Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 331-338

BINDING TO ERYTHROCYTE MEMBRANE IS THE PHYSIOLOGICAL MECHANISM FOR ACTIVATION OF Ca^{2+}-DEPENDENT NEUTRAL PROTEINASE

S. Pontremoli, E. Melloni, B. Sparatore, F. Salamino, M. Michetti, O. Sacco and B.L. Horecker*

Institute of Biological Chemistry, University of Genoa, Genoa, Italy

*Roche Institute of Molecular Biology, Roche Research Center, Nutley, N.J. 07110

Received February 28, 1985

In the presence of micromolar concentrations of Ca^{2+} the catalytic 80 kDa subunit of human erythrocyte procalpain binds to the cytosolic surface of the erythrocyte membrane. Binding is rapid, highly specific and is reversed by the removal of Ca^{2+}. In the bound form the 80 kDa catalytic subunit undergoes a rapid conversion to calpain, the active 75 kDa Ca^{2+}-requiring proteinase. The activated proteinase produces extensive degradation of membrane components, particularly of band 4.1 and 2.1 proteins. Binding to membranes may represent an obligatory physiological mechanism for the conversion of procalpain to calpain.

The Ca^{2+}-dependent neutral proteinase is present in human erythrocytes as an inactive 110 kDa proenzyme (procalpain) composed of two non-identical subunits (1). The larger 80 kDa subunit gives rise to active calpain (75 kDa); the function of the smaller 30 kDa subunit remains unknown (2, 3). Inactive procalpain is converted to active calpain on exposure to high (mM) concentrations of Ca^{2+} or to low (μM) concentrations of Ca^{2+} in the presence of a digestible substrate such as denatured globin or casein (3, 4). However, the nature of the endogenous substrate that might affect this conversion and the physiological mechanism for the activation of procalpain remain unknown.

Pertinent to these questions are the observations that an increase in the concentration of intracellular Ca^{2+} results in degradation of erythrocyte membrane proteins (5) and that traces of immunoreactive calpain can be detected in human erythrocyte membranes (6). In addition, we have recently shown

Abbreviations: CM-: carboxymethyl-; G3PD: glyceraldehyde 3-P dehydrogenase.

0006-291X/85 $1.50

that calpain plays a dual role in the degradation of excess ß-hemoglobin chains in the intact erythrocyte (7). It catalyzes the first step in the degradation process by removing a small peptide from the NH_2-terminus of the globin chains (8). Formation of acid soluble products from the modified hemoglobin chains requires their binding to the erythrocyte membrane, and the action of intrinsic membrane acid proteinases. Binding to the membranes occurs only after modification of inner surface of the membrane by calpain (7).

We now report evidence for the Ca^{2+}-mediated binding of 80 kDa subunit of procalpain to the erythrocyte membrane, followed by a rapid conversion of the bound subunit to the active 75 kDa proteinase. Both binding and activation require only micromolar concentrations of Ca^{2+}. These observations provide an understanding of the biochemical mechanisms that mediate the cellular response to the mobilization of Ca^{2+}. Formation of a ternary complex involving Ca^{2+}, procalpain and membrane components may fulfill the two essential requirements for conversion of procalpain to the active proteinase.

MATERIALS AND METHODS

Procalpain was purified from freshly collected human erythrocytes and converted to the active calpain as described (3). The unit of activity was defined as the quantity required to produce 1 µmol of free NH_2-groups under the specified assay conditions (3) with human acid-denatured globin (9) as the substrate. The inactive 80 kDa catalytic subunit was prepared from the enzyme--inhibitor complex (10). Iodo[^{14}C]acetate (58 Ci/mol) was purchased from Amersham International. The endogenous inhibitor was isolated and assayed as described (1). One unit was defined as the quantity that inhibits one unit of calpain activity.

Carboxymethylation of the purified procalpain and calpain subunits was carried out as described (2). The specific activities of procalpain were 985 cpm/µg and 600 cpm/µg for the CM-80 kDa procalpain and 75 kDa calpain subunits, respectively.

