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Activation of erythrocyte membrane Ca^{2+}-ATPase by calpain

K.S. Au

Department of Biochemistry, University of Hong Kong, Hong Kong (Hong Kong)

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Ca^{2+}-ATPase of erythrocyte membranes, prepared from erythrocytes substantially removed of contaminating leukocytes, was found to be activated by calpain isolated from the same source. Saponin or glycodeoxycholate treatment of membranes was essential for elicitation of the calpain response. Unlike the membrane bound ATPase, solubilized ATPase was inactivated by calpain. Digestion of membranes with the protease did not affect the K_m (ATP) of Ca^{2+}-ATPase though stimulation of the membrane ATPase by calmodulin could be partially substituted by calpain treatment. As compared with control, Ca^{2+}-ATPase of calpain-digested membranes attained maximal activity at a lower free Ca^{2+} concentration.

Introduction

Activity of the erythrocyte membrane Ca^{2+}-ATPase is known to be regulated by protein modulators. The calcium-binding protein, calmodulin, plays the part of a protein activator of the transport enzyme [1,2]. Its action is opposed by an endogenous inhibitor protein of the ATPase [3–5]. This Ca^{2+}-ATPase inhibitor protein, however, does not function as a specific protease to bring about irreversible degradation and hence inactivation of the ATPase [6]. Proteases, especially those that are stimulated by Ca^{2+}, are in fact known to activate various enzymes by limited proteolysis. Thus, for example, rat brain protein kinase C can be activated by a Ca^{2+} proteinase purified from the same source [7]. Tryptophan hydroxylase, one of the regulatory enzymes in the biosynthesis of serotonin in rat brain, is known to be activated by Ca^{2+}-dependent proteolysis [8]. In rat erythrocytes, a Ca^{2+}-activated protease is implicated in the regulation of pyruvate kinase [9]. A latent Ca^{2+}-stimulated ATPase of gastric microsomes can be unmasked after trypsinization, and a trypsin-like protease that can unmask the microsomal Ca^{2+}-stimulated ATPase is demonstratable in the cytosolic fraction of fundic cells [10]. The red cell membrane Ca^{2+}-ATPase has also been demonstrated to be activated by mild treatment with trypsin, which simultaneously abolishes calmodulin dependence of the ATPase [11,12]. No endogenous protease that can influence the activity of the transport ATPase, however, has so far been reported in the erythrocyte.

The mammalian erythrocyte in fact contains a Ca^{2+}-activated neutral protease, also known as calpain I [13,14], which is active at micromolar concentrations of Ca^{2+}. Its precise function in the red cell is not yet clear in spite of reports on its degradative action on band 3 [13] and other cytoskeletal proteins [15]. In view of the fact that

Correspondence: K.S. Au, Department of Biochemistry, University of Hong Kong, Sassoon Road, Hong Kong, Hong Kong.

Ca^{2+}-ATPase can be activated by protease, that calpain is known to activate a variety of enzymes and that calpain I functions at concentrations of Ca^{2+} that calmodulin and Ca^{2+}-ATPase operate, it appears desirable to test if the Ca^{2+}-activated neutral protease qualifies as another protein modulator of the erythrocyte Ca^{2+} transport ATPase besides calmodulin and the Ca^{2+}-ATPase inhibitor protein.

Materials and Methods

Materials

Vanadium-free ATP, leupeptin hemisulfate, antipain dihydrochloride, Reactive Red 120-agarose (type 3000-CL), α-cellulose, Type 50 microcrystalline cellulose and sodium glycodeoxycholate were purchased from Sigma Chemical Co. (St. Louis, MO). Saponin was from Calbiochem (San Diego, CA) while bovine testis calmodulin was a product of Pharmacia Fine Chemicals (Uppsala).

Methods

Preparation of erythrocyte membranes. Fresh heparinised adult pig blood was first removed of leukocytes and platelets by passage through a bed composed of microcrystalline cellulose and α-cellulose after the manner described by Beutler et al. [16]. The blood was then centrifuged to remove plasma and any remaining buffy coat and further washed three times with 8 vol. of saline.

