Site-directed activation of calpain is promoted by a membrane-associated natural activator protein

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Human erythrocytes contain a calpain activator protein with a molecular mass of approx. 40 kDa. The activator is present in association with the plasma membrane and promotes expression of calpain activity at a concentration of Ca^{2+} close to physiological values. The initial step of the activating mechanism involves association of the activator with calpain, followed by autoproteolytic activation of the proteinase in the presence of 1 μM Ca^{2+}, at a rate identical to that induced by 1 mM Ca^{2+}. In a reconstituted system, the activator binds to erythrocyte membranes, but not to phospholipid vesicles, suggesting the participation of an intrinsic membrane protein(s). In its membrane-associated form the activator selectively binds calpain, thus favouring interaction of the proteinase with the inner surface of plasma membranes. These results further confirm the importance of a natural activator protein in promoting intracellular activation of calpain under physiological conditions through a site-directed mechanism, which explains the high specificity of the proteinase for membrane of cytoskeletal proteins.

INTRODUCTION

Two isoforms of the Ca^{2+}-dependent neutral proteinase, calpain (EC 3.4.22.17), have been identified in many mammalian tissues (for reviews, see [1–3]). Although the two isoforms, named calpain I and II (μ and m), are quite similar in structure, both being heterodimers composed of an 80 kDa catalytic subunit and a 30 kDa subunit, their catalytic properties are different, particularly with respect to the concentration of Ca^{2+} required for the expression of catalytic activity. Thus calpain I requires a concentration of Ca^{2+} between 2 and 10 μM, whereas calpain II requires a higher Ca^{2+} concentration, i.e. close to 1 mM. Human erythrocytes contain a single calpain form, which can be classified as a type I form on the basis of its molecular and chromatographic properties; however, its catalytic properties are intermediate between those typical of calpain I and calpain II [4]. The concentration of Ca^{2+} required by the erythrocyte calpain is approximately two orders of magnitude greater than that present in the cells. It is reasonable, therefore, to assume that conditions capable of increasing the Ca^{2+} affinity of the proteinase must be present in these cells in order to promote the activity of the enzyme.

We have shown previously that erythrocyte calpain undergoes an autoproteolytic conversion which produces a new proteinase form capable of digesting target proteins at much lower Ca^{2+} concentrations [5]. Based on its catalytic properties [4], the autolysed calpain could be considered as the intracellular active form, but the conditions necessary to promote the interconversion of the high-[Ca^{2+}]-requiring form to the low-[Ca^{2+}]-requiring form are still very different from those found *in vivo*.

Although the association of calpain with membranes can also promote its autoproteolytic interconversion at low [Ca^{2+}], the kinetics of this activation are not consistent with the actual rate measured in cells [6,7]. In human [8] and bovine brain [9], and more recently in human platelets [10], human neutrophils [11] and rat skeletal muscle [12], an endogenous calpain activator protein has been described. The properties of these stimulating factors differ, however, with respect their effect on the $V_{max.}$ or on the Ca^{2+} requirement of the proteinase. Thus activators from brain and platelet have been shown to increase preferentially the efficiency of calpain catalytic activity, without altering its affinity for Ca^{2+}, whereas in neutrophils and skeletal muscle the activator has been found to increase by more than 100-fold and Ca^{2+}-sensitivity of calpain II. We have demonstrated that, in rat skeletal muscle, the activator is highly specific for calpain II, promoting autoproteolytic activation of the proteinase at very low concentration of Ca^{2+} [13].

The experiments reported in the present paper were designed to verify the presence and the mechanism of action of the activator protein in cells lacking the calpain II isoform and containing a single calpain type [4]. We report here that human erythrocytes contain an activator protein having molecular properties and a mechanism of action similar to those characteristic of the stimulating factor present in rat skeletal muscle [13]. The broad specificity of the activator with proteinases from different sources suggests the existence of a general mechanism for calpain activation by this protein.

MATERIALS AND METHODS

Materials

Sephadex G-200 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and DEAE-cellulose resin (DE52) was from Whatman, Maidstone, UK. Leupeptin, phenylmethanesulphonyl fluoride (PMSF), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin, fluorescamine, beef liver catalase, rabbit muscle aldolase, yeast glucose-6-phosphate dehydrogenase (G6PD), rabbit muscle phosphorylase *b*, cytochrome *c*, bovine pancrease trypsin, bovine pancrease chymotrypsin and Pronase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The electrophoresis reagents and silver stain kit were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Abbreviations used: CM-calpain, [^{14}C]carboxymethylated calpain; PMSF, phenylmethanesulphonyl fluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidyl-L-serine; G6PD, glucose-6-phosphate dehydrogenase; K_D, distribution coefficient.

