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# An endogenous activator of the $Ca^{2+}$-dependent proteinase of human neutrophils that increases its affinity for $Ca^{2+}$

(calpain/calpain activator/calpain inhibitor/cytoskeleton)

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ABSTRACT **An endogenous activator of the $Ca^{2+}$-dependent proteinase (calpain) has been identified in human neutrophils. In the presence of the activator, the affinity of calpain for $Ca^{2+}$ is increased by >100-fold and maximum catalytic activity is observed with $Ca^{2+}$ concentration below 1 $\mu$M. The activator is a heat-stable protein having an apparent molecular mass of ≈40 kDa. It appears to be associated with the cytoskeletal fraction of human neutrophils. Neutrophils also contain an endogenous cytosolic calpain inhibitor (calpastatin), which is readily separated from the activator by size-exclusion chromatography. The effects of the activator and inhibitor appear to be antagonistic and may constitute a physiological mechanism for modulating intracellular calpain activity.**

Most animal cells and tissues thus far examined contain cytosolic $Ca^{2+}$-dependent neutral proteinases (1)—CANP (2) or calpain (ref. 3; for reviews, see refs. 4 and 5). These are usually isolated as heterodimers (2, 5) composed of a large catalytic subunit (70–80 kDa) and smaller (25–30 kDa) subunit; the latter may have a regulatory function. Some calpains appear to lack the smaller subunit and are isolated as monomers (6, 7) or dimers containing only the 80-kDa subunit (8).

Calpains also differ in requiring either millimolar (type I) or micromolar (type II) concentrations of $Ca^{2+}$ (4, 5). Conversion of calpain I to calpain II, often referred to as activation, may occur by limited autoproteolysis; in human erythrocytes, this type of activation of the catalytic subunit is observed after dissociation of the "regulatory" subunit (9, 10) or after association of calpain with the cell membrane (4, 11). Calpain isolated from human neutrophils, on the other hand, is reversibly activated by binding to the cell membrane without autoproteolysis (12). Activation of calpains I and II by a protein present in bovine brain, without altering their affinity for $Ca^{2+}$, has also been reported (13).

Endogenous inhibitors—calpastatins (4)—first isolated in the laboratories of Suzuki (14) and Murachi (15) also appear to function as physiological regulators of calpain activity. The calpain inhibitor of rabbit liver was isolated as a tetramer containing four subunits with a molecular mass of ≈60 kDa (8); cloning the cDNA for this inhibitor established a subunit mass of 68,113 Da for the polypeptide and revealed the presence of four consecutive internal repeats (17).

In the present report, we describe the isolation from human neutrophils of a protein activator that is a component of the cytoskeletal fraction. This activator, which has a molecular mass of ≈40 kDa, increases the $Ca^{2+}$ sensitivity of neutrophil calpain by >100-fold. We also describe the isolation of an endogenous inhibitor and its effects on the activated and nonactivated forms of calpain.

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## METHODS

Neutrophils were separated from freshly collected human blood (18) and lysates were prepared as described in the figure legends. Calpain was purified from the cytosolic fraction (18) and its activity was routinely measured in the presence of 1 mM $Ca^{2+}$ using fluorescamine (Hoffmann–La Roche) to measure the release of acid-soluble peptides (19) from acid-denatured human globin (7, 8). One unit of proteinase activity was defined as the amount that generates 1 $\mu$mol of free amino groups per min under these conditions. The same assay was used to measure calpastatin activity with reaction mixtures containing 1 unit of calpain, with or without appropriate amounts of the inhibitor solution. One unit of calpastatin was defined as the quantity required to inhibit 1 unit of the proteinase activity under the specified conditions. For assay of calpain activator, the concentration of $Ca^{2+}$ was reduced to 1 $\mu$M. At this concentration of $Ca^{2+}$, the activity in the absence of activator was negligible. The unit of calpain activator was defined as the amount that produces 1 unit of proteolytic activity under these conditions. Before assay, the solutions containing the activator were heated for 2 min in a water bath at 100°C to inactivate any calpain that may have been present. Heating does not affect the activity of either the activator or of calpastatin. Protein was determined by the method of Bradford (20).

