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Activation of a Calmodulin-Dependent Phosphatase by a Ca^{2+}-Dependent Protease†

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ABSTRACT: A calmodulin-dependent protein phosphatase (calcineurin) was converted to an active, calmodulin-independent form by a Ca^{2+}-dependent protease (calpain I). Proteolysis could be blocked by ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, leupeptin, or *N*-ethylmaleimide, but other protease inhibitors such as phenylmethanesulfonyl fluoride, aprotinin, benzamidine, diisopropyl fluorophosphate, and trypsin inhibitor were ineffective. Phosphatase proteolyzed in the absence of calmodulin was insensitive to Ca^{2+} or Ca^{2+}/calmodulin; the activity of the proteolyzed enzyme was greater than the Ca^{2+}/calmodulin-stimulated activity of the unproteolyzed enzyme. Proteolysis of the phosphatase in the presence of calmodulin proceeded at a more rapid rate than in its absence, and the proteolyzed enzyme retained a small degree of sensitivity to Ca^{2+}/calmodulin, being further stimulated some 15–20%. Proteolytic stimulation of phosphatase activity was accompanied by degradation of the 60-kilodalton (kDa) subunit; the 19-kDa subunit was not degraded. In the absence of calmodulin, the 60-kDa subunit was sequentially degraded to 58- and 45-kDa fragments; the 45-kDa fragment was incapable of binding ^{125}I-calmodulin. In the presence of calmodulin, the 60-kDa subunit was proteolyzed to fragments of 58, 55 (2), and 48 kDa, all of which retained some ability to bind calmodulin. These data, coupled with our previous report that the human platelet calmodulin-binding proteins undergo Ca^{2+}-dependent proteolysis upon platelet activation [Wallace, R. W., Tallant, E. A., & McManus, M. C. (1987) *Biochemistry 26*, 2766–2773], suggest that the Ca^{2+}-dependent protease may have a role in the platelet as an irreversible activator of certain Ca^{2+}/calmodulin-dependent reactions.

A Ca^{2+}/calmodulin-dependent protein phosphatase, also known as calcineurin, was originally isolated as the major calmodulin-binding protein in bovine brain (Wang & Desai, 1976; Klee & Krinks, 1978; Wallace et al., 1978). It was subsequently identified as a protein phosphatase on the basis of its similarity to a Ca^{2+}/calmodulin-dependent phosphatase purified from rabbit skeletal muscle (protein phosphatase 2B) (Stewart et al., 1982; Yang et al., 1982). A similar enzyme has also been isolated from bovine heart (Krinks et al., 1983) and human platelets (Tallant & Wallace, 1985) and appears to be widely distributed in other tissues (Wallace et al., 1980a; Ingebritsen et al., 1983). The brain enzyme is a heterodimer (M_r 80000) composed of a 60-kilodalton (kDa)[1] subunit which binds calmodulin in a Ca^{2+}-dependent manner (Richman & Klee, 1978) and contains the catalytic site (Winkler et al., 1984) and a 19-kDa subunit which contains four high-affinity Ca^{2+}-binding sites (Klee et al., 1979). Although the phosphatase dephosphorylates a wide variety of substrates in vitro (Tallant & Cheung, 1986; King et al., 1984), it exhibits a high catalytic efficiency toward only a few phosphoproteins (King et al., 1984), suggesting a more limited specificity in situ.

In addition to its reversible activation by Ca^{2+}/calmodulin, the phosphatase can be irreversibly activated and made calmodulin-independent upon limited proteolysis by either trypsin (Manalan & Klee, 1983; Tallant & Cheung, 1984b; Tallant & Wallace, 1985) or chymotrypsin (Kincaid et al., 1986). Limited trypsinization of the phosphatase in the absence of calmodulin results in degradation of the 60-kDa subunit to a 43–45-kDa fragment which no longer binds calmodulin (Manalan & Klee, 1983; Tallant & Cheung, 1984b); proteolysis in the presence of calmodulin proceeds at an altered rate (Manalan & Klee, 1983; Tallant & Cheung, 1984b) and produces a series of proteolytic fragments of 57, 55, 54, 46, and 40 kDa, all of which are still capable of binding calmodulin (Manalan & Klee, 1983). Other calmodulin-dependent enzymes are also irreversibly activated and made calmodulin-independent by limited proteolysis including cyclic nucleotide phosphodiesterase (Lin & Cheung, 1980; Kincaid et al., 1985), skeletal muscle phosphorylase kinase (Depaoli-Roach et al., 1979), erythrocyte Ca^{2+}-ATPase (Niggli et al., 1981), myosin light-chain kinase (Walsh et al., 1982), NAD kinase (Meijer & Guerrier, 1982), and adenylate cyclase (Keller et al., 1980). However, proteolytic activation has not been considered to be an important physiological mechanism for activation of calmodulin-dependent enzymes, because no calmodulin-dependent enzyme has been shown to be activated either in vitro or in

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; CaM, calmodulin; kDa, kilodalton(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CANP, calcium-activated neutral protease(s); EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TCA, trichloroacetic acid.

