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Irreversible Effects of Calcium Ions on the Plasma Membrane Calcium Pump

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Abstract. The calcium pump of human red cells can be irreversibly activated by preincubation of the membranes in the presence of calcium ions, with a pattern reminiscent of that produced by controlled trypsin attack. With 1 mM Ca^{2+}, the activity of the basal enzyme increases three to fourfold over 30 to 60 min, to levels about half those obtained in the presence of calmodulin. On the whole, the effect occurs slowly, with a very low Ca^{2+} affinity at 37°C and is unaffected by serine-protease inhibitors. The activation caused by 1 mM Ca^{2+} is little affected by leupeptin (a thiol-protease inhibitor) and that obtained at 10 μM Ca^{2+} is not inhibited. Preincubations at 0°C also lead to activation, to a level up to half that seen at 37°C, and the effect is not affected by leupeptin or antipain. No activation is observed by preincubating soluble purified Ca,Mg-ATPase in Ca^{2+}-containing solutions at 37°C. Instead, calcium ions protect the detergent-solubilized enzyme from thermal inactivation, the effect being half-maximal between 10 and 20 μM Ca^{2+}. We conclude that the activation of the membrane-bound Ca,Mg-ATPase by Ca^{2+} should result from an irreversible conformational change in the enzyme and not from attack by a membrane-bound protease, and that this change presumably arises from the release of inhibitory particles existing in the original membrane preparations.

Key words: Ca,Mg-ATPase — Erythrocytes — Ca^{2+} activation — Detergents — Thermal inactivation

Introduction

The plasma-membrane Ca,Mg-ATPase or calcium pump transports calcium ions out of the cell against a steep electrochemical gradient and can maintain the cytoplasmic Ca^{2+} concentrations at levels in the 10^{-8} M range (Lew et al., 1982; Schatzmann, 1982, 1989). The enzyme is stimulated by calmodulin, which induces a conformation with high Ca^{2+} affinity and turnover number (Bond & Clough, 1973; Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1978; Scharff & Foder, 1978). The enzyme can be purified close to homogeneity in detergent solution, by affinity chromatography on calmodulin-Sepharose 4 B columns (Niggli, Penniston & Carafoli, 1979; Gietzen & Kolandt, 1982), and several isoforms have been cloned and sequenced (Shull & Greeb, 1988; Verma et al., 1988). In the red cell membrane, the enzyme is present as the dimer of the single polypeptide chain (Minocherhomjee et al., 1983; Cavieres, 1984).

The plasma-membrane Ca,Mg-ATPase can also be activated by proteolytic cuts with trypsin, chymotrypsin and calpain, an intracellular Ca^{2+}-activated protease (Enyedi et al., 1980; Taverna & Hanahan, 1980; Stieger & Schatzmann, 1981; Sarkadi et al., 1986; Papp et al., 1989; Wang, Roufogalis & Villalobo, 1989; Zvaritch et al., 1990). This type of activation appears as the calmodulin-binding peptide (found at the C-terminal extension of the primary structure) is removed and consists of broad kinetic changes that are apparently similar to those elicited by calmodulin binding. A third mode of activation of the pump, comparable in its effects, is obtained with fatty acids and acidic phospholipids, which are effective with both the cell membranes and the purified enzyme (Taverna & Hanahan, 1980; Niggli, Adunyah & Carafoli, 1981).

Yet a fourth form of activation arises from simply preincubating calmodulin-depleted red cell membranes in the presence of calcium ions (Klinger et al., 1981; Cavieres, 1987*a*; Au, Lee & Siu, 1989; Roufogalis et al., 1990). This modification is irreversible in the membrane-bound Ca,Mg-ATPase and also leads to a state of high Ca^{2+} affinity. Because

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of the possibility of a regulatory potential in this effect, the present study was carried out to decide whether or not a Ca^{2+}-activated protease could be responsible for the activation at near-physiological Ca^{2+} concentrations and to examine additional features of the activation process.

Materials and Methods

Materials

ATP was purchased from Boehringer Mannheim (Lewes, UK) and radioisotopes from New England Nuclear (Stevenage, UK). Bovine brain calmodulin (CaM), Sephadex G-50, trypsin, soybean trypsin inhibitor, benzamidine hydrochloride, leupeptin, antipain, bestatin, E-64 and other biochemicals were from Sigma (London) and *p*-toluene-sulphonyl fluoride (pTSF) was from Aldrich (Gillingham, UK). Calmodulin-Sepharose 4 B was obtained from Pharmacia LKB (Milton Keynes, UK) and purified egg-yolk phosphatidylcholine from Lipid Products (Nutfield, UK). All other chemicals were Analytical Reagent grade from Fisons (Loughborough, UK) or AnalaR from BDH Merck (Poole, UK). Double-distilled water was further purified with a Milli-Q Plus system (Millipore UK, Watford).

Preparation of Calmodulin-depleted Red Cell Membranes

Broken membranes from human erythrocytes were prepared from fresh heparinized blood from healthy donors or bank blood, as described (Cavieres, 1984). The membranes were resuspended in a *Mg/HEPES solution*, containing (mM): HEPES 15 (adjusted with NaOH to pH 7.4 at 20°C), $MgCl_2$ 0.1, frozen in aliquots at 8–10 mg protein/ml (200% hematocrit on the original cells) and kept at −80°C.

Preincubation of Red Cell Membranes

The membranes were mixed in microcentrifuge tubes on an ice-bath with 11 or 12 volumes of a solution containing $CaCl_2$. In early experiments, this was a phosphate-buffered saline (*PBS*), made up with (mM): NaCl 137, KCl 2.7, KH_2PO_4 1.5, Na_2HPO_4 8.1 (pH 7.45 at 20°C), $MgCl_2$ 0.4 and $CaCl_2$ 0.9, plus NaN_3 1 (which later proved to be an unnecessary precaution). Most experiments used *solution NK*, which contained (mM): NaCl 100, KCl 100, HEPES 15 (pH 7.40 at 20°C, 7.20 at 37°C and 7.69 at 0°C (Good & Izawa, 1972)), $MgCl_2$ 0.1 and to which fixed or varying concentrations of $CaCl_2$ were added, using calcium buffers at and below 10 μM Ca^{2+} (*see below*). The controls were (i) for the PBS preincubation: membranes kept at 0°C in Mg/HEPES solution and (ii) for the NK plus Ca^{2+} preincubation: membranes kept at 0°C in NK solution plus 0.5 mM ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid (EGTA). Preincubations at 37°C were stopped by cooling the tubes on an ice-bath for 5 min, followed by centrifugation of the membranes; 0°C preincubations were in fact terminated by the resuspension of the membranes in Mg/HEPES solution after the first spin.

Preincubated and control membranes were centrifuged at 20,000 × *g* and 5°C in an Ole Dich refrigerated microcentrifuge (Camlab, Cambridge). The membranes were resuspended with chilled Mg/HEPES solution and washed with the same solution. The tube walls were wiped with a roll of Whatman 1 filter paper and the pellet was finally resuspended with Mg/HEPES, ready for the Ca,Mg-ATPase assay.

Assay of the Ca,Mg-ATPase Activity of Red Cell Membranes

Fresh, control or preincubated membranes were assayed for their ATPase activity as previously described (Cavieres, 1984). This was done in 40 μl of a medium containing 2 mM ATP (plus [τ^{32}P]-ATP), 20 μM $CaCl_2$ (3 μM Ca^{2+}), 1.4 mM $MgCl_2$, 60 mM NaCl, 60 mM KCl, 15 mM HEPES (adjusted with NaOH to pH 7.2 at 37°C) and 0.1 mM ouabain (to inhibit Na,K-ATPase), with or without 0.5 mM EGTA, in triplicate initial and final tubes. The membrane suspension was equivalent to 12% hematocrit on the original cells (*ca.* 0.4 mg protein/ml). The final tubes were incubated at 37°C for 30 to 90 min, while the initials remained on the ice-bath. When calmodulin was used, this was at a concentration of 0.4 μM and the incubation was for 20 or 30 min. In these conditions, ATP hydrolysis does not exceed 25% and linear time courses are obtained (Cavieres, 1987*b*). The released $^{32}P_i$ was extracted and counted together with acid-hydrolyzed [τ^{32}P]-ATP standards (Brown, 1982; Cavieres, 1984). The Ca,Mg-ATPase activity was calculated as the difference between the enzymic activities measured in the absence and the presence of 0.5 mM EGTA.

Purification of Ca,Mg-ATPase

This was done by CaM-affinity chromatography in the presence of Triton X-100 and phosphatidylcholine, essentially as described by Niggli et al. (1979, 1981), except for the addition of thiol-protease inhibitors (500 μM leupeptin and bestatin). The red cell membranes were given a wash in a solution consisting of (mM) KCl 300, TES-triethanolamine 10 and dithiothreitol 2, before solubilizing with 5 mg Triton X-100 per ml and at 5 mg protein/ml. The solubilizate was cleared by centrifugation at 100,000 × *g* in a Beckman L8-80 ultracentrifuge, before calmodulin affinity chromatography. The fractions eluted with 5 mM EDTA were supplemented with 5 mM $CaCl_2$ and glycerol (to 20% v/v) before freezing and storage at −80°C. On average, the specific activity of the pooled fractions was 1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at 54 μM Ca^{2+} and 37°C.