Membrane vesicles were prepared from human erythrocytes as described (7) except that 0.1 mM EDTA, pH 8.0, replaced the 5 mM $NaPO_4$ buffer, pH 7.0, as the final medium in which the vesicles were suspended. The yield of inside--out vesicles, as determined by assay for acetylcholinesterase in the absence and in the presence of 0.1% Triton X100 (11), ranged from 65-70% of the total. Right-side out vesicles were prepared as described by Steck and Kant (11).

Binding of the carboxymethylated proteins to vesicles was measured in reaction mixtures (0.2 ml) containing vesicles (0.2 mg protein), and 10 µg of carboxymethylated protein in 50 mM sodium borate, pH 8.0 with or without $CaCl_2$ as indicated. After incubation at 25°C for 10 min, at which time binding was found to be complete, the suspension was centrifuged and the pellet washed with 1 ml of borate buffer containing the same concentration of $CaCl_2$. The washed vesicle pellet was suspended in 0.2 ml of 0.1% Triton X100 and 0.1 ml aliquots taken for radioactivity measurement.

Binding of native 80 kDa subunits was measured under the same conditions with vesicles (0.25 mg of protein) and subunit (25 µg). After removal of the vesicles by centrifugation, aliquots of the clear supernatant were assayed for proteinase activity in the presence of 5 µM and 1 mM Ca^{2+}. At the lower concentration only active calpain is detected; at the higher concentration procalpain is immediately converted to active calpain and the total activity is measured. The pellet, washed with 0.25 ml of borate buffer, was resuspended in 0.5 ml of buffer and divided into two aliquots. One was immediately treated with 0.1 mM EDTA to release the bound enzyme; the other was incubated for 2 h at 25°C in the presence of 10 µM Ca^{2+} and then treated with 0.1 mM EDTA.

RESULTS

Requirement of Ca^{2+} for the binding of the 80 kDa subunit to the erythrocyte membrane. Binding of CM-procalpain to inside-out vesicles was not detected at concentrations of Ca^{2+} below 50 µM and showed a linear increase as the concentration of Ca^{2+} was increased to 0.1 mM (Fig. 1). On the other hand, binding of the isolated CM-80 kDa procalpain subunit was observed with the lowest concentrations of Ca^{2+} tested, reaching the same maximal value. The CM-75 kDa subunit of active calpain did not bind to the membranes at any concentration of Ca^{2+}. These results identify the 80 kDa subunit as the only form capable of binding, because we have previously shown that at concentrations of Ca^{2+} above 50 uM procalpain is dissociated into its component subunits (3). No binding was observed with right-side out vesicles (data not

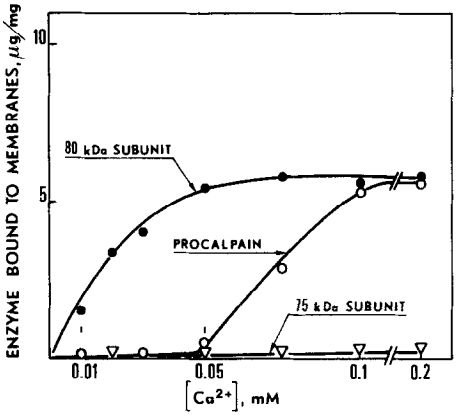

Fig 1. Requirement of Ca^{2+} for the binding of the neutral proteinase to inside-out vesicles. Binding of carboxymethylated procalpain to inside-out vesicles was carried out as described under Methods. For each enzyme form, 10 µg, corresponding to 9,850 cpm for the 80 kDa subunit or 6,000 cpm for activated calpain were incubated in 0.2 ml of borate buffer containing 0.1 mg (as protein) of inside-out vesicles and $CaCl_2$ as indicated. From the results we estimated that approximately 20,000 binding sites are present per erythrocyte.