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situ by an intracellular protease subject to regulation.

An increase in the intracellular concentration of Ca^{2+} results in the activation not only of calmodulin-dependent enzymes but also of Ca^{2+}-dependent proteases (Imahori, 1982; Murachi, 1983; Suzuki et al., 1984; Suzuki, 1987). The calpains, or calcium-activated neutral proteases (CANP), are present in a wide variety of mammalian tissues. They are most active at neutral pH, require a free thiol group for activity, and are absolutely dependent upon Ca^{2+} for activity. Two isozymes exist with different Ca^{2+} sensitivities: calpain I is active at micromolar concentrations of Ca^{2+} whereas calpain II requires millimolar Ca^{2+} concentrations. However, both isozymes undergo Ca^{2+}-dependent autolysis, which greatly increases their sensitivity to Ca^{2+}; the Ca^{2+} requirement of calpain II is reduced to the micromolar range in the presence of phospholipids (Coolican & Hathaway, 1984; Imajoh et al., 1986). The calpains are composed of an 80-kDa catalytic subunit and a 30-kDa regulatory subunit, both of which undergo limited degradation during autolysis (Imahori, 1982; Murachi, 1983; Suzuki et al., 1984; Suzuki, 1987). Ca^{2+}-dependent proteases act upon a limited number of substrates including protein kinase C (Inoue et al., 1977), phosphorylase kinase (Meyer et al., 1964), and several cytoskeletal proteins (Phillips & Jaka'bova', 1977; White, 1980; Murachi, 1983; Suzuki et al., 1984; Fox et al., 1985).

We have shown that specific calmodulin-binding proteins are degraded upon addition of Ca^{2+} to a platelet homogenate and in intact platelets upon activation with agents which increase the intraplatelet concentration of Ca^{2+} (Wallace et al., 1987). One of the calmodulin-binding proteins which is degraded is the calmodulin-dependent phosphatase. In this paper, we report that the phosphatase is converted into an active, calmodulin-independent form upon degradation by a Ca^{2+}-dependent protease. These data suggest that limited proteolysis may be a mechanism in the platelet for chronically activating certain Ca^{2+}/calmodulin-dependent enzymes.

Experimental Procedures

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) and Na^{125}I (13–17 mCi/μg) were purchased from Amersham, and vitamin-free casein was from ICN Nutritional Biochemicals. Bovine serum albumin, EGTA, EDTA, leupeptin, PMSF, aprotinin, benzamidine hydrochloride, DFP, soybean trypsin inhibitor, NEM, and protein A were obtained from Sigma Chemical Co. Electrophoresis reagents and bovine serum albumin (for use as a protein standard) were from Bio-Rad Laboratories, and nitrocellulose sheets (0.2 μm) were from Schleicher & Schuell, Inc. All other reagents were of highest analytical grade.

Calmodulin-dependent phosphatase was isolated from bovine brain according to published procedures (Tallant et al., 1983). Ca^{2+}-dependent protease (calpain I) was purified from human red blood cells by the original procedure of Murakami et al. (1981) as modified by Brumley and Wallace.[2] The catalytic subunit of cAMP-dependent protein kinase was isolated from bovine heart (Sugden et al., 1976) and used to prepare phosphorylated casein (5.3×10^4 cpm/nmol) as previously described (Tallant & Cheung, 1984a). Calmodulin was purified from porcine brain by fluphenazine–Sepharose affinity chromatography (Wallace et al., 1980b) followed by gel filtration chromatography on Sephadex G-100. Calmodulin was iodinated by the lactoperoxidase–glucose oxidase procedure (LaPorte & Storm, 1978); ^{125}I-calmodulin was subsequently isolated by affinity chromatography on phenyl-Sepharose (Gopalakrishna & Anderson, 1982). Protein A was iodinated by the chloramine T procedure (Hunter & Greenwood, 1962).

Enzyme Assays. Calmodulin-dependent phosphatase was degraded by the Ca^{2+}-dependent protease in a reaction mixture containing 83.3 mM Tris-HCl (pH 7), 0.17% (v/v) β-mercaptoethanol, 150–300 μM $CaCl_2$, and 1.7 mg/mL bovine serum albumin in the presence or absence of calmodulin (0.4 μM) at 30 °C. Prior to addition of the phosphatase, the protease was preincubated for 5 min in the presence of Ca^{2+} to allow sufficient time for its activation by autolysis. After the indicated time of proteolysis, an aliquot (30 μL) of the reaction mixture was added to an SDS stop solution to provide a final concentration of 1% SDS, 5 mM EDTA, 5 mM EGTA, 5% β-mercaptoethanol, 5% glycerol, 0.01% bromphenol blue, and 125 mM Tris-HCl (pH 6.8) and boiled for 2 min. A second aliquot (30 μL) was added to a reaction mixture (final volume 50 μL) containing ^{32}P-casein (15 μM which corresponds to 40000 cpm at the time of phosphorylation), leupeptin (50 μM) to inhibit proteolysis of the ^{32}P-casein, and EGTA (3 mM) to assay for calmodulin-independent phosphatase activity. A third aliquot (30 μL) was added to a reaction mixture (final volume 50 μL) containing ^{32}P-casein and leupeptin, as indicated above, and calmodulin (0.4 μM) to assay for total phosphatase activity. Calmodulin-dependent phosphatase activity was calculated as the difference between activity in the presence of Ca^{2+}/calmodulin and of EGTA. Phosphatase activity was assayed by the release of [^{32}P]P_i from the ^{32}P-casein, as previously described (Tallant & Cheung, 1984a). The released [^{32}P]P_i was extracted from the TCA-soluble ^{32}P radioactivity into ammonium molybdate (Martin & Doty, 1949), to separate it from ^{32}P-phosphopeptides released by residual protease activity; consistently, 2–3% of the total amount of ^{32}P-casein added was released as ^{32}P-phosphopeptides.