Preincubation and Assay of Purified Ca,Mg-ATPase

Existing Ca^{2+} and EDTA were removed from soluble-purified Ca,Mg-ATPase by gel filtration (*see below*) through spun Sephadex G50 columns (Penefsky, 1977) which had been pre-equilibrated in *medium S* consisting of (mM): NaCl 100, KCl 100, HEPES 15 (adjusted with NaOH to pH 7.4 at 20°C), dithiothreitol 1, and also phosphatidylcholine 0.8 mg/ml, Triton X-100 4 mg/ml and 10% (v/v) glycerol. Aliquots of the gel-filtered enzyme were preincubated for 10 min at 37°C at varying Ca^{2+} concentrations (using buffers at 10 μM Ca^{2+} and below) in the medium above, supplemented with 500 μM leupeptin and 500 μM antipain.

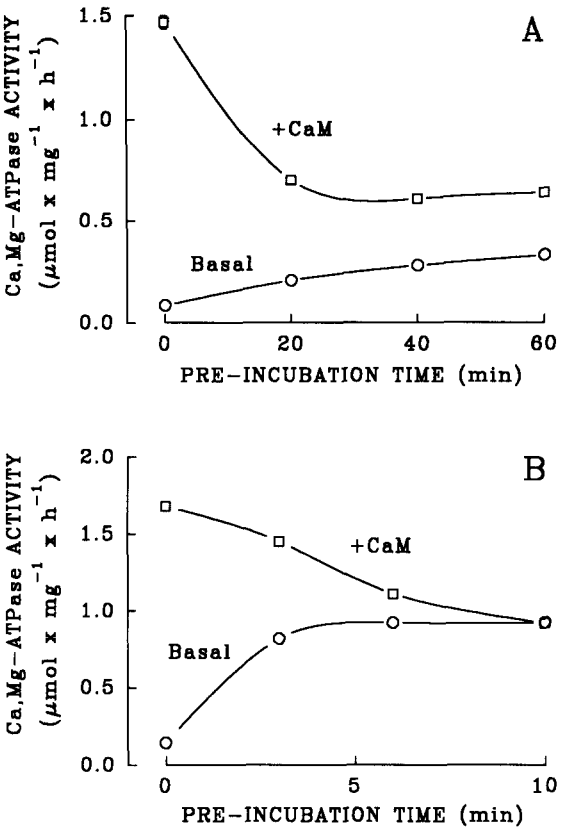

Fig. 1. (*A*) Effect of preincubation of calmodulin-depleted red cell membranes with a Ca^{2+}-containing solution (PBS) at 37°C. After washing in the refrigerated centrifuge, the membranes were assayed for Ca,Mg-ATPase activity at 37°C in the presence and in the absence of calmodulin (CaM), as detailed in Materials and Methods. Vertical bars indicate ±SEM on triplicate initials and finals, with and without 3 μM Ca^{2+}. (*B*) Effect of preincubation with 1 μg trypsin/ml at 37°C, in the presence of 20 μM Ca^{2+}. Protease action stopped by 100-fold excess of soybean trypsin inhibitor. The treated membranes were diluted in the cold and the Ca,Mg-ATPase activity assayed as in *A*.

The tubes were returned to the ice-bath and the enzyme solution (0.12 ml) was gel-filtered once or twice at 100 × *g* through 0.7 ml pre-equilibrated spun Sephadex G-50 columns made in disposable 1-ml syringes. Preliminary tests with $^{45}Ca^{2+}$, with or without an excess EGTA, had shown that not more than 0.4% of the loaded ^{45}Ca-EGTA or $^{45}Ca^{2+}$, respectively, remained in the solution after one pass through the column in these conditions. The ATPase activity of the filtered enzyme was assayed at 37°C in medium S, also containing (final concentrations) 0.5 mM ATP (plus [γ^{32}P]-ATP), 0.5 mM $MgCl_2$ (167 μM Mg^{2+}), 0.1 mM $CaCl_2$ (54 μM Ca^{2+}) and 22 nM enzyme. Equal volumes of enzyme and substrate were mixed at 0°C, transferred to the 37°C bath and after a 3 min equilibration period, a sample (20 μl) was removed for "zero-time" and then successive samples were removed up to 8 min. The samples were stopped on an ice-bath and the [^{32}P]-P_i was extracted as indicated above. The ATPase activity and associated error were routinely calculated from linear regressions on the release of [^{32}P]-P_i against time. Some time courses (and the lower curve in Fig. 1*A*) were fitted to exponentials of the form $y = A \cdot (1 - e^{-k \cdot t})$, where t = time, k is an apparent rate constant and A is a scaling factor. Since

$$dy/dt = A \cdot k \cdot e^{-k \cdot t}, \quad (1)$$

initial rates were calculated as the product $A \cdot k$. The results are the mean of duplicate incubations and are presented as percent of the specific activity of nonincubated controls kept on ice without Ca^{2+} and with 0.5 mM EGTA. The errors, therefore, have been compounded twice.

Curve Fitting

Least-squares linear fitting was done on a Hewlett-Packard programmable calculator. Exponential and hyperbolic curve fitting was done by nonlinear regression with the Sigmaplot 4 package (Jandel, Corte Madera, CA) on an IBM PS2 microcomputer.

Calcium Buffers

N-hydroxyethylethylene-diamine triacetic acid (HEDTA) was used at 5 mM to obtain Ca^{2+} concentrations between 0.2 and 10 μM and 2 mM EGTA for 0.1 μM Ca^{2+} and below, together with adequate amounts of $CaCl_2$, with or without ATP as the case required. When ATP was used in the presence of another chelator, the calculation of total Ca and Mg concentrations took both ligands into account (Fabiato & Fabiato, 1979). In the case of experiments at 2 mM Mg^{2+}, the HEDTA concentration was raised to 10 mM to make the buffer preparation feasible. Nominally "Ca^{2+}-free" conditions were obtained by adding 0.5 mM EGTA and no $CaCl_2$ to the solutions. As the sum total of reagents will contribute a few micromolar of contaminating Ca^{2+}, a value of 9 in the pCa^{2+} axes and figure legends has been used solely to signify that fact. The following apparent stability constants were calculated (Sillén & Martell, 1971; Martell & Smith, 1974) for 37°C and pH 7.20: HEDTA, $\log K_{Ca}$ 5.59, $\log K_{Mg}$ 4.69; EGTA, $\log K_{Ca}$ 6.93, $\log K_{Mg}$ 1.70 and ATP, $\log K_{Ca}$ 3.84, $\log K_{Mg}$ 4.21. For HEDTA at 0°C and pH 7.69, we obtained $\log K_{Ca}$ 6.09 and $\log K_{Mg}$ 4.25, using the reaction enthalpy changes at ionic strength = 0.1 (Martell & Smith, 1974).

Protein Determinations

Protein in the red cell membranes was assayed by the method of Lowry et al. (1951), using crystallized bovine serum albumin as standard. The membranes were precipitated with trichloroacetic acid (7% final concentration) and the precipitates were redissolved with 1 M NaOH for 30 min before adding the reagents. With the purified Ca,Mg-ATPase, the more sensitive bicinchoninic acid assay (Smith et al., 1985) was used, as described previously (Ward & Cavieres, 1993).

Results

Effects of Preincubating Membrane-Bound Ca,Mg-ATPase at 37°C

The time courses of the irreversible changes in the plasma-membrane calcium pump that result from preincubating red cell membranes in PBS at 37°C

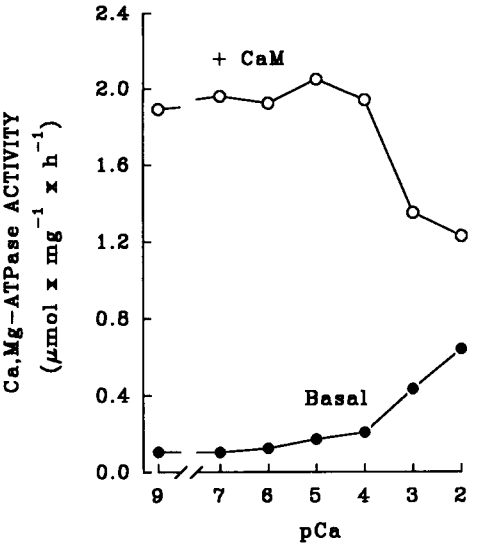

Fig. 2. Dependence of the modification of Ca,Mg-ATPase on the concentration of Ca^{2+} during the preincubation. The membranes were preincubated for 30 min in NK medium plus the Ca^{2+} concentrations indicated, using Ca^{2+} buffers at 10 μM Ca^{2+} and below (*see* Materials and Methods). After washing at 20,000 × *g* in the refrigerated microcentrifuge, the Ca,Mg-ATPase activity of the membranes was assayed as in Fig. 1. Errors comprised within the symbols.

are shown in Fig. 1*A*. The progress of the activation of the basal Ca,Mg-ATPase activity has been fitted to an exponential with an apparent rate constant of 0.023 min^{-1} and a maximal activation of 0.33 μmol · mg^{-1} · hr^{-1} above the initial ATPase activity. This represents a fivefold increase in activity (fourfold at 60 min). The calmodulin stimulation of the Ca,Mg-ATPase activity decreases both in absolute and relative terms, but the modified enzyme can still be stimulated after 60 min of preincubation. That calcium ions are responsible for these changes was established by selective removal of the various components in the solution (*not shown*). The results are representative of six experiments using PBS or Ca^{2+}-containing NK solution and agree with those reported by Klinger et al. (1981) and Roufogalis et al. (1990). The pattern bears at least a superficial resemblance to that resulting from mild trypsin treatment of the red cell membrane Ca,Mg-ATPase (Sarkadi, Enyedi & Gárdos, 1980; Taverna & Hanahan, 1980; Stieger & Schatzmann, 1981; Rossi & Schatzmann, 1982) and one such experiment is shown in Fig. 1*B*, for illustrative purposes. The Ca^{2+}-concentration dependence of the preincubation effects are shown in Fig. 2. It is apparent that neither the activation of the basal activity nor the inhibition of the calmodulin-stimulated activity are saturated at 10 mM Ca^{2+}. Higher Ca^{2+} concentrations were not explored, to avoid the uncertainties associated with the increase in ionic strength as its magnitude, with a 2:1 electrolyte, is considerable. With the CaM-stimulated activity, the extent of the inhibition by preincubation at 1 mM Ca^{2+} was variable and the stimulation at 10 μM was not always observed.

