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RATE CONSTANTS FOR CALMODULIN BINDING TO Ca^{2+}-ATPase IN ERYTHROCYTE MEMBRANES

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The Ca^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) in human erythrocyte membranes, which is part of the Ca^{2+} pump, can be activated by binding of calmodulin. Rate constants (k_1) for association of calmodulin and enzyme, which depends on the Ca^{2+} concentration, have been determined by the aid of an enzyme model. k_1 increased from $0.25 \cdot 10^6$ to $17.3 \cdot 10^6$ $M^{-1} \cdot min^{-1}$ (70 times) when the free Ca^{2+} concentration was raised from 0.7 to 20 μM. The binding of calmodulin to the Ca^{2+}-ATPase is reversible. The rate constants (k_{-1}) for dissociation of enzyme-calmodulin complex decreased from 6.0 to 0.044 min^{-1} (135 times) when the free Ca^{2+} concentration was increased from 0.1 to 2–20 μM. The apparent dissociation constant $K_d = k_{-1}/k_1$ accordingly increased from 2.5 nM to 25 μM (or higher) when the Ca^{2+} concentration was reduced from 20 to 0.1 μM. Therefore, at 10^{-7} M free Ca^{2+} most of the Ca^{2+}-pump enzyme will not bind calmodulin. For the intact cell the time dependences of activation and deactivation of the Ca^{2+}-pump enzyme have been estimated from the rate constants above. The results suggest that the Ca^{2+} pump is well suited to maintain a cytosolic concentration of 10^{-7} M free Ca^{2+} (or lower) in the unstimulated cell and, when the cell is stimulated, to allow transient Ca^{2+} signals up to approx. 10^{-5} M in the cytosol.

Introduction

The Ca^{2+} pump in the erythrocyte plasma membranes depends on Ca^{2+} and calmodulin (for reviews, see Refs. 1,2). It has recently been shown that in the presence of calmodulin the Ca^{2+} pump exhibits high Ca^{2+}-affinity (apparent K_d about 1 μM), high maximum activity (optimum at 10–50 μM Ca^{2+}), and inhibition at higher Ca^{2+} concentrations, whereas in the absence of calmodulin the Ca^{2+} affinity, the maximum activity, and the Ca^{2+} inhibition of the Ca^{2+} pump are all reduced [3–5].

The enzymatic basis for the Ca^{2+} pump is a Ca^{2+}-stimulated ATPase in erythrocytes (for reviews, see Refs. 1,2,6). Possibly, this enzyme, like other calmodulin-dependent enzymes, may assume two different conformations, either an open and relaxed form with high activity, sustained by Ca^{2+}-dependent binding of calmodulin, or a closed and constrained form with low activity that is characteristic for the calmodulin-dissociated enzyme [7].

It has been shown recently that calmodulin dissociates from the Ca^{2+}-ATPase in the range of 10^{-7}–10^{-6} M Ca^{2+}, even in the presence of physiological concentrations of calmodulin (3–5 μM) [8]. Apparently, the intracellular concentration of ionized calcium in unstimulated cells, including erythrocytes, is in the range of 10^{-8} to 10^{-7} M [9–11]. Therefore, the Ca^{2+} pump probably exists in the low-activity state (ground state or A-state) in the resting cell and, consequently, a shift of the pump to the activated state (B-state) requires that the intracellular Ca^{2+} concentration rises [12], for instance caused by increased physiological shear

stress of the erythrocytes [13] or by addition of the divalent cation ionophore A23187 [14].

Previously, it has been demonstrated that the activation of Ca^{2+}-ATPase due to calmodulin-binding proceeds very slowly at low concentrations of Ca^{2+} or calmodulin [12,15–17]. The deactivation caused by calmodulin-dissociation also proceeds slowly but accelerates strongly when the Ca^{2+} concentration is reduced from 10^{-6} to 10^{-7} or 10^{-8} M [18].

