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Ca^{2+}-mediated activation of human erythrocyte membrane Ca^{2+}-ATPase

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Ca^{2+}-ATPase of human erythrocyte membranes, after being washed to remove Ca^{2+} after incubation with the ion, was found to be activated. Stimulation of the ATPase was related neither to fluidity change nor to cytoskeletal degradation of the membranes mediated by Ca^{2+}. Activation of the transport enzyme was also unaffected by detergent treatment of the membrane, but was suppressed when leupeptin was included during incubation of the membranes with Ca^{2+}. Stimulation of the ATPase by a membrane-associated Ca^{2+}-dependent proteinase was thus suggested. Much less 138 kDa Ca^{2+}-ATPase protein could be harvested from a Triton extract of membranes incubated with Ca^{2+} than without Ca^{2+}. Activity of the activated enzyme could not be further elevated by exogenous calpain, even after treatment of the membranes with glycodeoxycholate. There was also an overlap in the effect of calmodulin and the Ca^{2+}-mediated stimulation of membrane Ca^{2+}-ATPase. While K_m(ATP) of the stimulated ATPase remained unchanged, a significant drop in the free-Ca^{2+} concentration for half-maximal activation of the enzyme was observed.

Introduction

Besides direct stimulation by Ca^{2+}, the activity of various membrane Ca^{2+}-stimulated ATPases, including that of the human erythrocyte, is known to be influenced by changes in membrane lipid fluidity [1–3], which in turn could be affected by Ca^{2+} [4]. Ca^{2+} is also the ion required for the functioning of red cell calpain, whether cytosolic [5] or membrane-associated [6]. Furthermore, the Ca^{2+}-dependent proteinase preferentially cleaves band 3 as well as band 4.1 and spectrin of the human red cell membrane cytoskeleton [7]. Such proteolytic events could lead to change in membrane fluidity [8,9], which in turn could affect Ca^{2+}-ATPase activity. One of the aims of the present study is thus to find out whether such changes induced by Ca^{2+} can affect Ca^{2+}-ATPase activity of human erythrocyte membranes.

Au [10] recently reported a higher Ca^{2+}-ATPase activity in pig erythrocyte membranes digested with erythrosolic calpain with added Ca^{2+}, as compared with Ca^{2+}-ATPase activity in membranes incubated with Ca^{2+} alone. Wang et al. [11] on the other hand, reported activation of human erythrocyte membrane Ca^{2+}-ATPase by erythrosolic calpain with added Ca^{2+}. It is important to note, however, that unlike Au's study [10], their stimulation of ATPase was observed only in comparison with membranes incubated with calpain but in the absence of Ca^{2+}. Whether there is activation of Ca^{2+}-ATPase in comparison with controls incubated with Ca^{2+} alone without added calpain is not known.

In the human red cell, 2% of the total pool of calpain is associated with the membrane [6]. Like cytosolic calpain, this pool of membrane-associated Ca^{2+}-dependent proteinase can also be stimulated by Ca^{2+} and hence may be involved in Ca^{2+}-ATPase activation. Physiologically, such a mode of ATPase stimulation would actually be more important than stimulation by cytosolic calpain, since membrane-associated Ca^{2+}-protease is not susceptible to calpastatin inhibition [6]. Another aim of the present study is therefore to see if Ca^{2+}-dependent proteinase associated with the human erythrocyte membrane indeed participates in the regulation of Ca^{2+}-ATPase present in the same membrane.

Materials and Methods

Materials

Vanadium-free ATP, leupeptin hemisulphate, Reactive-Red 120-agarose (type 3000-CL), α-cellulose, Type 50 microcrystalline cellulose and sodium glycode-

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oxycholate were purchased from Sigma (St. Louis, MO) while bovine testis calmodulin was a product of Pharmacia (Uppsala). 1,6-Diphenyl-1,3,5-hexatriene was from Aldrich (Dorset, U.K.).

Methods

Preparation of erythrocyte membranes. Fresh heparinised human or animal blood was employed. Removal of leukocytes and platelets by passage through a cellulose column and the subsequent preparation of membranes were performed as described by Au [10]. These membranes were freed of leukocyte contamination and deficient in calmodulin. All membranes prepared were stored at −70 °C for about 1 month before use.

Ca^{2+}-ATPase assay. Ca^{2+}-ATPase activity was measured at 37 °C according to the method of Au [10].