The possibility that the results of the 37°C preincubation were due to the effect of a membrane-associated protease was explored using serine-protease inhibitors (Cavieres, 1987*a*). Table 1 shows that these were ineffective, apart from a slight inhibition by pTSF. It has been reported that calpain, an endogenous thiol-protease, can activate the plasma-membrane calcium pump to levels similar to those attained with calmodulin (Au, 1987; Wang, Villalobo & Roufogalis, 1988; James et al., 1989; Wang et al., 1989). The effect of the thiol-protease inhibitors leupeptin and antipain (Umezawa, 1972; Susuki, Tsuji & Ishiura, 1981) on the changes brought about by preincubation of the membranes at 1 mM Ca^{2+} and 37°C are shown in Fig. 3. The inhibitors protect the calmodulin-stimulated Ca,Mg-ATPase from inactivation (only in part in other experiments), and leupeptin has a slight effect in preventing the activatory Ca^{2+} effect on the basal Ca,Mg-ATPase. Leupeptin, antipain and E-64 (Susuki et al., 1981) at 20 μM were just as effective at partially reversing the activation of the basal Ca,Mg-ATPase, but 20 μM bestatin was less so. When tested at a concentration of 100 μM for a direct effect on the basal and calmodulin-stimulated Ca,Mg-ATPase activities (*not shown*), only E-64 produced a slight inhibition. Figure 4 shows one of two similar experiments where 500 μM leupeptin or antipain cannot prevent the irreversible activation obtained at 10 μM Ca^{2+} which, in this experiment, is also manifest with the calmodulin-stimulated Ca,Mg-ATPase activity. The concentrations of leupeptin and antipain were about 1,000 and 250 times, respectively, their K_i towards calpain (Susuki et al., 1981). Besides, taking an upper figure of 20,000 calpain binding sites per red cell (Pontremoli et al., 1985) and an estimate of 2.4 · 10^9 original cells/ml during the preincubations (*see* Materials and Methods), the inhibitor/calpain molar ratio would have been greater than 6,000 if all the protease binding sites had been occupied.

Figure 5 shows the result of one of two similar experiments to examine the effect of leupeptin across the Ca^{2+} concentration range during preincubation. Preincubation at 10 mM Ca^{2+} activates the pump by a factor of 5.5 but only 12% of this (20% at 1 mM Ca^{2+}, 4% at 10 μM Ca^{2+}) can be suppressed by leupeptin at a concentration that is supramaximal for calpain inhibition. Panel *B* shows that a putative protease-induced activation requires about 25 μM Ca^{2+} for its effect to be half-maximal. This is higher

Table 1. Lack of effect of serine-protease inhibitors on the calcium pump modification caused by preincubating red cell membranes at 37°C in Ca^{2+}-containing solutions

Experimental condition	Ca,Mg-TPase activity (μmol · mg^{-1} · hr^{-1})	
	Basal	CaM-stimulated
EXP. 1		
Control	0.037 ± 0.005	0.887 ± 0.036
Ca^{2+} preinc., no add.	0.196 ± 0.016	0.445 ± 0.045
Ca^{2+} preinc. plus		
STI[a] (200 μg/ml)	0.197 ± 0.012	0.406 ± 0.019
EXP. 2		
Control	0.112 ± 0.023	1.453 ± 0.045
Ca^{2+} preinc., no add.	0.335 ± 0.015	0.701 ± 0.012
Ca^{2+} preinc. plus		
STI[a] (200 μg/ml)	0.337 ± 0.011	0.689 ± 0.014
Aprotintin (200 μg/ml)	0.388 ± 0.013	0.717 ± 0.009
Benzamidine (0.1 mM)	0.336 ± 0.016	0.658 ± 0.017
EXP. 3		
Control	0.193 ± 0.004	2.280 ± 0.045
Ca^{2+}-free preinc.	0.226 ± 0.005	2.396 ± 0.015
Ca^{2+} preinc.	0.892 ± 0.008	2.117 ± 0.021
Ca^{2+} pre-inc. plus		
pTSF[b] (0.1 mM)	0.836 ± 0.005	2.104 ± 0.037

When present, Ca^{2+} was at 0.9 mM (Exps. 1 and 2) or 1 mM (Exp. 3). The preincubation with or without inhibitors was for 30 min (Exp. 1), 60 min (Exp. 2) or 20 min (Exp. 3). All control membrane suspensions (kept at 0°C) and the Ca^{2+}-free condition of exp. 3, contained 0.5 mM EGTA. Preincubated and control membranes were washed by centrifugation at 5°C, resuspended in Mg/HEPES and assayed for Ca,Mg-ATPase activity with and without calmodulin as in the experiment of Fig. 1.
[a] Soybean trypsin inhibitor. [b] *p*-toluene-sulphonyl fluoride.

than the requirement for calpain I (10 μM), the form found in red cells (Murachi, 1983), but the Ca^{2+} effect could as well represent a binding site on Ca,Mg-ATPase.

The extent of a possible protease contribution to the observed changes seems then quite insignificant at near-physiological Ca^{2+} concentrations. However, the magnitude and time scale of the *overall* activation of the basal Ca,Mg-ATPase activity is such that it raises the issue of the linearity of pump reaction time courses. Indeed, one would expect the enzyme's specific activity to be steadily and irreversibly increasing as the reaction progresses in the presence of Ca^{2+} in the range of concentrations used in vitro, a situation which would lead to parabolic time courses. Our standard assay with the membrane enzyme, for instance, is carried out at 3 μM Ca^{2+} and the results in Fig. 5*A* show that a 25% increase in specific activity has occurred after a 30 min preincubation at 1 μM Ca^{2+} and 37°C. However, a consistent observation in this (Cavieres, 1984, 1987*b*) and other laboratories (Jarrett & Kyte, 1979; Waisman et al., 1982) has been that hydrolytic and exchange reactions of the membrane-bound Ca,Mg-ATPase are linear with time over periods of at least one hour, provided that ATP hydrolysis does not exceed 20–25%. The possibility that the enzyme did not experience the Ca^{2+}-induced activation while it was turning over was examined with the experiment of Fig. 6. The results show that at 2 mM Mg^{2+} and 1 mM Ca^{2+} (concentrations at which the basal Ca,Mg-ATPase activity should be about 50% optimal (Schatzmann, 1982)), turnover not only fails to prevent the changes but enhances them. Klinger et al. (1981) proposed that the observed linearity of the time course of the ATPase reaction arose from a progressive and concomitant inhibition by the ADP being released. This was unlikely, because the hydrolytic reaction proceeds quite linearly when the ATPase reaction is measured in the presence of 1.5 mM ATP and 1.5 mM ADP (Cavieres, 1987*b*), despite the dampening that the pre-existing ADP will exert on any effects by the ADP released from ATP. Nonetheless, we show in Table 2 the effect of 0.3 mM ADP from the beginning of the reaction. It is apparent that, whereas ADP can inhibit the Ca,Mg-

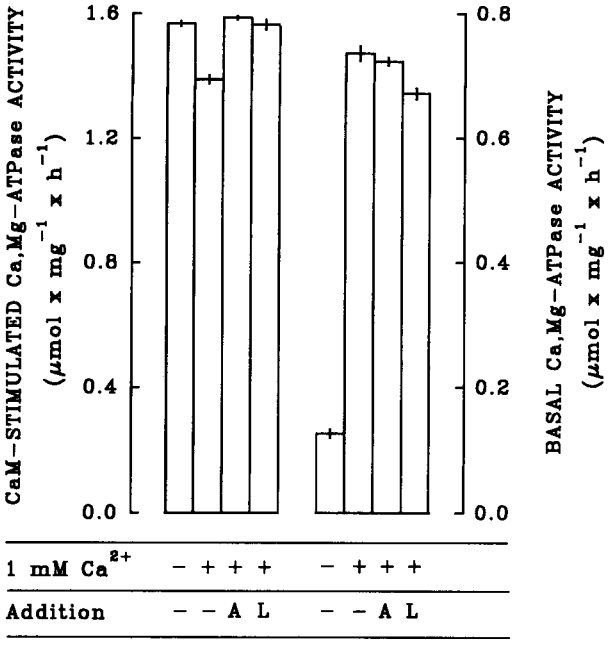

Fig. 3. Effect of 500 μM antipain (*A*) or leupeptin (*L*) on the irreversible modification of red-cell membrane Ca,Mg-ATPase by 1.0 mM Ca^{2+}. Protease inhibitors were added during preincubation with Ca^{2+} (30 min at 37°C). The nonincubated control membranes were kept at 0°C, without added Ca^{2+} and with 0.5 mM EGTA. The Ca,Mg-ATPase activity of the washed membranes was assayed with and without calmodulin at 37°C.

