL) dispersed in Ca^{2+}- and Mg^{2+}-free Tyrodes buffer received either Ca^{2+} (2 mM) (left) or EGTA (2 mM) (right) and were maintained at 37 °C with constant stirring. Each platelet sample then received either no addition, thrombin (1 unit/mL), ADP (50 μM) plus fibrinogen (0.2 mg/mL), collagen (200 μg/mL), A23187 (0.5 μM), or ionomycin (0.5 μM) as indicated in the figure and was allowed to incubate with stirring for an additional 10 min. The platelets were then made 1% in SDS, incubated in boiling water for 2 min, subjected to SDS gel electrophoresis, and Western blotted with ^{125}I-calmodulin as described under Materials and Methods. The molecular weight ($\times 10^{-3}$) of each calmodulin-binding protein (right) or calmodulin-binding proteolytic fragment (left) is indicated.

proteolysis of calmodulin-binding proteins, we were concerned that the proteolysis observed upon platelet activation could be due to lysis of the platelets during activation. To test this possibility, we measured the release of the cytosolic enzyme lactate dehydrogenase (Bergmeyer et al., 1965) upon platelet activation in parallel with the experiments shown in Figure 4. There was no cell lysis by the various activating agents at the concentrations used in our studies (data not shown).

Discussion

Ten calmodulin-binding proteins have been identified in the human platelet. We consider each of these proteins to be a specific Ca^{2+}/calmodulin-regulated enzyme or protein because each binds ^{125}I-calmodulin in a Ca^{2+}-dependent manner and this binding is blocked by the addition of either nonradioactive calmodulin or the calmodulin antagonist trifluoperazine. These calmodulin-binding characteristics are consistent with those which have been described for well-characterized Ca^{2+}/calmodulin-regulated enzymes and proteins (Cheung, 1979). Although we cannot rule out the possibility that some of these proteins may bind calmodulin in a nonspecific manner, this is unlikely on the basis of our recent attempts to identify calmodulin-binding proteins in *Escherichia coli*. We found that protein extracts from *E. coli*, an organism which does not contain calmodulin (Clark et al., 1980), do not contain calmodulin-binding proteins detectable with the Western blotting and ^{125}I-calmodulin overlay techniques used in this investigation.[3] The lack of calmodulin-binding proteins in an organism which does not contain calmodulin suggests that the structural requirements needed for a protein to bind calmodulin under the conditions of our experiments do not occur frequently in a random fashion but are conservative structural domains associated primarily with Ca^{2+}/calmodulin-regulated proteins and enzymes. Whether all of these platelet calmodulin-binding proteins play an important role in platelet function remains to be determined. Some of the calmodulin-binding proteins may be remnants of proteins which were important for the function of the megakaryocyte.

Due to the denaturing effects of SDS, the calmodulin-binding proteins which we have described represent the minimal number in the platelet; other proteins may be present in which the calmodulin-binding domain is not effectively renatured during Western blotting. In addition, differential rates of renaturation, transfer from the polyacrylamide gel to the nitrocellulose, and affinity for the nitrocellulose matrix make it impossible to determine the relative proportion of the various calmodulin-binding proteins from autoradiographs of Western blots. However, it is possible to compare the amount of a particular calmodulin-binding protein in different lanes of a gel as long as the electrophoresis and Western blotting conditions are constant for each lane.

Other investigators have also identified platelet calmodulin-binding proteins. Kosaki et al. (1983) reported four proteins of 100, 90, 60, and 40 kDa. Le Peuch et al. (1983) reported soluble calmodulin-binding proteins of 94, 87, 60, and 43 kDa and particulate proteins of 69, 57, 39, and 37 kDa which bound lower amounts of ^{125}I-calmodulin. The four calmodulin-binding proteins identified by Kosaki et al. (1983) and the soluble calmodulin-binding proteins described by Le Peuch et al. (1983) are most likely the same as the 90-, 82-, 60-, and 41-kDa calmodulin-binding proteins which we have identified. The particulate calmodulin-binding proteins described by Le Peuch et al. (1983) may correspond to the calmodulin-binding proteolytic fragments which we observed. Neither of these investigators observed the 245-, 225-, 190-, and 150-kDa calmodulin-binding proteins, which could be because these proteins bind lower amounts of ^{125}I-calmodulin, requiring a longer exposure period for detection by autoradiography. Alternatively, these investigators used a different technique—^{125}I-calmodulin overlays of polyacrylamide gels instead of nitrocellulose after Western blotting; the larger proteins may be more difficult to renature in the gel than on nitrocellulose due to steric hindrance from the polyacrylamide matrix. Grinstein and Furuya (1982) reported a 149-kDa calmodulin-binding protein associated with the platelet plasma membrane. Although we find a 150-kDa calmodulin-binding protein which is predominantly soluble, the particulate calmodulin-binding protein reported by Grinstein and Furuya (1982) most likely corresponds to a proteolytic fragment of the particulate 225-kDa calmodulin-binding protein; in the presence of micromolar Ca^{2+} levels, the 225-kDa protein is rapidly proteolyzed to a 150-kDa calmodulin-binding protein which remains associated with the particulate fraction.[4]

