Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 87-94

CALCIUM-INDUCED ALTERATIONS IN THE LEVELS AND SUBCELLULAR DISTRIBUTION OF PROTEOLYTIC ENZYMES IN HUMAN RED BLOOD CELLS

M. [illegible]asso, A. Morelli, and A. De Flora

Institute of Biochemistry, University of Genoa, Genoa, Italy

Received May 21, 1986

SUMMARY: Human red cells were treated with 100 μM Ca^{2+} and ionophore A 23187. This treatment induces remarkable changes in the activities of the two major proteolytic systems of red cells, i.e. Ca^{2+}-dependent neutral proteinase and acid endopeptidases. Ca^{2+}-dependent neutral proteinase undergoes intracellularly preliminary activation of the inactive proenzyme species, followed by eventual inactivation through self-proteolysis. Transient activation is shown by selective degradation of cytoskeletal proteins known to be targets of this enzyme system. Concomitantly, acid endopeptidase activity is substantially released from the membrane into the cytosol. Preliminary inactivation of the Ca^{2+}-dependent neutral proteinase by exposure of Glucose 6-phosphate dehydrogenase-deficient red cells to autoxidizing divicine prevents alterations induced by Ca^{2+} loading on cytoskeletal membrane proteins, while leaving solubilization of acid endopeptidase activity unaffected. The two events, although dependent on Ca^{2+} loading, are therefore unrelated to each other.

Mature human red blood cells (RBC) contain a highly integrated proteolytic system (1). This includes a Ca^{2+}-activated neutral proteinase (calpain), present in the cytosol, and three acid endopeptidases (forms I, II and III), mostly associated with the membrane in normal conditions (2, 3). Two aminopeptidases and two dipeptidylaminopeptidases are also present in the RBC cytosol (3).

The best known among proteolytic enzymes of RBC is calpain, whose regulation is extraordinarily sophisticated (1). "In vitro", the major control mechanism of this purified protein is related to the concentration of Ca^{2+}, an increase of which will promote conversion of an inactive, heterodimeric pro-

ABBREVIATIONS: RBC, red blood cells; G6PD, Glucose 6-phosphate dehydrogenase; TES, 2- |2-hydroxy-1,1-bis(hydroxymethyl)ethyl| amino ethanesulfonic acid; PBS, 20 mM Na phosphate, pH 7.4, containing 146 mM NaCl; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/86 $1.50

enzyme species (procalpain) to the fully active form (calpain) through the proteolytic removal of a 5 KD peptide from the 80 KD subunit (4). In turn, in the presence of high levels of Ca^{2+}, active calpain undergoes further autoproteolytic processing with attendant irreversible loss of activity (1). Accordingly, mobilization of intracellular Ca^{2+} or increased Ca^{2+} levels within RBC are expected to trigger a two-step activation-inactivation mechanism of calpain. The results reported in this paper demonstrate the occurrence of such mechanism inside RBC treated with Ca^{2+} and ionophore A 23187. An additional consequence of Ca^{2+} loading in RBC is the remarkable solubilization of membrane acid endopeptidases that are extensively released into the cytosol. Experiments with Glucose 6-phosphate dehydrogenase (G6PD)-deficient RBC indicate that the Ca^{2+}-dependent release of acid endopeptidases from RBC membranes into the cytosol is totally unrelated to the concomitant regulation of the procalpain-calpain system by intracellular Ca^{2+}.

MATERIAL AND METHODS

RBC from normal donors and from G6PD-deficient male subjects were obtained from blood samples defibrinated with glass beads and processed according to Beutler et al. (5) in order to remove leukocytes and residual platelets. The G6PD-deficient subjects were asymptomatic and had the Mediterranean type of the deficiency (6), as indicated by levels of G6PD activity of 0.001-0.004 I.U./g Hb (7).