Incubation of membranes with Ca^{2+} or cytosolic calpain. Membranes (600 μg protein) were incubated at 30 °C for 30 min in a freshly prepared medium containing 50 mM imidazole/5 mM cysteine/0.4 mM free Ca^{2+} (pH 7.4) in a final volume of 0.8 ml. Calpain, when present, amounted to 0.61 units. One unit of calpain is defined as the amount of the proteinase preparation catalysing an increase of 1.0 absorbance unit at 750 nm when assayed by the method of Murakami et al. [5] using casein as substrate. Calpain I was prepared from human erythrosol after the manner described by Au [10].

In some experiments, membranes were first treated with 15 μM glycodeoxycholate at 25 °C for 6 min, then washed to remove glycodeoxycholate before they were incubated with Ca^{2+} or calpain. After incubation, all membranes were washed twice again to remove Ca^{2+} or calpain before their Ca^{2+}-ATPase activities were determined. No Ca^{2+} remained in the washed membrane preparations to affect the subsequent measurement of Ca^{2+}-ATPase activity. Membrane protein concentration was also not significantly affected after incubation of the membranes with Ca^{2+}.

Measurement of fluorescence anisotropy. After labeling membranes with 1,6-diphenyl-1,3,5-hexatriene according to the method described by Jarolim and Mirčevová [12], steady-state anisotropy of fluorescence, r_s, was measured using a Hitachi 650-60 fluorescence spectrofluorimeter adapted for fluorescence polarization measurement. Determination of r_s, calculation of r_∞ (hindered anisotropy) and S (order parameter component of membrane fluidity) were performed as described by Lowe and Coleman [13]. Corrections for light scattering due to membranes and the fluorescence in the ambient medium were also made. The combined corrections were less than 3% of the total fluorescence intensity. Moreover, no significant difference in the excited-state lifetimes, as assessed by total fluorescence intensity, was observed for diphenylhexatriene incorporated in the various membranes.

Purification of human erythrocyte membrane Ca^{2+}-ATPase. Membrane solubilisation and purification of the ATPase from membranes incubated with or without 0.4 mM free Ca^{2+} were carried out after the manner described by Graf et al. [14].

SDS-polyacrylamide gel electrophoresis. Ca^{2+}-ATPase purified from the same amount of membrane incubated with or without Ca^{2+}, and membranes incubated under other conditions, were first boiled for 3 min with 2% SDS/15 mM dithiothreitol before being subjected to electrophoresis on a gradient slab gel (5–15% acrylamide) with the discontinuous system of Laemmli [15] and visualised by staining with Coomassie brilliant blue R-250.

Results

Ca^{2+}-ATPase activity of human erythrocyte membranes, washed after incubation at 30 °C for 30 min with 5 mM cysteine and 0.4 mM free Ca^{2+}, was found to be significantly higher than the Ca^{2+}-ATPase activity of membranes incubated in the absence of Ca^{2+} (Table I). Inclusion of 0.61 units of cytosolic calpain during incubation could not bring about any further increase in Ca^{2+}-ATPase activity of the Ca^{2+}-treated membranes. Furthermore, while with pig erythrocyte membranes, prior treatment with saponin or glycodeoxycholate is essential for calpain activation of Ca^{2+}-ATPase [10], with human erythrocyte membranes, detergent treatment could not elicit the calpain response, nor could the treatment affect basal Ca^{2+}-ATPase activity (Table I). The observed Ca^{2+}-ATPase activation in human mem-

TABLE I

Activation of human erythrocyte membrane Ca^{2+}-ATPase after incubation of the membranes with Ca^{2+}

Membranes were prepared as described in Materials and Methods. They were incubated at 30 °C for 30 min in the presence of 5 mM cysteine/0.4 mM free Ca^{2+} along with Ca^{2+}-free controls. Exogenous calpain, if present, was at a concentration of 0.61 units. Some membranes were treated with 15 μM glycodeoxycholate before incubation. All membranes were washed twice after incubation before their Ca^{2+}-ATPase activities (μmol/h per mg membrane protein) were compared. Means ± S.E. for n different membrane samples are presented. [a] $P < 0.01$ when compared with the respective Ca^{2+}-free controls.