Two different preparations of affinity-purified IgG directed against myosin light chain kinase were used to identify specific platelet calmodulin-binding proteins as myosin light chain kinase. Both preparations of IgG labeled proteins with mobilities identical with the 225- and 90-kDa calmodulin-binding proteins. The 90-kDa calmodulin-binding protein and the corresponding protein labeled by the antibody were found predominantly in the soluble fraction, while the 225-kDa calmodulin-binding protein and the corresponding protein labeled by the antibody were found exclusively in the particulate fraction. In addition, both the 225-kDa calmodulin-

[3] R. W. Wallace, unpublished data.

[4] R. W. Wallace, E. A. Tallant, and G. A. Piazza, manuscript in preparation.

binding protein and the 225-kDa protein labeled by the antibody are incorporated into the cytoskeleton upon platelet activation.[4] On the basis of these data, we conclude that the 225- and 90-kDa calmodulin-binding proteins are likely the same two proteins which are labeled by the myosin light chain kinase antisera. Myosin light chain kinase isolated from the human platelet soluble fraction has a polypeptide molecular weight of 105 000 (Hathaway & Adelstein, 1979). The difference in molecular weight between it and the 90-kDa calmodulin-binding protein may be due to partial proteolysis during sample preparation; platelet myosin light chain kinase was originally reported to be a 78-kDa polypeptide (Daniel & Adelstein, 1976) which was later found to be a proteolyzed form of the 105-kDa enzyme. Alternatively, the differences may be due to variations in mobility on SDS gels in the presence of the other platelet proteins; the molecular weight of 105 000 was obtained by SDS gel electrophoresis of the purified enzyme (Hathaway & Adelstein, 1979). Our immunochemical data raise the possibility that the 225-kDa calmodulin-binding protein is a particulate form of myosin light chain kinase. Alternatively, this protein may share antigenic determinants with myosin light chain kinase on the basis of common structural features involved in binding calmodulin; however, none of the other calmodulin-binding proteins cross-react with the antibody.

We have previously demonstrated that the 60-kDa platelet calmodulin-binding protein is the calmodulin-binding subunit of a phosphoprotein phosphatase which has been partially purified from human platelets and is similar to calcineurin, the well-characterized form of the enzyme from bovine brain (Tallant & Wallace, 1985). In this paper, we confirm the identity of the 60-kDa calmodulin-binding protein using an antiserum that is specific for the 60-kDa subunit of the brain enzyme.[2]

Caldesmon is a heat-stable protein (Bretcher, 1984) originally isolated from smooth muscle as a calmodulin-binding protein which binds to actin in the absence, but not in the presence, of Ca^{2+}/calmodulin (Sobue et al., 1981). Antisera to smooth muscle caldesmon labeled a platelet protein with a mobility identical with the 82-kDa calmodulin-binding proteins. Originally, we were puzzled that the caldesmon antiserum labeled the 82-kDa protein because caldesmon from smooth muscle has a molecular weight of 150 000 (Sobue et al., 1981). However, it has recently been reported that nonmuscle forms of caldesmon have molecular weights of 70 000–90 000 (Nagi & Walsh, 1985), and Dingus et al. (1985) have purified caldesmon from human platelets and found that it has a molecular weight of 80 000. A portion of the anti-caldesmon immunoreactive protein at 82 kDa was stable to heat-treatment, but the 82-kDa calmodulin-binding protein was not found in the heat-stable fraction. However, it has been shown that caldesmon is difficult to detect on polyacrylamide gels by ^{125}I-calmodulin overlay techniques (Kakiuchi et al., 1983). The heat treatment may have completely denatured the calmodulin-binding domain while leaving the antigenic determinants intact. Although additional experiments are required to establish the identity of the 82-kDa calmodulin-binding proteins, we suggest that one of them may be caldesmon on the basis of the close correlation in their electrophoretic mobilities with the anti-caldesmon immunoreactive protein and the recent report that purified platelet caldesmon has a similar molecular weight (Dingus et al., 1985).

