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THE ROLE OF CALPAIN IN THE SELECTIVE INCREASED PHOSPHORYLATION OF THE ANION-TRANSPORT PROTEIN IN RED CELL OF HYPERTENSIVE SUBJECTS

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SUMMARY.The phosphorylation of the anion-transport protein (band 3) is selectively increased in human red cell membrane, following exposure of intact cells to ionophore and micromolar calcium. The phosphorylation is catalyzed by a membrane associated protein kinase distinct from either protein kinase C or Ca2+/calmodulin dependent protein kinase. We show that the increase in phosphorylation of band 3 is abolished if red cells had been pre-loaded with an inhibitor of calpain or with an anticalpain monoclonal antibody. Our findings suggest that calpain activity may control, both at a functional and at a structural level, the activity of this important transmembrane protein through the modulation of its susceptibility as a substrate of membrane bound protein kinase(s). Based on previous observations indicating the presence in erythrocytes from hypertensive patients of an uncontrolled intracellular calpain-mediated proteolytic system accompanied by an increased phosphorylation of band 3 protein(s), we suggest that our results may shed light on the type of molecular alteration which is associated with the hypertensive state.

Red cells from patients with essential hypertension have been shown to be characterized by: a) an altered Na/K cotransport system (1-4); b) a modification of the structure of the red cell membrane (5-7); and c) an unbalanced intracellular proteolytic system resulting from the presence of normal amounts of calpain accompanied by decreased levels of the endogenous calpain inhibitor (8). In addition we have recently shown that phosphorylation of band 3 protein(s), by membrane associated protein kinase(s), is significantly higher in inside-out vesicles prepared from erythrocytes of hypertensive subjects as compared to control cells from normotensive subjects (9).

Abbreviations: Bt2cAMP, dibutyryl cyclic 3'-5' AMP; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PAS, perodic acid-Schiff; mAb, monoclonal antibody.

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Increased phosphorylation of band 3 can be induced in control cells, following exposure of inside-out vesicles to minute amounts of calpain (9). With these observations in mind we have further investigated the role of calpain in promoting enhanced ^{32}P incorporation in membrane proteins by the action of membrane associated protein kinase(s). We report here that in intact red cells activation of calpain results in increased phosphorylation of band 3 to an extent comparable to that which spontaneously occurs in red cells of hypertensive subjects. Moreover we provide evidence that the proteolytic modification responsible for such selective susceptibility to an increased phosphorylation occurs during the life span of the erythrocyte and is not a result of proteolysis occurring during cell lysis. We also provide preliminary identification of the type of kinase responsible for phosphorylation of the band 3 protein and suggest that this phosphoprotein is identical to the well characterized anion-transport protein.

MATERIALS AND METHODS

DEAE-cellulose (DE52) was obtained from Whatman. Calcium ionophore A 23187, trifluoperazine, phosphatidylserine (bovine brain), dioleoylglycerol, histone type III-S, pepstatin A and the various nucleotides were purchased from Sigma Chemical Co. γ-32P ATP (3000 Ci/mmol) was from Amersham. Triton X-100 was from Rohm and Haas and the electrophoresis reagents from Bio Rad. Purified glycophorin was a gift of Dr. Balduini of the University of Pavia. All other chemicals were reagent grade. Anticalpain monoclonal antibody (mAb C 56.3) was prepared as previously described (10). Erythrocyte membranes and inside-out vesicles were prepared as described (11) from freshly collected human blood obtained from essential hypertensive patients who never received antihypertensive treatment and from normotensive volunteers. Phosphorylation of the vesicles was carried out for 5 min as previously reported (9). In conditions used 32P incorporation into membrane proteins was linear up to 10 min.

Assay of protein kinase activity. The incubation mixture (0.2 ml), contained 25 mM imidazole-HCl, pH 7.5, 5 mM MgCl2, 10 µM γ-32P ATP (7.5 Ci/mmol), 100 µg of histone type III-S and the appropriate amount of enzyme source. The mixture was incubated for 10 min at 30 °C and the reaction stopped by addition of one ml of 10% trichloroacetic acid. The amount of radioactivity incorporated into acid insoluble material was evaluated as described (12). One unit of protein kinase activity was defined as the amount of enzyme that causes the incorporation of 1 nmol of 32P into histone under these conditions.

