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Calpain I Activates Ca^{2+} Transport by the Reconstituted Erythrocyte Ca^{2+} Pump

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Summary: Calpain I purified from human erythrocyte cytosol activates both the ATP hydrolytic activity and the ATP-dependent Ca^{2+} transport function of the Ca^{2+}-translocating ATPase solubilized and purified from the plasma membrane of human erythrocytes and reconstituted into phosphatidylcholine vesicles. Following partial proteolysis of the enzyme by calpain I, both the initial rates of calcium ion uptake and ATP hydrolysis were increased to near maximal levels similar to those obtained upon addition of calmodulin. The proteolytic activation resulted in the loss of further stimulation of the rates of Ca^{2+} translocation or ATP hydrolysis by calmodulin as well as an increase of the affinity of the enzyme for calcium ion. However, the mechanistic Ca^{2+}/ATP stoichiometric ratio was not affected by the proteolytic treatment of the reconstituted Ca^{2+}-translocating ATPase. The proteolytic activation of the ATP hydrolytic activity of the reconstituted enzyme could be largely prevented by calmodulin. Different patterns of proteolysis were obtained in the absence or in the presence of calmodulin during calpain treatment: the 136-kDa enzyme was transformed mainly into a 124-kDa active ATPase fragment in the absence of calmodulin, whereas a 127-kDa active ATPase fragment was formed in the presence of calmodulin. This study shows that calpain I irreversibly activates the Ca^{2+} translocation function of the Ca^{2+}-ATPase in reconstituted proteoliposomes by producing a calmodulin-independent active enzyme fragment, while calmodulin antagonizes this activating effect by protecting the calmodulin-binding domain against proteolytic cleavage by calpain.

Key Words Ca^{2+}-ATPase · Ca^{2+} pump · calpain · calmodulin dependent

Introduction

In the human erythrocyte the submicromolar intracellular calcium concentration is maintained by the plasma membrane-bound Ca^{2+}-translocating ATPase (*see* Al-Jobore et al., 1981). Both the Ca^{2+}-transport and the ATP-hydrolytic activities of this enzyme are stimulated by the ubiquitous Ca^{2+}-binding protein, calmodulin. Recently, we reported that the ATP-hydrolytic activity of the Ca^{2+} pump was increased following proteolysis by calpain I in the absence of calmodulin (Wang, Villalobo & Roufogalis, 1988*b*). The fragmented form(s) of the enzyme obtained in the absence of calmodulin lose the ability to bind, and to be stimulated by, calmodulin. However, the fragmented form(s) of the enzyme obtained in the presence of calmodulin preserved the capacity to bind and to be stimulated by calmodulin (Wang, Roufogalis & Villalobo, 1988*a*). To infer a physiological importance of this process it was essential to establish whether calpain also increased the Ca^{2+} transport function of the Ca^{2+} pump. In the present study the purified Ca^{2+}-translocating ATPase was reconstituted into phosphatidylcholine vesicles and subsequently incubated with calpain I. The kinetic properties and transport stoichiometry of both the control and the calpain-treated Ca^{2+} pump activity have been characterized. The protective effect of calmodulin on the activity and fragmentation pattern of the reconstituted Ca^{2+} pump by calpain I is also described.

Materials and Methods

Chemicals

Egg yolk phosphatidylcholine (grade I) was obtained from Lipid Products, Surrey, England, and asolectin from MCB Manufacturing Chemical, USA. Lactate dehydrogenase (rabbit muscle, type II), pyruvate kinase (rabbit muscle, type II), calmodulin-agarose gel, Arsenazo III, cholic acid (sodium salt) and alamethicin were from Sigma, USA. Calmodulin (bovine brain) and A23187 were from Calbiochem, USA. Sephacryl S-200 was from Pharmacia, Sweden; X-OMAT AR X-ray film was from Kodak. Molecular weight markers for SDS-polyacrylamide gel electrophoresis were from Bio-Rad, USA. All other reagents used in this work were of analytical grade.

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Preparation of Calmodulin-Depleted Erythrocyte Membranes and Solubilization and Purification of the Ca^{2+}-ATPase

The procedures for the preparation of calmodulin-depleted plasma membrane from human erythrocytes and the solubilization and purification of the Ca^{2+}-ATPase from these membranes have been described previously (Wang, Roufogalis & Villalobo, 1988*a*).

Reconstitution of the Purified Ca^{2+}-ATPase

A cholate-dialysis method (Kagawa & Racker, 1971), as modified by Villalobo and Roufogalis (1986), was used for the reconstitution of the purified enzyme. Purified Ca^{2+}-ATPase (0.1–0.3 mg protein) was added to a 7–15 ml presonicated phosphatidylcholine-sodium cholate mixture. The final mixture contained 1.5% (wt/vol) phosphatidylcholine, 1% (wt/vol) sodium cholate, 100 mM KCl, 20 mM potassium-HEPES, 2 mM dithiothreitol, 1 mM $MgCl_2$ and 50 μM $CaCl_2$ (pH 7.4). The mixture was placed inside two to four Spectropore dialysis bags (50 kDa cutoff) and dialyzed at 4°C for 24 to 36 hr against 7–11 liters of the buffer described above, except that it did not contain phosphatidylcholine or cholate.

Determination of the Ca^{2+}-ATPase and Ca^{2+}-Transport Activities

The ATP-hydrolytic activity of the reconstituted enzyme was determined either by measuring the formation of inorganic phosphate by a colorimetric method (Raess & Vincenzi, 1980) or by monitoring the rate of NADH oxidation at 340–400 nm in a dual-wavelength spectrophotometer using an ATP-regenerating system consisting of pyruvate kinase and lactate dehydrogenase. The first method (Method A) was chosen when determination of the initial rate of ATP hydrolysis was not an essential requirement. Forty-μl proteoliposome suspensions (0.6–1.2 μg protein plus 0.65 mg phospholipids) were incubated at 37°C for 30 min in 0.4 ml of a medium containing a final concentration of 55 mM Tris-maleate (pH 7.2), 66 mM KCl, 6.5 mM $MgCl_2$, 120 nM calmodulin (when added), 2 mM ATP and various concentrations of $CaCl_2$ (around 70 μM) and EGTA (around 110 μM) to attain 0.4 μM free calcium ion (unless stated otherwise). The second method (Method B) was employed when determination of the initial rate of ATP hydrolysis was required. Proteoliposomes (6–12 μg protein plus 7 mg phospholipids) were incubated at 37°C in 1.5 ml of a medium containing a final concentration of 73 mM KCl, 17 mM potassium-HEPES (pH 7.4), 5 mM dithiothreitol, 730 μM $MgCl_2$, 130 μM NADH, 5 mM phosphoenolpyruvate, 1 μM calmodulin (when added), 20 units pyruvate kinase, 90 units lactate dehydrogenase, and various concentrations of $CaCl_2$ (240–280 μM) and EGTA (300–370 μM) to obtain 0.4 μM free calcium ion. The reaction was initiated by the addition of 27 μM ATP. Ca^{2+} uptake by the proteoliposomes was monitored at 650–720 nm with a dual-wavelength spectrophotometer in the conditions described above in the presence of the metallochromic dye Arsenazo III (10 μM). To determine rates of ATP hydrolysis and Ca^{2+} transport in a 1.5-ml total volume, a 3-ml cuvette was used under magnetic stirring and the upper part of the light path of the spectrophotometer was blocked with black tape to avoid interference by the turbulence produced in the liquid/air interphase.

Treatment of the Reconstituted Ca^{2+}-ATPase with Purified Calpain I

Proteoliposomes (20–60 μg protein · ml^{-1} plus 11 mg phospholipid · ml^{-1}) were incubated at 25°C for 120 min (unless stated otherwise) in a medium containing 67 mM KCl, 50 mM potassium-HEPES (pH 7.4), 0.67 mM $MgCl_2$, 10 mM dithiothreitol, 2 U · ml^{-1} calpain I (unless stated otherwise), 40–100 μM EGTA (from calpain sample) and various concentrations of $CaCl_2$ (440–500 μM) to attain 400 μM free calcium ion. Proteolysis was arrested by the addition of 200 μM leupeptin.