Different preparations of bovine brain phosphatase had basal levels of activity in the presence of EGTA of 0.5–1.2 nmol (mg of protein)$^{-1}$ min^{-1} which could be stimulated 20–40-fold by Ca^{2+}/calmodulin to activities of 18–24 nmol mg^{-1} min^{-1}. The variations in the basal levels of activity in the different enzyme preparations are probably due to a slight degree of proteolysis during preparation.

Western Blotting Procedures. Proteins were separated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and electrophoretically transferred to nitrocellulose (Towbin et al., 1979). Calmodulin-binding proteins were identified by incubation with ^{125}I-calmodulin in the presence of Ca^{2+} (Wallace et al., 1987); the phosphatase and the protease and their degradation products were identified by incubation with the affinity-purified anti-phosphatase and anti-protease sera, as described (Tallant & Wallace, 1985), using ^{125}I protein A and autoradiography to visualize the immunoreactive bands. Antibodies against bovine brain calmodulin-dependent phosphatase were raised in rabbits (Wallace et al., 1980a) and purified by affinity chromatography on phosphatase–Sepharose (Tallant & Wallace, 1985). Antibodies against human erythrocyte Ca^{2+}-dependent protease were also raised in rabbits and isolated by protease–Sepharose affinity chromatography[2].

Protein Determination. Protein was determined according to Lowry et al. (1951) after precipitation with 10 volumes of 10% perchloric/1% phosphotungstic acid. Bovine serum albumin was used as a standard.

Results

To determine whether the calmodulin-dependent phosphatase is activated upon Ca^{2+}-dependent proteolysis, the Ca^{2+}-dependent protease (calpain I) was purified from human

[2] L. M. Brumley and R. W. Wallace, unpublished results.

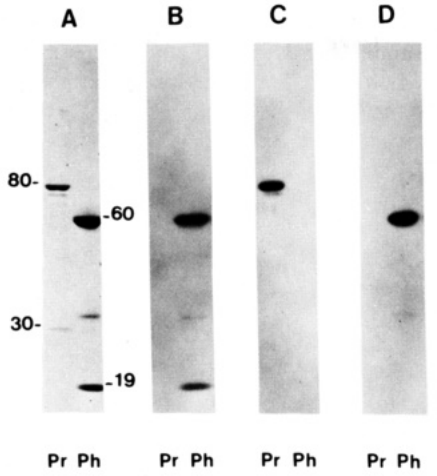

FIGURE 1: Determination of the subunit composition, immunoreactivity, and ^{125}I-calmodulin binding of the purified protease (Pr) and phosphatase (Ph). SDS–polyacrylamide gel electrophoresis, transfer to nitrocellulose, and blotting procedures are described under Experimental Procedures. Panel A is a gel stained for total protein with Coomassie brilliant blue, panel B is an autoradiograph of an immunoblot with antiserum against the phosphatase, panel C is an autoradiograph of an immunoblot with antiserum against the protease, and panel D is an autoradiograph of an ^{125}I-calmodulin overlay. Panel A, 4 μg of phosphatase and 4 μg of protease; panels B, C, and D, 0.2 μg of phosphatase and 0.2 μg of protease. The molecular weight ($\times 10^{-3}$) is indicated for each of the two subunits of the phosphatase and the protease.

Table I: Effect of Limited Proteolysis on Phosphatase Activity[a]

	phosphatase act. [nmol (mg of protein)$^{-1}$ min^{-1}]		
	EGTA	Ca^{2+}	Ca^{2+}/calmodulin
phosphatase	1.21	1.45	23.63
protease	0.00	0.06	0.12
phosphatase + protease	24.13	24.18	28.22

[a] Phosphatase (37 nM), protease (37 nM), and phosphatase plus protease were incubated for 5 min, and aliquots were subsequently assayed for phosphatase activity in the presence of EGTA, Ca^{2+}, or Ca^{2+}/calmodulin, as described under Experimental Procedures.

erythrocytes,[2] the calmodulin-dependent phosphatase was prepared from bovine brain, and affinity-purified antibodies were prepared for each enzyme. Human erythrocyte calpain I contains an 80- and 30-kDa subunit, and the brain phosphatase consists of 60- and 19-kDa subunits (Figure 1, panel A). The affinity-purified antiserum prepared against the brain phosphatase reacts with both phosphatase subunits but shows no cross-reactivity with the purified protease (panel B); the affinity-purified antiserum against the protease only labels its 80-kDa subunit, showing no reactivity with its 30-kDa subunit or the phosphatase subunits (panel C), and only the 60-kDa subunit of the phosphatase binds ^{125}I-calmodulin (panel D).