## RESULTS

**Purification of the Calpain Activator.** Filtration of concentrated lysates of human neutrophils (see *Methods*) on Sephadex G-200 revealed the presence of a calpain activator that emerged in a symmetrical peak following the fractions containing the endogenous inhibitor (Fig. 1). Based on its elution volume the molecular mass of the activator was estimated to be 35–40 kDa. The fractions from gel chromatography containing the calpain activator were concentrated and chromatographed on a DEAE-52 column, which completed the separation of the activator from the inhibitor activity (Fig. 2).

**Effect of the Activator on the $Ca^{2+}$ Requirement of Neutrophil Calpain.** In the presence of 1.0 $\mu$M $Ca^{2+}$, calpain activity in the absence of the activator was barely detectable, <1% of that observed with 1.0 mM $Ca^{2+}$ (Fig. 3). The addition of 4 $\mu$g of activator to an assay mixture containing 1.0 $\mu$M $Ca^{2+}$ and 3 $\mu$g of purified calpain resulted in activity that was comparable to, and often significantly greater than, that observed in the assay with 1.0 mM $Ca^{2+}$ alone. Very little effect of the activator was observed in the presence of 1.0 mM $Ca^{2+}$.

The activator appears to greatly increase the affinity of neutrophil calpain for $Ca^{2+}$. Nearly maximal activity was

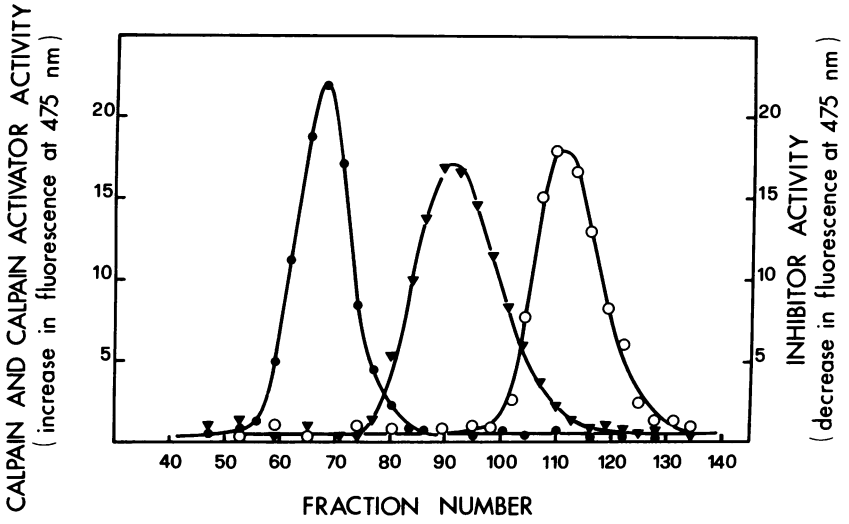


FIG. 1. Separation of calpain inhibitor and activator by Sephadex G-200 chromatography. Neutrophils ($2.5 \times 10^9$ cells) were suspended in 10 ml of 0.25 M sucrose containing 10 mM Hepes, 10 mM mercaptoethanol, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and lysed by sonication. The particulate fraction was discarded after centrifugation of the lysates at 100,000 x *g* for 10 min. The clear supernatant was collected, concentrated to 2 ml by ultrafiltration on an Amicon YM-10 membrane, and loaded onto a Sephadex G-200 column (1.5 × 120 cm) previously equilibrated with 50 mM sodium borate (pH 7.5) containing 0.1 mM EDTA. The flow rate was 12 ml/hr and 2-ml fractions were collected. Calpain activity (●) was assayed on 0.05-ml aliquots of the indicated fractions. The calpain inhibitor (▼) and activator (○) activities were evaluated using 0.2-ml aliquots of the indicated fractions previously heated to 100°C for 2 min. The column was standardized with the following proteins: rabbit muscle aldolase, 160 kDa; bovine serum albumin, 67 kDa; horse myoglobin, 17 kDa.

observed with less than micromolar concentrations of $Ca^{2+}$ (Fig. 4 *Inset*) compared to 100 $\mu$M $Ca^{2+}$ required for full activity in its absence.