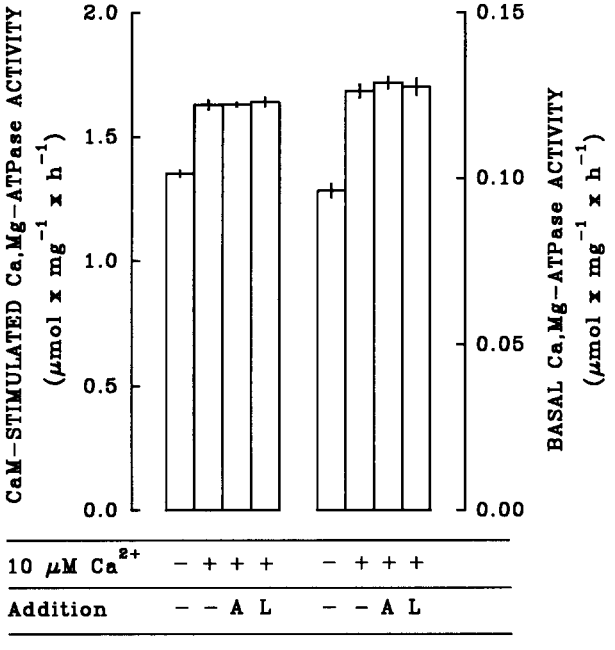

Fig. 4. Lack of effect of 500 μM antipain (*A*) or leupeptin (*L*) on the irreversible modification of red-cell membrane Ca,Mg-ATPase by 10 μM Ca^{2+} (5 mM HEDTA buffer). Experiment similar to that of Fig. 3, except for the Ca^{2+} concentration during the preincubation.

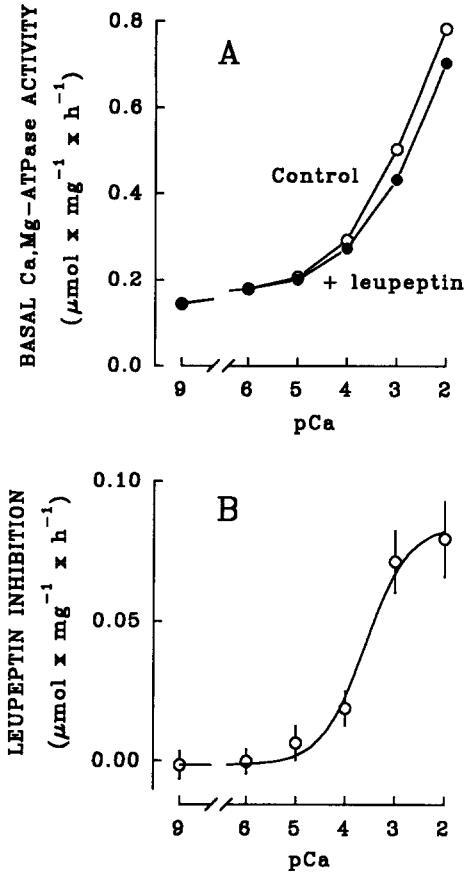

Fig. 5. (*A*) Ca^{2+}-concentration dependence of the 37°C modification of the basal Ca,Mg-ATPase activity of red cell membranes (30 min preincubation) and the effect of 500 μM leupeptin. (*B*) Ca^{2+} dependence of the extent of the leupeptin effect in *A* (difference between control and leupeptin curves).

ATPase activity by about 40% when the reaction is running with 10 μm [γ^{32}P]-ATP, it is ineffective at 2 mM [γ^{32}P]-ATP, i.e., when the ADP represents 15% breakdown product. It seems therefore not possible that a concomitant ADP inhibition can explain the sustained linearity of reactions measured at 1.5 mM ATP and above. An alternative hypothesis will be discussed below.

Effects of Preincubating Membrane-Bound Ca,Mg-ATPase at 0°C

Inconsistencies arising in some experiments encouraged us to investigate whether preincubation with Ca^{2+}-containing solutions at 0°C resulted in any irreversible changes in the activity of the calcium pump. The result of one of those experiments is shown in Fig. 7. Whereas the activity of the Ca,Mg-ATPase assayed in the presence of calmodulin changes little, there is in this case a twofold irreversible activation

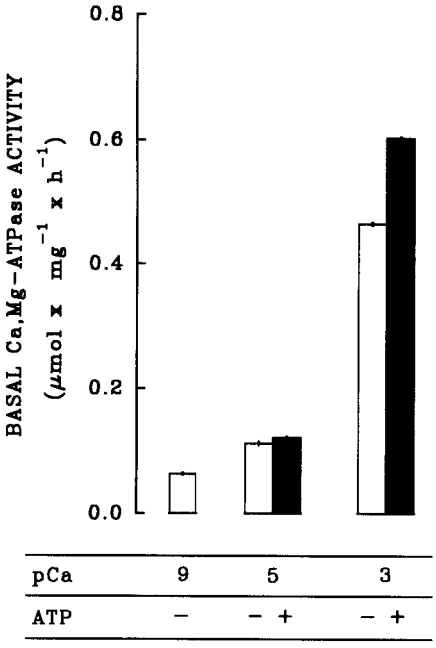

Fig. 6. Effect of ATP on the irreversible calcium pump activation by calcium ions. Red cell membranes were incubated for 30 min at 37°C in medium NK but at 1 mM Mg^{2+}: at nominally zero Ca^{2+} (pCa 9) and at 10 μM and 1 mM Ca^{2+}, with and without 2 mM total ATP. The Ca,Mg-ATPase activity was determined at 37°C after four washes in the refrigerated microcentrifuge.

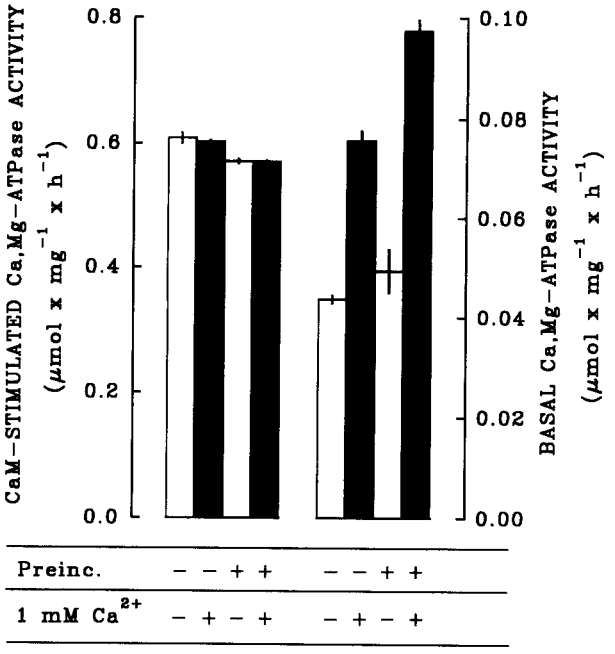

Fig. 7. Effect of a 90 min preincubation at nominally zero or with 1 mM Ca^{2+} at 0°C, on the Ca,Mg-ATPase activity of the membranes, measured with and without calmodulin. In the "no pre-incubation" condition, the membranes were defrosted and delivered to the preincubation medium at 0°C just before washing twice in the refrigerated microcentrifuge.

Table 2. Effect of 0.3 mM ADP on the Ca,Mg-ATPase activity of red cell membranes at 10 μM and 2 mM ATP

ATP Concentration	Basal Ca,Mg-ATPase activity (μmol · mg^{-1} · hr^{-1})	
	Control	+ADP
10 μM	0.0213 ± 0.0007	0.0076 ± 0.0002
2 mM	0.056 + 0.002	0.052 ± 0.004

Reactions measured over 20 min with 20 μM $CaCl_2$ plus 2 mM ATP and 1.5 mM $MgCl_2$ or 10 μM ATP and 0.1 mM $MgCl_2$, with and without 0.3 mM ADP. At 2 mM ATP, the net hydrolysis during the reaction period was 5%.

Table 3. Lack of effect of leupeptin on the calcium-induced modification at 0°C of the calcium pump of red cell membranes

Preincubation condition	Basal Ca, Mg-ATPase activity (μmol · mg^{-1} · hr^{-1})	
	Control	Leupeptin
0.5 mM EGTA	0.136 ± 0.004	0.147 ± 0.006
1.0 mM $CaCl_2$	0.919 ± 0.009	0.923 ± 0.006

The membranes were incubated for 90 min at 0°C with or without 1 mM $CaCl_2$ and with or without 500 μM leupeptin. After two washes in the cold and resuspension in "Mg/HEPES," the Ca,Mg-ATPase activity was determined in the absence of calmodulin.

of the basal Ca,Mg-ATPase after 90 min at 0°C in NK medium containing 1 mM Ca^{2+}. The membranes were spun out for 10 min at 5°C in the refrigerated centrifuge and some 5 more min elapsed at 0°C before the pellets were washed with Mg^{2+}/HEPES solution. It is likely that this additional period in 1 mM Ca^{2+} was responsible for the activation seen in the "no-preincubation" condition. The experiment shown in Table 3 demonstrates that the activation obtained in the cold is not inhibited by 500 μM leupeptin and is thus unlikely to be caused by the action of a Ca^{2+}-activated protease.