Procalpain activity was assayed in the presence of 1 mM Ca^{2+} (8, 9). Assays were carried out in 1.0 ml aliquots of packed RBC, following removal of the endogenous protein inhibitor (9) by chromatography of the hemolysates on a column (0.7 x 10 cm) of DEAE-cellulose (8). Removal of the inhibitor was verified as described (9). Activity was expressed as units/ml of packed RBC and 1.0 ml of RBC was assumed to contain 330 mg Hb as measured before chromatography.

Assays of acid endopeptidase activity were carried out as described (2). Membrane-bound activity was expressed as units/ml of packed RBC, i.e. referred to 5.0 mg of membrane proteins. Cytosolic acid activity was expressed as units/ml of packed RBC. Both the butanol-extracted (2) acid activity from RBC membranes and the cytosolic acid activity were analyzed for their content of proteinases I, II and III by gel chromatography on Ultrogel AC A44 (2). In the case of the cytosolic activity, this step was preceded by fractionation of the hemolysates on DEAE-cellulose DE 32 (3).

RBC were incubated at a final 10% hematocrit for various times at 37°C in 0.04 M TES, pH 7.4, containing 5 mM glucose, NaCl to a final osmolarity of 310 mOsm/liter H_2O, 5 µM A 23187 and varying concentrations (usually 100 µM) of $CaCl_2$. Samples not containing A 23187 or $CaCl_2$ were run in parallel. Incubations were terminated by addition of 1 mM EDTA and the RBC were washed three times with PBS and hemolyzed with 10 vols of 5 mM Na phosphate, pH 8.0. Both the supernatants and the membrane pellets (after 5 washings with 5 mM Na phosphate, pH 8.0) were assayed for endopeptidase activities, as described above.

Analysis of membrane proteins was carried out on SDS polyacrylamide slab gels by the procedure to Fairbanks et al. (10).

Incubations of RBC (10%, final hematocrit) with divicine were carried out in 0.04 M TES-glucose, pH 7.4, as reported above, using divicine solutions freshly prepared by hydrolysis of commercial vicine, as described (11).

RESULTS

Effects of Ca^{2+} loading on the activity of the procalpain-calpain system. Exposure of intact normal RBC to ionophore A 23187 and to increasing concentrations of Ca^{2+} results in a progressive and irreversible loss of intracellular procalpain activity (Fig. 1 A). No inactivation is observed when either Ca^{2+} or the ionophore is omitted. That such inactivation follows conversion of the proenzyme to the active calpain form, is shown by the attendant changes in membrane polypeptide patterns (Fig. 1 B). Thus, both the increased content of band 2.3 and the moderately decreased amount of band 4.1 are proteolytic events (12) consistent with transient intracellular expression of calpain activity (13).