For preparation of membranes, washed cells were lysed with 8 vol. of 10 mM Tris/1 mM EDTA (pH 7.4), then washed five times with the same buffer before three more washes with 10 mM Tris-HCl (pH 7.4). Membrane suspensions were adjusted to about 2 mg protein/ml and were kept at −70 °C before use. In some experiments, membranes were extracted to yield Ca^{2+}-ATPase. This was performed by treating membranes for 10 min at 4 °C with 50 mM imidazole/100 mM NaCl/5 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/0.5% Triton X-100/1% Tween 20/0.1% phosphatidylcholine (pH 7.4). After treatment, the mixture was centrifuged at 100 000 × *g* for 60 min to sediment residual membranes and the supernatant containing the ATPase was concentrated by ultrafiltration.

Ca^{2+}-ATPase assay. Ca^{2+}-ATPase activity was measured at 37 °C according to the method of Au et al. [6]. A membrane protein concentration of 100 μg/ml was employed. Membrane protein concentration was determined by the method of Lowry et al. [17]. Concentration of free Ca^{2+} in the assay medium was determined with a Ca^{2+} electrode (Radiometer, Copenhagen, Denmark). When solubilized Ca^{2+}-ATPase was assayed, the assay medium contained in addition 1% Tween 20.

Preparation of calpain I from pig erythrocytes. Removal of leukocytes, platelets and plasma from red cells has been described earlier. Washed erythrocytes were lysed with 10 vol. of 10 mM Tris/maleate (pH 6.6). The resulting hemolysate was then centrifuged at 48 000 × *g* for 45 min to remove membranes. Membrane-free hemolysate was then concentrated by ultrafiltration through Amicon PM-10 membrane, dialyzed against 20 mM Tris/maleate (pH 6.6) before chromatography on a carboxymethyl-Sephadex (C-50) column to remove hemoglobin. The column had a bed volume 10-times that of the volume of washed cells and was eluted with the 20 mM buffer. Protein moving ahead of hemoglobin was collected, concentrated by ultrafiltration through PM-10 membrane and dialyzed against 20 mM Mops/0.5 M NaCl/1 mM EDTA/1 mM EGTA/5 mM β-mercaptoethanol (pH 7.5). It was then further purified by Reactive Red-agarose affinity chromatography after the manner described by Clark et al. [18]. One unit of calpain is defined as the amount of the protease preparation catalyzing an increase of 1.0 absorbance unit at 750 nm when assayed by the method of Murakami et al. [13] using casein as substrate.

Digestion of membranes with calpain. Membranes were first treated with saponin (0.27 mg/ml) or glycodeoxycholate (10 μM or 3.9 mM) for 6 min at 25 °C. They were then washed to remove saponin or glycodeoxycholate. The pelleted membranes (570 μg protein) were resuspended in a freshly prepared medium containing 50 mM imidazole/5 mM cysteine/1 mM $CaCl_2$/0.1 mM EDTA (pH 7.4) and calpain in a final volume of 0.8 ml for incubation at 30 °C for 30 min. After incubation, calpain was removed from the membranes by repeated washing with the suspension buffer for the membranes.

Results

Ca^{2+}-ATPase of erythrocyte membranes, prepared from pig red cells washed four times with 8 vol. of isotonic buffer to remove plasma and buffy coat but without thorough removal of leukocytes by the method of Beutler et al. [16], was found to be sensitive to calpain inhibitors. 0.2 mM freshly prepared leupeptin suppressed Ca^{2+}-ATPase activity of such membranes by 40–70%, while the same concentration of antipain gave 30–40% inhibition. The degree of inhibition varied from one membrane preparation to another, fluctuating with the amount of contaminating leukocytes that were present. On the other hand, after removal of leukocytes by the method of Beutler et al. and subsequent washing of erythrocytes as described in Methods, 0.2 mM and even 0.4 mM leupeptin could only suppress Ca^{2+}-ATPase activity of the resulting erythrocyte membranes by about 10%. These findings suggest the presence of Ca^{2+}-activated neutral proteases derived from contaminating leukocytes in erythrocyte membrane preparations, and that such proteases could influence erythrocyte membrane Ca^{2+}-ATPase activity.