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Table 1 Purification of calpain activator from human erythrocyte membranes

Erythrocytes (10 ml of packed cells) were lysed and plasma membranes were isolated as described in the Materials and methods section. For experimental details of the purification, see the text. Protein concentration was determined by the procedure described by Lowry et al. [29] for steps 1–3, and by the procedure described by Bradford [30] for steps 4–6. Activator activity was assayed as described in the Materials and methods section

Step	Total protein (mg)	Activator activity Total (units)	Specific activity (units/mg)	Yield (%)
1. Extraction in 1 M NaCl	30	1200	40	100
2. Heat at 90 °C for 3 min	6	980	163	82
3. Ammonium sulphate precipitation	3.6	710	197	59
4. DE52 chromatography, pH 6.7	1.4	415	296	35
5. DE52 chromatography, pH 7.5	0.6	340	566	28
6. Sephadex G-200 chromatography	0.032	180	5600	15

[^{14}C]Iodoacetic acid (56 mCi/mmol) was purchased from Amersham International. Human denatured globin was obtained by using the procedure of Winterhalter and Huehns [14]. Calpain was purified from freshly collected human erythrocytes as previously reported [5]. Muscle calpains I and II were purified from rat skeletal muscle as previously reported [15]. All other chemicals were reagent grade.

Purification of calpain activator

The calpain activator from human erythrocyte membranes was purified according to the following procedure. Freshly collected human erythrocytes (10–50 ml of packed cells), after removal of leukocytes [16], were lysed with 10 vol. of cold 5 mM sodium phosphate buffer, pH 8.0, containing 0.1 mM leupeptin and 2 mM PMSF, and centrifuged at 10000 ***g*** for 15 min. The membrane fraction was washed five times in 5 mM sodium phosphate buffer, pH 8.0, resuspended in 1 M NaCl, incubated for 20 min at 4 °C and centrifuged at 40000 ***g*** for 10 min. The supernatant, containing the activator protein, was heated at 90 °C for 3 min; the insoluble material was removed by centrifugation and solid ammonium sulphate was added to the clear supernatant at a final concentration of 80 % saturation. The precipitated proteins were collected by centrifugation at 10000 ***g*** for 10 min and then dissolved in 5 ml of 50 mM sodium acetate, pH 6.7, containing 0.5 mM 2-mercaptoethanol and 0.1 mM EDTA. The solution was dialysed overnight against the same buffer and loaded on a DE52 column (1 cm × 10 cm) previously equilibrated with 50 mM sodium acetate, pH 6.7, containing 0.5 mM 2-mercaptoethanol and 0.1 mM EDTA. With this ion-exchange chromatography the calpain activator is recovered in the washing fractions. This step increases the purity of the sample by adsorbing contaminating proteins. The ion-exchange chromatography was then repeated using a DE-52 column equilibrated with 50 mM sodium borate, pH 7.5, containing 0.5 mM 2-mercaptoethanol and 0.1 mM EDTA. The washing fractions were then collected, concentrated by dialysis against 80 % ammonium sulphate and the precipitated materials were dissolved in 50 mM sodium borate buffer, pH 7.5, and loaded on a Sephadex G-200 column (1 cm × 150 cm) previously equilibrated with 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA and 0.5 mM 2-mercaptoethanol. The proteins were eluted with the same buffer at a flow rate of 0.1 ml/min and fractions of 1 ml were collected. The fractions containing activator protein activity were pooled and used as the source of the activator. A typical activator preparation from 10 ml of packed erythrocytes is reported in Table 1.

Assay of calpain and activator protein activities

Human erythrocyte calpain activity was routinely assayed as previously reported [5]. A unit of activity was defined as the amount that produced 1 μmol of free α-amino groups under the specified assay conditions using acid-denatured human globin as a substrate.

Activator protein activity was assayed by adding appropriate amounts of the activator preparation to the calpain assay mixture in the presence of 5 μM Ca^{2+}. At this concentration of Ca^{2+}, calpain activity in the absence of the activator is negligible. A unit of activator protein activity is defined as the amount producing 1 unit of proteolytic activity of calpain under the specified conditions.

Preparation of [^{14}C]carboxymethylated calpain (CM-calpain)

Carboxymethylation of purified erythrocyte calpain was carried out using [^{14}C]iodoacetic acid (56 mC/mmol), as previously reported [17]. The specific radioactivity of labelled calpain was 9×10^4 c.p.m./mg. Carboxymethylation causes inactivation of calpain by insertion of a single carboxymethyl group at the active site, without affecting other properties of the enzyme [17].