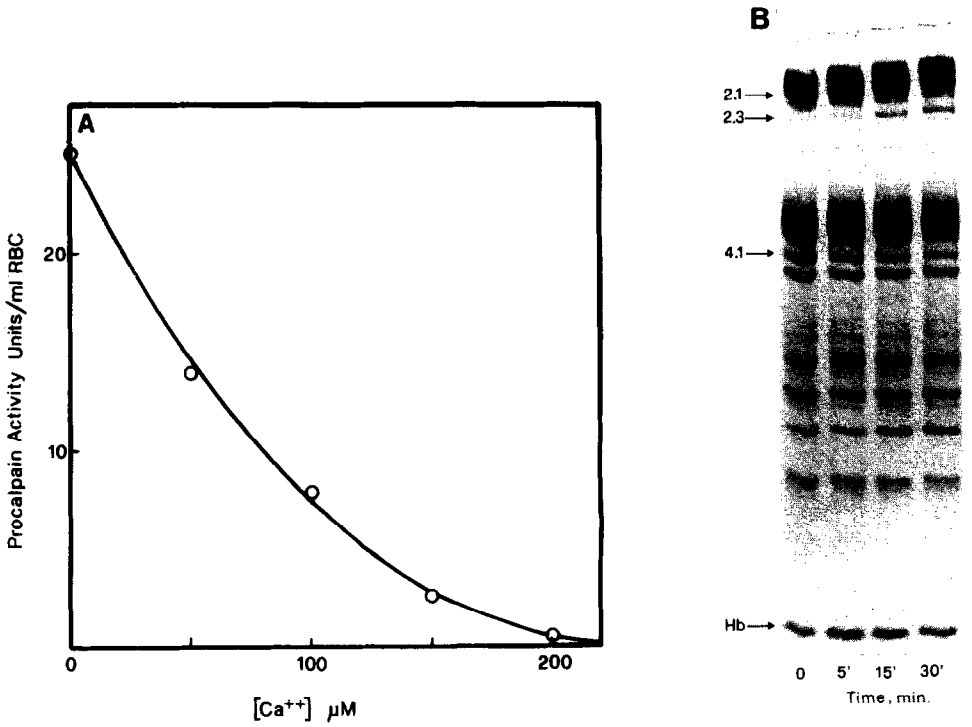

Fig. 1. Effects of Ca^{2+} loading on the levels and on membrane-targeted activity of the procalpain-calpain system. (A) Levels of procalpain activity in normal RBC exposed for 30 min at 37°C to 5 μM A 23187 and increasing Ca^{2+}. (B) SDS-PAGE patterns of the same RBC exposed to 5 μM A 23187 and 100 μM Ca^{2+} at 37°C for varying time intervals (zero, 5, 15 and 30 min, respectively).

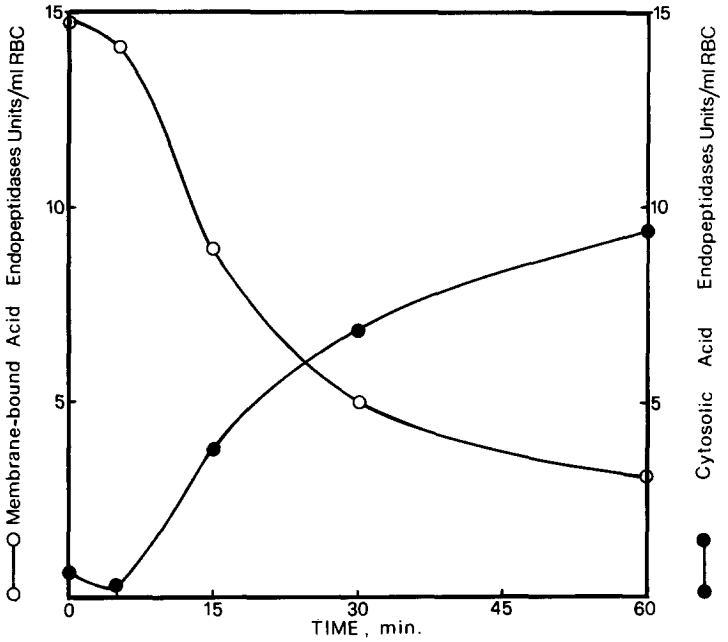

Fig. 2. Levels and subcellular distribution of total acid endopeptidase activity in RBC treated for 60 min at 37°C with 5 μM A 23187 and 100 μM Ca^{2+}. Membrane-bound and cytosolic total acid endopeptidase activities were assayed as reported under "Materials and Methods" and referred to 1.0 ml of packed RBC.

Effects of Ca^{2+} loading on the subcellular localization of acid endopeptidases. Besides modulating the procalpain-calpain system, loading with 100 μM Ca^{2+} induces a remarkable loss of membrane-bound acid endopeptidase activity, paralleled by appearance of this activity in the cytosol. Accordingly, total acid endopeptidase activity of the Ca^{2+}-loaded RBC keeps constant, yet its subcellular distribution is completely reversed (Fig. 2). Release into the

TABLE I. Solubilization of acid endopeptidases upon Ca^{2+} loading in human RBC

	Proteolytic activity (Units/ml RBC)			
	Untreated RBC		Ca^{2+}-loaded RBC	
	Membrane-bound	Cytosolic	Membrane-bound	Cytosolic
Proteinase I	0.9	traces	0.6	0.2
Proteinase II	9.6	0.42	3.2	6.5
Proteinase III	4.7	0.18	3.1	1.2

Normal RBC were incubated for 30 min at 37°C with 5 μM A 23187 and 100 μM Ca^{2+} as described in Materials and Methods. The three acid proteinase activities were isolated by gel chromatography and assayed according to refs. 2 and 3.