Calmodulin-dependent phosphatase activity was measured in the presence of EGTA, Ca^{2+}, and Ca^{2+}/calmodulin after the phosphatase had been incubated with the protease in the presence of Ca^{2+} (Table I). The phosphatase alone had a specific activity of 1.45 nmol (mg of protein)$^{-1}$ min^{-1} in the presence of Ca^{2+} and was stimulated 16-fold by Ca^{2+}/calmodulin; the addition of sufficient EGTA to reduce the Ca^{2+} concentration to submicromolar levels slightly decreased the specific activity. The protease had no significant phosphatase activity under any of the assay conditions. When the protease was preincubated with the phosphatase for 5 min, phosphatase activity determined in the presence of either EGTA or Ca^{2+} was increased to the level of activity of the untreated enzyme in the presence of Ca^{2+}/calmodulin. Ca^{2+}/calmodulin stimulated the proteolyzed enzyme to some extent (1.2-fold), suggesting that either the time of proteolysis was insufficient to allow full proteolytic activation of the phosphatase or the proteolyzed enzyme was still sensitive to calmodulin.

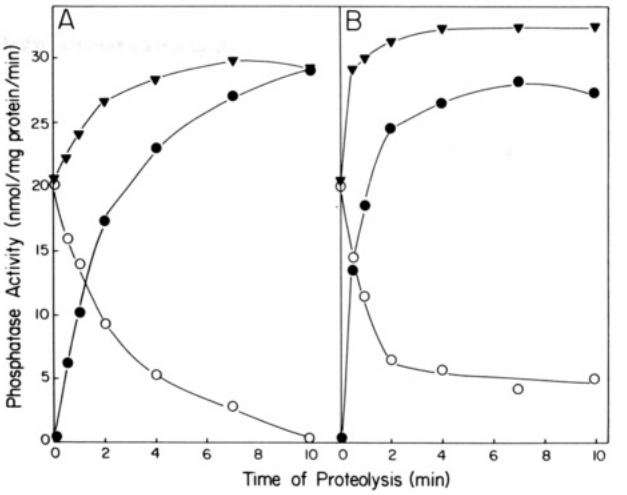

FIGURE 2: Stimulation of phosphatase activity by proteolysis. Phosphatase (37 nM) was incubated with protease (37 nM) in the absence (panel A) or the presence (panel B) of calmodulin (370 nM). At the times indicated, aliquots were assayed for calmodulin-independent phosphatase activity (●), total phosphatase activity (▼), and calmodulin-dependent activity (○), as described under Experimental Procedures. Another aliquot was prepared for gel electrophoresis, as shown in Figure 3. Control experiments established that in the absence of protease the activities of the phosphatase and the calmodulin–phosphatase complex were unchanged during the time course of the experiment (data not shown).

The time course of proteolytic activation of the phosphatase in both the absence and presence of calmodulin is shown in Figure 2. At the times indicated, an aliquot of the reaction mixtures was removed, leupeptin was added to stop proteolysis, and phosphatase activity was determined in the presence of EGTA (calmodulin-independent activity) and in the presence of Ca^{2+}/calmodulin (total activity); the difference between these two activities is the calmodulin-dependent activity. Prior to proteolysis, the phosphatase exhibits a very low level of calmodulin-independent activity (0.5 nmol mg^{-1} min^{-1}) which could be stimulated 40-fold upon addition of Ca^{2+}/calmodulin. When the phosphatase was incubated with the protease in the absence of calmodulin (panel A), calmodulin-independent phosphatase activity increased during the time of proteolysis with a concomitant decrease in Ca^{2+}/calmodulin-dependent activity. Ca^{2+} alone had no effect on the proteolyzed phosphatase (data not shown). After a 3-min treatment with the protease, phosphatase activity assayed in the presence of EGTA was equivalent to the activity of the untreated enzyme when fully stimulated by Ca^{2+}/calmodulin. However, calmodulin-independent activity increased even further as a result of longer proteolysis, reaching a plateau after 10 min, at which time the phosphatase was no longer sensitive to calmodulin.

Proteolysis of the phosphatase in the presence of calmodulin also produced calmodulin-independent phosphatase activity (panel B). However, the proteolytic activation occurred at a much faster rate; calmodulin-independent activity was almost equivalent to the Ca^{2+}/calmodulin-dependent activity of the native enzyme after only 1 min of treatment and reached a maximum by about 4 min. Maximal calmodulin-independent activity was approximately the same after proteolysis in the presence or absence of calmodulin. However, the phosphatase proteolyzed in the presence of calmodulin was still sensitive to calmodulin; Ca^{2+}/calmodulin further stimulated the fully proteolyzed enzyme 1.2-fold.