Loading of erythrocytes. Erythrocytes (1.8 ml of cells with 70% hematocrit) from normotensive or hypertensive subjects, were loaded with 0.2 mM leupeptin, 0.2 mM pepstatin A, a mixture of 0.2 mM leupeptin and 0.2 mM pepstatin A or 0.1 mg/ml mAb c56.3, following the procedure described by Ropars et al. (13). As controls erythrocytes were submitted to the same treatment with the omission of the proteinase inhibitors. Following resealing erythrocytes (0.2 ml) were loaded with 5 µM Ca2+ in the presence of 5 µM

A23187 as reported (11). As controls, resealed erythrocytes were submitted to identical treatment in the absence of calcium. The cells were finally lysed and inside-out vesicles were prepared using buffers containing the initial concentration of the proteinase inhibitors. Phosphorylation of inside-out vesicles was performed and evaluated as described above.

Separation of membrane bound protein kinases by ion- exchange chromatography. Erythrocyte membranes, obtained from 25 ml of packed cells, (2 mg of proteins/ml, 31.3 ml), were diluted with one volume of 5mM sodium phosphate buffer, pH 8.0, containing 0.2% Triton X-100, 5mM 2-mercaptoethanol, 1 mM EDTA and 0.1 mM pepstatin A. The mixture was incubated for 15 min at 0 °C and the first supernatant, obtained by centrifugation at 12,000xg for 10 min, was collected. The precipitate was treated with 10 ml of 0.2 M NaCl and the second supernatant was obtained following centrifugation as above. The two supernatants were pooled, concentrated to 10 ml by ultrafiltration on an Amicon Y10 membrane and dialyzed extensively against 5 mM sodium phosphate buffer, pH 7.6, containing 0.5 mM 2-mercaptoethanol and 1 mM EDTA (buffer A). The sample was loaded onto a DEAE-52 column (1x12 cm) previously equilibrated with buffer A and the adsorbed proteins were eluted with 300 ml of a linear gradient of NaCl from 0 to 0.4 M. The flow rate was 0.5 ml/min and fractions of 2.5 ml were collected. Protein kinase activity was assayed using 50 µl of the eluted fractions. Two peaks of kinase activity were eluted (fraction 27-35, peak I kinase and fraction 40-52, peak II kinase) separately pooled, concentrated to 3 ml by ultrafiltration and dialyzed against buffer A. The specific activities were 38.5 units/mg for peak I kinase and 1142 units/mg for peak II kinase with a purification of 24.7 and 732 fold respectively. A recovery of 83% of the total activity (97.6 units) loaded onto the column was obtained. Peak I kinase accounted for 60% (49 units) of the protein kinase activity recovered from the column, peak II kinase for the remaining 40% (32 units).

Purification of spectrin, band 4.1 and band 3 protein. Spectrin was prepared from human erythrocyte membranes as previously described (14), band 4.1 was purified as reported by Palfrey and Waasem (15). The preparation of band 3 protein was performed according to Steck et al. (16).

RESULTS

To provide direct evidence for a role of calpain in the increased susceptibility to phosphorylation of band 3 protein, we have investigated the rate of phosphorylation of membrane proteins in inside-out vesicles prepared from red cells from normotensive and hypertensive subjects pre-loaded by exposure to ionophore and micromolar calcium. In vesicles from control cells the rate of phosphorylation of band 3 protein increased 1.6 fold; in vesicles from erythrocytes of hypertensive patients 1.2 fold (Table I). In all the experiments the rate of phosphorylation of band 2 remained unchanged. These results indicate that the selective increase in susceptibility to phosphorylation of band 3 protein observed in red cells of hypertensive subjects, can be reproduced in red cells from normotensive subjects by increasing the intracellular concentration of free calcium. That the cal-

TABLE I

Effect of inhibition of intracellular proteinases on the phosphorylation of band 2 and band 3 protein

ADDITIONS		NORMOTENSIVE SUBJECTS		HYPERTENSIVE SUBJECTS		RATIO (B/A)
Proteinase inhibitor	Ca^{2+} (μM)	Band2 cpm	Band3(A) cpm	Band2 cpm	Band3(B) cpm	
None	0	890	4600	850	7250	1.58
None	5	800	7480	930	8800	1.18
Leupeptin	5	900	4390	890	7100	1.62
Pepstatin	5	850	7100	840	9080	1.28
Leupeptin + Pepstatin	5	910	4520	910	7800	1.73
mAb C 56.3	5	880	4750	860	7250	1.53