The Ca^{2+} concentration dependence of the activation at 0°C is shown in Fig. 8. A threefold activation of the basal Ca,Mg-ATPase was observed at 10 mM Ca^{2+}. However, in addition to the low-affinity effect seen at 37°C, a high-affinity component of the activation can be noticed in this and a second experiment at 0°C. As the experiments of Figs. 6 and 8 were carried out simultaneously and using red cell membranes from the same donor, a comparison

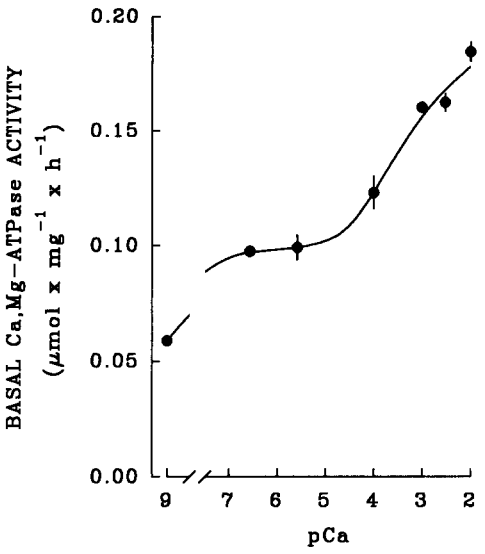

Fig. 8. Ca^{2+}-concentration dependence of the modification of the Ca,Mg-ATPase of the membranes caused by preincubation for 60 min at 0°C (pH 7.69), using solutions similar to those of Fig. 2. Membranes were washed four times in the refrigerated centrifuge.

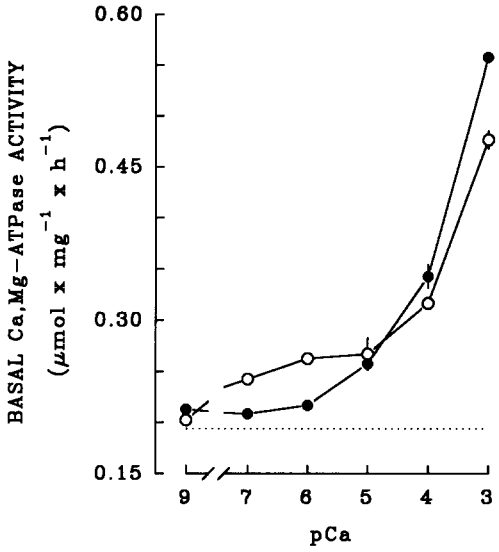

Fig. 9. Ca^{2+}-concentration dependence of the modification of the membrane-bound calcium pump by preincubation at 37°C for 10 min (open symbols) and 30 min (filled symbols). The 10-min mixes waited for 20 min on ice, before transferring to the 37°C bath. All samples cooled down for 5 min before spinning. The dotted line indicates the enzyme activity of nonincubated controls.

Table 4. Activation of the membrane-bound Ca,Mg-ATPase by Ca^{2+} at 0 and 37°C in two simultaneous experiments

Preincubation conditions	Basal Ca,Mg-ATPase activity (μmol · mg^{-1} · hr^{-1})	
	Control (0.5 mM EGTA)	1 mM Ca^{2+}
60 min at 0°C	0.059 ± 0.003	0.160 ± 0.002
30 min at 37°C	0.063 ± 0.002	0.465 ± 0.003

Comparison of data from the experiments of Figs. 6 and 8, run in parallel at different temperatures. The 37°C preincubation (Fig. 6) was started 25 min after the 0°C preincubation (Fig. 8), using membranes from the same stock (no added Ca^{2+}) kept at 0°C after defrosting, and was terminated by a 5 min cooling period. Thereafter, the four washes and the assays were done side by side.

of the Ca^{2+} effects at both temperatures can be made and is shown in Table 4. The preincubation temperature had no effect on the activity of the controls in 0.5 mM EGTA, as can also be seen in the two controls in Exp. 3 of Table 1. The preincubation at 1 mM Ca^{2+} leads to more than a sevenfold activation after 30 min at 37°C and to 2.7 times the control activity after 60 min at 0°C. One can estimate a 10-fold activation after 60 min at 37°C, by projecting the result at 30 min with the aid of the rate constant fitted in Fig. 1. Taking this as an upper limit, and assuming that, at 1 mM Ca^{2+}, the different magnitude of the modification at the two temperatures only arises from different rate constants for the process, an upper estimate of 25 kJ/mol ($Q_{10} < 1.6$) can be ventured for the Arrhenius activation energy associated with the irreversible modification of Ca,Mg-ATPase by 1 mM Ca^{2+}.

The effect of the preincubation at 0°C helps us understand the result of the experiment in Fig. 9. Here, the Ca^{2+} dependence after a 10 min incubation at 37°C shows a high-affinity component not dissimilar from that at 0°C. As both sets of incubations were terminated by cooling for 5 min at 0°C, it is unlikely that the high-affinity component appeared after the 10 min incubation at 37°C. It is more probable that this developed during the 20 min period which these aliquots spent at 0°C before joining the others at 37°C and/or during the initial stage of the 37°C incubation. In any case, the high-affinity activation should only be transient, as it gives way in time to a single, low-affinity pattern (filled circles).

Effect of Preincubating Soluble Purified Ca,Mg-ATPase at 37°C

It has been shown by Roufogalis et al. (1990) that the activation induced by preincubating with Ca^{2+} at 37°C disappears after solubilization of Ca,Mg-ATPase with Triton X-100. As it was not certain whether a second incubation of the solubilized enzyme would not lead to renewed activation, we decided to preincubate the soluble purified enzyme at

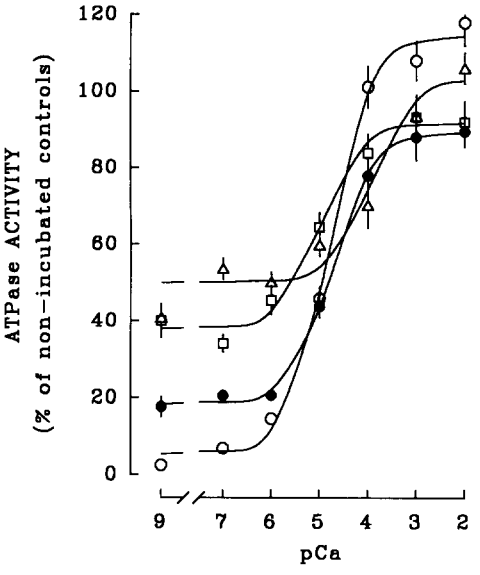

Fig. 10. Four experiments showing the effect on soluble purified Ca,Mg-ATPase of a 10 min preincubation at 37°C at varying Ca^{2+} concentrations, in the presence of Triton X-100 (1–2 mg/ml) and phosphatidylcholine (0.2–0.4 mg/ml). The curves represent least-squares hyperbolic fitting on the data. Filled symbols; 100 μM Mg^{2+}, no ATP ($K_{0.5}$ = 19 μM). Open symbols: 2 mM total ATP during preincubation, plus (○) 5 μM Mg^{2+} ($K_{0.5}$ = 16 μM), (□) 100 μM Mg^{2+} ($K_{0.5}$ = 11 μM) and (△) 2 mM Mg^{2+} ($K_{0.5}$ = 122 μM). After an initial gel filtration of the enzyme batch, duplicate preincubated and control enzyme samples were cooled on an ice-bath and filtered again through spun Sephadex columns (Penefsky, 1977) before the Ca,Mg-ATPase activity was assayed at 54 μM Ca^{2+} (100 μM total calcium) as detailed in Materials and Methods. The activity is plotted as percent of that of gel-filtered controls which were kept at 0°C without added Ca^{2+} during the preincubation period.

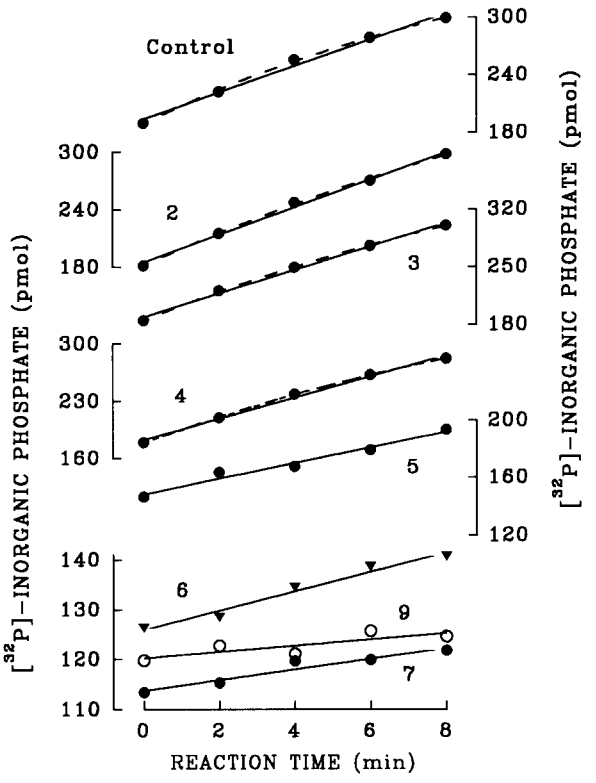

Fig. 11. ATPase reaction time courses of one out of two duplicates for each Ca^{2+} concentration in the preincubation with 2 mM ATP and 3 μM Mg^{2+} of Fig. 10 (open circles). Numbers in the curves indicate pCa during preincubation. Unbroken lines: linear regressions; dashed lines: exponential fitting. The linear regression coefficient and the exponential parameters A and k are (pmol/min, pmol and min^{-1}, respectively): 14, 220 and 0.085 (control); 14, 320 and 0.056 (pCa 2); 14, 280 and 0.066 (pCa 3) and 13, 230 and 0.073 (pCa 4).

various Ca^{2+} concentrations at 37°C. The results are presented in Fig. 10. To wash the enzyme free from Ca^{2+} and buffers before and after the preincubation, the solution was gel-filtered through spun Sephadex G-50 columns (Penefsky, 1977), which could remove more than 99.6% of the Ca^{2+} and buffers (*see* Materials and Methods). Experiments with one or two filtrations after the preincubation gave essentially similar results, although the additional handling involved with the second filtration led to a greater scatter in the data (e.g., triangles in Fig. 10). The basic outcome of these experiments is that a 10 min preincubation with Ca^{2+} at 37°C does not lead to activation, but that calcium ions protect the detergent-solubilized enzyme from the thermal inactivation associated with the preincubation. This is emphasized by the fact that the controls (which were used as a 100% reference for the preincubated samples, to allow for specific activity differences from experiment to experiment) were kept with 0.5 mM EGTA and without added Ca^{2+} at 0°C. ATP in the preincubation medium affords some protection when the Mg^{2+} concentration is 100 μM and above (squares and triangles), but at 5 μM Mg^{2+} there seems to be an increased inactivation at low Ca^{2+} levels (open circles). The curves were reasonably well fitted by hyperbolae with $K_{0.5}$ between 10 and 20 μM Ca^{2+}, except for the curve at 2 mM Mg^{2+} ($K_{0.5}$ = 122 μM).