Equilibrium distribution of calpain–activator complex in Sephadex G-200

These experiments were carried out according to the procedure of Ackers [18]. CM-calpain (10 μg; 900 c.p.m.) was mixed in a final volume of 0.5 ml of 50 mM sodium borate, pH 7.5, with an excess of activator protein (20 units) in the absence or presence of increasing concentrations of Ca^{2+} (from zero to 2 mM). The mixtures were then added to 0.5 ml of wet Sephadex G-200 resin previously equilibrated with 50 mM sodium borate, pH 7.5, mixed end-over-end for 1 h and centrifuged at 200 ***g*** for 2 min. Aliquots (0.2 ml) of the supernatant were collected for determination of the radioactivity. The distribution coefficient (K_D) was defined as $K_D = (V_f - V_a)/(V_t - V_a)$, where V_f is the penetration volume of CM-calpain, calculated from the total CM-calpain c.p.m., added to the mixture divided by the c.p.m./ml recovered in the supernatant. V_t is the total volume of the system. V_a, the volume of the aqueous phase, was calculated from a parallel experiment using the same quantity of Sephadex G-200 and the same volume of buffer containing Blue Dextran 2000 as a marker. The standards beef liver catalase (240 kDa), rabbit muscle aldolase (160 kDa), yeast G6PD (100 kDa) and cytochrome *c* (12.5 kDa) were used to calibrate the procedure.

Determination of free Ca^{2+} concentration

The free Ca^{2+} concentration was attained using an EDTA buffer and a dissociation constant of 1.2×10^{-8} M [19].

Preparation of human erythrocyte membranes

Inside-out membranes were obtained from erythrocytes as described [6,20]. The yield of inside-out vesicles, determined by the assay of acetylcholinesterase activity in the absence and presence of 0.1 % Triton X-100 [6], ranged from 65 to 80 % of the total.

Stripped erythrocyte membranes were prepared by mixing inside-out vesicles with 10 vol. of ice-cold 0.1 M NaOH [21], immediately followed by centrifugation at 200000 *g* for 10 min. The pellet was washed three times in 5 mM sodium phosphate buffer, pH 8.0, and resuspended in the same buffer. Stripped membranes were incubated with trypsin (at a ratio of 20:1, w/w) for 30 min in 50 mM sodium borate, pH 8.0. The digested membranes were collected by centrifugation at 100000 *g* for 10 min. The pellet, washed three times in 5 mM sodium phosphate buffer, pH 8, was resuspended in the same buffer.

Extraction of phospholipids from stripped erythrocyte membranes

Phospholipids were extracted from membranes with a mixture of chloroform/methanol (2:1, v/v) following the procedure reported by Reed et al. [22], and stored under N_2 at −20 °C. The amount of phospholipids was determined by measuring P_i after hydrolysis with 70 % $HClO_4$ at 190 °C, as previously described [23]. Immediately before use, an aliquot (75 μl, 0.4 mg) of the phospholipid fraction was dried under N_2 and dispersed in 1 ml of 50 mM sodium borate buffer, pH 7.5. The mixture was sonicated for 5 min, with intervals of 30 s, in an ice bath.

Preparation of phospholipid vesicles

Commercial phosphatidylcholine (PC), PS and PE from bovine brain were dissolved in chloroform/methanol (19:1, v/v) at 10 mg/ml and stored at −20 °C. Immediately before use, aliquots (100 μl) of the stock solution were dried under N_2, dispersed in 1 ml of 50 mM sodium borate buffer, pH 7.5, and sonicated for 5 min, with intervals of 30 s, in an ice bath.

Assay of G6PD and aldolase activities

G6PD activity was assayed as reported by Beutler [24]. Aldolase activity was measured out as described in [25].

RESULTS

Identification and isolation of a calpain activator protein from human erythrocytes

As shown in Figure 1, when human erythrocyte lysate was submitted to chromatography on a Sephadex G-200 column, in addition to the two peaks of identified components of the Ca^{2+}-dependent proteolytic system, i.e. calpain and calpastatin [4], a third peak of calpain activator was identified. A similar activating factor has also been found under the same chromatographic conditions in human neutrophils [11] and rat skeletal muscle [12].

The calpain activator was purified from human erythrocytes following the procedure described in the Materials and methods section and summarized in Table 1. A sample of the purified activator, with a specific activity of approx. 5.6 units/mg, reveals on SDS/PAGE the presence of a single major band with a mobility corresponding to a molecular mass of approx. 40±4 kDa (Figure 2). In a parallel electrophoretic run the gel was cut into slices and the eluted protein assayed for calpain activator activity. The peak of the activator activity coincided with the position of the stained band (see Figure 2).

The protein nature of the activator was also established by digestion with trypsin, chymotrypsin or Pronase (results not shown). Under these conditions, all activator activity was lost; it is interesting, however, to note the complete resistance of the activator to digestion by purified calpain (results not shown).