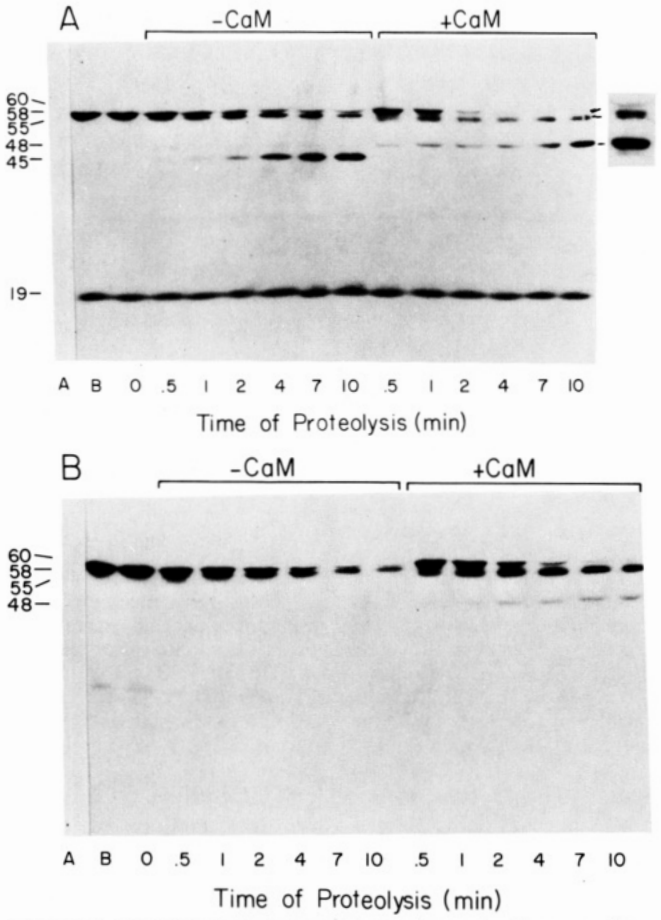

FIGURE 3: Effect of proteolysis on phosphatase subunit composition and ability to bind calmodulin. Aliquots of the phosphatase proteolyzed in the absence or presence of calmodulin (from Figure 2) were denatured in SDS, resolved by gel electrophoresis, and Western blotted with either antibodies against the phosphatase (panel A) or ^{125}I-calmodulin (panel B), as described under Experimental Procedures. Each lane contains 110 ng of phosphatase and/or 150 ng of protease. A and B are controls, containing only protease (A) or phosphatase (B). The molecular weight ($\times 10^{-3}$) is indicated for each reactive protein. The faint immunoreactive band in the 30-kDa region is an impurity in the phosphatase preparation. The insert to the right of Panel A shows an enlargement of a more resolved gel of the 55-kDa immunoreactive region. CaM, calmodulin.

The activity of the fully proteolyzed phosphatase was 1.4–1.6-fold higher than the activity of the native enzyme in the presence of Ca^{2+}/calmodulin (Figure 2), suggesting that the phosphatase contains latent activity which is only expressed upon proteolysis. The production of a higher level of enzyme activity following trypsinization was previously observed for the phosphatase isolated from bovine brain (Manalan & Klee, 1983; Kincaid et al., 1986) and from platelets (Tallant & Wallace, 1985).

A previous report (DeMartino & Blumenthal, 1982) suggests that some preparations of calmodulin may contain a low molecular weight, heat-stable protein factor which stimulates the activity of the Ca^{2+}-dependent protease. This could account for the increased rate of proteolytic activation of the phosphatase in the presence of calmodulin. To test this possibility, we measured protease activity in the presence and absence of calmodulin, using ^{125}I-labeled casein as a substrate (Zimmerman & Schlaepfer, 1984); calmodulin had no effect on the caseinolytic activity of the protease (data not shown).

The time course of proteolysis was also monitored by Western blotting with antiserum against the phosphatase (Figure 3, panel A). Proteolysis of the phosphatase in the absence of calmodulin results in an immediate reduction in the amount of the 60-kDa subunit and the formation of a 58-kDa proteolytic product. With increasing time of proteolysis, the labeling of both the 60-kDa subunit and the 58-kDa degradation product declined with a corresponding accumulation of a 45-kDa immunoreactive fragment. After 10 min of proteolysis, the major immunoreactive protein was the 45-kDa fragment although a small amount of the 60- and 58-kDa proteins remained. The 19-kDa subunit of the phosphatase was not degraded. Both the 60-kDa subunit of the phosphatase and its 58-kDa proteolytic product bound ^{125}I-calmodulin when an identical Western blot was incubated with ^{125}I-calmodulin in the presence of Ca^{2+} (Figure 3, panel B); the reduction in their levels of immunoreactivity was paralleled by a loss of ^{125}I-calmodulin binding. The 45-kDa proteolytic product did not bind ^{125}I-calmodulin. No smaller proteolytic fragments were detected by immunoreactivity or ^{125}I-calmodulin binding; however, it is possible that an immunoreactive fragment may have an electrophoretic mobility similar to that of the 19-kDa subunit.