Formation of the Phosphorylated Intermediate of the Reconstituted Ca^{2+}-ATPase

Proteoliposomes (60–120 μg protein plus 32 mg phospholipid) were incubated at 4°C for 15 sec in 400 μl of 50 mM KCl, 25 mM potassium-HEPES, pH 7.4, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 100 μM $LaCl_3$, 300 nM calmodulin and 4 μM ATP containing 12 mCi · $mmol^{-1}$ [γ-^{32}P]ATP. The reaction was initiated by the addition of [γ-^{32}P]ATP and terminated by the addition of ice-cold 10% (wt/vol) trichloracetic acid. The mixture was spun down at 10,000 × g_{max} for 5 min. The pellet was then treated with 500 μl chloroform to extract the phospholipid. The mixture was spun down again and the chloroform phase was removed. The remaining protein precipitate was then processed for acid-gel electrophoresis at pH 6.6 as described before (Wang, Roufogalis & Villalobo, 1988*a*). The dried gel was exposed to X-ray film for 72–144 hr at −70°C.

Other Methods

Calpain I was purified from human erythrocytes as described by Wang, Roufogalis and Villalobo (1988*a*). Calpain samples were routinely assayed using casein as substrate, as previously described. One unit of calpain activity produces an increase of one absorbance unit at 278 nm under the standard assay conditions (Wang et al., 1988*b*). These preparations of calpain have 20–100 U · mg prot.$^{-1}$ and are stored at 4°C in 50 mM Tris-HCl (pH 7.4), 1 mM EGTA and 1 mM dithiothreitol until use. Polyacrylamide linear gradient (5–20%, wt/vol) slab-gel electrophoresis in the presence of sodium dodecylsulfate was run at pH 8.3 following the method of Laemmli (1970). Protein concentrations were determined by the method of Lowry et al. (1951). Free Ca^{2+} concentrations were determined as previously described (Villalobo, Brown & Roufogalis, 1986), employing a computer program described by Goldstein (1979).

Abbreviations

SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Mops, morpholinopropanesulfonic acid; and CaM, calmodulin.

Results

Effect of A23187, Alamethicin and Triton X-100 on the ATP-Hydrolytic Activity of the Reconstituted Ca^{2+}-ATPase

Since the majority of previous work on the reconstituted Ca^{2+}-ATPase was carried out on asolectin vesicles (Haaker & Racker, 1979; Niggli et al., 1981; Benaim, Clark & Carafoli, 1986; Villalobo & Roufogalis, 1986), it was necessary to establish the degree of coupling and the sidedness of the Ca^{2+}-pump in the phosphatidylcholine liposomes. Figure 1 shows that the reconstituted Ca^{2+}-ATPase activity was stimulated about threefold by A23187, indicating satisfactory tightness of the proteoliposomes to Ca^{2+}. The low degree of coupling with highly purified phosphatidylcholine was noted previously (Villalobo & Roufogalis, 1986). Under similar conditions, using asolectin instead of phosphatidylcholine, the Ca^{2+}-ATPase activity was stimulated six to sevenfold by A23187 (*results not shown*). Figure 1*A* also illustrates that alamethicin, at concentrations higher than 0.5 $\mu g \cdot ml^{-1}$, further increased the Ca^{2+}-ATPase activity (assayed in the presence of A23187) by about 25%. Alamethicin is known to increase permeability of phospholipid membranes (Mueller & Rudin, 1968) and, therefore, it appeared about 20% of the Ca^{2+} pump molecules were oriented with the catalytic site in the lumen of the liposome, whereas the majority of the enzyme had the catalytic site(s) oriented to the outside (inward Ca^{2+}-pumping orientation). At higher concentrations of alamethicin (≥ 10 $\mu g \cdot ml^{-1}$) the proteoliposomes appeared to be completely permeable to ATP and Ca^{2+}, since the Ca^{2+}-ATPase activity was maximal and no longer required A23187 to express its full activity. Similarly, Ca^{2+}-ATPase activity in the presence of A23187 was increased about 25% by Triton X-100 at concentrations greater than 0.005% (wt/vol), while higher concentrations ($\geq 0.1\%$) of Triton X-100 also abolished the requirement of A23187 for full Ca^{2+}-ATPase activity (Fig. 1*B*), further supporting the predominance of the inwardly Ca^{2+}-transporting, Ca^{2+}-pump orientation.

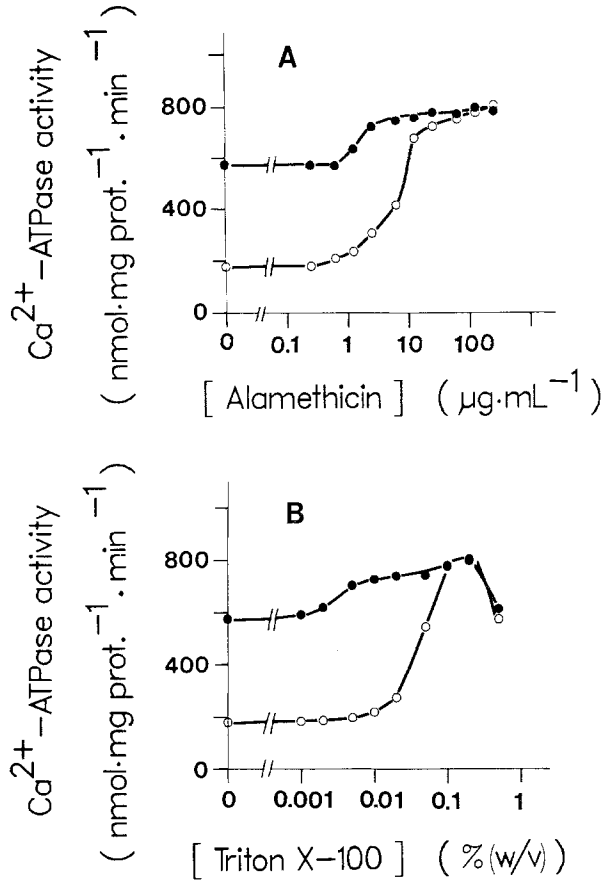

Fig. 1. Effect of A23187, alamethicin and Triton X-100 on the reconstituted Ca^{2+}-ATPase activity. Proteoliposomes (0.8 μg protein and 0.65 mg phospholipid) were incubated at 37°C for 30 min with 4.3 μM of free Ca^{2+}, 2 mM ATP, 120 nM calmodulin, 66 mM KCl, 6.5 mM $MgCl_2$, 5 mM potassium-HEPES, 55 mM Tris-maleate, pH 7.2 in the absence (open circles) or the presence (filled circles) of 120 nM A23187. (*A*) Various amounts of alamethicin or (*B*) Triton X-100 were also added as indicated. Results presented here are typical of two separate experiments

Effect of Calpain on the Ca^{2+}-Transport and ATP-Hydrolytic Activities of the Reconstituted Ca^{2+}-ATPase (Ca^{2+} Pump)

In this series of experiments, proteoliposomes were preincubated either with or without calpain. After the treatment, leupeptin was added to the mixture to arrest proteolysis. Leupeptin alone did not affect either Ca^{2+} uptake or the ATP hydrolysis of the Ca^{2+} pump (*results not shown*). Figure 2*A* shows