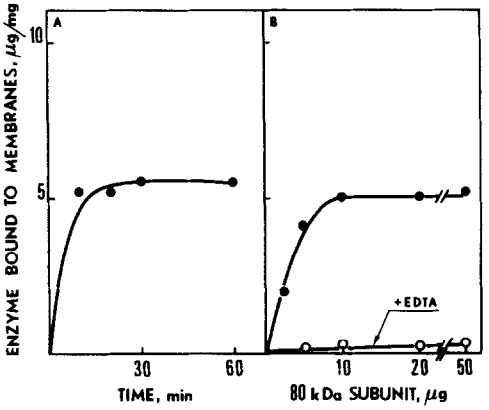

Fig 2. Binding to the erythrocyte membrane of the 80 kDa catalytic subunit: effect of time and of enzyme concentration. A. The carboxymethylated 80 kDa (50 ug, 49,250 counts) was incubated at 25°C in 1.0 ml of sodium borate, pH 8.0, containing 0.5 mg of inside-out vesicles and 50 μM Ca^{2+}. At the times indicated 0.2 ml aliquots were removed, and the amount of proteinase bound was determined as described in Methods. B. The inside-out vesicles (0.2 mg) were incubated at 25°C for 10 min in 0.4 ml of 50 mM sodium borate, pH 8.0, containing 50 μM Ca^{2+} and the indicated amounts of ^{14}C-carboxymethylated 80 kDa catalytic subunit. The washed vesicles were resuspended in 0.4 ml of 50 mM sodium borate , pH 8.0, containing 50 μM calcium and 0.2 ml aliquots were removed and the amount of proteinase bound determined as described in Methods. To the remaining 0.2 ml samples, 0.1 mM EDTA was added and following incubation for 5 min at 4°C, the amount of proteinase bound was evaluated as described in Methods.

shown). Binding of the 80 kDa subunit was very rapid; was saturated when the quantity bound reached 5.5 μg of subunit/mg of membrane protein (Fig 2A and B) and fully reversed by removal of Ca^{2+} with EDTA (Fig 2B).

Specificity of Ca^{2+}-dependent binding. Only CM-procalpain and the CM-80 kDa procalpain subunit showed Ca^{2+}-dependent binding to the membrane vesicles (Table I). No binding of hemoglobin or ß-hemoglobin chains was observed either with or without Ca^{2+}. Binding of aldolases and glyceraldehyde 3-phosphate dehydrogenase, proteins previously shown to bind to erythrocyte membrane proteins (14, 15) was not affected by the addition of Ca^{2+}.

Activation of the 80 kDa catalytic subunit on association with the inside-out membrane vesicles. Association of the native 80 kDa subunit with the membranes results in its conversion to the active proteinase. When assayed directly with 5 μM Ca^{2+}, the 80 kDa subunit shows only 2% of the activity observed in the assay with 1 mM Ca^{2+}, a condition that results in immediate and complete activation (4). After incubation for 20 min in the presence of 10 uM Ca^{2+}, approximately 15% conversion to the active form is observed (Table II).

TABLE I

Specificity of the binding of the neutral proteinase to inside-out vesicles

Protein	Amount of protein bound to vesicles (µg/mg)	
	$-Ca^{2+}$	+0.2 mM Ca^{2+}
Procalpain	0	5.5
Procalpain subunit (80 kDa)	0	5.7
Active calpain (75 kDa)	0	0
Hemoglobin	0	0
ß-hemoglobin chains	0	0
Glyceraldehyde 3-P dehydrogenase	22.2	22.5
Aldolase	20.4	20.7

Procalpain, 80 kDa catalytic subunit and active calpain subunit were carboxymethylated with ^{14}C-iodoacetate as previously described. ^{3}H-labelled hemoglobin chains were prepared from human reticulocytes incubated with ^{3}H-leucine as previously described (12). Isolated ß-hemoglobin chains were obtained from ^{3}H-hemoglobin as described by Bucci and Fronticelli (13). 10 ug of the indicated proteins were incubated for 10 min at 25°C with 0.1 mg of inside-out vesicles in 0.2 ml of sodium borate, pH 8.0, in the absence or in the presence of 0.1 mM Ca^{2+}. The protein bound to membranes was evaluated as reported in Methods. The amount of G3PD and aldolase was estimated by the enzyme activity associated to the vesicles.