Glycodeoxycholate treatment	During incubation		n	Ca^{2+}-ATPase activity
	Ca^{2+}	calpain		
−	−	−	6	1.574 ± 0.216
−	+	−	6	2.820 ± 0.425 [a]
−	+	+	6	2.809 ± 0.416 [a]
+	−	−	7	1.474 ± 0.212
+	+	−	7	2.802 ± 0.442 [a]
+	+	+	7	2.784 ± 0.334 [a]

TABLE II

Leupeptin supression of Ca^{2+}-ATPase activation

Human erythrocyte membranes used were prepared as described in Materials and Methods. They were incubated at 30 °C for 30 min in the presence of 5 mM cysteine/0.4 mM free Ca^{2+} along with Ca^{2+}-free controls. Leupeptin, when present, was at a concentration of 0.4 mM. All membranes were washed twice after incubation and the Ca^{2+}-ATPase activities (μmol/h per mg membrane protein) of the treated membranes were then compared with that of the control. Leupeptin had no direct effect on Ca^{2+}-ATPase activity of control membranes but significantly inhibited ($P < 0.02$) Ca^{2+}-ATPase activity of membranes incubated with Ca^{2+}. Means ± S.E. for six differnt membrane samples are presented. n.s., not significant when compared with Ca^{2+}-free control.

During incubation		Ca^{2+}-ATPase activity	P
Ca^{2+}	leupeptin		
–	–	1.155 ± 0.127	
+	–	1.767 ± 0.178	< 0.01
+	+	1.440 ± 0.173	n.s.

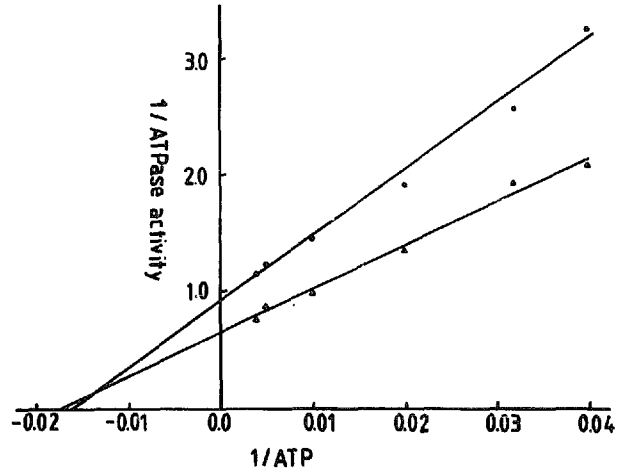

Fig. 1. Lineweaver-Burk plots for Ca^{2+}-ATPase of human erythrocyte membranes incubated with 5 mM cysteine/0.4 mM free Ca^{2+} (▲) and control membranes incubated without Ca^{2+} (●). After incubation, both membranes were washed twice before their Ca^{2+}-ATPase activities (μmol/h per mg membrane protein) at various ATP concentrations (μM) were compared.

branes was found to be significantly suppressed when 0.4 mM leupeptin was included during incubation of the membranes with Ca^{2+} (Table II), thus suggesting the involvement of an erythrocyte membrane-associated Ca^{2+}-dependent proteinase in activation of the human red cell Ca^{2+}-ATPase. The possibility of stimulation of ATPase by contaminating leukocyte proteinase is ruled out because the red cell preparations had been freed of contaminating leukocytes by passage through cellulose columns as recommended by Beutler et al. [16].

When pig erythrocyte membranes were similarly incubated with Ca^{2+} without added capain, it was, however, found that their membrane Ca^{2+}-ATPase activity could not be stimulated, irrespective of whether the membranes had been subjected to prior treatment with glycodeoxycholate or not (results not shown). Furthermore, it was found that, while 1% Triton X-100 could extract calpain from human erythrocyte membranes, as was reported by Hatanaka et al. [6], the same treatment could not release any membrane-associated calpain activity from pig erythrocyte membranes, thus suggesting the presence of membrane-associated Ca^{2+}-dependent proteinase in human, but not in pig, red cell membranes for Ca^{2+}-ATPase activation.