Erythrocytes were previously loaded with proteinase inhibitors and resealed as reported in Methods. The concentrations of leupeptin and pepstatin were 0.2 mM and that of mAb C 56.3 was 0.1 mg/ml. Aliquots (0.2 ml) of the resealed cells were incubated with 5 μM A23187 and 5 μM Ca2+. Inside-out vesicles were prepared and incubated with γ32 P-ATP (see Methods).32P incorporation in band 2 and band 3 was evaluated as described in Fig. 1. The values reported are the means of three separated experiments carried out with different subjects.

cium effect is mediated by activation of calpain (9) is supported by the observation that if erythrocytes were loaded with the calpain inhibitor leupeptin or with mAb C 56.3, prior to the addition of calcium and ionophore, the rate of phosphorylation of band 3 protein in inside-out vesicles was maintained at constitutive levels in each group of subjects (Table I). Preloading with pepstatin A, an inhibitor of the membrane bound acid endopeptidases, did not affect the increased phosphorylation induced by the presence of micromolar calcium. On the basis of these results we suggest that in red cell from hypertensive subjects, due to the presence of low levels of calpain inhibitor (8) associated with increased intracellular concentration of Ca^{2+} (18,19), activation of calpain occurs, causing alterations in the membrane structure that are responsible for the increased phosphorylation of band 3 protein. The protein phosphorylated corresponds to the anion-transport protein, based on the observation that glycophorin, also present in the band 3 fraction, appears not to be a substrate for the

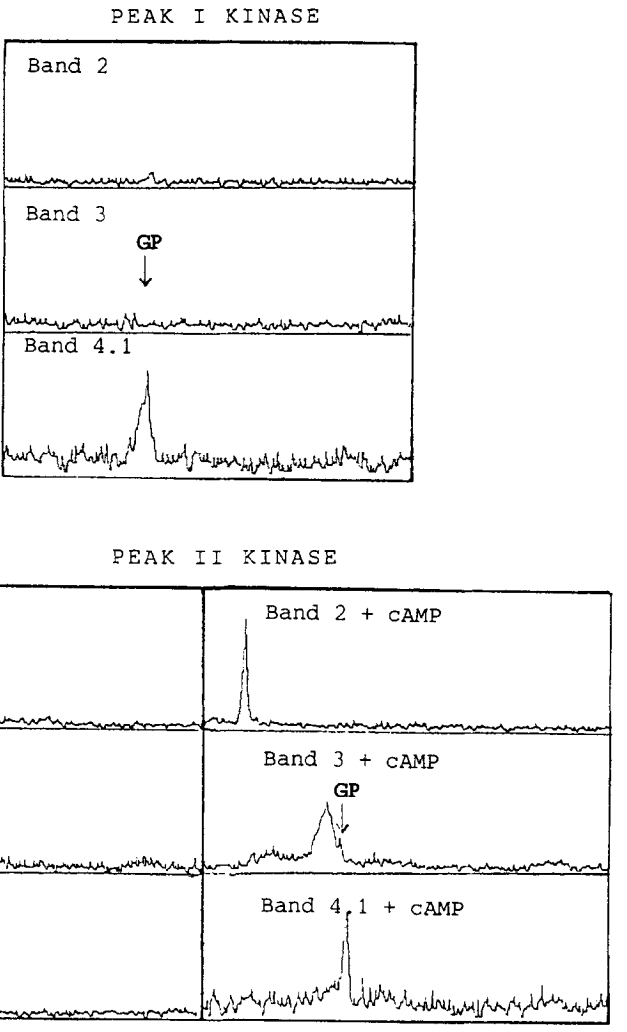

Fig.1 Phosphorylation of purified erythrocyte proteins by peak I and peak II kinases. Spectrin, band 4.1 and band 3 protein (0.02 mg) were incubated in 0.2 ml of 25 mM imidazole HCl, pH7.5, containing 5 mM MgCl2, 10 µM γ-32P ATP and 0.2 units of each partially purified protein kinase. In the case of peak II kinase the assay was also performed in the presence of 10 µM cAMP. The mixtures were incubated for 5 min at 30°C, the reaction stopped by the addition of 50 µl of 50 mM Tris-HCl, pH 8.0, containing 5% SDS, 50% glycerol, 5mM EDTA and 5% 2-mercaptoethanol, and heating at 100°C for 2 min. An aliquot (70 µl) of the mixture was then submitted to SDS-PAGE (17). The gel was stained with Coomassie-blue and dried. The labelled proteins were identified with an Ambis Beta Scanning System. Glycophorin (GP) migration was established by PAS staining (17) of band 3 lanes.

kinases of the erythrocyte membrane (Fig. 1) and by the fact that the α and β subunits of the calmodulin-binding protein are virtually absent in our preparations of inside-out vesicles (20).