TABLE II. Susceptibility of procalpain and of acid endopeptidase activity of G6PD-deficient RBC to autoxidizing divicine

Proteolytic activities (Units/ml RBC)	Divicine (mM)			
	0	0.75	2.5	5.0
Procalpain (cytosolic)	22.5	1.2	zero	zero
Acid endopeptidases				
a) Membrane-bound	11.8	10.9	8.8	7.6
b) Cytosolic	0.4	0.6	1.0	1.2

G6PD-deficient RBC were incubated for 30 min at 37°C in the presence of divicine at the concentrations indicated (see Materials and Methods).

cytosol affects proteinase II much more than proteinases I and III (Table I) and is not accompanied by any change in the pH optimum (2, 3).

Correlations between effects on procalpain-calpain and on acid endopeptidases. Procalpain has been recently demonstrated to be a selective target of divicine cytotoxicity (14). Thus, divicine, a pyrimidine aglycone implicated in the pathogenesis of favism (15), inactivates procalpain both in the purified system and within RBC (14). Inactivation is caused by autoxidation of divicine and is much greater in G6PD-deficient than in normal RBC, as a result of distinctive lability of GSH in the defective cells which yet have normal levels of procalpain (14). This specific effect of divicine has now been used as a tool to investigate the role of calpain in the multiple changes elicited by loading with 100 μM Ca^{2+}.

Table II confirms the high susceptibility of the procalpain activity of G6PD-deficient RBC to divicine (14). The activity of membrane-bound acid endopeptidase is little affected, with a limited extent of solubilization which is likely to reflect partial disruption of the membrane architecture, at least at the highest concentrations of divicine.

Fig. 3 shows the influence of pre-incubating G6PD-deficient RBC with various divicine concentrations on the two effects induced by subsequent Ca^{2+} loading, i.e. (A) solubilization of acid endopeptidases and (B) alterations of membrane polypeptides. Preliminary inactivation of procalpain does not affect appreciably the release of acid endopeptidases into the cytosol that follows loading of RBC with 100 μM Ca^{2+} (Fig. 3 A): this was deliberately limited in extent, as compared with the experiment of Fig. 2, by a shorter exposure of