Kosaki et al. (1983) found that under in vitro conditions platelet calmodulin-binding proteins are degraded by an endogenous Ca^{2+}-dependent protease. We also found that specific calmodulin-binding proteins are degraded when Ca^{2+} is added to a platelet homogenate; this degradation could be blocked by the protease inhibitors leupeptin and *N*-ethylmaleimide which is characteristic of the Ca^{2+}-dependent protease (Phillips & Jakabova, 1977; Marachi, 1983). We have extended the findings of Kosaki et al. (1983) by demonstrating that degradation of calmodulin-binding proteins also occurs upon activation of intact platelets. This degradation only occurs when the platelets are activated in the presence of extracellular Ca^{2+}; moreover, when thrombin, ADP plus fibrinogen, and collagen were used to activate the platelets, degradation was greatly enhanced by stirring the platelets. The 90-kDa calmodulin-binding protein was almost completely degraded upon activation of intact platelets with the Ca^{2+} ionophores A23187 and ionomycin but was degraded only slightly when Ca^{2+} was added to a platelet homogenate. This may indicate that the relative cellular orientation and compartmentalization of specific calmodulin-binding proteins and the Ca^{2+}-dependent protease are important factors in determining which proteins are degraded by the protease.

Platelet shape change and secretion do not require extracellular Ca^{2+}; the Ca^{2+} needed to trigger these reactions may be derived from intraplatelet stores. However, aggregation requires extracellular Ca^{2+} and is enhanced by stirring the platelet solution (Holmsen et al., 1977). Thus, proteolysis of calmodulin-binding proteins appears to correlate with platelet aggregation. Fox et al. (1983, 1985) found that proteolytic degradation of platelet actin-binding protein and P_{235} by the Ca^{2+}-dependent protease also occurs during platelet aggregation. They proposed that activation of the protease may be triggered by the uptake of extraplatelet Ca^{2+} which has been observed during aggregation (Massini & Luscher, 1976). A similar mechanism may be involved in the proteolysis of calmodulin-binding proteins during thrombin, ADP plus fibrinogen, or collagen induced aggregation. Such a mechanism would be consistent with our observation that proteolytic degradation of the calmodulin-binding proteins upon platelet activation by Ca^{2+} ionophores does not require stirring; the ionophores cause an influx of extracellular Ca^{2+} which otherwise would not occur until aggregation is initiated.

Proteolytic activation has not been considered as an important physiological mechanism for the regulation of Ca^{2+}/calmodulin-dependent enzymes for a variety of reasons. First, there was no evidence that a protease capable of activating calmodulin-dependent enzymes is regulated by any of the intracellular messengers such as cAMP, cGMP, or micromolar concentrations of Ca^{2+}; second, most of the well-charcterized intracellular regulatory mechanisms, such as phosphorylation–dephosphorylation reactions, are reversible, and third, there has been no indication that Ca^{2+}/calmodulin-dependent enzymes are proteolyzed upon cell activation.

Two forms of a Ca^{2+}-dependent protease, calpain I and calpain II, have been found in many cell types (Murachi, 1983). Calpain I may be important in intracellular Ca^{2+}-regulatory mechanisms since it is activated by micromolar levels of Ca^{2+}; the role of calpain II is unclear since it requires millimolar levels of Ca^{2+} for activity (Murachi et al., 1981). A form of calpain I termed Ca^{2+}-activated protease has been identified in human platelets (Phillips & Jakabova, 1977) and has been shown to be involved in platelet activation through proteolysis of proteins which are associated with the platelet cytoskeleton (Fox et al., 1985). It makes some intuitive sense that irreversible reactions such as proteolysis might play an important role in the platelet reaction sequence; unlike a

muscle cell or neuron, the fully activated platelet does not revert to the resting or inactivated state. Such irreversible activation reactions may also occur in other biological situations, such as during cellular differentiation, during transformation of a cell from a normal to a neoplastic state, or during activation of other blood cells such as neutrophils or lymphocytes. In these situations, the irreversible activation of key enzymes and proteins may occur in order to elicit irreversible responses involved in cellular differentiation or physiology.

In conclusion, we have identified 10 distinct platelet calmodulin-binding proteins and have shown that proteolysis of specific calmodulin-binding proteins occurs during platelet activation. Two of the calmodulin-binding proteins which are degraded correspond to myosin light chain kinase and calmodulin-dependent phosphatase. Both of these Ca^{2+}/calmodulin-regulated enzymes are irreversibly activated upon limited proteolysis by a variety of proteolytic enzymes in vitro (Walsh et al., 1982; Manlan & Klee, 1983; Tallant & Cheung, 1984). If they are found to also be activated by Ca^{2+}-dependent proteolysis, the data presented in this paper suggest a physiological role for the Ca^{2+}-dependent protease as an irreversible activator of Ca^{2+}/calmodulin-dependent reactions.