The mechanism for the observed Ca^{2+}-ATPase activation was investigated by measuring K_m (ATP) and K_m for Ca^{2+} (the free Ca^{2+} concentration for half-maximal activation of the Ca^{2+}-ATPase). While V of the Ca^{2+}-ATPase of human erythrocyte membranes incubated with Ca^{2+} was found to be higher than that of membranes incubated with Ca^{2+} (Fig. 1), the K_m(ATP) determined for Ca^{2+}-ATPase of the Ca^{2+}-treated membranes (58.6 μM) was not significantly different from that determined for membranes incubated in the absence of Ca^{2+} (61.5 μM). Measurement of the free-Ca^{2+} concentration for half-maximal activation of the Ca^{2+}-ATPase, on the other hand, revealed a significantly lower K_m value for Ca^{2+} of 1.15 μM for Ca^{2+}-ATPase of membranes incubated with Ca^{2+} as compared with a K_m value for Ca^{2+} of 13.6 μM for Ca^{2+}-ATPase of membranes incubated without Ca^{2+} (Fig. 2). The above findings suggest that the observed Ca^{2+}-ATPase stimulation is due to an increase in V and

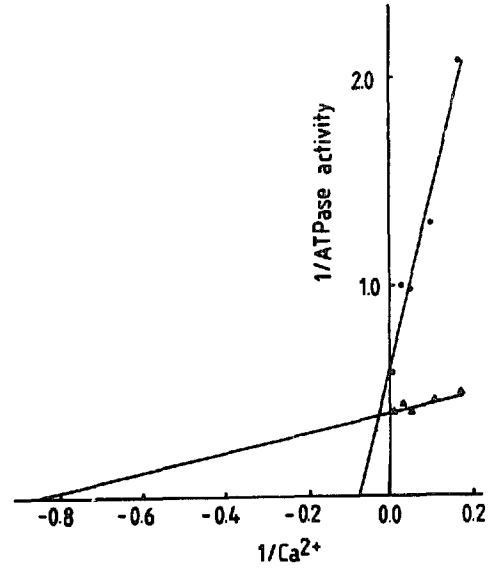

Fig. 2. Double-reciprocal plots of human erythrocyte membrane Ca^{2+}-ATPase activity (μmol/h per mg membrane protein) vs. free-Ca^{2+} concentration (μM) for determination of the concentration of Ca^{2+} giving half-maximal activation of the enzyme. ▲, membranes incubated with 5 mM cysteine/0.4 mM free Ca^{2+}; ●, control membranes incubated without Ca^{2+}. After incubation, both membranes were washed twice before their Ca^{2+}-ATPase activities (μmol/h per mg membrane protein) were compared at various free-Ca^{2+} concentrations (μM).

TABLE III

Fluorescence anisotropy from diphenylhexatriene in human erythrocyte membranes after incubation with Ca^{2+} or calpain

Membranes were prepared as described in Materials and Methods. They were incubated at 30 °C for 30 min in the presence of 5 mM cysteine/0.4 mM free Ca^{2+} along with Ca^{2+}-free controls. Exogenous calpain, if present, was at a concentration of 0.61 units. Some membranes were treated with 15 μM glycodeoxycholate before incubation. All membranes were washed twice after incubation before they were labeled with diphenylhexatriene for measurement of fluorescence anisotropy. Means ± S.E. for n different membrane samples are presented.

Temperature (°C)	Glycodeoxycholate treatment	During incubation		n	r_s	r_∞	S
		Ca^{2+}	calpain				
26	–	–	–	8	0.226 ± 0.003	0.201 ± 0.004	0.709 ± 0.008
	–	+	–	8	0.226 ± 0.006	0.202 ± 0.007	0.709 ± 0.013
	–	+	+	8	0.230 ± 0.007	0.207 ± 0.009	0.718 ± 0.016
	+	–	–	4	0.222 ± 0.005	0.196 ± 0.006	0.700 ± 0.010
	+	+	–	4	0.212 ± 0.008	0.182 ± 0.010	0.674 ± 0.019
	+	+	+	4	0.219 ± 0.006	0.192 ± 0.009	0.692 ± 0.016
37	–	–	–	8	0.207 ± 0.005	0.175 ± 0.007	0.660 ± 0.013
	–	+	–	8	0.210 ± 0.006	0.180 ± 0.007	0.670 ± 0.013
	–	+	+	8	0.202 ± 0.007	0.170 ± 0.010	0.650 ± 0.019
	+	–	–	4	0.203 ± 0.006	0.170 ± 0.009	0.651 ± 0.016
	+	+	–	4	0.194 ± 0.002	0.159 ± 0.003	0.630 ± 0.006
	+	+	+	4	0.198 ± 0.006	0.164 ± 0.008	0.639 ± 0.016

a decrease in K_m for Ca^{2+} but not the result of a change in the affinity of the ATPase for its substrate, ATP.