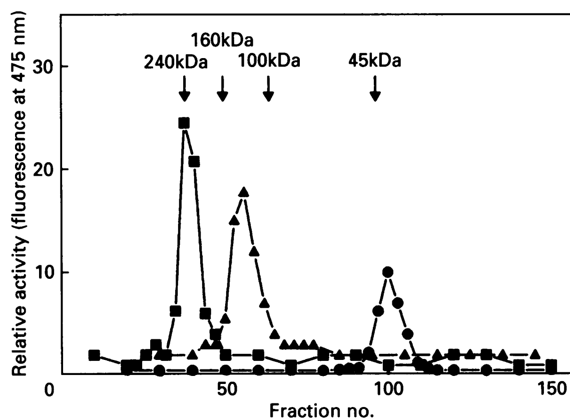

Figure 1 Identification of a calpain activator protein in human lysates

Erythrocytes (5 ml) were lysed in 10 vol. of 1 mM EDTA, pH 7.5, followed by sonication (six bursts of 10 s each). The membranes were removed by centrifugation at 100000 *g* for 20 min and the clear supernatant was collected and concentrated to 4 ml by ultrafiltration on a YM-10 Diaflo ultrafiltration membrane. The concentrated lysate was then applied to a Sephadex G-200 column (1 cm × 150 cm), previously equilibrated with 50 mM sodium borate buffer, pH 7.5, containing 0.1 mM EDTA and 2-mercaptoethanol, and eluted with the same buffer. The flow rate was 0.1 ml/min and fractions of 1 ml were collected. Calpain activity (▲) was assayed in the eluted fractions using globin as substrate (see the Materials and methods section). Calpastatin (■) and calpain activator activity (●) were assayed, using 0.025 ml and 0.1 ml respectively of the eluted fractions, previously heated at 90 °C for 3 min to inactivate possible contamination by proteolytic activities. The arrows indicate the elution volume of standard proteins (beef liver catalase, 240 kDa; rabbit muscle aldolase, 160 kDa; yeast G6PD, 100 kDa; chicken egg albumin, 45 kDa).

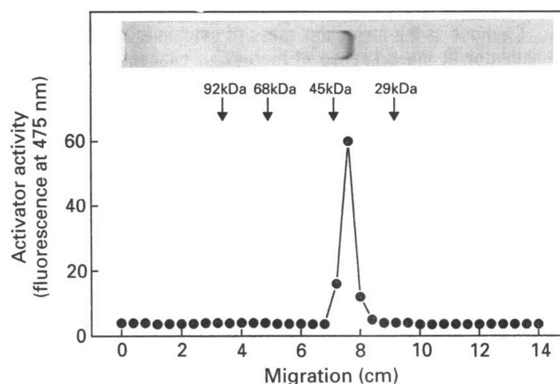

Figure 2 SDS/PAGE of the purified erythrocyte activator

The activator (1.2 μg), purified as described in the Materials and methods section, was loaded on a SDS/PAGE gel in two different lanes. The electrophoretic run was carried out as previously described [31]. The gels were then immersed in 0.02 M phosphate buffer, pH 7.0, containing 20% methanol, to remove excess SDS. After 20 min the gels were transferred to the same buffer without methanol and gently stirred for an additional 20 min. One lane was stained with silver [32]; the other lane was cut into 0.4 cm slices. Each slide was homogenized in 0.3 ml of 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA and 0.5 mM 2-mercaptoethanol. The gel was removed by centrifugation and the clear supernatants were collected and tested for the activator activity (●). The arrows indicate the migration, in a distinct lane, of standard proteins (rabbit muscle phosphoryliase *b*, 92 kDa; BSA, 68 kDa; chicken egg albumin, 45 kDa), stained by the silver staining method.

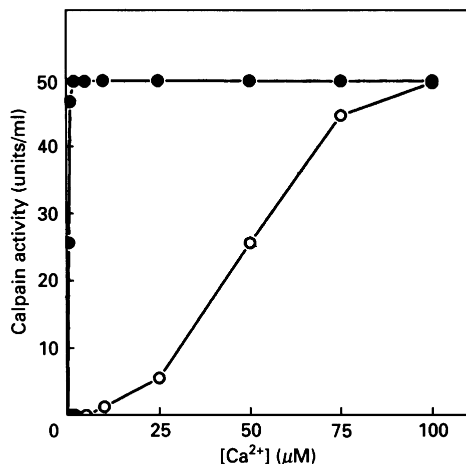

Figure 3 Effect of the activator protein on the Ca^{2+} requirement of human erythrocyte calpain

Erythrocyte calpain activity (2.2 units) was measured under standard conditions in the absence (○) or the presence (●) of erythrocyte activator (2.2 units) at the indicated concentrations of Ca^{2+}.