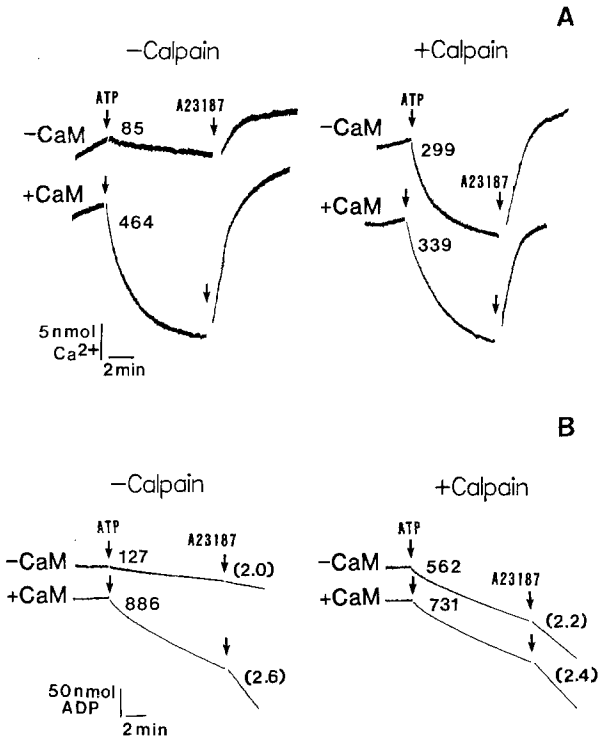

Fig. 2. Effect of calpain on the initial rate of both calcium uptake and ATP hydrolysis of the reconstituted Ca^{2+}-ATPase. Proteoliposomes (55 μg protein and 42 mg phospholipid) were incubated at 25°C for 120 min in a medium containing 67 mM KCl, 50 mM potassium-HEPES (pH 7.4), 0.67 mM $MgCl_2$, 10 mM dithiothreitol, 400 μM free Ca^{2+} in the absence or the presence of calpain (as indicated). Leupeptin (200 μM) was added at the end of the incubation and the proteoliposome suspensions were processed for: (*A*) calcium uptake and (*B*) ATP-hydrolytic activity measurement. (*A*) The time course of calcium uptake was monitored as in Materials and Methods, in the absence or the presence of 1 μM calmodulin (as indicated). Additions of 27 μM ATP and 60 nM A23187 were as indicated on the traces. Numbers beside the traces represent initial rates of Ca^{2+} uptake in nmol · mg prot.$^{-1}$ · min^{-1}. (*B*) The time course of ATP hydrolysis was monitored according to Method A in Materials and Methods, in the absence or the presence of 1 μM calmodulin (as indicated). Additions of 27 μM ATP and 60 nM A23187 are shown on the traces. Numbers beside the traces represent initial rates of ATP hydrolysis. Numbers in brackets are the ATP hydrolysis control ratio (ATP_{HCR}). Results presented here are typical of three separate experiments

that the initial rate of Ca^{2+} uptake into the reconstituted Ca^{2+} pump proteoliposomes is stimulated fivefold by calmodulin at 0.4 μM free Ca^{2+}, which to our knowledge is one of the first published demonstrations of the stimulatory effect of calmodulin on Ca^{2+} transport by the reconstituted Ca^{2+} pump. After calpain treatment the initial rate of Ca^{2+} uptake was also increased about threefold (299 compared to 85 nmol · mg prot.$^{-1}$ · min^{-1}) and became almost insensitive to calmodulin (Fig. 2*A*).

In parallel, the initial rate of ATP hydrolysis was also measured under almost identical conditions, except that Arsenazo III was omitted. The initial rate of ATP hydrolysis by the untreated Ca^{2+} pump (127 nmol · mg prot.$^{-1}$ · min^{-1}) was stimulated sevenfold by calmodulin (to 886 nmol · mg prot.$^{-1}$ · min^{-1}) (Fig. 2*B*). Calpain treatment increased the initial rate of ATP hydrolysis in the absence of calmodulin to 562 nmol · mg prot.$^{-1}$ · min^{-1}, approaching the calmodulin-stimulated initial rate of 731 nmol · mg prot.$^{-1}$ · min^{-1} (Fig. 2*B*).

We also attempted to quantitate the degree of coupling (q), by following nonequilibrium thermodynamic formulations (Rottenberg, 1979). q can be expressed as

$$q = \left[1 - \frac{(J_{ATP})_{J_{Ca^{2+}}} = 0}{(J_{ATP})_{\Delta\mu_{Ca^{2+}}} = 0}\right]^{1/2} \quad (1)$$

$(J_{ATP})_{J_{Ca^{2+}}} = 0$ is the rate of ATP hydrolysis when the net Ca^{2+} uptake becomes zero (static head state of the reconstituted enzyme) while $(J_{ATP})_{\Delta\mu_{Ca^{2+}}} = 0$ is the rate of ATP hydrolysis when the electrochemical Ca^{2+} potential difference ($\Delta\mu_{Ca^{2+}}$) across the membrane becomes zero (level flow state of the enzyme). Normally, the reconstituted Ca^{2+} pump has a progressively decreasing rate of ATP hydrolysis following initiation of the transport by ATP, which is due to the build-up of an electrochemical gradient of Ca^{2+} across the liposome membrane. Eventually, it reaches a point where the rate of ATP hydrolysis approaches linearity (and the rate of Ca^{2+} uptake approaches zero). At this point, the rate of ATP hydrolysis (Rate of ATP hydrolysis (−A23187)) is taken as an approximation of $(J_{ATP})_{J_{Ca^{2+}}} = 0$ (static head state). A23187 is then added, and the linear rate of ATP hydrolysis (Rate of ATP hydrolysis (+A23187)) now obtained is taken as $(J_{ATP})_{\Delta\mu_{Ca^{2+}}} = 0$ since A23187 collapses the electrochemical gradient of Ca^{2+} (level flow state). Therefore, the expression of q can be rewritten as

$$q = \left[1 - \frac{\text{Rate of ATP hydrolysis (−A23187)}}{\text{Rate of ATP hydrolysis (+A23187)}}\right]^{1/2}. \quad (2)$$

Another term used to quantitate coupling is the ATP hydrolysis control ratio

$$ATP_{HCR} = \frac{\text{Rate of ATP hydrolysis (+A23187)}}{\text{Rate of ATP hydrolysis (−A23187)}}. \quad (3)$$

Table 1. Effect of calpain treatment on the degree of coupling and Ca^{2+}/ATP ratio of the liposome-reconstituted Ca^{2+} pump

Treatment	Calmodulin	ATP_{HCR}[a]	Degree of coupling (q)[b]	Experimental Ca^{2+}/ATP ratio[c]	Mechanistic Ca^{2+}/ATP ratio[d]
None	−	2.0 ± 0.1	0.71 ± 0.01	0.60 ± 0.10	1.20 ± 0.17
	+	2.2 ± 0.3	0.73 ± 0.04	0.44 ± 0.08	0.83 ± 0.16
Calpain	−	2.0 ± 0.2	0.71 ± 0.02	0.52 ± 0.03	1.04 ± 0.07
	+	2.0 ± 0.4	0.70 ± 0.05	0.41 ± 0.05	0.84 ± 0.15

[a] ATP_{HCR}, ATP hydrolysis control ratio, *see* text for calculation.
[b] q, degree of coupling, see text for calculation.
[c] Experimental Ca^{2+}/ATP ratio $= \dfrac{\text{Initial rate of } Ca^{2+} \text{ transport}}{\text{Initial rate of ATP hydrolysis}}$.
[d] Mechanistic Ca^{2+}/ATP ratio $= \dfrac{\text{Experimental } Ca^{2+}/\text{ATP ratio}}{q^2}$.

Liposomes (54 μg protein plus 49 mg phospholipid) were incubated with or without calpain, as described in Fig. 2. Ca^{2+} uptake and ATP-hydrolysis measurements were carried out as described in Materials and Methods. The values ± SE in this table were obtained from three separate experiments.