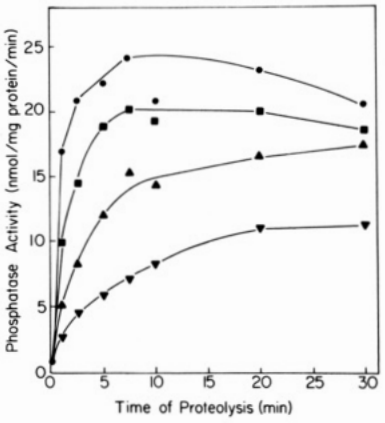

FIGURE 4: Effect of protease concentration on proteolytic activation of the calmodulin–phosphatase complex. A reaction mixture containing phosphatase (37 nM) and calmodulin (370 nM) was incubated with the following concentrations of protease: 37 nM (●), 18.5 nM (■), 9.25 nM (▲), and 3.7 nM (▼). At the times indicated, aliquots were assayed for calmodulin-independent phosphatase activity, as described under Experimental Procedures.

Proteolysis of the phosphatase in the presence of calmodulin resulted in the production of a different group of protein fragments. The 60-kDa subunit was immediately degraded, and after 4 min of proteolysis, its immunoreactivity had almost totally disappeared (Figure 3, panel A). Although a 58-kDa proteolytic product was distinguishable at the earlier times of proteolysis, its labeling was similarly absent by 4 min. ^{125}I-Calmodulin labeling in the area of the blot containing these proteins was also lost as a function of the time of proteolysis (panel B). In contrast, proteolytic products of 55 and 48 kDa were present at low levels at the earliest time point (0.5 min) and accumulated during proteolysis (panel A). The 55-kDa region is actually composed of two distinct immunoreactive bands. Although this is suggested by the immunoreactive labeling of the 55-kDa region at the earlier times of proteolysis, it is more apparent upon magnification of a more resolved gel, as shown at the side of panel A in Figure 3. Both the 55- and 48-kDa fragments bound ^{125}I-calmodulin (panel B); however, a larger amount of ^{125}I-calmodulin was bound in the 55-kDa region. Again, the 19-kDa subunit of the phosphatase was not altered nor were any smaller molecular weight peptide fragments detected.

The effect of various concentrations of protease on the activation of the calmodulin–phosphatase complex is shown in Figure 4. In all of the preceding experiments, the protease and the phosphatase were incubated at equimolar concentra-

Table II: Effect of Various Protease Inhibitors on Proteolytic Stimulation of Phosphatase Activity[a]

addition	phosphatase act. [nmol (mg of protein)$^{-1}$ min^{-1}]	
	EGTA	Ca^{2+}/calmodulin
none	12.7	20.4
EGTA (1 mM)	0.3	4.5
leupeptin (50 μM)	0.4	17.6
PMSF (100 μM)	10.3	18.4
aprotinin (1 TIU/mL)	15.1	20.5
benzamidine (2 mM)	11.9	19.8
DFP (1 mM)	10.2	17.8
trypsin inhibitor (2 μM)	12.3	19.9
NEM (1 mM)	0.2	13.1

[a] Phosphatase (37 nM) was incubated with protease (37 nM) for 3 min in the presence of various protease inhibitors, as indicated. Prior to the addition of the phosphatase, the protease was preincubated with the inhibitors for 5 min. Aliquots were subsequently assayed for phosphatase activity in the presence of EGTA (calmodulin-independent activity) or in the presence of Ca^{2+}/calmodulin (total activity), utilizing ^{32}P-casein as a substrate. The assay was as described under Experimental Procedures, with the exception of the incubation with NEM, in which β-mercaptoethanol was omitted from the standard reaction mixture. A third aliquot was prepared for gel electrophoresis, as shown in Figure 5. Prior to proteolysis, phosphatase activity in the presence of EGTA was 0.6 nmol mg^{-1} min^{-1}, and total activity in the presence of Ca^{2+}/calmodulin was 17.8 nmol mg^{-1} min^{-1}.

tions, in which case the phosphatase is fully active after 10 min of proteolysis (panel A, Figure 2, and the upper curve of Figure 4). With substoichiometric levels of protease, both the initial rates and the final level of activation were decreased. However, even with 10-fold less protease in the reaction mixture (Figure 4, lower curve), approximately 40% of the maximal phosphatase activity could be obtained. The inability of substoichiometric levels of protease to provide maximal phosphatase activity was due to the inability of dilute solutions of the protease to be fully activated by autolysis and the instability of the protease under our reaction conditions; after treatment with substoichiometric concentrations of protease, the phosphatase could be activated by Ca^{2+}/calmodulin to the same level of activity (data not shown).