We have now determined the rate constants for the calmodulin-dependent activation and deactivation of the erythrocyte Ca^{2+}-ATPase at different Ca^{2+} concentrations, in order to estimate the time dependence of a shift between the two pump states in the intact cell. To make sure that the observed time-phenomena are not artifacts caused by reduced access of calmodulin or Ca^{2+} to the membrane-bound enzyme [19], the rate constants were determined both in the absence and presence of either saponin or A23187.

Methods

Preparation of erythrocyte membranes. Two types of membranes were prepared [8].

A-membranes. The erythrocytes were hemolyzed with 9 vol. of a solution containing 6.7 mM sodium phosphate and 1.0 mM EGTA, pH 7.4.

B-membranes. The erythrocytes were hemolyzed with a solution containing 6.7 mM sodium phosphate and a calcium-nitrilotriacetic acid buffer, pH 7.4. The concentration of ionized calcium was about 30 μM.

The hemolysis was succeeded by two washings with Tris-HCl buffer and the membranes were stored at −25°C.

Saponin and A23187 pretreated membranes. Membranes (3.5 mg protein/ml) were incubated with saponin (Calbiochem, 100 μg/ml) or the divalent cation ionophore A23187 (35 μg/ml) in 70 mM Tris-HCl, pH 7.6 for 15 min at room temperature.

ATPase assay. The ATPase activity was measured at 37°C as ADP production which was linked to oxidation of NADH and monitored continuously at 366 nm. The content of the assay medium was as described in Ref. 8, except that only EGTA was used as the calcium-chelating agent in the calcium buffers.

Determination of ATPase parameters. Mg^{2+}-stimulated ATPase was assayed with 1 mM EGTA and no addition of calcium. Ca^{2+}-ATPase activity was determined as the difference between (Ca^{2+} + Mg^{2+})-dependent and Mg^{2+}-stimulated activity, expressed as μmol · min^{-1} per g protein. Calmodulin-dependent Ca^{2+}-ATPase activity (Δv) was determined as the difference between the activity in the presence of calmodulin (v) and the activity of A-membranes (v_A).

Values of maximum activity of calmodulin-dependent Ca^{2+}-ATPase (ΔV) were determined from double reciprocal plots, depicting Δv versus total calmodulin concentration (Z_t). Values of Z_t required for half-maximum activation ($K_{0.5}$) were determined from Hill plots, i.e., $\log(\Delta v/(\Delta V - \Delta v))$ vs. $\log Z_t$. The apparent dissociation constant (K_d) for the enzyme-calmodulin complex was calculated from $K_d = K_{0.5} - 0.5 \cdot E_t$ (see Ref. 8). The enzyme concentration (E_t) was determined to be 5 nmol/l assay medium (10 nmol/g membrane protein) [8].

The rate constants for the binding of calmodulin to the Ca^{2+}-ATPase were calculated as described in the Appendix.

The purification of calmodulin from bovine brain and the determinations of protein and ionized calcium were as described in Ref. 8.

Results and discussion

Enzyme model

As previously suggested, a shift between two states (A and B) of the erythrocyte Ca^{2+}-ATPase, dependent on calcium and calmodulin, may be described by a two-step model [8,20]:

$$i\,Ca^{2+} + Z \rightleftarrows Ca_iZ^* \quad (1)$$

$$E_A + Ca_iZ^* \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E_B(Ca_iZ^*) \quad (2)$$

where Z refers to calmodulin, Ca_iZ^* to calmodulin that is activated by binding of Ca^{2+} (i mol/mol). E_A and E_B represent the Ca^{2+}-ATPase in A-state, characterized by low maximum activity (V), low sensitivities to Ca^{2+} and monovalent cations, low K_m for ATP, and B-state that shows

high V, high sensitivities to Ca^{2+} and monovalent cations, and higher K_m than A-state [7,12].

k_1 and k_{-1} are the rate constants for calmodulin-dependent activation and deactivation of the Ca^{2+}-ATPase. In the present work, the rate constants for the reactions in Eqn. 1 were disregarded, since they seem to be much higher [21] than k_1 and k_{-1}.