The effect of the activator is completely reversible and does not involve autoproteolytic activation of the proteinase, since after removal of $Ca^{2+}$ with chelating agents and filtration through a Sephadex G-100 column, the two proteins can be separated and the recovered calpain exhibits the catalytic properties of the native form (Table 1).

**Effects of the Endogenous Calpastatin.** In the presence of the activator, the calpastatin suppressed the calpain activity assayed with either millimolar or micromolar $Ca^{2+}$ (Fig. 5). At the higher concentration of $Ca^{2+}$, the same effect of calpastatin on the calpain activity was observed in an experiment carried out in the absence of the activator (data not shown). Thus, in the presence of millimolar $Ca^{2+}$, the activator had little or no effect on either the calpain activity (see Fig. 3) or on its sensitivity to calpastatin. Opposing effects of the activator and inhibitor are observed only at the low, more physiological, concentrations of $Ca^{2+}$. In support of this hypothesis, the activator could be shown to fully reverse the effects of calpastatin in the presence of micromolar but not millimolar concentrations of $Ca^{2+}$.

**Characterization of the Activator and Its Subcellular Distribution.** The activator was fully sensitive to chymotrypsin and partially inactivated by incubation with trypsin (data not

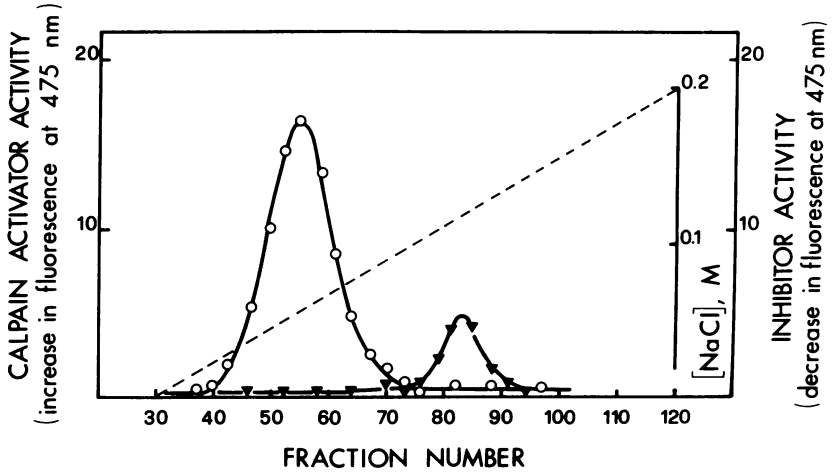


FIG. 2. Purification of the calpain activator by ion-exchange chromatography. Fractions 105–120 containing the bulk of the calpain activator activity (see Fig. 1) were pooled and concentrated to 6 ml by ultrafiltration on an Amicon YM-10 membrane. The concentrated solution was loaded onto a DEAE-cellulose (type DE 52) column (1 × 10 cm) equilibrated with 50 mM sodium borate (pH 7.5) containing 0.1 mM EDTA. The flow rate was 1 ml/min and 2-ml fractions were collected. The column was washed with 6 column vol of buffer and the absorbed proteins were eluted with a linear gradient of sodium chloride from 0 to 0.2 M (100 ml + 100 ml) in the same buffer. Calpain inhibitor (▼) and activator (○) activities were assayed on 0.2-ml aliquots of the indicated fractions, previously heated to 100°C for 2 min. The fractions containing the highest activator activity were pooled (fractions 45–65), concentrated to 2 ml, heated for 2 min at 100°C, and centrifuged at 25,000 x *g* for 10 min. The resulting clear solution was used as the source of the calpain activator. Fractions 76–86, containing the endogenous calpastatin, were pooled and concentrated to 2 ml and heated and centrifuged as described above. This solution was used as the source of calpastatin.