The effect of various protease inhibitors on the proteolytic stimulation of phosphatase activity is shown in Table II. In the absence of protease inhibitors, the protease stimulated the phosphatase activity from 0.6 to 12.7 nmol mg^{-1} min^{-1}. When excess EGTA was added to the reaction mixture during proteolysis, the calmodulin-independent phosphatase activity was not increased; leupeptin and NEM were also effective in blocking the stimulation of calmodulin-independent activity. None of the other protease inhibitors (PMSF, aprotinin, benzamidine, DFP, or trypsin inhibitor) was effective. To determine whether any of the protease inhibitors interfere with the measurment of phosphatase activity, the ability of the proteolyzed phosphatase to be stimulated by Ca^{2+}/calmodulin was determined. In the absence of protease inhibitors, the activity of the proteolyzed phosphatase was stimulated by Ca^{2+}/calmodulin from 12.7 to 20.4 nmol mg^{-1} min^{-1}. With the exception of EGTA and NEM, both of which inhibited the Ca^{2+}/calmodulin-dependent phosphatase activity, none of the other protease inhibitors had any significant effect on the phosphatase activity. In a separate experiment, EGTA and NEM also inhibited the Ca^{2+}/calmodulin-dependent activity of the native phosphatase while the other protease inhibitors had no effect (data not shown).

The effect of the various protease inhibitors on the subunit composition of the phosphatase after its incubation in the proteolysis reaction mixture was determined by immunoblotting (Figure 5, panel A). The 60-kDa subunit of the

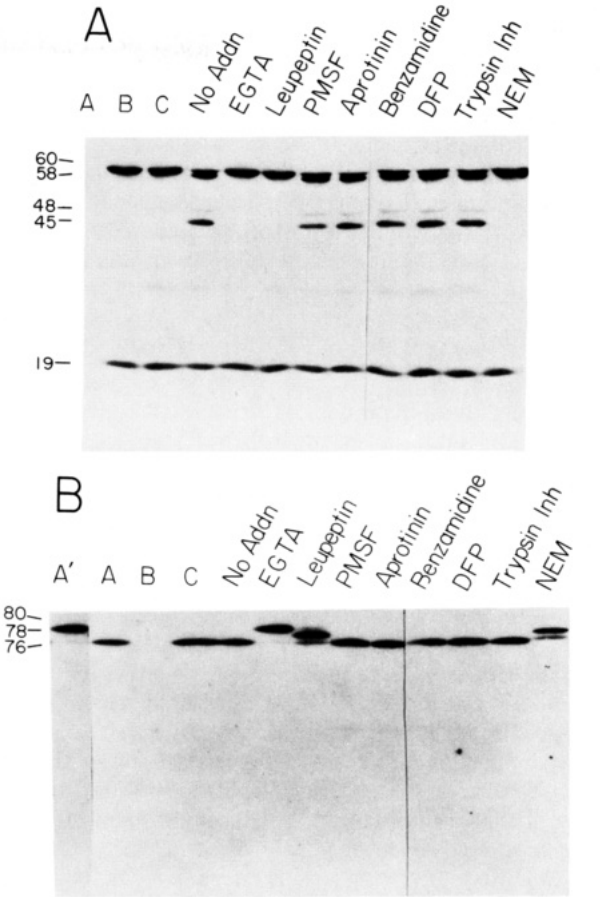

FIGURE 5: Effect of protease inhibitors on proteolysis of the phosphatase and the protease. Aliquots of the phosphatase proteolyzed in the presence of various protease inhibitors (from Table II) were denatured in SDS, resolved by gel electrophoresis, and Western blotted with antibodies against the phosphatase (panel A) or the protease (panel B) as described under Experimental Procedures. The concentrations of the protease inhibitors are indicated in Table II. Each lane contains 110 ng of phosphatase and/or 150 ng of protease. A′, A, B, and C are controls; protease alone prior to autolysis (A′), protease following autolysis (A), phosphatase alone (B), or protease and phosphatase denatured prior to incubation (C). The molecular weight ($\times 10^{-3}$) is indicated for each immunoreactive protein.

phosphatase (shown in lanes B and C, Figure 5) was degraded to peptides of 58, 48, and 45 kDa (shown in the lane labeled "No Addn"); a similar pattern of proteolysis was observed in the presence of PMSF, aprotinin, benzamidine, DFP, and trypsin inhibitor. However, in the presence of EGTA, leupeptin, and NEM, the 60-kDa subunit remained intact, with no apparent degradation. The 45-kDa proteolytic fragment did not bind ^{125}I-calmodulin while ^{125}I-calmodulin binding to the intact 60-kDa subunit was retained when the proteolysis reaction mixture contained EGTA, leupeptin, or NEM (data not shown).