These results also introduce a difficulty with respect to the conditions to measure ATPase activity, in this case with the purified enzyme. In fact, they predict that when following ATP hydrolysis at Ca^{2+} concentrations below 10 μM, there will be substantial enzyme inactivation as the reaction progresses. Even in our assays at 54 μM Ca^{2+}, one would predict a 10–15% decrease of the ATPase activity after 10 min. This is examined in more detail in Fig. 11, which shows time courses corresponding to one of the experiments in Fig. 10. The analysis was done for the data of the four upper time courses, obtained

at higher enzyme activities and therefore more reliable. Despite the fact that the correlation coefficients for the linear regressions were all better than +0.994, a slight downward curvature of the time courses can be discerned. The exponentials shown by the dashed lines do indeed fit the points better. An estimate of the initial rates was obtained from the product of the fitted A and k parameters (*see* Materials and Methods) and a comparison with the rates obtained a linear regression coefficients shows that the latter underestimate the initial rates by beween 19 and 27%. This is not large enough to invalidate the results of Fig. 10 where, besides, all the assays were performed at the same Ca^{2+} concentration, but the difficulty would be certainly compounded when one measured rates at variable Ca^{2+} or ATP concentrations, or both, as when doing a kinetic analysis.

The exponential fitting presents an apparent quantitative inconsistency. Since Eq. (1) can be rewritten as $(dy/dt)_{t=t} = (dy/dt)_{t=0} \cdot e^{-kt}$, it follows that the instantaneous reaction rate must decrease on account of inactivation with an apparent rate constant as fitted for the progress curve. Using the apparent rate constants in the legend to Fig. 11, we calculate that a 10 min preincubation at 54 μM Ca^{2+} should have inactivated the enzyme between 43 and 57% and yet from Fig. 10 one can interpolate not more than 10–15% inactivation at that concentration. The discrepancy probably arises from slightly different conditions during preincubation and assay of the enzyme and the implications of this will be discussed in the next section.

Discussion

Exposure of calmodulin-depleted red cell membranes to Ca^{2+}-containing solutions at 0 or 37°C leads to a large and irreversible activation of the basal Ca,Mg-ATPase activity of their calcium pumps. The basic process is independent of the action of membrane-bound proteases and these make a very small contribution at 10 μM Ca^{2+}, i.e., the maximal concentration which might be considered physiological in mammalian cells (Marban et al., 1980; Cannell, Berlin & Lederer, 1987). Although the experiments have been conducted with broken membranes in which sidedness has been lost, the Ca^{2+} effects are no doubt at the intracellular aspect of the membranes: the normal red cell Ca^{2+} concentration is 10–30 nM (Lew et al., 1982), but erythrocytes are normally exposed to an extracellular concentration of about 1 mM. Thus, an extracellular Ca^{2+} effect would have led to membrane preparations whose Ca^{2+} pumps were irreversibly activated from the start.

One type of activation of the plasma-membrane calcium pump results from removal of the calmodulin-binding domain (Papp et al., 1989; Zvaritch et al., 1990). It seems that this C-terminal segment can be normally parked at either of two sequences: one at or near the active site (Falchetto et al., 1991) and another in a different region on the same chain or, perhaps, on a neighboring subunit in a dimer (Vorherr et al., 1991). The first interaction inhibits the ATPase activity of a calpain-truncated enzyme and the second stimulates the activity of the intact enzyme, presumably by promoting dimerization (Falchetto et al., 1991; Vorherr et al., 1991). The other relevant mode of activation is by fatty acids and acidic phospholipids acting specifically at two sequences, one of which is the calmodulin-binding domain (Papp et al., 1989; Brodin et al., 1992; Filoteo, Enyedi & Penniston, 1992). These topological relationships may explain the fact that both controlled protease attack and acidic phospholipids decrease the calmodulin stimulation as they increase the basal activity of the enzyme with a pattern not too different from that obtained by previous exposure to calcium ions (Fig. 1).

These structural correlates should help us understand the present results, but the inferences are not immediately obvious nor free from difficulty. For one thing, the greater part of the irreversible Ca^{2+} activation does not result from a cut in the polypeptide chain (Roufogalis et al., 1990) in conditions such that added calpain would have clipped the calmodulin-binding peptide (James et al., 1989; Wang et al., 1989). The overall effect of leupeptin (Figs. 3–5) is quite small and a minor degree of proteolysis on account of membrane-bound calpain might not show in the gels in these conditions. It seems plausible, therefore, that the Ca^{2+} activation instead results from an irreversible conformational change in the calcium pump (Roufogalis et al., 1990) but, distinctively, the effect has a slow time course, a low activation energy and a low Ca^{2+} affinity. These features might offer some clues about the nature of the activating process.

Perhaps the slowest rate constant for a conformational change in a P-type ATPase is that measured for the transition of the occluded K^+ form of Na,K-ATPase, 0.05–0.4 sec^{-1} at 20–22°C (Glynn & Richards, 1982; Forbush, 1987; Faller et al., 1991) and this is at least 130 times faster than the apparent rate constant fitted in Fig. 1 for the preincubation at 37°C. The activation energy for calcium transport has been estimated between 57 and 105 kJ/mol (Schatzmann, 1982), and those for E_2/E_1 transitions in Na,K-ATPase and H,K-ATPase are between 71 and 120 kJ/mol (Faller et al., 1991), which are much higher than the upper estimate of 25 kJ/mol for the

irreversible Ca^{2+} activation. The high-affinity component (transient at 37°C) bears the hallmark of the intracellular activatory Ca^{2+}-binding sites, where half-maximal binding occurs at 0.06 μM Ca^{2+} ($pK_{0.5}$ = 7.2), as estimated from intrinsic fluorescence titrations with the purified soluble enzyme (Kosk-Kosicka & Inesi, 1985). However, the apparent affinity of the "site" promoting the *major* effect seems well above 1 mM in these experiments (around 0.5 mM according to Roufogalis et al. (1990)), and this excludes the activatory intracellular Ca^{2+} sites. All these factors seem to suggest that the effect on the enzyme is an indirect one, arising from a slow nonenzymic process.

A crucial feature is that if one is to observe linear time courses with the membrane-bound Ca,Mg-ATPase reaction at Ca^{2+} concentrations in the micromolar range, the Ca^{2+} activation must be such that it becomes apparent not during but after the first Ca^{2+} exposure. This condition would be satisfied if a Ca^{2+} preincubation removed tightly bound inhibitory factors from the enzyme. If the "basal" enzyme had two or more bound particles which conferred the low-affinity, low-V_{max} characteristics of the calmodulin-free state (Scharff & Foder, 1978), either the release of those particles or the binding of calmodulin could move the kinetic characteristics of the pump towards the calmodulin-liganded state. Such a removal process would have to occur at least in two stages:

$$\text{CMA} \cdot \text{I} \cdot \text{P} \overset{Ca^{2+}}{\rightleftharpoons} \text{CMA} \cdots \text{I} + \text{P} \quad \text{(a)}$$

$$\text{CMA} \cdots \text{I} \longrightarrow \text{CMA} + \text{I} \quad \text{(b)}$$

where CMA is Ca,Mg-ATPase, I is an inhibitor and P is a second particle. If the I and P particles remain in the medium, they might still inhibit the enzyme by binding to it at equilibrium (*see below*). To expose an irreversible activation, therefore, the released particles would have to be washed away before assaying for enzymic activity. In the scheme above, *stage a* would take place during the preincubation of the enzyme in the present experiments, and would consist of the slow Ca^{2+}-activated release of the P particle (presumably a peripheral membrane protein) occurring with a low activation energy. The low-affinity Ca^{2+} site might be located on the I or P particles. The release of P would have the effect of "de-occluding" I or labilizing ($\cdots$) the contacts between the enzyme and the I particle. If turnover was taking place and the kinetic behavior of CMA · I · P and CMA $\cdots$ I did not differ much, ATP hydrolysis would continue to be linear with time. *Stage b*, leading to irreversible activation of Ca,Mg-ATPase, would be introduced by the experimenter at the washing stage by releasing the I particle and disposing of I plus P. From our data (and assuming 1,000 molecules each of pump and I particle per leaky red-cell ghost), we calculate that if *stage b* were reversible at all times and had a dissociation constant between 10^{-10} and 10^{-11} M, then *ca.* 90% of the enzyme would still be binding the I particle during the preincubation and yet would release it and expose activation after the washes. Alternatively, the release of a tightly bound I particle could be caused by mechanical disruption of the CMA $\cdots$ I complex, as a result of the shear forces generated during centrifugation and resuspension of the pellet.