According to Eqn. 2, the activation of Ca^{2+}-ATPase by calcium-calmodulin complex is a second order reaction whereas the deactivation can be characterized as a first order reaction. However, the reversibility of the reaction complicates the kinetics of the shift between the two enzyme states, and derivation of the equations for determination of k_1 and k_{-1} is shown in the Appendix.

Accessibility of membrane-bound Ca^{2+}-ATPase

The calmodulin-deficient A-state can be studied in the so-called A-membranes that are prepared in the presence of EGTA, whereas the calmodulin-saturated B-state is found in the B-membranes that are prepared in the presence of Ca^{2+} (see Methods).

In order to make sure that impermeability or resealing during assay of A-membranes or B-membranes did not disturb the determination of the rate constants k_1 and k_{-1}, we examined the time

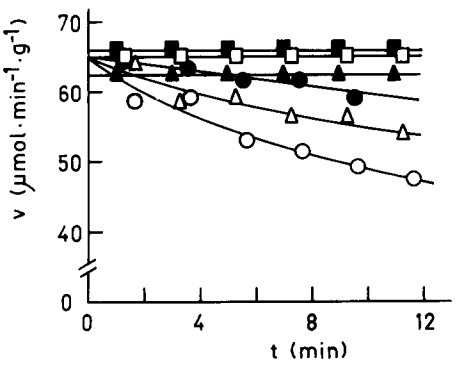

Fig. 1. Ca^{2+}-ATPase activity (v) of pretreated calmodulin-associated erythrocyte membranes dependent on time (t). Pretreatments: saponin (■, □), A23187 (▲, △), none (●, ○). Black and white points refer to calmodulin-associated A-membranes and B-membranes, respectively. The A-membranes were preincubated with 140 nM purified calmodulin and 1.6 mM Ca^{2+} for 10 min in order to obtain calmodulin-associated Ca^{2+}-ATPase. Assayed in the presence of 21 μM free Ca^{2+} and 86 nM purified calmodulin. Single experiment.

dependence of the Ca^{2+}-ATPase activity in different membrane preparations. The membrane preparations were assayed in the presence of 21 μM free Ca^{2+} and 86 nM calmodulin. These conditions should ensure that no calmodulin dissociated from the enzyme during assay, i.e., enzyme activity should be constant. Prior to assay, the A-membranes were preincubated with Ca^{2+} and calmodulin in order to convert the enzyme to B-state.

Fig. 1 shows that the B-state Ca^{2+}-ATPase activity of both calmodulin-associated A-membranes and B-membranes declined during assay. Pretreatment of the membranes (A and B) with saponin prevented the decline of activity, whereas pretreatment with the divalent cation ionophore A23187 ensured constant activity of the A-membranes but did not prevent reduction of the B-membrane activity.

These results suggest that the membranes resealed to some extent in the presence of high Ca^{2+} concentration (21 μM), and that the effect of saponin, at these conditions, was to make the membranes more permeable to calmodulin and calcium (cf. Ref. 19). However, the results do not exclude that saponin, in addition, influenced the enzyme conformation, as it has been shown that the conformation of Ca^{2+}-ATPase is strongly dependent on lipids [22].

The possible resealing during assay is remarkable, since the membranes have been frozen/thawed and exposed to shear stress during preparation (continuous-flow centrifugation). This combined treatment has been shown to be necessary and sufficient to ensure the accessibility of the membrane-bound Ca^{2+}-ATPase at high Ca^{2+} concentrations [23]. However, in earlier investigations we were only interested in the initial enzyme activity and, as it appears from Fig. 1, the initial activity was high, in spite of the decreasing activity during assay.

Furthermore, Fig. 1 shows that the decrease of enzyme activity was higher in the B-membranes than in the A-membranes. This difference could be due to a higher content of spectrin in the B-membranes which were prepared in the presence of Ca^{2+}, since it has been shown that the loss of resealing ability is associated with the release of spectrin and that the presence of Ca^{2+} prevents the loss of spectrin [24].