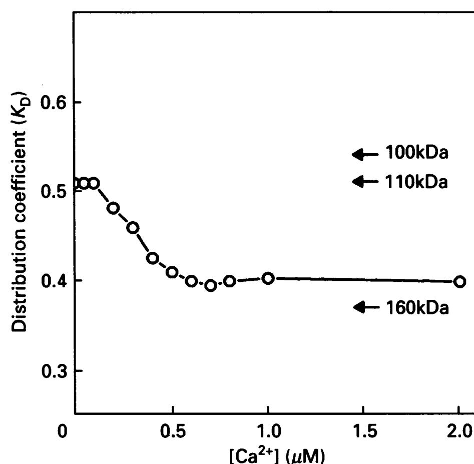

Figure 4 Changes in the molecular mass of calpain induced by incubation with the activator in the presence of increasing concentrations of Ca^{2+}

The changes in K_D values, following exposure of CM-calpain to the activator protein in the presence of the indicated concentrations of Ca^{2+} were evaluated as described in the Materials and methods section. The correlation between K_D and the molecular mass of the protein samples was determined using standard proteins: rabbit muscle aldolase (160 kDa), human erythrocyte calpain (110 kDa), yeast G6PD (100 kDa). Each point is the mean of three different experiments.

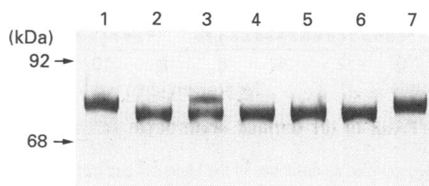

Figure 5 Effector of the activator on the rate of autoproteolysis of human erythrocyte calpain

Native clapain (lane 1) was incubated in 50 mM sodium borate, pH 7.5, with 1 mM Ca^{2+} for 20 s (lanes 2 and 6) or with 1 μM Ca^{2+} and saturating amounts of activator for 10 s (lane 3), 20 s (lane 4) or 30 s (lane 5). In lane 7, calpain was incubated with 1 μM Ca^{2+} alone. To all incubations were added (final concentrations) 0.06 M Tris buffer, pH 6.8, 2% SDS, 20% glycerol and 0.275 M 2-mercaptoethanol; the samples were heated at 100 °C for 2 min and submitted to SDS/PAGE as previously described [33].

Effect of the activator on the Ca^{2+} requirements of calpain

As shown in Figure 3, human erythrocyte calpain activity becomes appreciable only at concentrations of Ca^{2+} above 20 μM, whereas full catalytic efficiency is reached at approx. 100 μM Ca^{2+} (with $\frac{1}{2}V_{max.}$ at 50 μM Ca^{2+}). When the activator was present, calpain activity became detectable at Ca^{2+} concentrations below 1 μM and reached a maximal rate at approx. 2 μM Ca^{2+}. Thus an increased sensitivity to Ca^{2+} is apparently the mechanism by which this activating protein produces its effects. This conclusion is further supported by the results of experiments designed to establish the concentration of Ca^{2+} required for the formation of the calpain–activator complex. As shown in Figure 4, complex formation occurred only in the presence of Ca^{2+}, became detectable at concentrations of Ca^{2+} of approx. 0.2 μM, and reached completion at 0.5–0.6 μM. On the basis of its molecular mass (approx. 150 kDa), it has been calculated that the complex contains one molecule each of calpain and the activator protein.

Identification of the mechanism of action of the calpain activator

The basic mechanism of activation of native calpain has been shown to proceed through autoproteolytic conversion to an active calpain form [2,5]. In order to verify whether the activator effect is to decrease the amount of Ca^{2+} required to trigger the autoproteolytic activation, or whether it results from a direct protein–protein interaction, we have evaluated the efficiency of the activator protein in promoting autoproteolysis of calpain at low concentrations of Ca^{2+}.

As shown in Figure 5, following exposure for a short period of time (20 s) to 1 mM Ca^{2+}, calpain undergoes autoproteolytic conversion to a new enzyme form, displaying a molecular mass of approx. 75 kDa. If the concentration of Ca^{2+} was reduced to 1 μM, no conversion of the native form could be detected, even after a prolonged period of time. However, when the activator was added to the same incubation mixture, the rate of autoproteolysis increased significantly and became comparable with that observed in the presence of 1 mM Ca^{2+}. The finding that, in the presence of the activator or of 1 mM Ca^{2+}, an identical molecular species is produced, is also supported by the observation that in both cases the new calpain form is irreversibly activated and is able to express maximal catalytic activity at micromolar Ca^{2+} concentrations (results not shown).

Specificity of the calpain activator

The properties of the calpain activator protein from human erythrocytes so far described are very similar to those of the corresponding protein previously identified and isolated from rat skeletal muscle [12]. This activator, however, was shown to possess an absolute specificity, being effective only with calpain II and not with calpain I isoforms. In order to establish if the human erythrocyte activator has similar specific properties, we have tested its efficiency with respect to calpain I and II isolated from different sources. As shown in Table 2, the activator had no effect on the $V_{max.}$ of any of the calpains tested, independent of their type or origin. However, the activator reduced to a significant extent the Ca^{2+} requirement of calpain II from rat skeletal muscle and vascular smooth muscle, and of calpain I forms characterized by a Ca^{2+} requirement approx. 10-fold higher than that of typical calpain I isoenzymes.