To determine the effect of the protease inhibitors on autolysis of the protease, the degradation of the 80-kDa protease subunit was also monitored by immunoblotting (Figure 5, panel B). The immunoreactive 80-kDa subunit, shown in lane A′, was sequentially degraded to 78- and 76-kDa proteolytic fragments during autolysis; after 5 min of autolysis, the protease was fully active as determined by its ability to degrade casein (data not shown), and the 80-kDa subunit had been quantitatively converted to the 76-kDa fragment (lane A). In the absence of protease inhibitors (No Addn) or in the presence of PMSF, aprotinin, benzamidine, DFP, or trypsin inhibitor, the 80-kDa subunit was degraded to the catalytically active 76-kDa proteolytic fragment. EGTA completely blocked

autolysis of the protease. Leupeptin and NEM also blocked the formation of the 76-kDa fragment; however, the 80-kDa subunit was almost quantitatively degraded to a 78-kDa fragment. Because our antiserum does not react with the 30-kDa subunit of the protease, it was not possible to follow its degradation during autolysis. These results are consistent with our interpretation that EGTA, leupeptin, and NEM block the proteolytic stimulation of the calmodulin-dependent phosphatase by inhibiting the autolytic activation of the protease.

Discussion

The data presented in this paper demonstrate that the Ca^{2+}/calmodulin-dependent phosphatase is converted to an active, calmodulin-independent form upon Ca^{2+}-dependent proteolysis. Activation of the phosphatase by the protease was correlated with the degradation of the 60-kDa subunit of the phosphatase; there was no alteration of the 19-kDa subunit. The presence or absence of calmodulin in the reaction mixture during proteolysis resulted in alterations in the rate of proteolysis, the proteolytic fragments generated, and the sensitivity of the proteolyzed enzyme to Ca^{2+}/calmodulin. Since calmodulin did not affect the activity of the protease itself, the interaction of calmodulin with the phosphatase most likely produces a conformational change that exposes alternate cleavage sites to the protease. Trypsinization of the phosphatase in the presence versus the absence of calmodulin also proceeded at an altered rate, produced different proteolytic fragments some of which still bound calmodulin, and changed the sensitivity of the enzyme to calmodulin (Manalan & Klee, 1983; Tallant & Cheung, 1984). We presume that the Ca^{2+}/calmodulin/phosphatase complex would be the most likely substrate for the protease within a cell because the intracellular concentration of calmodulin is greater than that of the phosphatase. However, the concentration of calmodulin relative to the total complement of calmodulin-binding proteins and the relative subcellular distribution of calmodulin and the phosphatase within most cells have yet to be determined.

Proteolytic conversion of calmodulin-dependent enzymes to chronically active, calmodulin-independent forms may be due to the removal of the calmodulin-binding domain which has an inhibitory influence on the rate of catalysis of the enzyme. Proteolytic activation of the phosphatase in the absence of calmodulin yields a 45-kDa fragment which does not bind ^{125}I-calmodulin. Proteolytic activation of the phosphatase in the presence of calmodulin produces two predominant fragments—a major one of 48 kDa and a minor one of 55 kDa. The 55-kDa fragment still binds ^{125}I-calmodulin and may account for the fraction of total activity which is stimulated by Ca^{2+}/calmodulin; the 48-kDa fragment binds only a minor amount of ^{125}I-calmodulin. These data suggest that the 45- and 48-kDa proteolytic fragments constitute the catalytic core of the enzyme from which the domain responsible for the regulation by calmodulin has been removed. Thus, the 60-kDa subunit of the phosphatase contains at least three domains. Two of these—the catalytic domain and the regulatory domain, which is responsible for interacting with the 19-kDa subunit—are relatively resistant to proteolysis; the proteolytic fragments of the 60-kDa subunit still bind to the 19-kDa subunit.[3] The third domain, which is sensitive to proteolysis, is the inhibitory domain which contains the calmodulin-binding site; its interaction with calmodulin removes inhibition in a Ca^{2+}-dependent, reversible manner while its removal by limited proteolysis converts the enzyme into an active, calmodulin-independent form.

The experiments described in this paper were initiated because we found that the activation of human platelets under certain conditions is accompanied by Ca^{2+}-dependent proteolysis of calmodulin-binding proteins, including the Ca^{2+}/calmodulin-dependent phosphatase (Wallace et al., 1987). Previously, we had shown that the platelet calmodulin-dependent phosphatase shares many properties with the enzyme from bovine brain, including the ability to be converted into an active, calmodulin-independent form by limited trypsinization (Tallant & Wallace, 1985). We now demonstrate that the Ca^{2+}/calmodulin-dependent phosphatase can be activated and made calmodulin-independent by Ca^{2+}-dependent proteolysis. The activation of calmodulin-dependent enymes by Ca^{2+}-dependent proteolysis may be an alternative physiological mechanism for chronically activating enzymes which are otherwise regulated reversibly by Ca^{2+}/calmodulin. Such a mechanism may be important in certain cell types which undergo irreversible activation. It also has the potential to provide an explanation for long-term cellular responses which are known to occur in some cells in response to a highly transient Ca^{2+} signal.

[3] E. A. Tallant, L. M. Brumley, and R. W. Wallace, unpublished results.

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