Using calpain I isolated from the erythrocytes, it was indeed demonstrated that erythrocyte membrane Ca^{2+}-ATPase could be stimulated by this protease. Demonstration of calpain activation required that membranes were prepared from red cells substantially removed of contaminating leukocytes. Besides, treatment of membranes with saponin or glycodeoxycholate was essential for elicitation of the calpain response (Table I). The degree of calpain activation of membrane ATPase increased with increasing concentration of the protease (Fig. 1). Digestion of the membranes with calpain for 5 min at 30 °C resulted in the same degree of Ca^{2+}-ATPase stimulation as digestion of the membranes for 30 min.

Response of Ca^{2+}-ATPase of human erythrocyte membrane to calpain isolated from pig red cells was also tested. With saponin-treated membranes, 0.8 units of pig calpain could only activate the human ATPase by 15%. With glycodeoxycholate (3.9 mM)-treated membranes, 30% activation was observed. The relatively poor response of the

TABLE I

EFFECT OF CALPAIN ON ERYTHROCYTE MEMBRANE Ca^{2+}-ATPase

Erythrocytes used had been removed of contaminating leukocytes by the method of Beutler et al. [16] prior to preparation of membranes as described in Methods. Membranes (570 μg protein) were digested with 0.76 units of calpain before their Ca^{2+}-ATPase activities were compared with controls. ATPase activity was expressed as μmol/h per mg membrane protein.

Membranes	Ca^{2+}-ATPase activity		% activation
	control membranes	digested membranes	
Untreated	4.9	4.9	0
Saponin-treated (0.27 mg/ml)	4.66	9.23	98.1
Glycodeoxycholate-treated			
3.9 mM	3.23	6.69	107.1
10 μM	3.81	5.59	46.7

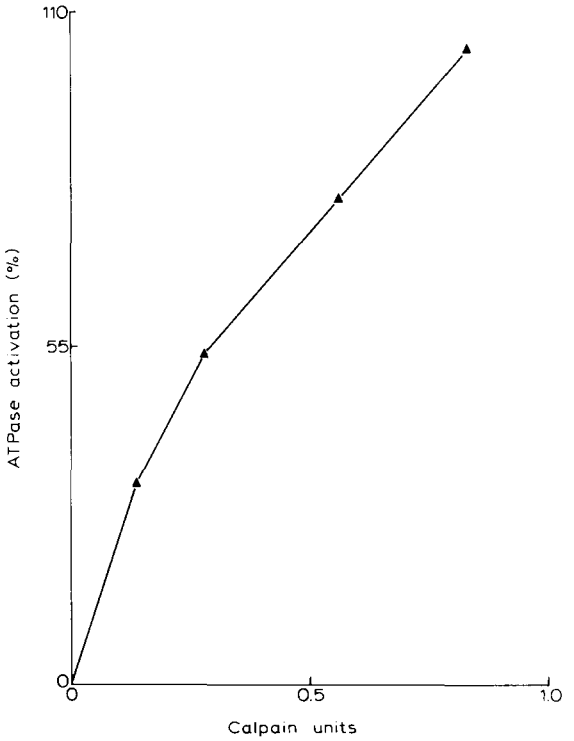

Fig. 1. Response of membrane Ca^{2+}-ATPase activity to varying concentrations of calpain. Membranes were treated with saponin as described in Methods before measurement of ATPase activity. Ca^{2+}-ATPase activity of undigested erythrocyte membranes was 4.7 μmol/h per mg membrane protein.

human enzyme to pig red cell calpain might be related to species difference.

The effect of calpain was also tested using Triton X-100 solubilized Ca^{2+}-ATPase. In an experiment in which 0.4 units of calpain were incubated at 30 °C for 5 min with the solubilized enzyme, instead of the 67% activation that was observed with ATPase bound to membranes from which the solubilized enzyme was derived, calpain decreased Ca^{2+}-ATPase activity of the solubilized enzyme by 51%.