As a consequence of the results above, we decided to determine the rate constants by using either A-membranes, untreated or pretreated with saponin or A23187, or, when necessary, B-membranes pretreated with saponin.

Calmodulin activation. K_d and ΔV

The maximum Ca^{2+}-ATPase activity dependent on calmodulin (ΔV) and the calmodulin concentration ($K_{0.5}$) required for half maximum activation were determined at four Ca^{2+} concentrations for non-pretreated, A23187-pretreated and saponin-pretreated membranes. The determinations were made from results like those in Fig. 2A by the aid of a double-reciprocal plot (ΔV) and a Hill plot ($K_{0.5}$), and the apparent dissociation constant of the enzyme-calmodulin complex (K_d) was calculated from $K_{0.5}$ as described in Methods.

In accordance with our previous experiments [8], $K_{0.5}$ increased drastically when the concentra-

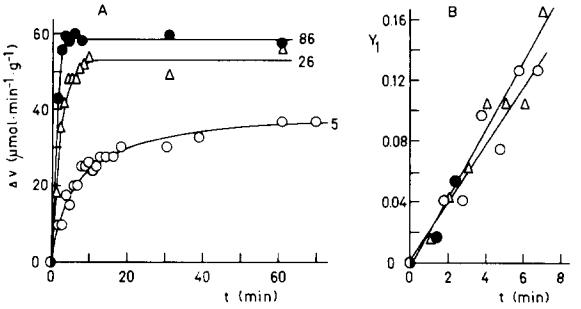

Fig. 3. (A) Calmodulin-dependent Ca^{2+}-ATPase activity (Δv) vs. time (t), assayed at 21.0 μM free Ca^{2+} and different calmodulin concentrations (5.2, 26, 86 nM). Single experiment with saponin-pretreated A-membranes. v_A was 12.4 μmol·min^{-1}·g^{-1}.
(B) Y_1 vs. time (t). The Y_1 values were calculated by Eqn. 6 in Appendix from the Δv values in Fig. 3A (same symbols), using $\Delta V = 58.2$ μmol·min^{-1}·g^{-1} and $K_d = 2.9$ nM, determined from an experiment like that in Fig. 2A and calculated as described in Methods. The slopes of the regression lines (only two are shown), indicating the rate constant k_1, were not significantly different, and therefore $k_1 = (20.8 \pm 0.8) \cdot 10^6$ M^{-1}·min^{-1} and $k_{-1} = K_d \cdot k_1 = 0.061 \pm 0.002$ min^{-1} (S.E., $n = 3$).

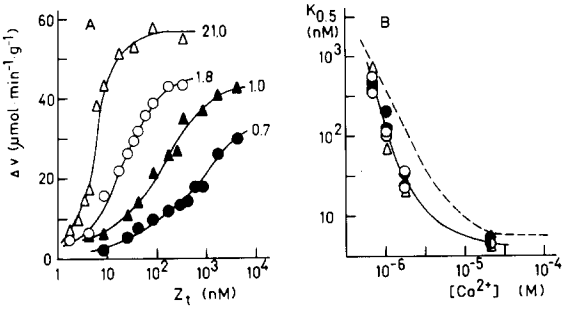

Fig. 2. (A) Calmodulin-dependent Ca^{2+}-ATPase activity (Δv) vs. total calmodulin concentration (Z_t) at various free Ca^{2+} concentrations during assay. Single experiment with A23187-pretreated A-membranes which were preincubated with calmodulin in the assay medium without ATP for 60 min at 37°C in order to obtain equilibrium. The reaction was started by addition of ATP. Four concentrations of free Ca^{2+} were used (0.7, 1.0, 1.8 and 21.0 μM) and the activities without addition of calmodulin (v_A) were 0.2, 1.1, 2.8 and 9.9 μmol·min^{-1}·g^{-1}, respectively.
(B) $K_{0.5}$ values vs. free Ca^{2+} concentration. The $K_{0.5}$ values were calculated (cf. Methods) from experiments as that in Fig. 2A, using membranes with different pretreatments: saponin (●), A23187 (△), none (○). There were no significant effects of pretreatment. The mean ±S.E. ($n = 6$) of $K_{0.5}$ (nM) at the four Ca^{2+} concentrations were: 0.7 μM, 473±53; 1.0 μM, 120±19; 1.8 μM, 27±3; 21.0 μM, 4.7±0.3. The dashed curve was that obtained previously by us [8].