Particles I and P could be cytoskeletal components closely associated with Ca,Mg-ATPase. The anion exchanger (Band 3) and the brain voltage-gated sodium channel (Luna & Hitt, 1992), as well as Na,K-ATPase in transporting epithelia (Koob et al., 1987; Nelson & Veshnook, 1987; Nelson & Hammerton, 1989) can be found associated with cytoskeletal elements. In the case of the plasma-membrane Ca,Mg-ATPase, radiation inactivation experiments show that, in addition to the dimers, a substantial fraction of the calmodulin-stimulated enzyme is associated with a particle of a size in excess of 1 MD (Cavieres, 1984).

Apart from the more general proposal above, it seems appropriate to consider also the reversal of activation observed after solubilization and purification of Ca,Mg-ATPase. It appears that acidic phospholipids stimulate Ca,Mg-ATPase mainly by binding at the calmodulin-binding domain, even enhancing calmodulin binding (Brodin et al., 1992). This seems quite plausible, as the calmodulin-binding "A" domain has the features of an amphipathic α-helix, rich in Arg^{+} residues on one side and hydrophobic residues on the other (Shull & Greeb, 1988). It is conceivable that the hydrophobic face of domain "A" is normally occupied by phosphatidylcholine in the initial membrane preparations and that calmodulin, acidic phospholipids or removal of the hypothetical I and P particles activate by displacing the inhibitory lecithin. During solubilization, the detergent or added phosphatidylcholine would occupy domain "A," now reversing the Ca^{2+}-induced activation (which is irreversible in membrane-bound form). Phospholipid binding does not necessarily imply an interaction between the helix and the lipid bilayer (or annulus phospholipid in the soluble enzyme), as even nonionic amphiphiles will bind at hydrophobic patches on the predominantly hydrophilic surface of globular proteins (Tanford, 1980). The hypotheses above, as well as a related earlier proposal (Roufogalis et al., 1990), have some elements that could be tested experimentally. Nevertheless, it seems likely that the overall effect could

arise from a more complicated process, as another activated form appeared at 0°C (Fig. 8) and at short times at 37°C (Fig. 9), with a higher Ca^{2+} affinity, and this might imply a contribution of binding sites on the enzyme. In this connection, it has recently been observed that preincubation of red cell membranes in the presence of Mg^{2+} and vanadate also results in an activation of Ca,Mg-ATPase (Romero, 1993).

The protective effect of Ca^{2+} on the thermal inactivation of the soluble purified enzyme is likely to result from Ca^{2+} binding at the high-affinity intracellular activatory site. Being 10–20 μM, however, the apparent dissociation constant is more than two orders of magnitude higher than that obtained from equilibrium experiments (Kosk-Kosicka & Inesi, 1985), and one order of magnitude greater than the $K_{0.5}$ values derived from turnover experiments at similar enzyme concentrations (Graf et al., 1982). The reason for this discrepancy is not known but it seems unlikely that these numbers reflect the intracellular or extracellular inhibitory Ca^{2+} sites, with a $K_{0.5}$ normally in the region of 1 and 9 mM, respectively (Kratje, Garrahan & Rega, 1985).

The rates of inactivation of the Triton enzyme at 37°C are fast and time courses should be mandatory, specially when the Ca^{2+} concentrations are being varied. Initial rates can nevertheless be calculated with the exponential fitting method used in Fig. 11, preferably with more detailed time courses.

Faster inactivation rates were apparent during the ATPase reactions in Fig. 11 than could be estimated from inspection of the curves in Fig. 10 at 54 μM Ca^{2+}. Considering that one of the latter curves was obtained in turnover conditions, the only difference between the two figures seems to be the enzyme concentration. This was around 24 nM during the assays (Fig. 11) but twice as high during the preincubations (Fig. 10). As the equilibrium constant for dimerization of the $C_{12}E_8$-solubilized enzyme seems to be in the region of 20 nM (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka & Bzdega, 1988; Kosk-Kosicka, Bzdega & Wawrzynow, 1989), during the preincubation the enzyme must have had a higher content of oligomeric forms than during the ATPase reaction. The different rates of inactivation should then depend on the oligomeric state of the soluble enzyme, with the monomeric form being the least thermally stable. One might therefore regard stability as one of the advantages of oligomerization, the other being activation of the enzyme in detergent solution to a calmodulin-liganded state but without calmodulin (Kosk-Kosicka & Bzdega, 1988). Perhaps increased stability is all that the membrane-bound enzyme gains when forming dimers as, unlike the situation with the purified enzyme (Kosk-Kosicka & Inesi, 1985), calmodulin is effective in increasing the enzymic activity of the membrane-bound dimer (Cavieres, 1984).

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References

Au, K.S. 1987. Activation of erythrocyte membrane Ca^{2+}-ATPase by calpain. *Biochim. Biophys. Acta* **905:**273–278

Au, K.S., Lee, M.F., Siu, Y.L. 1989. Ca^{2+}-mediated activation of human erythrocyte membrane Ca^{2+}-ATPase. *Biochim. Biophys. Acta* **978:**197–202

Bond, G.H., Clough, D.L. 1973. A soluble protein activator of (Mg^{2+} + Ca^{2+})-dependent ATPase in human red cell membranes. *Biochim. Biophys. Acta* **323:**592–599

Brodin, P., Falchetto, R., Vorherr, T., Carafoli, E. 1992. Identification of 2 domains which mediate the binding of activating phopholipids to the plasma-membrane Ca^{2+} pump. *Eur. J. Biochem.* **204:**939–946

Brown, A.M. 1982. ATP and ATPase determinations in red blood cells. *In:* Red Cell Membranes, A Methodological Approach. J.C. Ellory and J. Young, editors. pp. 223–238. Academic, London

Cannell, M.B., Berlin, J.R., Lederer, W.J. 1987. Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells. *Science* **238:**1419–1423

Cavieres, J.D. 1984. Calmodulin and the target size of the (Ca^{2+} + Mg^{2+})-ATPase of human red-cell ghosts. *Biochim. Biophys. Acta* **771:**241–244

Cavieres, J.D. 1987*a*. Calcium-dependent modification of the plasma membrane Ca,Mg-ATPase upon incubation at 37°C. *Biochem. Soc. Trans.* **15:**1067–1068

Cavieres, J.D. 1987*b*. Fast reversal of the initial reaction steps of the plasma membrane (Ca^{2+} + Mg^{2+})-ATPase. *Biochim. Biophys. Acta* **899:**83–92

Enyedi, A., Sarkadi, B., Szasz, I., Bot, G., Gárdos, G. 1980. Molecular properties of the red cell calcium pump. II. Effects of calmodulin, proteolytic digestion and drugs on the calcium-induced membrane phosphorylation by ATP in inside-out red cell membrane vesicles. *Cell Calcium* **1:**299–310

Fabiato, A., Fabiato, F. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J. Physiol. (Paris)* **75:**463–505

Falchetto, R., Vorherr, T., Brunner, J., Carafoli, E. 1991. The plasma membrane Ca^{2+} pump contains a site that interacts with its calmodulin-binding domain. *J. Biol. Chem.* **266:** 2930–2936

Faller, L.D., Diaz, R.A., Scheiner-Bobis, G., Farley, R.A. 1991. Temperature dependence of the rates of conformational changes reported by fluorescein 5′-isothiocyanate modification of H^+,K^+- and Na^+,K^+-ATPases. *Biochemistry* **30:** 3503–3510

Filoeto, A.G., Enyedi, A., Penniston, J.T. 1992. The lipid-binding peptide from the plasma membrane Ca^{2+} pump binds calmodulin, and the primary calmodulin-binding domain interacts with lipid. *J. Biol. Chem.* **267:**11800–11805

Forbush, B. 1987. Rapid release of ^{42}K or ^{86}Rb from two distinct

transport sites on the Na,K-pump in the presence of P_i or vanadate. *J. Biol. Chem.* **262:**11116–11127

Gietzen, K., Kolandt, J. 1982. Large-scale isolation of human erythrocyte Ca^{2+}-transport ATPase. *Biochem. J.* **207:**155–159

Glynn, I.M., Richards, D.E. 1982. Occlusion of rubidium ions by the sodium-potassium pump: its implications for the mechanism of potassium transport. *J. Physiol.* **330:**17–43

Good, N.E., Izawa, S. 1972. Hydrogen ion buffers. *Methods Enzymol.* **24B:**53–68

Gopinath, R.M., Vincenzi, F.F. 1977. Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+} + Mg^{2+})ATPase. *Biochem. Biophys. Res. Commun.* **77:** 1203–1209

Graf, E., Verma, A.K., Gorski, J.P., Lopaschuk, G., Niggli, V., Carafoli, E., Penniston, J.T. 1982. Molecular properties of calcium-pumping ATPase from human erythrocytes. *Biochemistry* **21:**4511–4516

James, P., Vorherr, T., Krebs, J., Morelli, A., Castello, G., McCormick, D.J., Penniston, J.T., Deflora, A., Carafoli, E. 1989. Modulation of erythrocyte Ca^{2+}-ATPase by selective calpain cleavage of the calmodulin-binding domain. *J. Biol. Chem.* **264:**8289–8296

Jarrett, H.W., Kyte. J. 1979. Human erythrocyte calmodulin. Further chemical characterization and the site of its interaction with the membrane. *J. Biol. Chem.* **254:**8237–8244