Table 1 summarizes the results of several Ca^{2+} transport and ATP hydrolysis experiments. It was shown that the ATP hydrolysis control ratio (ATP_{HCR}) (the ratio of the rate of ATP hydrolysis in the presence *versus* the absence of A23187) was maintained between 2 and 3, both in the absence and in the presence of calmodulin. Calpain treatment did not appear to alter this ratio. The calculated value of q was maintained at about 0.7 in the absence or the presence of calpain treatment, both with and without calmodulin.

Table 1 also shows that the Ca^{2+} transport to ATP hydrolysis stoichiometric ratio (Experimental Ca^{2+}/ATP ratio) immediately after the addition of ATP was around 0.60 ± 0.10 and 0.44 ± 0.08, in the absence or the presence of calmodulin, respectively. In a fully coupled system, the experimental Ca^{2+}/ATP ratio gives a good approximation of the actual number of calcium ions translocated per molecule of ATP hydrolyzed. However, due to the low degree of coupling in the present work, a significant fraction of total ATP hydrolysis was contributed by uncoupled Ca^{2+} pump molecules. The uncoupled fraction of the Ca^{2+} pump is defined as follows:

$$\text{Uncoupled fraction of } Ca^{2+} \text{ pump} = \frac{\text{uncoupled enzyme molecules}}{\text{Total enzyme molecules}}. \quad (4)$$

Since the Rate of ATP hydrolysis (−A23187) was measured when no apparent Ca^{2+} uptake was observed, its value represents the rate of ATP hydrolysis by uncoupled enzyme molecules. On the other hand, since the Rate of ATP hydrolysis (+A23187) was measured when the electrochemical gradient of Ca^{2+} had collapsed, its value represents the rate of ATP hydrolysis by all enzyme molecules (coupled and uncoupled). Therefore, the uncoupled fraction of the enzyme can be estimated by

$$\text{Uncoupled fraction of } Ca^{2+} \text{ pump} = \frac{\text{Rate of ATP hydrolysis (−A23187)}}{\text{Rate of ATP hydrolysis (+A23187)}} \quad (5)$$

and by combining Eqs. (1) and (5), we can obtain the coupled fraction of the Ca^{2+} pump as

$$\text{Coupled fraction of } Ca^{2+} \text{ pump} = 1 - \text{Uncoupled fraction of } Ca^{2+} \text{ pump} = q^2. \quad (6)$$

Interestingly, the coupled fraction of the Ca^{2+} pump equals q^2 in value (*see* Eqs. (2), (5) and (6)).

Only the coupled fraction of the Ca^{2+} pump would contribute to Ca^{2+} transport. Therefore, to obtain a good approximation of the mechanistic Ca^{2+}/ATP ratio, the initial rate of ATP hydrolysis must first be multiplied by the "coupled fraction of the Ca^{2+}-pump" (or by q^2). The corrected mechanistic Ca^{2+}/ATP ratio can now be calculated as

$$\text{Mechanistic } Ca^{2+}/\text{ATP ratio} = \frac{\text{Initial rate of } Ca^{2+} \text{ uptake}}{\text{Initial rate of ATP Hydrolysis} \cdot q^2} \quad (7)$$

or

$$\text{Mechanistic } Ca^{2+}/\text{ATP ratio} = (\text{Experimental } Ca^{2+}/\text{ATP ratio})/q^2. \quad (8)$$

The corrected Ca^{2+}/ATP ratio was 1.20 ± 0.17 and 0.83 ± 0.16, in the absence and presence of

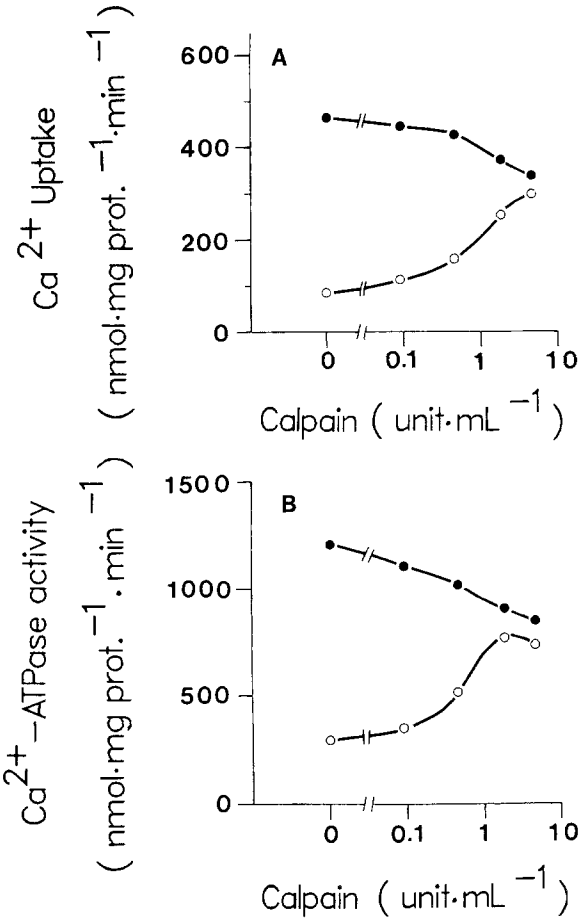

Fig. 3. Effect of increasing calpain concentration on the initial rate of Ca^{2+} uptake and the ATP-hydrolytic activity of the reconstituted Ca^{2+} pump. Proteoliposomes (128 μg protein and 112 mg phospholipid) were incubated at 25°C with the indicated amount of calpain for 60 min, as described in Fig. 2. (*A*) Calpain-treated proteoliposomes were used to determine the initial rate of Ca^{2+} uptake in the absence (open circles) or the presence (filled circles) of 1 μM calmodulin as described in Fig. 2. (*B*) Treated proteoliposomes were assayed for Ca^{2+}-ATPase activity (Method A) in the absence (open circles) or the presence (filled circles) of calmodulin. Results presented here are representative of two separate experiments

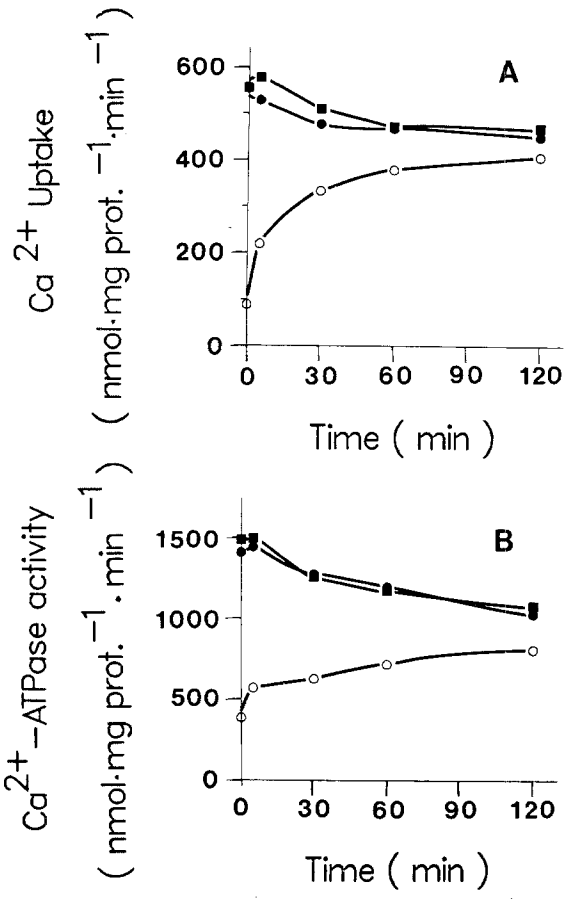

Fig. 4. Time course of the effect of calpain digestion on the initial rate of Ca^{2+} uptake and the ATP-hydrolytic activity. Proteoliposomes (156 μg protein plus 112 mg phospholipid) were treated with 2 U · ml^{-1} of calpain in the absence (circles) or the presence (squares) of calmodulin for the indicated period of time, as in Materials and Methods; calpain was not added for the zero-time values. After the addition of 200 μM leupeptin, the proteoliposomes were then used for (*A*) Ca^{2+} uptake measurement or (*B*) Ca^{2+}-ATPase activity determination in the absence (open symbols) or the presence of 300 nM calmodulin (filled symbols), as described in Fig. 3. Results presented here are typical of three separate experiments

calmodulin, respectively. These values were not significantly altered by calpain treatment (Table 1).