K_m (ATP), determined for pig erythrocyte membrane Ca^{2+}-ATPase was 3.64 μM (Fig. 2). This is in the same range as the K_m value determined for Ca^{2+}-ATPase of human erythrocyte membrane [19]. Though V of calpain-digested membrane Ca^{2+}-ATPase was found to increase by 78% as compared with control, the K_m value remained unchanged, thus suggesting that calpain stimulated ATPase not by affecting the affinity of the enzyme for its substrate, ATP.

Calmodulin stimulation of membrane Ca^{2+}-ATPase was also studied (Table II). With saponin-treated membranes, calmodulin alone activated the ATPase by 92.9% while calpain digestion stimulated the membrane enzyme by 100%, giving a total of 192.9% activation. The observed stimulation due to the combined action of calmodulin and calpain treatment, however,

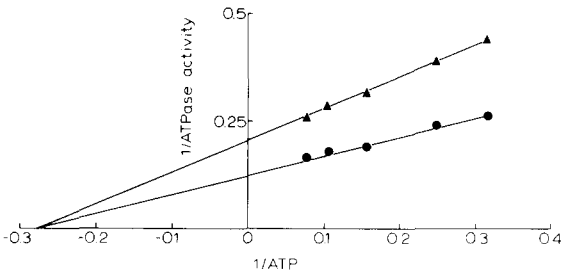

Fig. 2. Lineweaver-Burk plots for Ca^{2+}-ATPase of control (▲———▲) and calpain-digested (●———●) erythrocyte membranes. ATP (μM); ATPase activity (μmol/h per mg membrane protein). Saponin-treated membranes (570 μg protein) were digested with 0.54 units of calpain as described in Methods before the Ca^{2+}-ATPase activity was measured. The Ca^{2+}-ATPase activities at various ATP concentrations were compared with those measured for control membranes.

was only 161.9%. That the observed combined effect was lower than if the separate effects were added together suggests that there was an overlap in the effects of calmodulin and calpain on membrane Ca^{2+}-ATPase.

With glycodeoxycholate (3.9 mM) treated membranes, the observed combined effect due to calmodulin and calpain treatment was 114.9% stimulation of ATPase. This was identical to the activation of the enzyme due to calpain treatment alone. Treatment with 10 μM glycodeoxycholate was found to give an observed combined effect of

TABLE II

CALMODULIN STIMULATION OF Ca^{2+}-ATPase OF CALPAIN DIGESTED MEMBRANES

Membranes used were prepared as described in Methods. They were then further treated with saponin (0.27 mg/ml) or glycodeoxycholate (3.9 mM) before calpain digestion and subsequent assay of Ca^{2+}-ATPase activity (μmol/h per mg membrane protein). For calpain treatment, membranes (570 μg protein) and calpain (0.77 units) were used. Calmodulin, when present, was at a concentration of 0.6 μM.

Membranes	Calpain	Calmodulin	Ca^{2+}-ATPase activity	% activation		
				by calmodulin	by calpain	by calpain + calmodulin
Saponin-treated						
	−	−	4.2			
	−	+	8.1	92.9		
	+	−	8.4		100	
	+	+	11.0			161.9
Glycodeoxycholate-treated						
	−	−	3.23			
	−	+	6.37	97.2		
	+	−	6.94		114.9	
	+	+	6.94			114.9