Jarrett, H.W., Penniston, J.T. 1978. Purification of the Ca^{2+}-stimulated ATPase activator from human erythrocytes. Its membership in the class of Ca^{2+}-binding proteins. *J. Biol. Chem.* **253:**4676–4682

Klinger, R., Wetzker, R., Fleischer, I., Frunder, H. 1981. Transformation of (Ca^{2+} + Mg^{2+}) ATPase of calmodulin-depleted erythrocyte membranes into a high Ca^{2+}-affinity form by Ca^{2+}. *Cell Calcium* **2:**553–560

Koob, R., Zimmermann, M., Schoner, W., Drenckhahn, D. 1987. Colocalization and coprecipitation of ankyrin and Na+,K+-ATPase in kidney epithelial cells. *Eur. J. Cell Biol.* **45:**230–237

Kosk-Kosicka, D., Bzdega, T. 1988. Activation of the erythrocyte Ca^{2+}-ATPase by either self association or interaction with calmodulin. *J. Biol. Chem.* **263:**18184–18189

Kosk-Kosicka, D., Bzdega, T., Warwrzynow, A. 1989. Fluorescence energy transfer studies of purified erythrocyte Ca^{2+}-ATPase. *J. Biol. Chem.* **264:**19495–19499

Kosk-Kosicka, D., Inesi, G. 1985. Cooperative calcium binding and calmodulin regulation in the calcium-dependent adenosine triphosphatase purified from the erythrocyte membrane. *FEBS Lett.* **189:**67–71

Kratje, R.B., Garrahan, P.J., Rega, A.F. 1985. Two modes of inhibition of the Ca^{2+} pump in red cells by Ca^{2+}. *Biochim. Biophys. Acta* **816:**365–378

Lew, V.L., Tsien, R.Y., Miner, C., Bookchin, R.M. 1982. Physiological $(Ca^{2+})_i$ level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. *Nature* **298:**478–481

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Luna, E.J., Hitt, A.L. 1992. Cytoskeleton-plasma membrane interactions. *Science* **258:**955–964

Marban, E., Rink, T.J., Tsien, R.W., Tsien, R.Y. 1980. Free calcium in heart muscle at rest and during contraction measured with Ca^{2+}-sensitive microelectrodes. *Nature* **286:** 845–850

Martell, A.E., Smith, R.M. 1974. Critical Stability Constants. Plenum, New York and London

Minocherhomjee, A.M., Beauregard, G., Potier, M., Roufogalis, B.D. 1983. The molecular weight of the calcium-transport-ATPase of the human red blood cell determined by radiation inactivation. *Biochem. Biophys. Res. Commun.* **116:**895–900

Murachi, T. 1983. Calpain and calpastatin. *Trends Biochem. Sci.* **8:**167–169

Nelson, W.J., Hammerton, R.W. 1989. A membrane-cytoskeletal complex containing Na^+,K^+-ATPase, ankyrin and fodrin in Madin-Darby kidney (MDCK) cells. Implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* **108:**893–902

Nelson, W.J., Veshnook, P.J. 1987. Ankyrin binding to (Na^+,K^+)-ATPase and implications for the organization of membrane domains in polarized epithelial cells. *Nature* **328:**533–535

Niggli, V., Adunyah, E.S., Carafoli, E. 1981. Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase. *J. Biol. Chem.*, **256:**8588–8592

Niggli, V., Penniston, J.T., Carafoli, E. 1979. Purification of the (Ca^{2+}-Mg^{2+})-ATPase from human erythrocyte membranes using a calmodulin affinity column. *J. Biol. Chem.* **254:** 9955–9958

Papp, B., Sarkadi, B., Enyedi, A., Caride, A.J., Penniston, J.T., Gárdos, G. 1989. Functional domains of the in situ red cell membrane calcium pump revealed by proteolysis and monoclonal antibodies. *J. Biol. Chem.* **264:**4577–4582

Penefsky, H.S. 1977. Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **252:** 2891–2899

Pontremoli, S., Melloni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O., Horecker, B.L. 1985. Binding to erythrocyte membrane is the physiological mechanism for activation of Ca^{2+}-dependent neutral protease. *Biochem. Biophys. Res. Commun.* **128:**331–338

Romero, P.J. 1993. Synergistic activation of the human red cell calcium ATPase by magnesium and vanadate. *Biochim. Biophys. Acta* **143:**45–50

Rossi, J.P.F.C., Schatzmann, H.J. 1982. Trypsin activation of the red cell Ca^{2+}-pump ATPase is calcium-sensitive. *Cell Calcium* **3:**583–590

Roufogalis, B.D., Brzuszczak, I., Conigrave, A.D., Xu, Y.-H., Machan, C.L., Wang, K.K.W. 1990. Persistent Ca^{2+}-induced activation of erythrocyte membrane Ca^{2+}-ATPase unrelated to calpain proteolysis. *Arch. Biochem. Biophys.* **279:**78–96

Sarkadi, B., Enyedi, A., Földes-Papp, Z., Gárdos, G. 1986. Molecular characterization of the in situ red cell membrane calcium pump by limited proteolysis. *J. Biol. Chem.* **261,** 9522–9557

Sarkadi, B., Enyedi, A., Gárdos, G. 1980. Molecular properties of the red cell calcium pump. I. Effects of calmodulin, proteolytic digestion and drugs on the kinetics of active calcium uptake in inside-out red cell membrane vesicles. *Cell Calcium* **1:**187–198

Scharff, O., Foder, B. 1978. Reversible shift between two states of Ca^{2+}-ATPase in human erythrocytes mediated by Ca^{2+} and a membrane bound activator. *Biochim. Biophys. Acta* **509:** 67–77

Schatzmann, H.J. 1982. The plasma membrane calcium pump of erythrocytes and other animal cells. *In:* Membrane Transport of Calcium. E. Carafoli, editor. pp. 41–106. Academic, New York

Schatzmann, H.J. 1989. The calcium pump of the surface membrane and of the sarcoplasmic reticulum. *Annu. Rev. Physiol.* **51:**473–485

Shull, G.E., Greeb, J. 1988. Molecular cloning of two isoforms

of the plasma membrane Ca^{2+}-transporting ATPase from rat brain: structural and functional domains exhibit similarity to Na^+,K^+- and other cation transport ATPases. *J. Biol. Chem.* **263**:8646–8657

Sillén, L.G., Martell, A.E. 1971. Stability Constants of Metal-Ion Complexes. Suppl. No. 1. The Chemical Society, London

Smith, P.K., Krohn, R.I., Hermanson, G., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85

Stieger, J., Schatzmann, H.J. 1981. Metal requirement of the isolated red cell Ca-pump ATPase after elimination of calmodulin dependence by trypsin attack. *Cell Calcium* **2**:601–616

Susuki, K., Tsuji, S., Ishihura, S. 1981. Effect of Ca^{2+} on the inhibition of calcium-activated neutral protease by leupeptin, antipain and epoxysuccinate derivatives. *FEBS Lett.* **136**: 119–122

Tanford, C. 1980. The Hydrophobic Effect: Formation of Micelles and Biological Membranes, John Wiley & Sons, New York

Taverna, R.D., Hanahan, D.J. 1980. Modulation of human erythrocyte Ca^{2+}/Mg^{2+} ATPase activity by phospholipase A_2 and proteases. A comparison with calmodulin. *Biochem. Biophys. Res. Commun.* **94**:652–659

Umezawa, H. 1972. Enzyme Inhibitors of Microbial Origin. University of Tokyo, Tokyo

Note Added in Proof

In a contemporary study, J. Fermín and P.J. Romero (*J. Membrane Biol.* **137**: *in press*) have concluded that the Ca^{2+} activation of the plasma membrane Ca^{2+} pump results from neither proteolysis nor alteration of the membrane fluidity or phospholipid turnover.

Verma, A.K., Filoteo, A.G., Stanford, D.R., Wieben, E.D., Penniston, J.T., Strehler, E.E., Fischer, R., Heim, R., Vogel, G., Mathews, S., Strehler-Page, M.A., James, P., Vorherr, T., Krebs, J., Carafoli, E. 1988. Complete primary structure of a human plasma membrane Ca^{2+} pump. *J. Biol. Chem.* **263**:14152–14159

Vorherr, T., Kessler, T., Hofmann, F., Carafoli, E. 1991. The calmodulin-binding domain mediates the self-association of the plasma membrane Ca^{2+} pump. *J. Biol. Chem.* **266**:22–27

Waisman, D.M., Smallwood, J., Lafreniere, D., Rasmussen, H. 1982. The role of band III in calcium transport across the human erythrocyte membrane. *FEBS Lett.* **145**:337–340

Wang, K.K.W., Roufogalis, B.D., Villalobo, A. 1989. Calpain-I activates Ca^{2+} transport by the reconstituted erythrocyte Ca^{2+} pump. *J. Membrane Biol.* **112**:233–245

Wang. K.K.W., Villalobo, A., Roufogalis, B.D. 1988. Activation of the Ca^{2+}-ATPase of human erythrocyte membrane by an endogenous Ca^{2+}-dependent neutral protease. *Arch. Biochem. Biophys.* **260**:696–704

Ward, J.D., Cavieres, J.D. 1993. Solubilized $\alpha\beta$ Na,K-ATPase remains protomeric during turnover yet shows apparent negative cooperativity towards ATP. *Proc. Natl. Acad. Sci. USA* **90**:5332–5336

Zvaritch, E., James, P., Vorherr, T., Falchetto, R., Modyanov, N., Carafoli, E. 1990. Mapping of functional domains in the plasma membrane Ca^{2+} pump using trypsin proteolysis. *Biochemistry* **29**:8070–8076