The effect of calpain concentration on the reconstituted Ca^{2+} pump was determined at a constant incubation time (120 min) (Fig. 3). The initial rates of Ca^{2+} uptake and Ca^{2+}-ATPase activity became near maximal and almost insensitive to calmodulin at about 1 to 5 U · ml^{-1} calpain. Another approach taken was to follow the time course of calpain treatment. Figure 4 shows that the initial rate of Ca^{2+} uptake and the Ca^{2+}-ATPase activity were progressively increased following calpain treatment (in the absence of calmodulin) and became largely calmodulin insensitive after 120 min. On the other hand, irrespective of whether calmodulin was absent or present during calpain treatment, when measured in the presence of calmodulin the initial rates of Ca^{2+} uptake and Ca^{2+}-ATPase activity slowly declined with time of proteolysis. The decline was about 17–19% and 26–28%, respectively, of their initial (zero-time) values (Fig. 4).

To determine the molecular changes accompanying the activation by calpain, the fragmentation of the reconstituted Ca^{2+} pump, both in the absence and the presence of calmodulin, was examined. Figure 5*A* shows that with increasing time of calpain

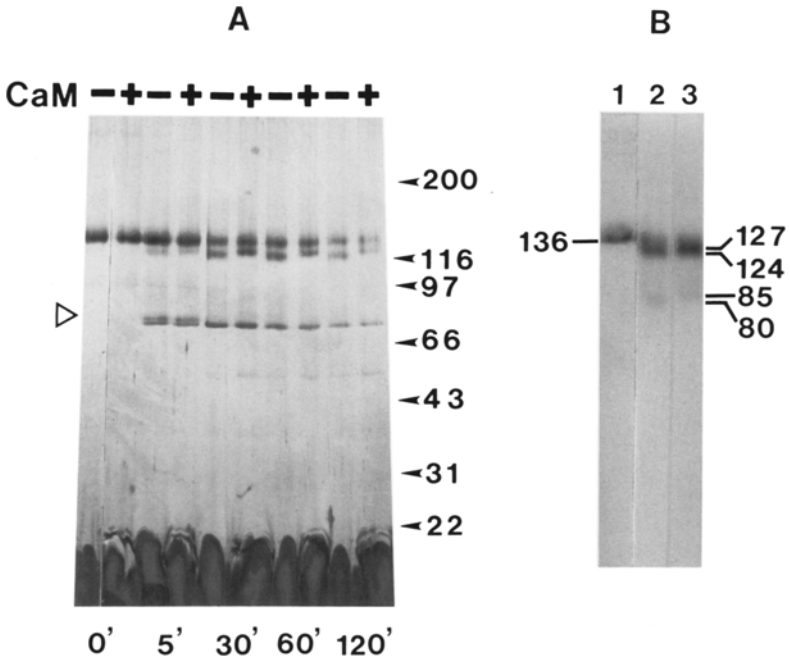

Fig. 5. Time course of calpain-induced proteolysis of the reconstituted Ca^{2+} pump. Proteoliposomes (156 μg protein plus 112 mg phospholipid) were treated with calpain for the indicated period of time in the absence (−) or the presence (+) of 130 nM calmodulin, as described in Fig. 4. After the proteolysis was arrested with 200 μM of leupeptin, the proteoliposomes were subjected to (*A*) SDS-gel electrophoresis followed by silver staining or (*B*) ^{32}P-phosphoenzyme formation followed by acid-gel electrophoresis and autoradiography. The numbers on the sides of the lanes represent relative molecular masses of protein standards in kDa. The open triangle indicates the bands of calpain and its fragment. (*B*) Proteoliposomes were treated with 0 (lane *1*) or 2 U · ml^{-1} calpain in the absence (lane *2*) or the presence (lane *3*) of 130 nM calmodulin. Results presented here are typical of five separate experiments

proteolysis, the reconstituted 136-kDa Ca^{2+}-ATPase was transformed mainly into a 124-kDa fragment in the absence of calmodulin and a 127-kDa fragment in the presence of calmodulin. Even after 120 min, a portion of unfragmented intact enzyme (136 kDa) was still present, which was likely to be contributed by the outward Ca^{2+}-pumping orientated Ca^{2+}-ATPase molecules and by Ca^{2+}-ATPase molecules not properly orientated in the liposome membranes. Acylphosphoprotein intermediate (E-P) formation by the control enzyme gave a single band of 136 kDa (Fig. 5*B*, lane *1*). After calpain treatment (120 min) in the absence of calmodulin, a major E-P band corresponding to the 124-kDa band stained with Coomassie blue was observed, together with minor bands of 136 and 80 kDa (lane *2*). Calpain treatment in the presence of calmodulin, however, produced a major acylphosphoprotein band corresponding to the 127-kDa band stained with Coomassie blue and less prominent bands of 136 and 85 kDa (lane *3*).

Protective Effect of Calmodulin Against Proteolytic Activation of the Ca^{2+} Pump by Calpain

In another series of experiments, methods were developed to allow determination of the effect of calmodulin preincubation on the Ca^{2+}-ATPase and Ca^{2+}-uptake activity following calpain treatment of the proteoliposomes, which required prior separation of the calmodulin (and calpain) from the reconstituted enzyme. The proteoliposomes were first incubated with calpain in the absence or the presence of calmodulin, while "control" proteoliposomes were incubated in the absence of calpain. In order to separate calmodulin and calpain from the proteoliposomes after treatment, individual samples were passed through a gel filtration column (Sephacryl S-200) in the presence of EGTA. The proteoliposomes eluting in the void volume were collected. Figure 6 shows that these proteoliposomes were calmodulin-free (lanes *b* and *d*) and also free of autolytic fragments of calpain (lane *a* to *d*). Calpain treatment in the absence of calmodulin produced the 124-kDa major fragment and lighter bands of 80, 55, 39 and 37 kDa, as described above (lane *c*). In the presence of calmodulin, calpain fragmented the Ca^{2+}-ATPase into a 127-kDa major fragment and minor fragments of 85, 55, 39 and 37 kDa (lane *d*). In a control experiment we measured the Ca^{2+}-ATPase activity of the proteoliposomes, preincubated with or without calmodulin alone before subjecting to gel filtration. The stimulation by calmodulin was found to be about 3.0- and 3.1-fold, respectively (in the presence of A23187) (Table 2). This again indicates that the calmodulin present in the preincubation medium had been completely separated from the proteoliposomes by the gel filtration step. Proteoliposomes pretreated with calpain alone had increased basal Ca^{2+}-ATPase activity (11.43 compared to 4.95 nmol · min^{-1} · ml^{-1}), while the