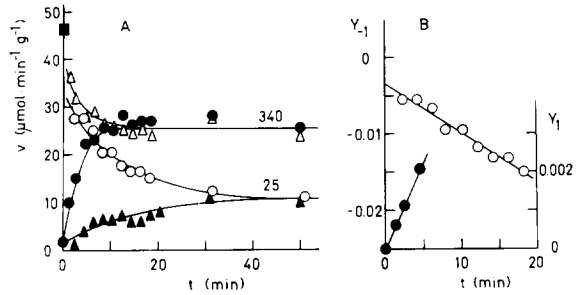

Fig. 4. (A) Ca^{2+}-ATPase activity (v) vs. time (t), assayed at 1.0 μM free Ca^{2+} and different calmodulin concentrations (25 and 340 nM). Single experiment with A23187-pretreated A-membranes which were either calmodulin-deficient (▲, ●) or calmodulin-associated (△, ○) at zero time. The maximum activity (V) determined from an experiment like that in Fig. 2A is shown (■). The calmodulin-associated membranes were preincubated with calmodulin (40 and 550 nM) at 1.4 mM Ca^{2+} at 37°C for 10 min.
(B) Y_1 and Y_{-1} vs. time (t). The Y_1 and Y_{-1} values were calculated by Eqns. 6 and 7 in Appendix using the Δv-values obtained from Fig. 4A (same symbols) and using $\Delta V = 44.4$ μmol·min^{-1}·g^{-1} and $K_d = 106$ nM. From the slopes, the rate constant k_1 was determined to be $0.45 \cdot 10^6$ (●) and $0.64 \cdot 10^6$ M^{-1}·min^{-1} (○), and then $k_{-1} = K_d \cdot k_1$ amounted to 0.047 and 0.067 min^{-1}. Y_1 and Y_{-1} values from the two remaining curves (▲, △) in Fig. 4A could not be calculated because the activities are too close to the equilibrium values.

tion of free Ca^{2+} was decreased from 21 to 0.7 μM (Fig. 2B).

The values of $K_{0.5}$ are somewhat lower than those found by us previously (see Fig. 2B), mainly because the membranes were preincubated with calmodulin for 60 min, instead of 10–20 min, before ATPase assay. The prolonged time of preincubation was necessary to obtain equilibrium of the reaction shown in Eqn. 2, especially at the lower concentrations of Ca^{2+} and calmodulin (see Figs. 3A and 4A).

No significant effects on $K_{0.5}$ of the pretreatment of membranes with the divalent cation ionophore A23187 or with saponin could be detected (Fig. 2B). However, the maximum activity (ΔV) of the membrane-bound Ca^{2+}-ATPase was increased slightly by the saponin-pretreatment, i.e. 9.0 ± 3.5% (S.E., $n = 7$, $P < 0.05$), whereas the A23187-pretreatment showed no significant effect on ΔV.

Time dependence of calmodulin binding at high Ca^{2+} concentration

When calmodulin-deficient erythrocyte membranes (A-membranes) are assayed in the presence of Ca^{2+} and appropriately low concentrations of calmodulin, curves depicting ADP-production vs. time are bending upwards (see Ref. 12), i.e. enzyme activity increases with time, as a result of the binding of calmodulin during the assay.