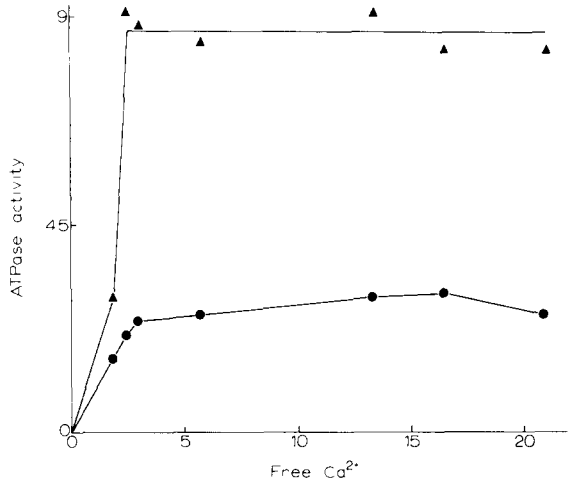

Fig. 3. Response of Ca^{2+}-ATPase of control (●———●) and calpain-digested (▲———▲) erythrocyte membranes to varying concentrations of free Ca^{2+}. ATPase activity (μmol/h per mg membrane protein); free Ca^{2+} concentration (μM). Saponin-treated membranes (570 μg protein) were digested with 2.1 units of calpain as described in Methods before measurement of Ca^{2+}-ATPase activity. The Ca^{2+} concentration required for half-maximal activation of control pig erythrocyte membrane Ca^{2+}-ATPase was found to be 2 μM.

53.2% activation of the enzyme versus 100.5% activation due to calmodulin and 42.9% activation due to calpain. That the observed combined effect due to calmodulin and calpain treatment in glycodeoxycholate-treated membranes was similar to the effect due to calpain treatment alone suggests a loss of calmodulin response in Ca^{2+}-ATPase of these membranes following calpain digestion.

While the concentration of Ca^{2+} required for half-maximal activation of human erythrocyte membrane Ca^{2+}-ATPase is about 20 μM [20], at the same concentration range of free Ca^{2+}, Ca^{2+}-ATPase of pig erythrocyte membrane showed maximal activity. With calpain digested membranes, maximal ATPase activity was attained already at 2.4 μM free Ca^{2+} concentration (Fig. 3).

Discussion

Proteolysis of enzymes normally depresses their catalytic activity. In some of the targets of calmodulin regulatory function, however, activity is enhanced at least on limited proteolysis [20]. Thus in the case of Ca^{2+}-pumping ATPase of erythrocyte membrane, mild treatment with trypsin activates the transport enzyme [11,12]. The present study shows that the Ca^{2+}-activated neutral protease, calpain I, isolated from red cells can stimulate membrane Ca^{2+}-ATPase of the same cells.

There are differences, however, in the way that trypsin and calpain act. While in the case of trypsin digestion, both membrane and solubilized ATPase are stimulated [11,21], with calpain digestion, it was found that the membrane enzyme was activated while the solubilized enzyme was inactivated. This suggests the importance of the membrane bilayer in ensuring specific protease action on membrane enzyme. Such specific action of calpain on the membrane Ca^{2+}-ATPase might be physiologically significant. Furthermore, while increasing concentration of trypsin gives less activation [12], increasing calpain concentration gives greater activation.

Elicitation of the calpain response requires prior exposure of the erythrocyte membrane to saponin or glycodeoxycholate. This suggests protection by the membrane lipid bilayer of a normally concealed site on Ca^{2+}-ATPase where calpain acts, but that such a site, possibly an inhibitory peptide of the ATPase, is exposed to calpain action through treatment of the membrane with glycodeoxycholate or saponin.

When high concentration of glycodeoxycholate (3.9 mM) was used, the calmodulin-binding site on membrane Ca^{2+}-ATPase presumably became more exposed and hence more susceptible to calpain degradation as compared with when saponin was employed as detergent. This might explain why Ca^{2+}-ATPase of saponin but not of glycodeoxycholate-treated membranes responded to calmodulin after calpain treatment. In the fully solubilized ATPase, further exposure of portions of the enzyme crucial for activity to the devastating action of calpain might account for inactivation rather than stimulation of the enzyme.

Though saponin and high concentration of glycodeoxycholate would be irrelevant physiologically, calpain stimulation of membrane Ca^{2+}-ATPase demonstratable at 10 μM glycodeoxycholate might be of some relevance. Calpain activation of the membrane ATPase may hence be viewed as one way that the red cell's defensive system acts to guard against any undesirable in-

crease in intracellular Ca^{2+}. The ability of calpain-treated membrane Ca^{2+}-ATPase to attain maximal activity at a free Ca^{2+} concentration of only 2.4 μM further emphasizes that calpain-activated ATPase can participate in handling Ca^{2+} changes in the micromolar range.

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