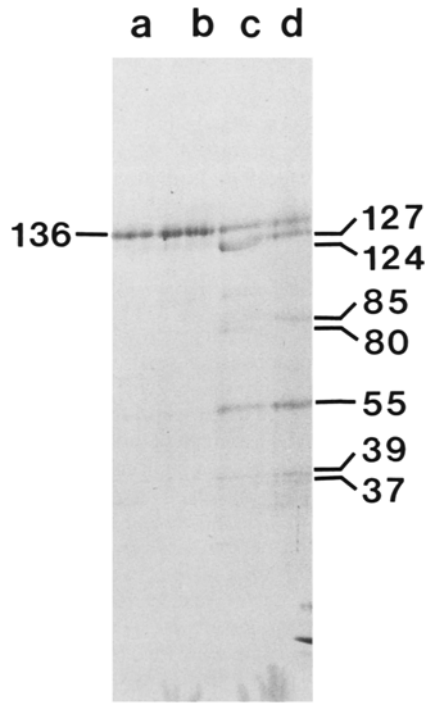

Fig. 6. Effect of calmodulin on the proteolysis of the reconstituted Ca^{2+}-ATPase. Proteoliposomes (129 μg protein plus 120 mg phospholipid) were incubated at 25°C for 90 min in a medium (2.7 ml) containing 75 mM KCl, 50 mM potassium-HEPES, pH 7.4, 10 mM dithiothreitol, 400 μM free Ca^{2+} in the presence of (lane *a*) no addition (lane *b*) 500 nM calmodulin (lane *c*) 2 U · ml^{-1} calpain or (lane *d*) 500 nM calmodulin and 2 U · ml^{-1} calpain. At the end of the incubation, 5 mM EGTA was added to each sample. After chilling on ice for 10 min, 2 ml of each sample was separately loaded onto a Sephacryl S-200 column (60 × 1.5 cm). The column was equilibrated and eluted with 100 mM KCl, 200 μM $MgCl_2$, 200 μM EGTA, 20 mM potassium-HEPES, pH 7.4 and 2 mM dithiothreitol at 20 ml · hr^{-1}. Two-ml fractions were collected. The liposome peak fractions (elution volume 38–41 ml) were pooled, assayed for Ca^{2+}-ATPase activity (*see* Table 2) and subjected to SDS-gel electrophoresis (lanes *a–d*). The gel was stained with Coommassie blue R-250. Numbers shown are apparent molecular mass in kDa. Results presented are typical of two separate experiments

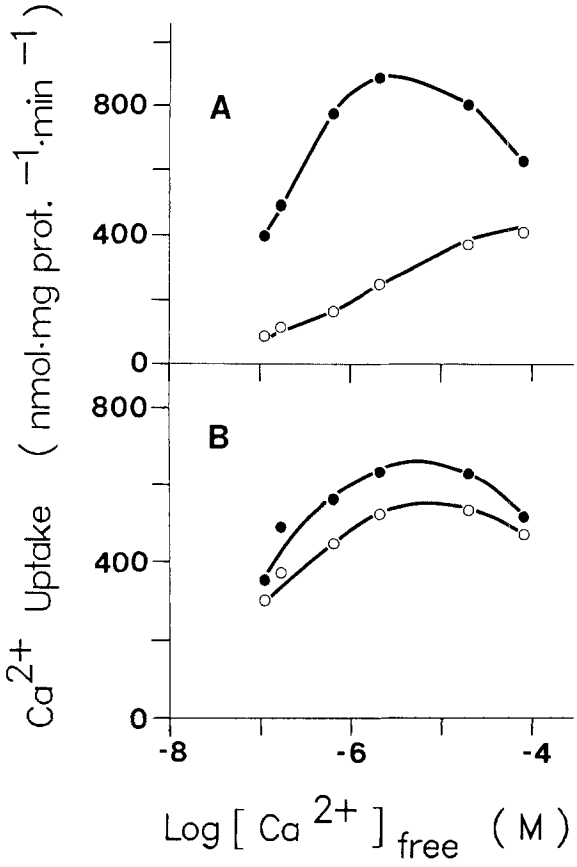

Fig. 7. Calcium dependence of the initial rate of Ca^{2+} uptake of the reconstituted Ca^{2+} pump. Proteoliposomes (237 μg protein plus 105 mg phospholipid) were treated with 0 (*A*) or 2 U · ml^{-1} calpain (*B*) at 25°C for 120 min as in Materials and Methods. The proteoliposomes were then subjected to Ca^{2+} uptake measurement in the absence (open circles) or the presence (filled circles) of 1 μM calmodulin at the indicated concentrations of free Ca^{2+}. Values presented are the means of two separate experiments

stimulation by calmodulin was reduced to 1.1-fold (in the presence of A23187). On the other hand, proteoliposomes pretreated with calpain in the presence of calmodulin maintained a 2.5-fold calmodulin stimulation of the Ca^{2+}-ATPase activity (assayed in the presence of A23187).

Kinetic Properties of the Intact and Calpain-Treated Ca^{2+} Pump in Phosphatidylcholine Vesicles

The intact and the calpain-treated reconstituted Ca^{2+} pump was studied with respect to its dependence on Ca^{2+} and ATP. Figure 7*A* illustrates, for the first time, that the affinity for Ca^{2+} of the initial rate of calcium uptake by the reconstituted Ca^{2+} pump was greatly increased by calmodulin ($K_{0.5}$ around 4 and 0.2 μM in the absence and the presence of calmodulin, respectively). From measurement of calcium uptake into inside-out membrane vesicles by the Ca^{2+} pump, Enyedi et al. (1987) obtained $K_{0.5(Ca)}$ of 5–10 μM and 0.5–0.7 μM, in the absence and the presence of calmodulin. Also, using inside-out vesicles, Smallwood, Giigi and Rasmussen (1988) reported $K_{0.5(Ca)}$ of 1.2 and 0.3 μM, in the absence and presence of calmodulin. Therefore, the reconstituted Ca^{2+} pump appears to have Ca^{2+} affinities similar to those of the native membrane-bound enzyme. It was also found that calmodulin increased V_{max} from 400 to 850 nmol · mg prot.$^{-1}$ · min^{-1}. After calpain treatment in the absence of

Table 2. Protective effect of calmodulin against proteolytic activation of the reconstituted Ca^{2+}-ATPase by calpain

Addition	Ca^{2+}-ATPase activity (nmol · min^{-1} · ml^{-1})				Fold stimulation by calmodulin	
	−A23187		+A23187			
	−CaM	+CaM	−CaM	+CaM	−A23187	+A23187
None	1.36	4.55	4.95	14.94	3.4	3.0
Calmodulin	1.37	4.99	5.04	15.86	3.6	3.1
Calpain	3.66	4.13	11.43	12.58	1.1	1.1
Calpain + calmodulin	1.70	4.86	5.34	13.26	2.9	2.5

Proteoliposomes (129 μg protein plus 120 mg phospholipid) were incubated in the presence of 10 mM dithiothreitol and 40 μM free Ca^{2+} under one of the additions indicated, as described in Fig. 6. After gel filtration (*see* Materials and Methods), the calmodulin-free proteoliposomes were assayed for Ca^{2+}-ATPase activity, as described in Materials and Methods. Ca^{2+}-ATPase activity is given in nmol per min per ml of pooled liposomes peak fractions (void volume). Values presented are typical of two separate experiments.

calmodulin, however, the Ca^{2+} pump had high affinity for Ca^{2+} ($K_{0.5}$ around 0.3 μM) even when assayed in the absence of calmodulin (Fig. 7*B*), and the addition of calmodulin did not further increase the affinity for Ca^{2+} and only slightly increased V_{max} (from about 550 to 650 nmol · mg prot.$^{-1}$ · min^{-1}). We also examined the Ca^{2+}-dependence of the ATP-hydrolytic activity of the reconstituted enzyme (Fig. 8). The intact Ca^{2+} pump exhibited the low Ca^{2+} affinity ($K_{0.5}$ around 2 μM), low V_{max} mode typical of the enzyme in the absence of calmodulin and the high Ca^{2+} affinity ($K_{0.5}$ around 0.2 μM), high V_{max} mode when assayed in the presence of calmodulin (Fig. 8*A*). The $K_{0.5(Ca)}$ values for Ca^{2+}-ATPase activity (both in the absence and presence of calmodulin) reported here are in good agreement with the $K_{0.5(Ca)}$ values for Ca^{2+} uptake in this work (*see above*). However, those values are slightly lower than those reported by Niggli et al. (1981) ($K_{0.5(Ca)}$ of 10–14 μM in the absence of calmodulin and 0.8–0.9 μM in its presence for the reconstituted Ca^{2+}-ATPase activity). This discrepancy could be due to a difference in the calculation of free-Ca^{2+} concentration using the Ca/EGTA buffering system. Very often, a larger error is present when the calculated free-Ca^{2+} concentration is in the micromolar range since the Ca^{2+} buffering capacity of EGTA is low in this range. The treatment with calpain again converted the enzyme into the predominantly high Ca^{2+} affinity ($K_{0.5}$ around 0.3 μM), high V_{max} mode even when it was assayed in the absence of calmodulin (Fig. 8*B*).