Fig. 3A shows the time dependence of enzyme activity, assayed at 21 μM Ca^{2+}. It appears that equilibrium was obtained within few minutes at the highest concentration of calmodulin (86 nM) but not until 60 min after start of assay at the low concentration (5 nM). The reaction is remarkably slow, considering the relatively high Ca^{2+} concentration. Similar curves have been demonstrated by Gietzen et al. [17].

The initial parts of the curves from Fig. 3A can be transformed into straight lines, being nearly identical (Fig. 3B), by insertion of enzyme activity vs. time into Eqn. 6 (see Appendix). As expected from the enzyme model (Eqn. 2), the rate constants, calculated from the slopes of the regression lines, were not significantly different at the different calmodulin concentrations.

The curves in Fig. 3A and B were obtained by assaying saponin-treated A-membranes. Similar curves were obtained with untreated or A23187-treated membranes (not shown). However, the rate constants were 2–3-times lower than those of saponin-treated membranes (see Table I), probably due to reduced accessibility of the membrane-bound Ca^{2+}-ATPase at the high Ca^{2+} concentration.

TABLE I

RATE CONSTANTS FOR CALMODULIN BINDING TO Ca^{2+}-ATPase AT HIGH Ca^{2+} CONCENTRATION

The rate constants are shown as mean ± S.E. (n) and the effects of the pretreatments were tested by Student's t-test. The enzyme (A-membranes) was assayed at 5–86 nM calmodulin and a free Ca^{2+} concentration of 21.0 ± 2.0 μM (S.E., $n = 4$), measured with Ca^{2+} electrode.

Pre-treatment of membranes	$k_1 (\times 10^{-6})$ ($M^{-1} \cdot min^{-1}$)	k_{-1} (min^{-1})	Significance
None	8.48 ± 0.74 (6)	0.020 ± 0.002 (6)	–
A23187	6.89 ± 0.22 (6)	0.015 ± 0.002 (6)	$P > 0.05$
Saponin	17.34 ± 2.18 (5)	0.046 ± 0.009 (5)	$P < 0.02$

The apparent lack of time dependence of calmodulin activation of saponin-treated erythrocyte Ca^{2+}-ATPase, reported by Downes et al. [19], was probably due to the high concentration of calmodulin (280 nM) used by these authors.

Recently, Wang et al. [25] found that the rate constants for the calmodulin-activated reaction of cyclic nucleotide phosphodiesterase from bovine brain at a saturating Ca^{2+} concentration amounted to approximately $k_1 = 4 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and $k_{-1} = 3 \cdot 10^{-3}\ s^{-1}$. According to Table I (saponin-pretreated membranes), these values are 13- and 4-times higher than the corresponding rate constants for the Ca^{2+}-ATPase reaction ($k_1 = 0.3 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and $k_{-1} = 0.8 \cdot 10^{-3}\ s^{-1}$). The different rate constants may be due to the uses of purified phosphodiesterase contrary to membrane-bound ATPase but may also reflect functional differences between the two cellular enzymes (cf. later).

Time dependence of calmodulin binding at low Ca^{2+} concentrations

Contrary to the results in Table I, obtained with 21 μM free Ca^{2+} during assay, the rate constants determined at lower Ca^{2+} concentrations (Table

TABLE II

RATE CONSTANTS FOR CALMODULIN BINDING TO Ca^{2+}-ATPase AT DIFFERENT Ca^{2+} CONCENTRATIONS

The rate constants are shown as mean ± S.E. (n) of all experiments with non-pretreated A-membranes and A-membranes pretreated with A23187 or saponin because no significant effects of pretreatments were found at these Ca^{2+} concentrations. The free Ca^{2+} concentrations are shown as mean ± S.E. ($n=4$). The calmodulin concentration was 25–860 nM. The differences between A→B and B→A, referring to association and dissociation experiments, were not significant when tested by Student's t-test.