With respect to the human red cell membrane cytoskeleton, while no degradation was observed for membranes incubated for 30 min with Ca^{2+} alone, degradation of spectrin, band 3 and band 4.1 was obvious when calpain was added to the incubation medium along with Ca^{2+}. With membranes incubated with Ca^{2+} alone, it was found that breakdown of band 3, for example, was apparent only after overnight incubation at 37 °C. Hatanaka et al. [6] also reported digestion of human erythrocyte membrane band 3 and band 4.1 in the presence of Ca^{2+} only after incubation for 5 h.

In order to see whether the observed Ca^{2+}-ATPase activation in human membranes incubated with Ca^{2+} is related to fluidity change in the membranes, the order parameter component of membrane fluidity, S, was measured at 26 °C and also at 37 °C, which was the temperature for assay of the ATPase. It was found that membranes washed after incubation with Ca^{2+}, or in combination with calpain, did not have S values significantly different from control membranes incubated in the absence of Ca^{2+} (Table III). Prior treatment of membranes with glycodeoxycholate also did not significantly alter S values under all incubation conditions.

Calmodulin stimulation of Ca^{2+}-ATPase in human erythrocyte membranes washed after incubation with Ca^{2+} was also studied. Calmodulin alone activated Ca^{2+}-ATPase by 195.4 ± 25.6%, while Ca^{2+} treatment of the membranes stimulated the ATPase by 72.9 ± 19.8%, giving a total of 266.8 ± 41.6% activation (Table

TABLE IV

Calmodulin response of human erythrocyte membrane Ca^{2+}-ATPase after incubation of the membranes with Ca^{2+}

Membranes were prepared as described in Materials and Methods. They were incubated at 30 °C for 30 min in the presence of 5 mM cysteine/0.4 mM free Ca^{2+} along with Ca^{2+}-free controls. All membranes were washed twice after incubation before their Ca^{2+}-ATPase activities (μmol/h per mg membrane protein) were compared in the presence or absence of 0.6 μM calmodulin in the ATPase assay medium. Means ± S.E. for six different membrane samples are presented.

Ca^{2+} during incubation	Calmodulin during assay	Ca^{2+}-ATPase activity	% activation		
			incubation with Ca^{2+}	calmodulin	incubation with Ca^{2+} + calmodulin
–	–	1.309 ± 0.145			
–	+	3.621 ± 0.323		195.4 ± 25.6 ($P < 0.001$)	
+	–	2.189 ± 0.252	72.9 ± 19.8 ($P < 0.02$)		
+	+	3.773 ± 0.580			163.4 ± 18.7

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified Ca^{2+}-ATPase from membranes (45 mg protein) (A) incubated with 5 mM cysteine/0.4 mM free Ca^{2+} and (B) incubated without Ca^{2+}. After incubation, both membranes were washed twice before they were employed for isolation of Ca^{2+}-ATPase by calmodulin-Sepharose affinity chromatography. Isolated Ca^{2+}-ATPase from both membranes were then subjected to electrophoresis as described in Materials and Methods.

IV). The observed stimulation due to the combined action of calmodulin and Ca^{2+} treatment, however, was only 163.4 ± 18.7%, which was significantly lower ($P < 0.05$) than if the separate effects of the two were added together, thus suggesting that there was an overlap in the effects of calmodulin and the Ca^{2+} treatment in the stimulation of Ca^{2+}-ATPase.

When the Triton extract of membranes incubated with Ca^{2+} was chromatographed on a calmodulin-Sepharose affinity column, it was found that much less of the 138 kDa Ca^{2+}-ATPase protein could be harvested by eluting protein bound to the column with EDTA-containing buffer (Fig. 3). The protein profile of proteins not bound to the column, on the other hand, was found to be the same for Triton extracts of membranes incubated with or without Ca^{2+}. The intensity of each individual unbound protein band from the two extracts, including those in the 120–150 kDa range, was identical.