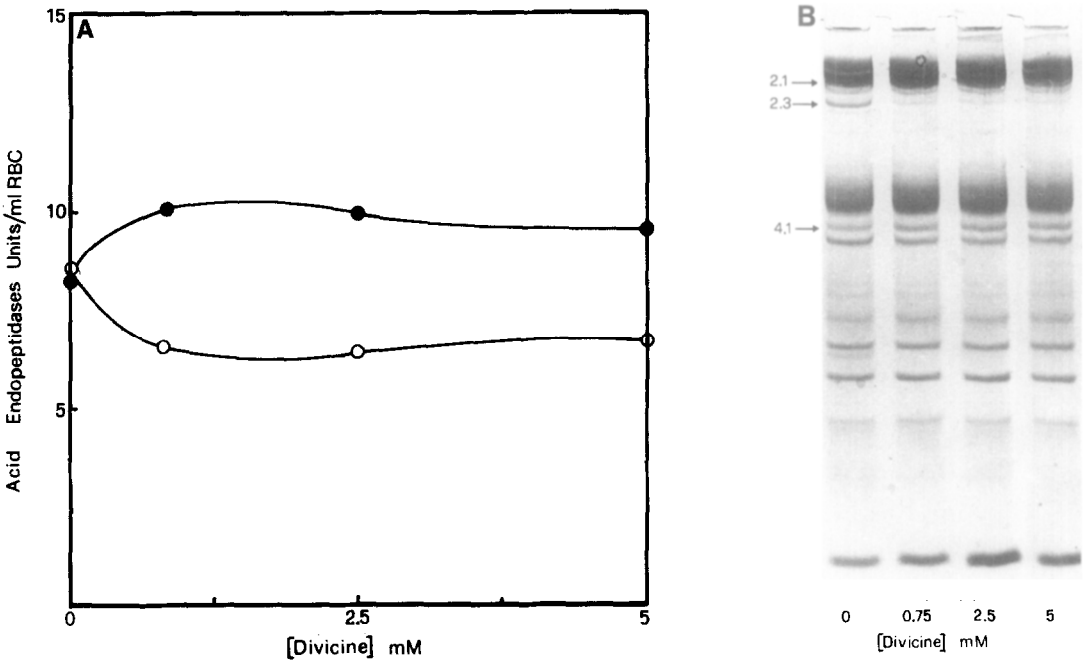

Fig. 3. Ca^{2+}-induced changes in acid endopeptidase activity and in membrane polypeptide patterns of G6PD-deficient RBC following inactivation of procalpain. RBC (0.002 I.U./g Hb) were pre-incubated for 30 min at 37°C with divicine at the concentrations indicated on the abscissa and, after three washings in PBS, re-incubated for 30 min at 37°C with 5 µM A 23187 and 100 µM Ca^{2+}. Procalpain activity was completely lost following treatment with divicine. (A) Membrane-bound (O) and cytosolic (●) total acid endopeptidase activities are referred to 1.0 ml of packed RBC. (B) SDS-PAGE patterns of the same RBC untreated or pre-treated with divicine and then incubated with A 23187 and Ca^{2+}.

RBC to Ca^{2+}. On the contrary, pre-incubation of RBC with divicine before Ca^{2+} loading prevents both the appearance of band 2.3 and the decrease of band 4.1 that are found in the same RBC directly submitted to treatment with Ca^{2+} and A 23187 (Fig. 3 B). These data confirm that the Ca^{2+}-induced changes in cytoskeletal proteins are the result of intracellular activation of procalpain. Moreover, the release of acid membrane-bound endopeptidases into the cytosol is independent of Ca^{2+}-mediated regulation of the procalpain-calpain system.

DISCUSSION

Loading of 100 µM Ca^{2+} in human RBC induces two major effects on the resident endopeptidase activities: 1) occurrence of the activation-autoinactivation mechanism of procalpain whereby specific cytoskeletal proteins undergo limited degradation, 2) a reversed subcellular distribution of the three acid endopeptidases that are released from RBC membranes into the cytosol. Evidence

has emerged that these two results, although being both strictly dependent on Ca^{2+} loading, are unrelated to each other from a mechanistic point of view. Moreover, the final outcome of Ca^{2+} loading seems to be the switching off of both endopeptidase systems since solubilized acid proteinases can hardly be expected to work at the intracellular pH values of RBC, while some activity is known to be present in the hydrophobic environment of RBC membranes (16). Also suggested by these data is the apparent localization of the three acid endopeptidases at the cytoplasmic face of the membrane.

