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CHARACTERIZATION OF THE DEFECTIVE CALPAIN-ENDOGENOUS CALPAIN INHIBITOR SYSTEM IN ERYTHROCYTES FROM MILAN HYPERTENSIVE RATS

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In mature red cells of rats from Milan normal (MNS) and hypertensive strains (MHS), the soluble Ca^{2+} dependent neutral proteinase (calpain) is present in similar amounts with identical M_r of 110 kDa and a dimeric structure composed of two unequal subunits of M_r of 84 and 26 kDa. Conversely, the amount of the endogenous inhibitor is now confirmed by analysis of the specific activity to be approximately 10 times less in red cells of MHS rats. The inhibitor is present in red cells of both strains in three different oligomeric forms of M_r of 240, 120 and 64 kDa. This last molecular species corresponds to the single basic constituent subunit which is the reacting inhibitor form. The apparent equilibrium between the three oligomeric structures is Ca^{2+}-dependent. The high (0.1 mM) Ca^{2+} requirement for the activity of calpain from erythrocytes of both strains is reduced to 1-5 μM in the presence of plasma membrane phospholipids. Activation of the enzyme in these conditions is prevented by the natural inhibitor. These results strongly support and further emphasize the hypothesis that the structural and functional abnormalities in MHS rats red cells result from an impairment in the modulation of intracellular calpain activity by interaction with its endogenous inhibitor.

In the Milan strain of genetically hypertensive rats, (1) the red cell proteolytic system has been shown to be present in an "unregulated condition" due to a profound unbalance between the levels of calpain activity and those of its endogenous inhibitor (2). This defect is clearly represented by the inhibitor/proteinase ratio (calculated on a unit basis) corresponding to 0.2 in red cells of MHS rats and to 2.0 in red cells of MNS rats (2). In the present paper we report data which indicate that in erythrocytes of MHS rats the inhibitor is indeed present in smaller quantities in a fully active molecular form. The results obtained concerning the mechanism of calpain activation and modulation by the endogenous inhibitor at physiological concentrations of Ca^{2+}, strongly support the hypothesis that the genetic defect in MHS rats,

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characterized by modification in structural and functional properties of the red cells, might be expressed at the phenotypic level by a reduced number of calpain inhibitor copies .

MATERIALS AND METHODS

Adult MNS or MHS rats, weighing 200-210 g, were obtained as described (1). Blood was collected and leukocytes were immediately removed as previously reported (2). Calpain was purified following the procedure previously described (3). Calpain inhibitor was purified according to (3), with the following modifications. The final gel-chromatography was substituted with an hydrophobic chromatography on butyl-agarose (4). Calpain and calpain inhibitor were routinely assayed as reported (2). One unit of enzyme activity is defined as the amount that releases 1 nmol/min of free amino groups in these conditions. The specific activity of calpain purifeid from MNS rats was 4500 unit/mg, that of calpain from MHS rats was 4200 units/mg.

One unit of calpain inhibitor is defined as the amount that inhibits one unit of calpain activity.

Preparation of phospholipids vesicles. Phospholipids were extracted from erythrocyte membranes and vesicles were prepared as described (5).

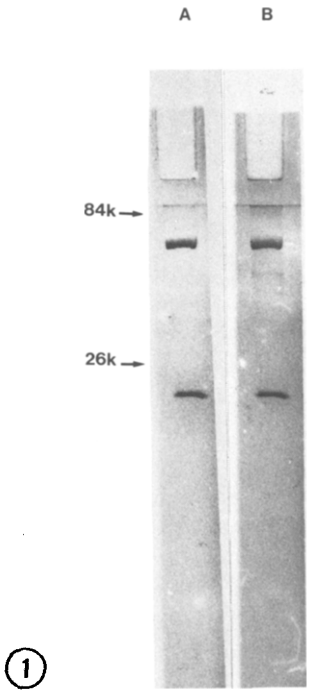
Analysis of molecular properties of calpain and its endogenous inhibitor. The molecular sizes in native conditions were determined by the elution volume from a Sephadex G-200 column (1.5x120cm) previously equilibrated in 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA. The flow rate was 9 ml/h and fractions of 1.5 ml were collected.

SDS-PAGE of purified calpain and calpain inhibitor. Aliquots of the enzyme or of the inhibitor preparations, containing approximately 5 μg of protein, were analyzed by SDS-PAGE according to the method of Laemmli (6) using 10% slab gels.

RESULTS

Molecular size of calpain. Calpain purified from red cells of MHS and MNS rats filtered through a Sephadex G-200 column emerged in single homogenous peaks of approximate 110 kDa molecular weight (data not shown). SDS-PAGE analysis of purified calpain samples revealed the presence of two bands with mobility corresponding to subunits of 84 kDa and 26 kDa (Fig. 1).