The effect of ATP on the initial rate of Ca^{2+} uptake by the reconstituted Ca^{2+} pump was also investigated. Figure 9*A* demonstrates for the first time that the reconstituted enzyme expresses two distinct affinities for ATP at 0.4 μM free Ca^{2+}, as in the case of the solubilized and purified enzyme (Villalobo, Brown & Roufogalis, 1986). The apparent $K_{0.5(ATP)}$ values are 1.4 and 289 μM in the absence of calmodulin and 1.1 and 205 μM in its presence (Fig. 9*A*). The calpain-fragmented Ca^{2+}-pump expresses comparable apparent $K_{0.5}$ values for ATP (0.9 and 245 μM), both in the absence and in the presence of calmodulin (Fig. 9*B*). Therefore, calpain treatment did not significantly modify the biphasic ATP-dependence of the Ca^{2+} pump.

Discussion

In recent years the purified Ca^{2+}-translocating ATPase from human erythrocytes has been extensively characterized (for reviews, *see* Al-Jobore et al., 1981; Carafoli & Zurini, 1982; Niggli, Zurini & Carafoli, 1987; Roufogalis & Villalobo, 1989). Fewer studies have been reported, however, on the liposome-reconstituted enzyme (Haaker & Racker, 1979; Niggli et al., 1981; Benaim, Clark & Carafoli, 1986; Villalobo & Roufogalis, 1986), and in most studies the reconstitution was performed with asolectin as the source of phospholipid. Due to the presence of acidic phospholipid components in asolectin, however, the calmodulin-stimulatory effect on Ca^{2+} translocation has not been reported, to our knowledge. In order for us to observe proteolytic activation of the reconstituted Ca^{2+} pump and the loss of calmodulin stimulation, as predicted from previous studies on membrane-bound and purified enzyme (Wang et al., 1988*a*; Wang, Villalobo & Roufogalis, 1988*b*), a calmodulin-sensitive, reconstituted Ca^{2+}-pump preparation was required. This

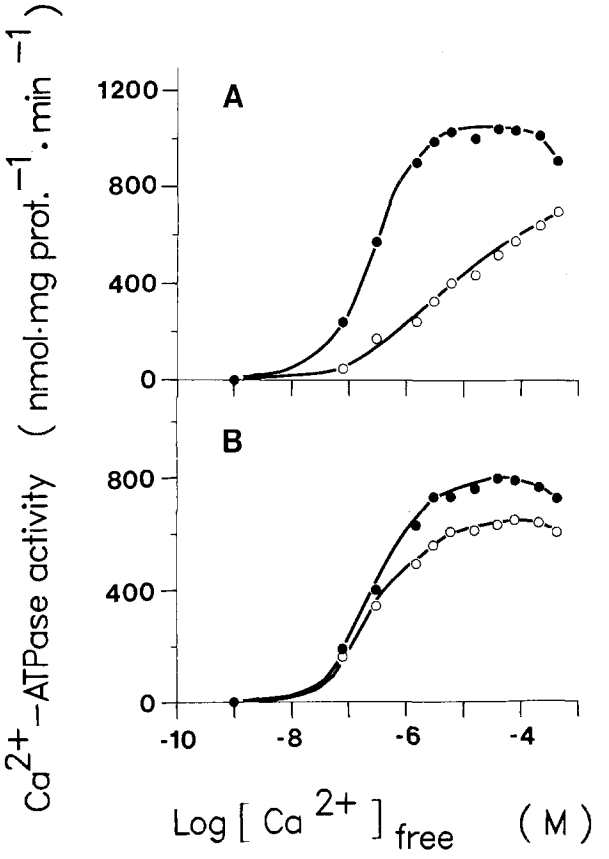

Fig. 8. Calcium dependence of the Ca^{2+}-ATPase activity in proteoliposomes. Proteoliposomes (50 μg protein plus 22 mg phospholipid) were treated with 0 (*A*) or 2 U · ml^{-1} calpain (*B*) at 25°C for 120 min, as in Materials and Methods. The proteoliposomes were then assayed for Ca^{2+}-ATPase activity (Method A) in the absence (open circles) or the presence (filled circles) of 120 nM calmodulin at the indicated concentrations of free Ca^{2+}. Values presented are the means of two separate experiments

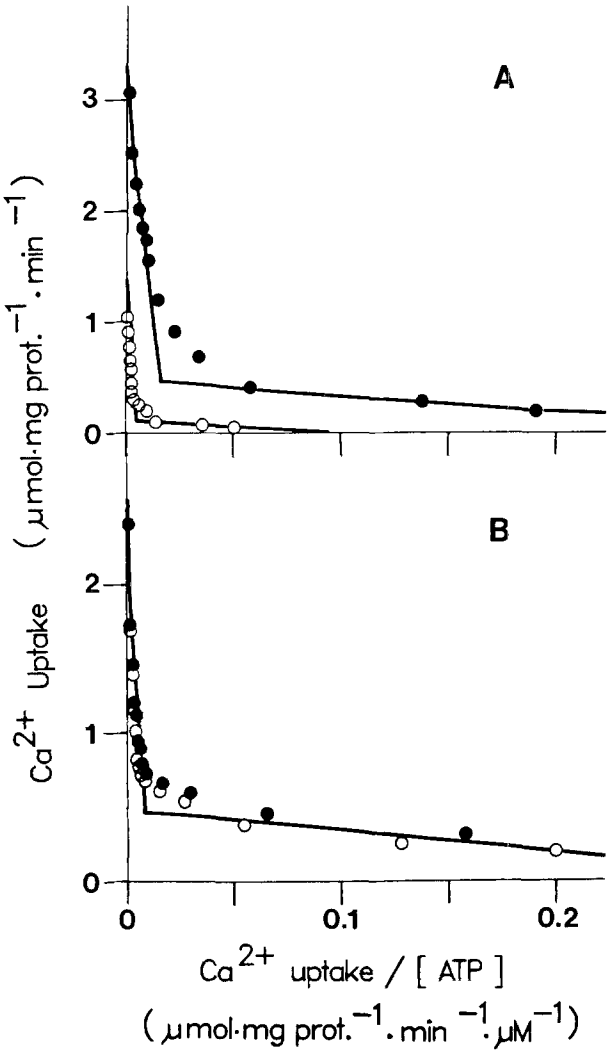

Fig. 9. Effect of ATP concentration on the initial rate of Ca^{2+} uptake of the reconstituted Ca^{2+} pump. Proteoliposomes (466 μg protein plus 210 mg phospholipid) were treated with 0 (*A*) or 2 U · ml^{-1} calpain (*B*) at 25°C for 120 min as in Materials and Methods. The proteoliposomes were then subjected to Ca^{2+} uptake measurement in the absence (open circles) or the presence (filled circles) of 1 μM calmodulin at various concentrations of ATP (0.5 to 2000 μM). Values presented are the means of two separate experiments

problem was resolved by using highly purified egg yolk phosphatidylcholine (Niggli et al., 1981) for reconstitution of the enzyme, with the compromise that vesicles so formed were more Ca^{2+} permeable. With the phosphatidylcholine-reconstituted Ca^{2+} pump, we demonstrated the following: (*i*) The initial rates of both calcium uptake and ATP-hydrolytic activity were stimulated at least three- to fourfold by calmodulin at 0.4 μM free Ca^{2+} (Fig. 2). Treatment of proteoliposomes with calpain increased these rates to levels close to those obtained upon addition of calmodulin before calpain treatment (Fig. 2). (*ii*) The initial rate of both Ca^{2+} uptake and ATP-hydrolytic activity of the intact Ca^{2+} pump exhibited two affinities for Ca^{2+}: low affinity in the absence of calmodulin and high affinity in its presence (Figs. 7 and 8). Calpain treatment shifted the enzyme into the high affinity mode regardless of whether calmodulin was absent or present in the assay medium (Figs. 7 and 8). (*iii*) The initial rate of Ca^{2+} uptake exhibited two affinities for ATP (approximately 1.1–1.4 and 245–289 μM), both in the absence and in the presence of calmodulin (Fig. 9*A*). This biphasic dependence on ATP was not altered by calpain treatment (Fig. 9*B*).