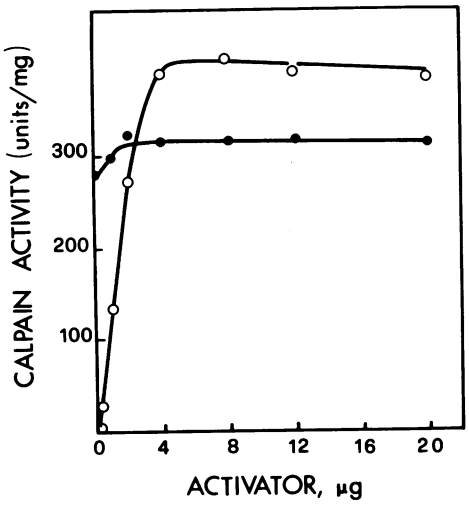


FIG. 3. Effect of increasing amounts of activator on the activity of calpain at 1 μM $Ca^{2+}$ and 1 mM $Ca^{2+}$. Calpain activity was assayed (see *Methods*) with 1 μM $Ca^{2+}$ (○) or with 1 mM $Ca^{2+}$ (●), using 1 unit (3 μg) of the enzyme and the indicated quantities of activator.

shown), supporting the conclusion that it is a protein. When neutrophil lysates were fractionated on a Percoll gradient, the bulk of the activator emerged in a fraction between the plasma membrane and granule/mitochondria fractions; this fraction did not contain any of the marker proteins used to characterize the subcellular fractions (Table 2, experiment 1, unidentified fraction). This unidentified fraction was suspected to contain the cytoskeletal proteins. To confirm this localization of the activator, the Triton X-100 insoluble fraction was prepared and analyzed (experiment 2). More than 80% of the calpain activator activity was recovered in this fraction. The effect of the activator was detectable in either the solubilized or the insoluble cytoskeletal fractions. Whether the activator corresponds to any of the known cytoskeletal proteins remains to be determined.

## DISCUSSION

We have previously characterized the calpain of human neutrophils as a monomeric protein having a molecular mass

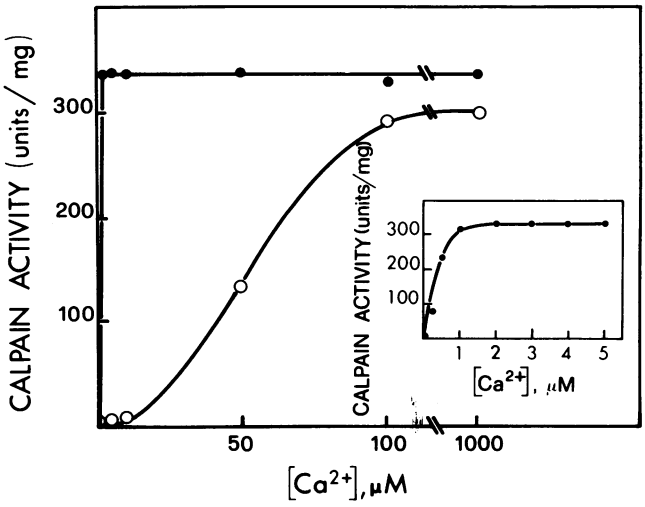


FIG. 4. Effect of the activator on the calcium requirement of neutrophil calpain. Calpain (1 unit, 3 μg) was assayed with the indicated concentrations of calcium in the absence (○) or in the presence (*Inset*; ●) of 4 μg of activator.

Table 1. Reversibility of the activation of neutrophil calpain

| Addition | Treatment | Calpain activity (units/mg) assayed at μM $Ca^{2+}$ | Calpain activity (units/mg) assayed at mM $Ca^{2+}$ |
|---|---|---|---|
| None* | None | <3 | 297 |
| Purified activator* | None | 315 | 307 |
| Purified activator† | EDTA and gel chromatography | <3 | 286 |

*Calpain activity (1 unit, 3 μg) was assayed in the presence of 1 μM $Ca^{2+}$ or 1 mM $Ca^{2+}$ with or without 4 μg of activator as described in *Methods*.