Activation of calpain by Ca^{2+} and phospholipids. In 0.1 mM Ca^{2+} calpain expresses maximum proteolytic activity but, at difference with the corresponding human enzyme (7) is not autoproteolytically converted to a low Ca^{2+} requiring form. In the presence of increasing concentrations of a mixture of phospholipids extracted from red cell plasma membrane, proteolytic activity was expressed also at μM Ca^{2+} and reached 80% of maximum catalytic activity at a concentration of phospholipids of 50 μg/ml (Fig. 2). Thus phospholipids



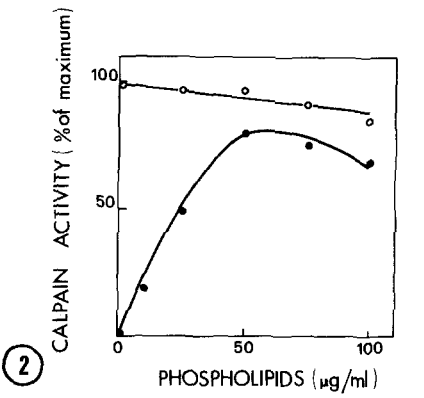


Fig. 1. SDS-PAGE of calpain purified from erythrocytes of MNS and MHS rats. Samples (5 µg) of calpain isolated from erythrocytes of MNS (lane A) and MHS (lane B) rats were submitted to SDS-PAGE (see Methods). The molecular weights were estimated using the following standard proteins: phosphorylase b (94 kDa), transferrin (74 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), myoglobin (17.4 kDa) and cytochrome C (12.5 kDa).
Fig. 2. Activation of calpain by erythrocytes membrane phospholipids. Purified calpain (10 µg) was incubated for 1 min at 4°C with different amounts of phospholipids vesicles described (5). The enzyme activity was assayed either in 0.1 mM (○) or 5 µM Ca^{2+} (●).

presumably promote a conformational change in the proteinase molecule inducing an increase in the affinity for Ca^{2+}.

Molecular properties of the calpain inhibitor. When the calpain inhibitors isolated from erythrocytes of normal and hypertensive rats were submitted to gel-chromatography on a Sephadex G-200 column, three peaks with approximately molecular mass of 250-, 120- and 64 kDa were detected in each inhibitor preparation (Fig. 3). The different calpain inhibitor forms were equally efficient, on a unit basis, when tested in the presence of 0.1 mM Ca^{2+}. The three inhibitor forms showed however different inhibitory capacity in the presence of micromolar Ca^{2+} (Table I). Whereas the 64 kDa species appears to be equally effective in both conditions, the higher M_r forms were less active at micromolar Ca^{2+} concentrations. These results could be explained assuming that the

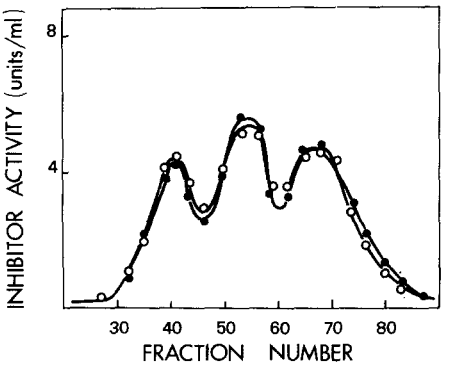


Fig. 3. Analysis of molecular size of calpain inhibitor. Calpain inhibitor (200 units) from MNS (●) and MHS (○) rats was submitted to gel filtration on a Sephadex G-200 column as described in Methods. The inhibitor activity was assayed using 100 ul aliquots of the eluted fractions. The fractions from 33 to 43 (250 kDa), from 48 to 56 (120 kDa) and from 64 to 75 (64 kDa) were separately collected and concentrated by ultrafiltration using an Amicon YM10 membrane.

monomeric species are the active form of the inhibitor and that higher Ca^{2+} concentrations exert a dissociating effect on the high M_r forms.

To test this hypothesis, we have analyzed the effect of Ca^{2+} on the oligomeric structure of the 250 kDa inhibitor form. Following gel chromatography on Sephadex G-200, in the presence of 5 μM Ca^{2+}, 64% of the inhibitor activity is recovered as 64 kDa form, 33% as 120 kDa form and only 3-4% as the tetrameric species (Table II). The concentration of the 64 kDa subunit found in these conditions corresponds almost precisely to the percent of inhibition expressed by the inhibitor when assayed in 5 μM Ca^{2+} (see Table I). These results indi-

TABLE I

Effect of Ca^{2+} on the efficiency of the different calpain inhibitor forms

Inhibitor form	Inhibitor activity (units/mg)	
	0.1 mM Ca^{2+}	5 μM Ca^{2+}
250 kDa	2,000	1,300
120 kDa	2,200	1,890
64 kDa	2,100	2,100

Calpain inhibitor was isolated from MNS rat erythrocytes as described in Methods. The different inhibitor forms were obtained as reported in the legend to Fig. 3 and assayed for their activity on phospholipid-treated calpain (see Methods) either in 0.1 mM or 5 μM Ca^{2+}.