Discussion

In the present study, we have shown that Ca^{2+} alone without added calpain can mediate the activation of human erythrocyte membrane Ca^{2+}-ATPase, even after the divalent cation has been removed from the membranes after incubation. This Ca^{2+}-mediated stimulation of membrane Ca^{2+}-ATPase is therefore not due to direct activation of the enzyme by Ca^{2+}, since the ion has been removed before enzyme assay. Furthermore, the ATPase stimulation cannot be explained on the basis of any fluidity change in the membrane nor is it related in any way to degradation of membrane cytoskeletal proteins and band 3 induced by Ca^{2+}, since breakdown of these proteins are seen only after prolonged incubation of the membranes with Ca^{2+}, while the Ca^{2+}-mediated activation of Ca^{2+}-ATPase is a much quicker event. Our observation of the suppression of ATPase stimulation by leupeptin, on the other hand, strongly supports the involvement of a membrane-associated Ca^{2+}-dependent proteinase in the stimulation of membrane Ca^{2+}-ATPase. In addition, though the red cell membrane cytoskeleton is a well-known target for action of cytosolic or membrane-associated calpain [6], our observation of Ca^{2+}-ATPase activation occurring before any cytoskeleton degradation is detectable suggests that membrane Ca^{2+}-ATPase might be an initial target protein of membrane-associated Ca^{2+}-dependent proteinase in human red cells.

Cytosolic calpain has been proven to have the ability to stimulate erythrocyte membrane Ca^{2+}-ATPase [10,11]. In human red cells, however, it is doubtful whether the erythrosolic Ca^{2+}-dependent proteinase can actually act on the membrane ATPase in vivo because of the large excess of cytosolic calpastatin over cytosolic calpain [5]. Membrane-associated Ca^{2+}-dependent proteinase in human erythrocytes, on the other hand, is not inhibited by calpastatin [6]. Furthermore, our present study shows that exogenous calpain isolated from human erythrosol cannot further activate Ca^{2+}-ATPase of membranes treated with Ca^{2+}. Thus, with reference to Ca^{2+}-ATPase stimulation in human erythrocytes, the alleged membrane-associated Ca^{2+}-activated proteinase is functionally more important than its cytosolic counterpart. The earlier claim by Wang et al. [11], concerning activation of Ca^{2+}-ATPase by exogenous calpain in the presence of Ca^{2+}, could be explained solely on the basis of Ca^{2+} activation of membrane-associated Ca^{2+}-dependent proteinase without involvement of any cytosolic calpain. In the case of degradation of the human red cell cytoskeleton by Ca^{2+}-dependent proteinase, the membrane-associated proteinase is again considered functionally more significant than the cytosolic proteinase [6]. A species difference does exist, however, in the response of erythrocyte membrane Ca^{2+}-ATPase to Ca^{2+}-dependent proteinase. Pig red cells, for example, were found to possess no membrane-associated calpain activity. Their membrane Ca^{2+}-ATPase can thus respond to cytosolic calpain [10]. Furthermore, while the presence of detergent is essential for elicitation of the calpain response in pig erythrocytes [10], membrane-as-

sociated Ca^{2+}-dependent proteinase and Ca^{2+}-ATPase might be in close proximity with each other in the human red cell membrane so that no detergent is required for exposing sites on Ca^{2+}-ATPase for proteinase action.

In view of the identification of Ca^{2+}-ATPase fragments of 128 and 85 kDa by Wang et al. [11] after treatment of membranes with Ca^{2+} and calpain, it is tempting to suggest that Ca^{2+}-ATPase activation by this proteinase might involve detachment of an inhibitory peptide from the transport enzyme, the loss of which results in an increase in Ca^{2+}-ATPase activity. The inhibitory peptide released by Ca^{2+}-dependent proteinase might also be the portion of the ATPase controlling Ca^{2+} affinity of the transport enzyme, and removal of this portion of Ca^{2+}-ATPase has the effect of increasing Ca^{2+} affinity. By removing this portion of the ATPase, Ca^{2+}-dependent proteinase would give rise to a decrease in K_m for Ca^{2+} of the ATPase, as was observed in the present study.

The much reduced amount of 138 kDa Ca^{2+}-ATPase protein that could be harvested from calmodulin-Sepharose affinity chromatography of Triton extract from membranes incubated with Ca^{2+} suggests that the calmodulin-binding domain of Ca^{2+}-ATPase isolated from such membranes was affected. Recently, James et al. [17] published the sequence of the calmodulin-binding domain of human erythrocyte Ca^{2+}-ATPase showing the existence of an

NH_2-Glu-Leu-Arg-Arg-Gly-Gln $\cdots$.