The enzyme bound to the membranes and then released by a further incubation in the presence of EDTA showed full activity in the presence of 5 µM Ca^{2+}, indicating complete conversion to active calpain. This activation was not observed

TABLE II

Activation of the 80 kDa catalytic subunit by binding to inside-out erythrocyte vesicles

Treatment	Proteinase activity (units/mg) assayed with		Ratio (A/B)
	5 µM Ca^{2+} (A)	1 mM Ca^{2+} (B)	
none[a]	12	600	0.02
20 min at 10 µM Ca^{2+} [b]	90	600	0.15
bound to membrane[c]	582	600	0.97
recovered as unbound fraction[d]	90	600	0.15

[a] 80 kDa catalytic subunit.

[b] 80 kDa catalytic subunit incubated for 20 min at 25°C in the presence of 10 µM Ca^{2+}.

[c] 80 kDa catalytic subunit released from the vesicles by 0.1 mM EDTA (see Methods).

[d] 80 kDa subunit remained unbound after incubation with the catalytic inside-out vesicles (see Methods).

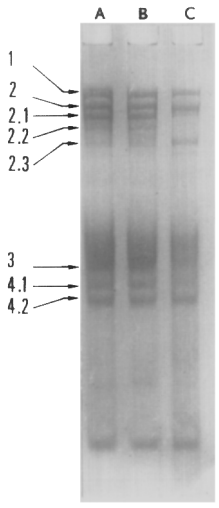

Fig 3. Digestion of membrane proteins by the neutral proteinase associated to the inside-out vesicles. The 80 kDa proteinase subunit was bound to inside-out vesicles as described in Methods and in footnotes to Table II. The vesicles (100 µl) were then incubated at 25°C for 2 h. The digestion was terminated by the addition of 100 µl of 20 mM tris/HCl pH 8.0 containing 2 mM EDTA, 10% sucrose, 2% sodium dodecyl sulfate and 80 mM dithiotreitol. The samples were heated at 100°C for 1 min and 0.1 ml of the solution was loaded on a polyacrilamide slab-gel as described by Fairbanks et al. (16). The electrophoresis was runned for 4 h at 100 mA and the proteins were stained with Coomassie Brilliant Blue. The bands were identified according to Fairbanks et al. (16). Lane A: inside-out vesicles; lane B: inside-out with bound 80 kDa subunit, at 0 time; lane C: as B, incubated for 2 hours.

with the enzyme that remained unbound and was recovered in the supernatant solution. Conversion to active calpain appears to be a rapid process since the enzyme recovered few minutes after its association to the membrane shows a molecular mass of 75 kDa (data not shown) which corresponds to the active calpain form (4).

Modification of erythrocyte membrane proteins. The membranes recovered after a 2 h incubation with the bound calpain showed a significant loss of band 4.1 and band 2.1 proteins (Fig 3). A similar decrease, particularly in the amount of band 4.1 protein, was obseved with red cell membranes recovered from erythrocytes after loading with Ca^{2+} in situ following incubation with Ca^{2+} and the Ca^{2+}-ionophore A-23187 (7).

DISCUSSION

The results presented here define a novel role for Ca^{2+} in the cellular localization and activation of the Ca^{2+}-dependent proteinase of human erythro-

cytes. We have previously shown that physiological concentrations of Ca^{2+} would promote the conversion of procalpain to the active proteinase only in the presence of a digestible substrate such as denatured globin or casein (3, 4). However, the nature of the endogenous activator remained unknown. The present evidence indicated that a component of the erythrocyte membrane will satisfy this requirement. Calcium ions appear to be required not only for activation but also for binding of the procalpain subunit to the membranes.