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Registry No. ADP, 58-64-0; Ca, 7440-70-2; myosin light chain kinase, 51845-53-5; phosphatase, 9013-05-2; thrombin, 9002-04-4; proteinase, 9001-92-7.

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Changes in the Levels of Translatable Glutaminase mRNA during Onset and Recovery from Metabolic Acidosis†

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ABSTRACT: The amount of the mitochondrial glutaminase present within rat kidney is increased 5-fold during chronic metabolic acidosis. This adaptive response is due to a corresponding increase in the relative rate of glutaminase synthesis. Poly(A+) RNA was purified from the kidneys of control, 7-day acidotic, and 2-day recovered rats and then fractionated by electrophoresis on a low melting temperature agarose gel. Translation of the fractionated RNA in a rabbit reticulocyte lysate yields a 72 000-dalton protein that is specifically precipitated by anti-glutaminase IgG. The level of this protein is at least 3-fold greater in the translation products of the fractionated poly(A+) RNA derived from the acidotic vs. control or recovered rats. Therefore, the 72 000-dalton product of translation is the apparent precursor to the 68 000- and 65 000-dalton proteins that are contained in the mitochondrial glutaminase. From its relative electrophoretic mobility, the size of the glutaminase mRNA was estimated to be approximately 6.5 kilobases. The relative levels of translatable glutaminase mRNA were determined by using unfractionated poly(A+) RNA prepared from rats at various times following onset and recovery from acidosis. The observed increase occurred gradually, requiring 7 days to reach a maximal induction of 4.2-fold. The increase could be due to the increased transcription of a stable mRNA ($t_{1/2} \sim 3$ days). However, 2 days of recovery was sufficient to return the level of translatable glutaminase mRNA to normal. Thus, the selective inactivation or the altered stability of the glutaminase mRNA must also contribute to the regulation of the glutaminase gene expression.

Plasma glutamine is an important substrate for oxidative metabolism and the biosynthesis of many nitrogen-containing compounds (Haussinger & Sies, 1984). The catabolism of glutamine occurs primarily in the small intestine, brain, liver, and kidney, where it is initiated by a mitochondrial glutaminase (Kovacevic & McGivan, 1983). The hepatic glutaminase is a unique isoenzyme that has a high K_M for glutamine and is dependent upon NH_4^+ ions (Patel & McGivan, 1984). The glutaminases contained in the three other tissues exhibit similar kinetics (Haser et al., 1985), require a polyvalent anion, and are structurally and immunologically related (Curthoys et al., 1976). However, only the renal glutaminase activity exhibits an adaptive increase in response to metabolic acidosis (Tong et al., 1986).

During normal acid–base balance, the rat kidney extracts very little, if any, of the plasma glutamine (Squires et al., 1976; Hughey et al., 1980). Renal extraction is increased rapidly following the onset of acute acidosis and is sustained during chronic acidosis. The initial increase in renal catabolism of glutamine results primarily from changes in the concentration of metabolites and H^+ that regulate flux through glutaminase, glutamate dehydrogenase, and α-ketoglutarate dehydrogenase (Tannen & Sastrasinh, 1984). The resulting increase in ammoniagenesis provides an expendable cation that facilitates the excretion of acids and conserves Na^+ and K^+ ions. The initial changes in renal metabolites and in plasma pH are largely compensated during chronic acidosis (Parry & Brosnan, 1978). The increased renal catabolism of glutamine is sustained by the gradual increase in the relative rate of glutaminase synthesis (Tong et al., 1986). The increased rate of synthesis reaches a plateau within 5 days that is 5.3-fold greater than normal. The apparent half-life for glutaminase degradation is unaltered during acidosis. As a result, the total renal glutaminase activity is increased approximately 5-fold within 7 days after the onset of acidosis.

In this study, renal poly(A+) RNA was purified from control, acidotic, and recovered rats and translated in a rabbit reticulocyte lysate. Specific immunoprecipitation procedures were used to identify the primary translation product of the mitochondrial glutaminase and to further characterize the mechanisms responsible for the regulated expression of the renal glutaminase.

Materials and Methods

Guanidinium thiocyanate and sodium *N*-laurylsarcosine were purchased from Fluka. DEAE Affi-Gel Blue and reagents for polyacrylamide gel electrophoresis were products of Bio-Rad. ^{14}C-Labeled protein molecular weight standards

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