Ca^{2+} concn. (μM)	Type of experiment	$k_1(\times 10^{-6})$ ($M^{-1}\cdot min^{-1}$)	k_{-1} (min^{-1})
0.72	A→B	0.27±0.02 (4)	0.121±0.015 (4)
±0.06	B→A	0.24±0.03 (8)	0.108±0.014 (8)
1.04	A→B	0.55±0.08 (8)	0.060±0.007 (8)
±0.08	B→A	0.54±0.04 (7)	0.060±0.008 (7)
1.78	A→B	1.52±0.08 (14)	0.035±0.004 (14)
±0.12	B→A	2.59±0.90 (8)	0.051±0.013 (8)

II) were not affected by the pretreatment of the A-membranes with A23187 or saponin (not shown). This suggests that the accessibility phenomena described above may be more significant at high Ca^{2+} concentrations, in agreement with an earlier investigation [23].

When the Ca^{2+} concentration during assay was only 10^{-6} M, the Ca^{2+}-ATPase activity of calmodulin-associated membranes decreased with time to a level of equilibrium that was dependent on the concentration of added calmodulin (Fig. 4A).

Conversely, the activity of calmodulin-deficient membranes increased with time, and the activity attained the same levels of equilibrium as the calmodulin-associated membranes (Fig. 4A). The equilibriums were attained in 10 and 40 min at 340 and 25 nM calmodulin, respectively.

Transformation of the initial parts of the curves into straight lines (Fig. 4B) by use of Eqns. 6 and 7 (see Appendix) allows calculation of k_1 and k_{-1}. The rate constants derived from the curves that depict shift of B to A state did not deviate significantly from those determined from the curves which depict the opposite shift, i.e. from A to B state, neither at this Ca^{2+} concentration nor in similar experiments conducted at other Ca^{2+} concentrations (Table II).

The initial Ca^{2+}-ATPase activity of the calmodulin-associated membranes in Fig. 4A was lower than the maximum activity of calmodulin-saturated membranes (see Fig. 4A), which indicates that the preincubation of the A-membranes in the presence of calmodulin and Ca^{2+} has not been sufficient to obtain calmodulin-saturation of the enzyme. Therefore, the interception on the vertical axis of the Y_{-1} curve in Fig. 4B deviates from zero. This phenomenon has no consequences for the determination of k_1 and k_{-1} (see Appendix).

The accordance between the rate constants calculated from the shifts of enzyme state in both directions (see Eqn. 2) supports the enzyme model used. The interaction of the enzyme with an equimolar amount of calmodulin has recently been confirmed by Graf and Penniston [26] who used pure, solubilized Ca^{2+}-ATPase from human erythrocyte membranes. However, their and our results do not exclude that m mol calmodulin react with m subunits of an oligomeric Ca^{2+}-ATPase [26,8]. The mathematical treatment of the results in the present investigation does not account for the possibility of positive and negative cooperativity between enzyme subunits by binding of calmodulin, a possibility suggested by Gietzen et al. [17]. Further experiments are needed to test this possibility.

The technique used above was not suitable for deactivation experiments showing dissociation of calmodulin at Ca^{2+} concentrations lower than 0.5 μM. Since the intracellular Ca^{2+} concentration in human erythrocytes is approx. 0.1 μM or lower [9,11] we adopted another technique [18] for the determination of k_{-1} at Ca^{2+} concentrations ranging from 0.1 to 0.5 μM.

The technique took advantage of the slow recombination of enzyme and low concentrations of calmodulin at 1 μM free Ca^{2+}. For these experiments we used the calmodulin-saturated B-membranes which were preincubated for various time periods at a given concentration of free Ca^{2+}. During the preincubation part of the calmodulin was released from the B-membranes, shifting the enzyme partly to the A-state. At the end of the preincubation the free Ca^{2+} concentration was