This is the first report where the liposome-reconstituted plasma membrane Ca^{2+} pump was subjected to limited proteolysis. The success of the approach used in the present study was in part due

to the fact that the fragmentation by calpain was more selected than that of trypsin or other proteases. The technique employed in this report can be applied to further studies on the properties of the Ca^{2+} pump in its purified and reconstituted state. A study of the fragmentation pattern coupled with ^{32}P-acylphosphoprotein formation (Fig. 5) revealed that the 124- and the 127-kDa forms of the reconstituted Ca^{2+} pump were the major active ATPase fragments produced by calpain in the absence or the presence of calmodulin, respectively. The formation of the 124-kDa fragment occurred concomitantly with the proteolytic activation of the initial rates of both the Ca^{2+} uptake and the ATP-hydrolytic activity of the enzyme (Figs. 3 and 5). The formation of the 124- and 127-kDa fragments were observed previously when the membrane-bound or the purified Ca^{2+}-ATPase was treated with calpain I (Wang et al., 1988*a*,*b*).

In our earlier work, we observed that the presence of calmodulin protected the membrane-bound Ca^{2+}-ATPase from proteolytic activation of the ATP-hydrolytic activity (Wang et al., 1988*b*). We also found that the 127-kDa fragment was still sensitive to calmodulin, whereas the 124-kDa fragment did not bind, nor was stimulated by, calmodulin (Wang et al., 1988*a*). Therefore, it was of interest to examine whether such a protective effect of calmodulin occurred on the reconstituted Ca^{2+} pump. By gel filtration chromatography (Sephacryl S-200), it was possible to separate calpain and calmodulin from the proteoliposomes after they were pretreated with calpain in the absence or in the presence of calmodulin. Besides the expected 124-kDa fragment formed in the absence of calmodulin, less prominent bands of 80, 55, 39 and 37 kDa were also observed (Fig. 6, lane *c*). In the presence of calmodulin, in addition to the prominent 127-kDa fragment, less prominent bands of 85, 55, 39 and 37 kDa were also identified (Fig. 6, lane *d*). These smaller fragments were also observed previously when the purified Ca^{2+}-ATPase was proteolyzed by calpain (Wang et al., 1988*a*). We observed that calpain alone produced proteolytic activation of the Ca^{2+}-ATPase activity and decreased the fold-stimulation by calmodulin from 3.4 to 1.1 (Table 2), whereas the calmodulin-stimulation after calpain treatment in the presence of calmodulin remained prominent (2.9-fold) (Table 2). Therefore, the protective effect of calmodulin against proteolytic activation of the reconstituted Ca^{2+}-ATPase was clearly demonstrated. Furthermore, since unlike the plasma membrane-bound or purified enzyme (Wang et al., 1988*a*,*b*), the 80- (in the absence of calmodulin) and the 85-kDa active fragments (in the presence of calmodulin) were only formed in small quantity from proteolysis of the liposome-reconstituted enzyme (Fig. 5), we may conclude that the 124-kDa fragment formed in the absence of calmodulin, was the major proteolytically activated and calmodulin-insensitive ATPase fragment, whereas the 127-kDa fragment formed in the presence of calmodulin was the major ATPase fragment which retained calmodulin sensitivity. As the calmodulin-binding domain is located near the C-terminal end of the Ca^{2+}-ATPase (Shull & Greeb, 1988; Verma et al., 1988), it seems likely that the 3-kDa region, represented by the difference between the 127- and 124-kDa fragments formed in the presence and absence of calmodulin, is part of the calmodulin-binding domain. Fragmentation of this 3-kDa region in the absence of calmodulin is, therefore, sufficient to activate the Ca^{2+}-ATPase and abolish its calmodulin-binding ability.

In summary, we have demonstrated that purified calpain I can effectively activate the Ca^{2+} transport function and ATP-hydrolytic activity of the liposome-reconstituted Ca^{2+} pump from human erythrocytes and render these activities calmodulin independent. Such irreversible activation was prevented by the presence of calmodulin. If these events can be translocated to the living cell (Fig. 10), at a resting state the cell pumps Ca^{2+} outward and hydrolyzes ATP at a slow rate, well below its maximum capacity. In response to certain stimuli, the intracellular calcium concentration may be elevated, and would normally trigger the calcium/calmodulin complex to bind and activate the Ca^{2+} pump. However, if the elevated calcium level persists, perhaps in association with other as yet undefined red cell stress, calpain may be activated and subsequently activate the Ca^{2+} pump irreversibly, possibly as a defensive mechanism against the uncontrolled, elevated intracellular calcium concentration. Intracellular calmodulin might also modulate the irreversible action of calpain if sufficient amounts of it are accessible to the Ca^{2+} pump at these elevated Ca^{2+} concentrations.

The calpain-mediated irreversible activation of the Ca^{2+} pump echoes the activating effect of calpain on two other calmodulin-dependent enzymes (cyclic nucleotide-phosphodiesterase and calcineurin) reported recently (Ito et al., 1987; Tallant, Brumley & Wallace, 1988; Wang, Roufogalis & Villalobo, 1989*a*). We theorize that a group of calmodulin-dependent enzymes are endogenous substrates for calpain in certain cell types (Wang, Villalobo & Roufogalis, 1989*b*). Upon activation of calpain by Ca^{2+}, these enzymes would become the targets of calpain-mediated proteolysis. The modified activities of these enzymes would subsequently produce amplified effects on their respective cata-

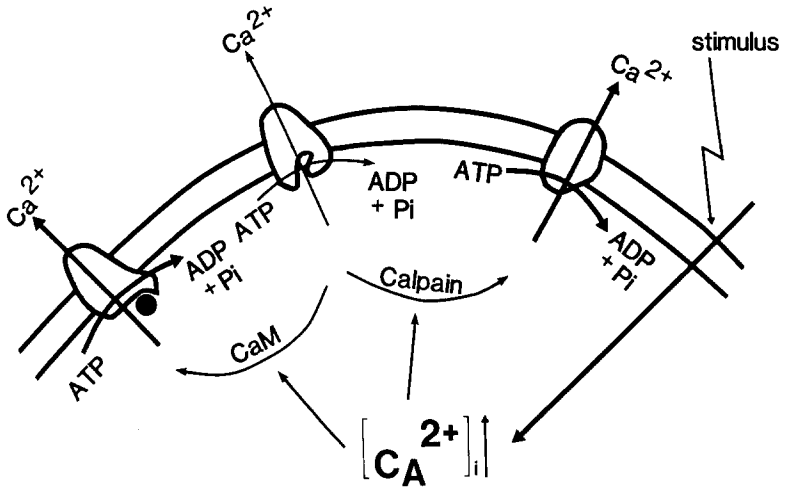

Fig. 10. Proposed model for the dual control of the plasma membrane Ca^{2+} pump by calmodulin and calpain in a living cell. Calmodulin is represented by the black dot. For detailed explanation, *see* text

lytic pathways. To turn off the signal-transducing calpain activity, intracellular calcium would have to be reduced to resting levels (submicromolar free Ca^{2+}). It is tempting to speculate that the calpain-activated plasma membrane Ca^{2+} pump might in turn participate in this process.

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