In terms of physiological significance of these observations, it is important that binding and activation occur at micromolar concentrations of Ca^{2+}. The two events may be part of a functional link between Ca^{2+} mobilization and the intracellular localization and activation of the Ca^{2+}-dependent neutral proteinase. We have shown (7) that degradation of excess hemoglobin chains requires two events catalyzed by the activated neutral proteinase: 1) limited proteolysis of the hemoglobin or globin chains and 2) modification of a protein on the inner surface of the membrane. The modified hemoglobin chains then bind to the modified membranes where they are degraded by intrinsic membrane proteinases. Activation of procalpain on the membrane and release of the active form into the cytosol may also play a role in the turnover of other erythrocyte proteins. Finally, the hypothesis that binding to the plasma membrane is required for normal activation of the proenzyme may provide a function for the endogenous inhibitor. We have observed that the binding of the 80 kDa subunit to inside-out vesicles in the presence of 10 μM Ca^{2+} is not affected by the presence of the inhibitor. At higher concentrations of Ca^{2+} (100 μM) an inhibitor enzyme complex is formed and binding of the 80 kDa catalytic subunit to the membranes is not observed (unpublished observations). The inhibitor would thus prevent the uncontrolled activation of procalpain that would occur if the concentration of Ca^{2+} should reach unphysiological levels.

The effect of activated calpain on the band 4.1 and band 2.1 membrane protein may also have physiological implication and require further study.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Consiglio Nazionale delle Ricerche, Progetto Finalizzato Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie.

REFERENCES

1. Melloni, E., Sparatore, B., Salamino, F., Michetti, M., and Pontremoli, S. (1982) Biochem. Biophys. Res. Commun. 106, 731-740.

2. Melloni, E., Sparatore, B., Salamino, F., Michetti, M., and Pontremoli, S. (1982) Biochem. Biophys. Res. Commun. 107, 1053-1059.
3. Melloni, E., Salamino, F., Sparatore, B., Michetti, M., and Pontremoli, S. (1984) Biochem. Int. 8, 477-489.
4. Pontremoli, S., Sparatore, B., Melloni, E., Michetti, M., and Horecker, B.L. (1984) Biochem. Biophys. Res. Commun. 123, 331-337.
5. Lorand, L., Berrum, O.J., Hawkins, M., Lowe-Krentz, L., and Siefring, J.E., Jr. (1983) J. Biol. Chem. 258, 5300-5305.
6. Hatanaka, M., Yoshimura, N., Murakami, T., Kannagi, R., and Murachi, T. (1984) Biochemistry 23, 3272-3276.
7. Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M., and Horecker, B.L. (1984) Proc. Natl. Acad. Sci. USA 81, 6714-6717.
8. Melloni, E., Salamino, F., Sparatore, B., Michetti, M., and Pontremoli, S. (1984) Biochim. Biophys. Acta 788, 11-16.
9. Winterhalter, K.H., and Huehns, E.R. (1984) J. Biol. Chem. 239, 3699-3705.
10. Pontremoli, S., Sparatore, B., Salamino, F., Michetti, M., and Melloni, E. (1985) Arch. Biochem. Biophys. In press.
11. Steck, T.L., and Kant, J.A. (1974) Methods Enzymol. 31, 172-180.
12. Melloni, E., Salamino, F., Sparatore, B., Michetti, M., and Pontremoli, S. (1982) Arch. Biochem. Biophys. 216, 495-502.
13. Bucci, E., and Fronticelli, C. (1965) J. Biol. Chem. 240, 551-552.
14. Yu, J., and Steck, T.L. (1977) J. Biol. Chem. 250, 9176-9184.
15. Strapazon, E., and Steck, T.L. (1977) Biochemistry 16, 2966-2971.
16. Fairbanks, J., Steck, T.L., and Wallack, D.F.H. (1971) Biochemistry 10, 2606-2617.