In order to characterize the protein kinase involved in the phosphorylation of band 3 protein, the erythrocyte membrane bound protein kinase activity

was solubilized and applied to a DEAE-column (see Methods). Two distinct peaks of protein kinase were eluted (see Methods), collected separately and the properties of the two partially purified kinase forms were examined. Both kinases were insensitive to calcium ions, phospholipids and dioleoylglycerol added either separately or in combination. Trifluoperazine, an inhibitor of calcium dependent protein kinases, also did not inhibit (Table II). Thus neither protein kinase belongs to the class of Ca^{2+}/calmodulin dependent or Ca^{2+}/phospholipid dependent protein kinases. Peak I kinase activity was found to be insensitive to cyclic nucleotides, while peak II kinase showed a 1.5-1.8 fold stimulation by cAMP (Table II).

Purified peak I kinase was found to phosphorylate only band 4.1 protein, whereas peak II kinase catalyzed the phosphorylation of band 2 (the β subunit of spectrin), of band 3 protein and of band 4.1 only in the presence of cAMP (Fig. 1). Stimulation of protein kinase by cAMP restricted to certain substrates is in agreement with previous report (21).

TABLE II

Catalytic properties of erythrocyte membrane protein kinases

ADDITION	PROTEIN KINASE ACTIVITY (%)	
	Peak I	Peak II
None	100	100
Ca^{2+}, 0.5 mM	86	100
Ca^{2+}, 0.5mM + Phosphatidylserine + dioleoylglycerol	82	140
Phosphatidylserine	110	130
Trifluoperazine, 50 µM	98	100
Bt $_2$cAMP, 10 µM	100	180
cAMP, 10 µM	100	158
cGMP, 10 µM	100	100
AMP, 10 µM	100	100
ADP 10 µM	73	76

Peak I and peak II kinases, 0.1 units, were assayed (see Methods) in the absence or in the presence of the indicated effectors.
The concentrations of phosphatidylserine and dioleoylglycerol were 50 µg/ml and 1 µg/ml, respectively.

DISCUSSION

Human erythrocytes have been shown to contain membrane bound protein kinase activities stimulated by either cAMP (21,22) or Ca^{2+} (23) and acting on several transmembrane (24-26) or membrane skeletal proteins (27-29). The present work provides important clues to a possible physiological role for these kinases. The specific increase in phosphorylation of band 3 protein by membrane-bound protein kinase(s) recently reported in erythrocytes of patients with essential hypertension (9) has tentatively been explained on the basis of unregulated calpain-mediated intracellular proteolysis (8). In the present studies the role of calpain is confirmed by the demonstration that phosphorylation of band 3 protein is increased when the intracellular concentration of Ca^{2+} is raised within the micromolar range and that this increase is not seen in cells preloaded with leupeptin or a monoclonal anticalpain antibody. The increased rate of phosphorylation of band 3: a) appears to be a constitutive property of red cells from hypertensive subjects, b) does not occur during cell lysis, c) is highly selective in red cell of hypertensive patients or in control cells following activation of calpain. These results suggest that the effects of calpain are not mediated through a proteolytic activation of the protein kinase. Preliminary identification of the membrane-bound protein kinase responsible for the enhanced phosphorylation of band 3 protein is also reported. On the basis of the characteristics of the kinases partially purified from red cell membrane, we have identified the one responsible for phosphorylation of band 3, and found to correspond to the one previously described as being stimulated by cAMP (21). Since the band 3 corresponds to the anion-transport protein, examination of the effects of the kinase on its properties may be important to establish if alteration in cell functions in hypertensive subjects can be due to an abnormal rate of phosphorylation affecting either the dynamic organization of the membrane skeleton or the function of the anion-transport transmembrane protein. In any case our results demonstrate that an important lesion in the metabolic and architectural organi-

zation of the cell, as it occurs in the hypertensive state, involves an abnormal regulation of calpain activity.

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