The present results may bear relevance to the pathophysiology of favism, an acute hemolytic disease affecting some G6PD-deficient subjects and triggered by fava bean components among which divicine is strongly implicated (15). Favism is characterized at the RBC level by severe oxidant damage, which at least in part is afforded by divicine autoxidation, and by markedly perturbed Ca^{2+} homeostasis as well (11, 17). The link between these two alterations, both affecting the procalpain-calpain system, is as yet uncertain, in spite of the report that divicine autoxidation inactivates the membrane Ca^{2+}-ATPase significantly (18). Although both divicine and enhanced intracellular Ca^{2+} are expected to produce eventual inactivation of calpain, the Ca^{2+}-dependent mechanism involves transient expression of proteolytic activity. Thus, in RBC from favic patients, significant alterations of bands 2.3 and 4.1 have been observed (19) as a proof of unrestrained calpain activity. However, the picture of biochemical dysfunctions of RBC proteolytic systems in favic patients may be complicated by additional effects including attainment of intracellular Ca^{2+} levels higher than those obtained in the present study, resulting in activation of the usually latent Ca^{2+}-dependent transglutaminase (20) and in the formation of disulfide bridges among membrane polypeptides. Analysis of the biochemical properties of RBC during the hemolytic crisis in favism requires careful consideration of this wide multiplicity of events.

ACKNOWLEDGMENTS

This work was supported by the P.F. Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie of the Italian C.N.R. and by the Ministry of Education. We are indebted to Professor S. Pontremoli for criticisms and discussions.

REFERENCES

1. Pontremoli, S. and Melloni, E. (1985) Annu. Rev. Biochem., in press.
2. Pontremoli, S., Salamino, F., Sparatore, B., Melloni, E., Morelli, A., Benatti, U. and De Flora, A. (1979) Biochem. J. 181, 559-568.

3. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M., Benatti, U., Morelli, A. and De Flora, A. (1980) Eur. J. Biochem. 110, 421-430.
4. Pontremoli, S., Sparatore, B., Melloni, E., Michetti, M. and Horecker, B.L. (1984) Biochem. Biophys. Res. Commun. 123, 331-337.
5. Beutler, E., West, C. and Blume, K. (1976) J. Lab. Clin. Med. 88, 328-333.
6. Kirkman, H.N., Schettini, F. and Pickard, B.M. (1964) J. Lab. Clin. Med. 63, 726-735.
7. Morelli, S., Benatti, U., Lenzerini, L., Sparatore, B., Salamino, F., Melloni, E., Michetti, M., Pontremoli, S. and De Flora, A. (1981) Blood 58, 642-644.
8. Melloni, E., Sparatore, B., Salamino, F., Michetti, M. and Pontremoli, S. (1982) Biochem. Biophys. Res. Commun. 106, 731-740.
9. Melloni, E., Salamino, F., Sparatore, B., Michetti, M. and Pontremoli, S. (1984) Biochem. Int. 8, 477-489.
10. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.
11. De Flora, A., Benatti, U., Guida, L., Forteleoni, G. and Meloni, T. (1985) Blood, 66, 294-297.
12. Allan, A. and Thomas, P. (1981) Biochem. J. 198, 433-440.
13. Pontremoli, S., Melloni, E., Sparatore, B., Michetti, M. and Horecker, B.L. (1984) Proc. Natl. Acad. Sci. USA 81, 6714-6717.
14. Morelli, A., Grasso, M. and De Flora, A. (1986) Arch. Biochem. Biophys., submitted.
15. Chevion, M., Navok, T., Glaser, G. and Mager, J. (1982) Eur. J. Biochem. 127, 405-409.
16. Melloni, E., Salamino, F., Sparatore, B., Michetti, M. and Pontremoli, S. (1982) Arch. Biochem. Biophys. 216, 495-502.
17. Turrini, F., Naitana, A., Mannuzzu, L., Pescarmona, G. and Arese, P. (1985) Blood 66, 302-305.
18. Benatti, U., Guida, L., Forteleoni, G., Meloni, T. and De Flora, A. (1985) Arch. Biochem. Biophys. 239, 334-341.
19. De Flora, A., Morelli, A., and Grasso, M. (1986) Glucose 6-Phosphate Dehydrogenase (Yoshida, A., and Beutler, E., eds.), Academic Press, New York, in press.
20. Lorand, L., Bjerrum, O.J., Hawkins, M., Lowe-Krentz, L. and Siefring, G.E., Jr. (1983) J. Biol. Chem. 258, 5300-5305.