These results indicate that the action of the activator protein represents a highly selective mechanism that allows all calpains having a Ca^{2+} requirement greatly above normal intracellular

Table 2 Effect of the activator on the catalytic properties of calpain isoforms from different sources

Calpain was purified from the different sources, as reported in the Materials and methods section. The Ca^{2+} requirement was determined by assaying calpain activity in the absence or the presence of saturating amounts of the activator and increasing concentrations of Ca^{2+} (from 0 to 1000 μM)

Source of calpain	Calpain type	V_{max} (units/mg)		$[Ca^{2+}]$ required for V_{max} (μM)	
		+Activator	−Activator	−Activator	+Activator
Human erythrocytes	I	1908±60	1968±68	120±25	2±0.4
Rat erythrocytes	I	1160±45	1205±50	110±15	2±0.4
Human neutrophils	I	607±20	670±30	120±10	5±1
Murine erythroleukemia cells	I	577±22	588±25	120	2±0.5
Rat skeletal muscle	I	550±30	578±36	7±2	7±2
Rat skeletal muscle	II	448±30	470±27	600±50	12±2
Vascular smooth muscle	I	255±10	260±12	6±2	6±1
Vascular smooth muscle	II	298±20	350±18	650±60	16±3

Table 3 Distribution and solubilization of calpain activator in human erythrocytes

Packed erythrocytes (10 ml) were lysed in 10 vol. of 5 mM sodium phosphate buffer, pH 8 (5P8), or in 1 mM EDTA followed by sonication (six bursts of 10 s each), and centrifuged at 40000 **g** for 10 min. The cytosolic fraction was treated at 90 °C for 3 min, and the denatured proteins were removed by centrifugation. Solid ammonium sulphate (80% final concentration) was then added to the clear supernatant. The precipitated proteins were collected by centrifugation and dissolved in 1 ml of 50 mM sodium borate buffer, pH 7.5, containing 0.5 mM 2-mercaptoethanol and 0.1 mM EDTA, and loaded on a Sephadex G-200 column (1 cm × 150 cm) equilibrated in the same buffer. The activator activity was assayed in aliquots (0.1 ml) of the eluted fractions, as described in the Materials and methods section. The membrane fraction was washed with the lysing buffer until the haemoglobin was completely removed, then resuspended in 1 ml of 1 mM EDTA and sonicated (three bursts, 10 s each). The clear supernatant, obtained after centrifugation at 200000 **g** for 10 min, was heated at 90 °C for 3 min and then treated as the cytosolic fraction. Membranes prepared from 10 ml of packed erythrocytes following lysis with 5 mM sodium phosphate buffer, pH 8, were utilized to investigate solubilization of the calpain activator. The following treatments all resulted in 100% solubilization: 1 mM EDTA plus sonication, 1 M NaCl, 0.2% Triton X-100, deoxycholate (10 mg/ml) and butanol/water (1:1, v/v).

Lysis conditions	Activator activity (units/ml of packed erythrocytes)	
	Cytosol	Membranes
Hypotonic solution	15	140
EDTA (1 mM) plus sonication	145	10

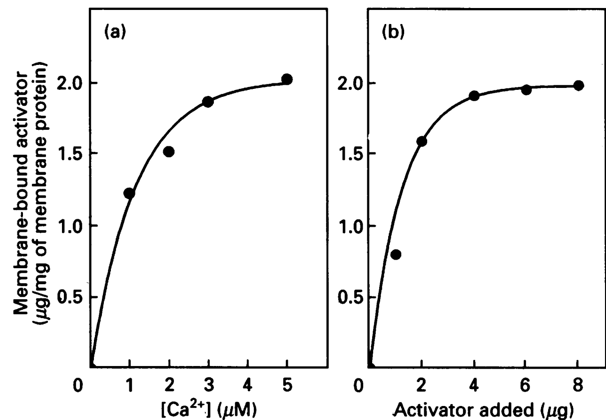

Figure 6 Binding of the activator to erythrocyte membranes

(**a**) Purified activator (20 units) was incubated with 1 mg of inside-out vesicles at 20 °C for 15 min at the indicated concentrations of Ca^{2+}. The incubation was then centrifuged, the supernatant removed and the pellet suspended in 0.3 ml of 0.1 mM EDTA. The mixture was kept at 20 °C for 10 min and then centrifuged at 200000 **g** for 10 min. Activator activity was assayed in the supernatants as described in the Materials and methods section. (**b**) Erythrocyte inside-out vesicles (1 mg of protein) were incubated with increasing amounts of the activator in the presence of 5 μM Ca^{2+}, following the procedure described in (**a**). The activator activity was assayed as in (**a**).

concentrations of the ion to express proteolytic activity at physiological concentrations. The precise mechanism by which this overall process occurs in the cell, and the precise intracellular roles of calpain I and II, are still to be defined.