TABLE II

Effect of Ca^{2+} on the oligomeric structure of the 250 kDa calpain inhibitor form

Addition	Inhibitor activity (% of total)		
	250 kDa	120 kDa	64 kDa
EDTA, 0.1 mM	85	10	5
Ca^{2+}, 5 μM	3	33	64

The 250 kDa inhibitor form was prepared from MNS rat erythrocytes as described in Fig. 3. The fractions from 33 to 43 (11.5 ml) were collected and concentrated to 1.5 ml by ultrafiltration. Aliquots (30 units) were then loaded on a Sephadex G-200 column (1.5x120 cm) previously equilibrated in 50 mM sodium borate, pH 7.5, containing 0.1 mM EDTA or 5 μM Ca^{2+}. The inhibitor activity was measured as described in Methods, using 0.2 ml of the eluted fractions. The amounts of the various inhibitor forms were calculated from the areas under each peak (see Fig. 3).

cate that the inhibitor is present in the cells in different oligomeric forms in an apparent equilibrium controlled by Ca^{2+}.

SDS-PAGE analysis of the levels of the endogenous inhibitor in red cells of MHS rats. In order to establish if the decreased level of inhibitor activity in erythrocytes of MHS rats was due to a lower amount of inhibitor protein or to the presence of a modified form with lower activity, samples of the inhibitor purified from red cells of both strains containing the same amounts of units were submitted to SDS-PAGE. As shown in Fig. 4 both samples appeared to contain an equal amount of protein inhibitor; thus indicating that the defect which characterizes the decreased level of inhibitor activity in MHS rat erythrocytes is due to a decreased amount of inhibitor protein.

DISCUSSION

The Milan hypertensive strain has been previously identified as a genetically determined experimental hypertensive condition (1), predominantly characterized in red blood cells by modifications of the volume and of the Na^+-K^+ cotransport (8). We have previously reported a decreased level of calpain inhibitor activity in red blood cells of MHS rats (2) this representing the first indication for the existence of a potentially unregulated intracellular proteinase in a mammalian cell.

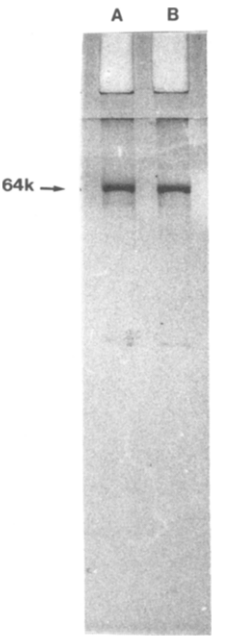


Fig. 4. SDS-PAGE of calpain inhibitor isolated erythrocytes of MNS and MHS rats. Samples (12 units) of calpain inhibitor purified (see Methods) from erythrocytes of MNS (lane A) and MHS (lane B) rats were submitted to SDS-PAGE as described in Methods. The molecular size was determined by comparison with the mobility of standard proteins.

The present data have established that in erythrocytes of hypertensive and normal rats calpain is present in a single dimeric form of 110 kDa composed of two non identical subunits of approximately 84 and 26 kDa each. In this respect the proteinase appears to be similar to that previously isolated and characterized from human red cells (4, 7). The rat calpain differs however from the corresponding human enzyme in that its activation process does never involve an irreversible autoproteolytic conversion from a high to a low Ca^{2+} requiring form (9). The proteinase has been shown to express full catalytic activity at 5 μM Ca^{2+} in the presence of a phospholipid mixture prepared from plasma membrane. This implying that activation of the proteinase occurs upon translocation to the plasma membrane with an identical mechanism to that described for the human neutrophils (10) and erythrocytes enzyme (5, 11). Of particular significance is the observation that the decreased level of endogenous inhibitor activity, previously described, in red cells from MHS rats is due to a decreased amounts of the inhibitor. The characterization of the inhibitor properties has indicated that the activation of calpain at 5 μM Ca^{2+} in

the presence of phospholipids is prevented by the addition of the natural inhibitor. This is the first evidence, at difference from the human red cell calpain system, for an interaction of the inhibitor with calpain at micromolar concentrations of Ca^{2+}. Although the inhibitor appears to be present in three oligomeric forms in a Ca^{2+} dependent apparent equilibrium, it has been proved that the 64 kDa constituent subunit is the species interacting with the proteinase.

In conclusion the occurrence in MHS rats red cells of an unregulated proteolytic system capable of limited proteolytic modifications of membrane and cytoskeletal proteins (12), may significantly contribute to the understanding of those intracellular biochemical mechanisms which might be responsible for the development of the hypertensive condition.

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