†Purified calpain (22 μg) was incubated for 10 min at 37°C in 0.5 ml of 50 mM sodium borate buffer (pH 7.5) containing 1 μM $Ca^{2+}$ and 30 μg of activator. EDTA, at the final concentration of 0.1 mM, was then added and the solution was applied onto a Sephadex G-100 column (0.8 × 50 cm) previously equilibrated with 50 mM sodium borate buffer (pH 7.5) containing 0.1 mM EDTA. The flow rate was 15 ml/hr and 1-ml fractions were collected. Calpain activity was recovered in fractions 15–20 and the activator was in fractions 20–27.

of 80 kDa (12). The purified enzyme requires 0.1 mM $Ca^{2+}$ for full catalytic activity but the affinity for $Ca^{2+}$ is greatly enhanced when the enzyme is tested in the presence of a neutrophil membrane fraction; under these conditions, 60% of the maximal catalytic activity was found to be expressed in the presence of 5 μM $Ca^{2+}$. This activation was reversed when the enzyme was released from the membranes (12). The observation that this activation could be reproduced by a mixture of phospholipids extracted from neutrophil membranes and the fact that erythrocyte calpain is similarly activated by binding to erythrocyte membranes, or in the presence of phospholipids extracted from erythrocyte membranes, supported the hypothesis that this mechanism operated *in vivo* (9, 22). Proteolytic modification of membrane proteins in erythrocytes (ref. 9; for a review, see ref. 23) or of membrane-bound protein kinase C in human neutrophils (18, 24, 25) and platelets (26) is attributable to this mechanism for the activation of calpain.

An additional mechanism for the activation of neutrophil calpain is provided by the results reported in the present work and may be responsible for a distinct set of biochemical responses (e.g., degranulation) in activated neutrophils. The

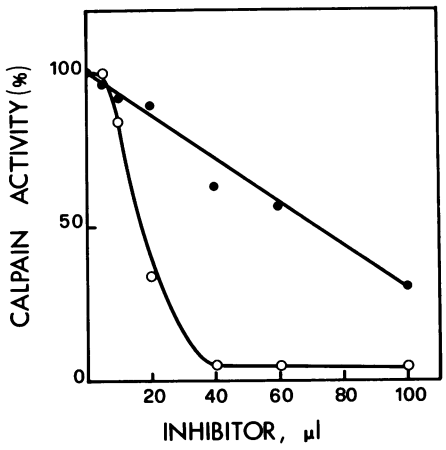


FIG. 5. Effect of the endogenous inhibitor purified from human neutrophils on activated neutrophil calpain. Purified calpain (see *Methods*; 1 unit, 3 μg) was assayed in the presence of 4 μg of activator and with 1 μM $Ca^{2+}$ (○) or with 1 mM $Ca^{2+}$ (●). Inhibitor purified from human neutrophils (see Fig. 2) was added as indicated. The values for 100% were 305 units/mg and 320 units/mg for the enzyme treated in the presence of millimolar or micromolar $Ca^{2+}$, respectively.

Table 2. Distribution of calpain activator and inhibitor activities in human neutrophils

| Subcellular fraction | Activator activity, unit(s) per $10^6$ cells | Inhibitor activity, unit per $10^6$ cells |
|---|---|---|
| Experiment 1 | | |
| Cytosol | 0.33 (15) | 0.40 (71) |
| Plasma membrane | 0.08 (4) | 0.03 (5) |
| Unidentified fraction | 1.60 (73) | 0.03 (5) |
| Granules | 0.04 (2) | 0.02 (4) |
| Nuclei | 0.06 (3) | 0.01 (2) |
| Experiment 2 | | |
| Soluble fraction | 0.42 (19) | 0.48 (85) |
| Solubilized cytoskeletal fraction | 1.60 (73) | 0.06 (11) |
| Intact cytoskeletal fraction | 1.38 | 0.06 |