Subcellular distribution of the calpain activator

We have established previously that, in human neutrophils, the calpain activator is recovered in association with the particulate fraction of the cell, probably the cytoskeletal network [11]. A similar subcellular distribution is also characteristic of the human erythrocyte calpain activator. As shown in Table 3, following lysis of the cells with a hypotonic solution, the activator molecule was recovered in association with the plasma membrane, whereas in the presence of chelating agents such as EDTA or high concentrations of salt, the activator was obtained in a soluble form.

These results are indicative of an ionic interaction of the activator with the membrane, but also suggest that the binding to specific site(s), probably protein(s), is mediated by the presence of a metal ion. Treatment with detergents such as deoxycholate or Triton X-100, both of which destroy the organization of the membrane, or with organic solvents, also promoted the release of the activator from its bound form.

The nature of the component(s) involved in the association between the activator and the plasma membrane was investigated in a reconstituted system containing the purified activator and various preparations of erythrocyte vesicles. The results obtained indicate that the activator binds to inside-out vesicles only in the presence of Ca^{2+} (Figure 6a). At a Ca^{2+} concentration of approx. 1 μM, 60% of maximal binding occurred; at 2–3 μM, binding was complete. The association with the membrane follows a saturating curve (Figure 6b), indicating the involvement of a discrete binding site. At saturation the amount of activator bound was approx. 2 μg/mg of membrane protein.

To identify the nature of the binding site on the plasma

Table 4 Binding of activator to different vesicle preparations as a function of Ca^{2+} concentration

The membrane vesicles (1 mg of membrane protein) were incubated with 20 units (corresponding to 3.8 μg) of purified activator as described in the Materials and methods section and in the legend to Figure 6, with the indicated additions. The membranes were then collected by centrifugation at 200000 **g** for 10 min and the supernatants were discarded. The membrane-bound activator was assayed following solubilization with 0.1 mM EDTA. Alternatively, membrane vesicles were substituted with 1 mg of the phospholipid mixtures. n.d., not detectable.

Vesicle preparation	Additions	Membrane-bound activator (units)
Inside-out membranes	EDTA, 1 mM	n.d.
	Ca^{2+}, 2μM	9.2
Stripped membranes	EDTA, 1 mM	n.d.
	Ca^{2+}, 2 μM	9.09
Stripped membranes digested with trypsin	EDTA, 1 mM	n.d.
	Ca^{2+}, 2 μM	0.55
Membrane lipid vesicles	EDTA, 1 mM	n.d.
	Ca^{2+}, 2 μM	n.d.
	Ca^{2+}, 5 μM	n.d.
	Ca^{2+}, 50 μM	n.d.
PS + PE + PC vesicles	EDTA, 1 mM	n.d.
	Ca^{2+}, 50 μM	n.d.

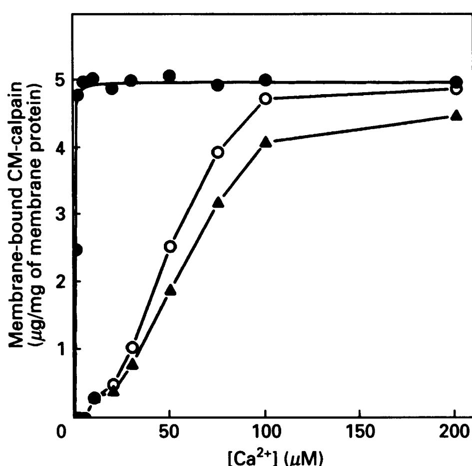

Figure 7 Effect of the activator on the binding of CM-calpain to various vesicle preparations

Calpain activator was bound to inside-out vesicles as reported in Figure 6; the resulting membranes contained 1.9 μg of activator/mg of membrane protein. CM-calpain (10 μg, 900 c.p.m.) was incubated with inside-out vesicles containing the activator (0.5 mg of protein) in the presence of the indicated concentrations of Ca^{2+} (●). As controls, inside-out vesicles without the activator (○) or a phospholipid vesicle preparation containing PS, PE and PC (see Table 4) (▲) was incubated as above with CM-calpain. After 5 min at 20 °C, the vesicles were recovered as pellets by centrifugation at 200000 **g** for 10 min. The amount of calpain bound to membranes was calculated by the radioactivity recovered in association with the vesicles, using the specific radioactivity of CM-calpain.

membrane, we have evaluated the association of the activator with different preparations of vesicles. As shown in Table 4, the activator associated with high efficiency both to inside-out vesicles and to stripped membranes, but only if Ca^{2+} was also present. Vesicles containing only erythrocyte membrane lipids, obtained following extraction in organic solvents (see the Materials and methods section), or containing equivalent mixtures of commercially available PS, PC and PE, bound negligible amounts of the activator. In agreement with the data reported in Table 3, these observations suggest that the association of the activator with the membrane is mediated by ionic interactions with intrinsic membrane protein(s), with the participation of Ca^{2+} ions. This conclusion is also confirmed by digestion of stripped membranes with trypsin, which abolished almost completely the association of the calpain activator with the membranes.