sequence. Furthermore, it is known that Ca^{2+}-dependent thiol proteinase selectively attacks the carboxyl side of an arginine residue in a protein, provided that the residue adjacent to arginine on the amino-terminal side is a hydrophobic amino acid, like leucine [18]. Thus, the alleged membrane-associated Ca^{2+}-dependent proteinase might attack the calmodulin-binding domain of membrane Ca^{2+}-ATPase at the site indicated. It should be noted, however, that if the calmodulin-binding domain of Ca^{2+}-ATPase was affected by the membrane-associated Ca^{2+}-dependent proteinase, one would expect that the resulting ATPase could no longer bind to the calmodulin-Sepharose affinity column and should therefore be able to be recovered from the initial washing of the column. This is, however, not found to be the case. Furthermore, the presence of still functional and calmodulin-sensitive Ca^{2+}-ATPase in membranes washed after incubation with Ca^{2+} suggests that the ATPase was not lost from the membranes after incubation with Ca^{2+}, but rather was rendered tightly bound onto the membrane so that even Triton extraction could not liberate the ATPase from the membrane. It follows that the amount of 138 kDa Ca^{2+}-ATPase protein that could be harvested from such a Triton extract should be much reduced. This was indeed found to be the case. Ca^{2+}-ATPase fragments of 128 and 85 kDa reported by Wang et al. [11] were also not found. This might be because exogenous calpain was not added in the present study to promote further digestion of the ATPase, or that these fragments remained tightly bound to the membranes and thus resisted extraction by Triton during the isolation of Ca^{2+}-ATPase.

Ca^{2+}-dependent proteinase might also activate Ca^{2+}-ATPase in vivo via prior stimulation of protein kinase C. This proposal is based on the ability of Ca^{2+}-dependent proteinase in stimulating protein kinase C [19] and a recent report by Smallwood et al. [20], who showed that protein kinase C, after association with the human erythrocyte membrane in the presence of Ca^{2+} and diacylglycerol, can activate erythrocyte membrane Ca^{2+}-ATPase. It should be noted, however, that in the present in vitro study, no protein kinase C was included in the incubation or assay medium. Besides, the erythrocyte membranes used had been washed with EGTA so that no protein kinase C should remain bound to the membranes to activate Ca^{2+}-ATPase.

References

1 Strittmatter, W.J., Hirata, F. and Axelrod, J. (1979) Biochem. Biophys. Res. Commun. 88, 147–153.
2 Hidalgo, C., Thomas, D.D. and Ikemoto, N. (1978) J. Biol. Chem. 253, 6879–6887.
3 Brasitus, T.A., Schachter, D. and Mamouneas, T.G. (1979) Biochemistry 18, 4136–4144.
4 Brasitus, T.A. and Dudeja, P.K. (1986) Biochem. J. 239, 625–631.
5 Murakami, T., Hatanaka, M. and Murachi, T. (1981) J. Biochem. 90, 1809–1816.
6 Hatanaka, M., Yoshimura, N., Murakami, T., Kannagi, R. and Murachi, T. (1984) Biochemistry 23, 3272–3276.
7 Croall, D.E., Morrow, J.S. and De Martino, G.N. (1986) Biochim. Biophys. Acta 882, 287–296.
8 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352.
9 Minetti, M. and Di Stasi, A.M.M. (1987) Biochemistry 26, 8133–8137
10 Au, K.S. (1987) Biochim. Biophys. Acta 905, 273–278.
11 Wang, K.K.W., Villalobo, A. and Roufogalis, B.D. (1988) Arch. Biochem. Biophys. 260, 696–704.
12 Jarolim, P. and Mirčevová, L. (1982) Biochim. Biophys. Acta 688, 460–464.
13 Lowe, P.J. and Coleman, R. (1982) Biochim. Biophys. Acta 689, 403–409.
14 Graf, E., Verma, A.K., Gorski, J.P., Lopaschuk, G., Niggli, V., Zurini, M., Carafoli, E. and Penniston, J.T. (1982) Biochemistry 21, 4511–4516.
15 Laemmli, U.K. (1970) Nature (London) 227, 680–685.
16 Beutler, E., West, C. and Blume, K.G. (1976) J. Lab. Clin. Med. 88, 328–333.
17 James, P., Maeda, M., Fischer, R., Verma, A.K., Krebs, J., Penniston, J.T. and Carafoli, E. (1988) J. Biol. Chem. 263, 2905–2910.
18 Sasaki, T., Kukuchi, T., Yumoto, N., Yoshimura, N. and Murachi, T. (1984) J. Biol. Chem. 259, 12489–12494.
19 Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7610–7616.
20 Smallwood, J.I., Gugi, B. and Rasmussen, H. (1988) J. Biol. Chem. 263, 2195–2202.