Experiment 1: Neutrophils ($2 \times 10^8$ cells) were disrupted in 2 ml of 0.25 M sucrose containing 1 mM EDTA using 50 strokes of a glass-Teflon homogenizer. The homogenate was loaded onto a 55% Percoll solution containing 2 mM $MgCl_2$ and 0.25 M sucrose and centrifuged at 40,000 rpm for 18 min in a Spinco type 65 rotor. The subcellular fractions were identified as described (21) with the following markers: 5'-nucleotidase for plasma membranes, lysozyme for granules, succinate dehydrogenase for mitochondria, DNA for nuclei, and NADH-ferricyanide reductase for endoplasmic reticulum. The indicated fractions were collected and Percoll was removed by a second centrifugation at 60,000 × *g* for 30 min. Each of the particulate fractions was solubilized by sonication in the presence of 0.2% Triton. Each fraction was concentrated and chromatographed directly on a DEAE 52 column as described in the legend to Fig. 2 and aliquots of the pooled fractions containing inhibitor or activator were assayed (see *Methods*). Experiment 2: Neutrophils ($5 \times 10^6$ cells) were lysed in 1 ml of 50 mM Tris·HCl (pH 7.5) containing 160 mM KCl, 10 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The cytoskeletal fraction was collected by centrifugation at 12,000 × *g* and washed twice with 5 ml of the same lysing buffer. The final pellet was resuspended in 0.5 ml of the same buffer without Triton X-100. Aliquots (0.2 ml) were solubilized by sonication (six strokes, 10 sec each). Assays for inhibitor and activator were carried out after chromatography on DEAE 52 as described for Experiment 1 except for the solubilized cytoskeletal fraction, which was assayed directly. The values in parentheses represent the percentage of recovery of the activator and inhibitor activities, based on their content in the crude extracts calculated from an experiment similar to that shown in Fig. 1. The levels were 2.20 units per $10^6$ cells and 0.56 unit per $10^6$ cells for the activator and inhibitor, respectively.

degranulation response appears to be correlated with phosphorylation of cytoskeletal components and the proteolytic degradation by calpain of at least one of these phosphorylated proteins (16, 27). Thus, in activated neutrophils, the degranulation response appears to involve phosphorylation of several cytoskeletal components by the proteolytically modified form of protein kinase C and digestion of the phosphorylated 20-kDa component by calpain. The ability of cytosolic calpain in activated neutrophils to digest this phosphorylated protein (16, 27), presumably in the presence of less than micromolar concentrations of $Ca^{2+}$, prompted the present search for a cytosolic activator and particularly for an activator associated with the cytoskeletal fraction. The cytoskeletal protein described in the present work fulfills the requirements for a physiological activator; it increases the affinity of neutrophil calpain for $Ca^{2+}$ by >100-fold, and in the presence of the activator significant calpain activity is expressed in the presence of 0.25 $\mu$M $Ca^{2+}$ (see Fig. 4). This is in contrast to the activator isolated from human brain (13), which increases the catalytic activity without altering the affinity for $Ca^{2+}$.

The presence of a soluble calpastatin that fully antagonizes the effects of the activator, but only at low concentrations of $Ca^{2+}$, raises interesting questions concerning the physiological role of these modulators of calpain activity. Calpastatin would protect cytosolic proteins from proteolysis, even in the presence of elevated cytosolic $Ca^{2+}$. Association of calpain with the plasma membrane or with the cytoskeletal fraction would result in digestion of specific membrane or cytoskeletal proteins—e.g., protein kinase C in the case of membranes (18) or a phosphorylated 20-kDa polypeptide associated with the cytoskeletal fractions (16, 27). Calpain and the proteolytically modified form of protein kinase C are emerging as important components of the signal transduction mechanism of human neutrophils.

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