Effect of the activator protein on the binding of calpain to plasma membranes

For these experiments, calpain was first carboxymethylated with [^{14}C]iodoacetate, in order to prevent its autoproteolytic activation and release from the membranes during the exposure to Ca^{2+}.

As shown in Figure 7, binding of calpain to inside-out vesicles or to lipid vesicles requires the presence of Ca^{2+}. Binding became appreciable only at a concentration of 30 μM Ca^{2+}, and reached maximal values at around 100 μM Ca^{2+}. If vesicles were pre-incubated with the activator protein, however, initial binding occurred at very low Ca^{2+} concentrations, reaching a maximum at approx. 2 μM. The different kinetics of binding induced by the presence of the activator at low concentrations of Ca^{2+} further emphasize the role of the activator in a site-directed activation of calpain under physiological conditions.

DISCUSSION

Calpain, the neutral thiol proteinase present in almost all mammalian cells, contains a built-in domain which covers the active site and therefore maintains the enzyme in an inactive state [26]. Binding to Ca^{2+} induces rearrangement of the molecular conformation and promotes expression of catalytic activity. Two classes of calpain, type I and type II, are frequently present in the same cell. Type I calpain requires for activity a concentration of Ca^{2+} that is close to physiological values (1–10 μM), whereas the concentrations required [1–4] by type II isoenzymes are much higher than those normally present in the cell, and only occur under pathological conditions. Because of these molecular and kinetic properties, a physiological role could be attributed to calpain I, whereas participation in the aspecific process of intracellular protein breakdown could be suggested as the predominant or exclusive function of calpain II. A variety of experimental evidence tends, however, to exclude such a strict distinction. The most relevant of these findings is the observation that in several cell types only a single calpain isoform exists, with intermediate Ca^{2+} requirements (50–100 μM), which are still higher, however, than occur under normal conditions [4].

Thus, in order to clarify the physiological role of high-[Ca^{2+}]-requiring calpains, the presence of a mechanism capable of decreasing the Ca^{2+} requirement of these isoforms has been carefully explored. As a result of these investigations, it has been established that the basic general mechanism that promotes the conversion of high-Ca^{2+}- to low-Ca^{2+}-requiring calpain forms is an autoproteolytic process which removes the so-called active site masking domain [2].

However, the problem still remained partially unresolved, since the concentration of Ca^{2+} required for autoproteolysis to occur corresponds exactly to that inducing the expression of catalytic activity of the native proteinase; probably the two events are sequential to each other. Thus the nature of the molecular mechanism promoting the autoproteolytic process under physiological conditions remained unknown, even though high concentrations of substrate [5] or association of calpain

with the phospholipid membrane bilayer [7,27,28] have both been shown to accelerate the autoproteolytic conversion. The first mechanism, however, is too slow, whereas the second mechanism can decrease Ca^{2+} requirements by only one-third and again is rather slow.

An important advancement in the overall understanding of the mechanism of calpain activation and of the physiological role of the proteinase was made by the discovery of a specific activatory protein factor. The activator was originally isolated from brain cells [8,9] and shown to affect preferentially the $V_{max.}$ of calpain, but more recently we have identified in human neutrophils [11] and rat skeletal muscle [12,13] a calpain-stimulating factor which decreases by approx. 100 times the Ca^{2+} concentration required for activation of the proteinase.

Since the activator was shown to be highly specific for the calpain II isoform, it was of interest not only to establish whether the activator is present in cells containing a single calpain form, but also to compare the properties of this stimulating factor with those of the activators identified in other cells and tissues [8–13]. In the present paper we demonstrate the presence of a calpain activator in human erythrocytes, and provide new information on its localization on the inner surface of the plasma membrane, in a Ca^{2+}-mediated association with an intrinsic membrane protein. The characteristics of the calpain–activator complex have been established. It was found to be composed of equimolar amounts of the two proteins and to produce, in a few seconds by autoproteolytic conversion, a calpain form which is constitutively active at micromolar concentrations of Ca^{2+}.

The mechanism of action of the erythrocyte activator is similar to that previously shown for the corresponding protein factor isolated from rat skeletal muscle [13]. These observations, taken together with the finding that the human erythrocyte activator can express its stimulatory activity with calpain II isoforms from other tissues, suggest that this natural activating factor is an essential component of the general activating mechanism of calpain. This is also supported by the fact that the level of calpain greatly exceeds that of the activator protein, both in human erythrocytes and in other cells [8–13], suggesting a catalytic role for this factor in the overall process.

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