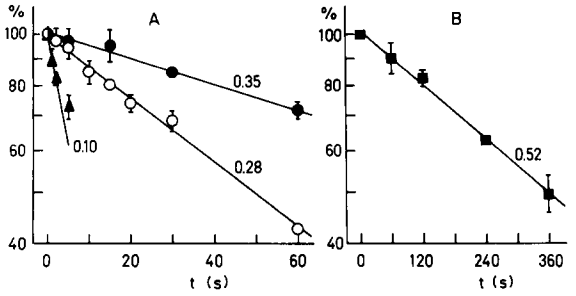

Fig. 5. Relative Ca^{2+}-ATPase activity (%) of preincubated B-membranes vs. time (t) of preincubation. The preincubations were conducted at different free Ca^{2+} concentrations (0.10, 0.28, 0.35 and 0.52 μM). Mean ± S.E. of three experiments are given. The preincubation was started by addition of saponin-pretreated B-membranes (300 μl suspension containing approx. 3.5 mg/ml protein) to the rest of the preincubation medium (1800 μl) that contained Ca^{2+}-EGTA buffer with the shown free Ca^{2+} concentration (0.1–0.5 μM). After the preincubation period (1–360 s), the assay of ATPase activity was started by addition of extra 100 μl $CaCl_2$ solution which raised the free Ca^{2+} concentration to 1 μM. The initial calmodulin-dependent Ca^{2+}-ATPase activity of the preincubated membranes was related to the initial activity ($\Delta v = 39.1 \pm 0.9$ μmol · min^{-1} · g^{-1}, S.E., $n = 24$) of non-preincubated saponin-pretreated B-membranes, also assayed at 1 μM free Ca^{2+}. The slopes of the linear regressions represent the rate constant k_{-1} (see Table III), as the re-binding of released calmodulin can be neglected.

raised by addition of $CaCl_2$ solution, and the initial ATPase activity was assayed at the resulting concentration of free Ca^{2+} (1 μM) before the released calmodulin (maximally 10–15 nmol/l of assay medium) reassociated with the enzyme. The reduced initial ATPase activity was related to the activity of non-preincubated B-membranes, and the relative activity then reflects the shift from B to A state (see Fig. 5). The resulting curves in Fig. 5 followed first order kinetics, as expected from Eqn. 2 above, and the k_{-1} values (see Table III) were then obtained from the slopes of the linear regressions. Since determinations of $K_{0.5}$ and K_d (cf. Fig. 2A) were not practicable at 0.1–0.5 μM Ca^{2+}, it was not possible to calculate k_1 from these experiments.

Ca^{2+} dependence of rate constants

It appears from Tables I (saponin-pretreated membranes) and II that the rate constant for association (k_1) of enzyme and calmodulin increased from $0.25 \cdot 10^6$ to $17.3 \cdot 10^6$ M^{-1} · min^{-1}, i.e. 70 times, when the Ca^{2+} concentration was increased from 0.7 to 21 μM. On the contrary, Tables I, II and III show that the rate constant for dissociation (k_{-1}) decreased from 6.0 to 0.044 min^{-1}, i.e. 135 times, when the Ca^{2+} concentration was increased from 0.1 μM to the range of 2–20 μM (see also Fig. 6).

TABLE III

RATE CONSTANTS AND HALF TIMES FOR CALMODULIN DISSOCIATION FROM Ca^{2+}-ATPase AT LOW Ca^{2+} CONCENTRATIONS

The results are shown as mean ± S.E. ($n = 3$). Calmodulin-saturated membranes (B-membranes), pretreated with saponin, were used for the experiments. The unit of half times ($t_{0.5}$) is seconds, in order to facilitate the comparison with Table IV. For experimental procedure, see Fig. 5.

Ca^{2+} concn. (μM)	k_{-1} (min^{-1})	$t_{0.5}$ (s)
0.10 [a]	6.0 ±0.9	7± 1
0.28±0.03	0.865±0.006	48± 1
0.35±0.03	0.339±0.021	124± 8
0.52±0.07	0.117±0.010	360±28

[a] Calculated as described previously [27].

The Ca^{2+} dependence of the rate constants implies that the equilibrium constant $K_d = k_{-1}/k_1$ decreased from approx. 25 μM to 2.5 nM, i.e. 10^4 times, when the Ca^{2+} concentration was increased from 0.1 to 20 μM. The K_d-value at 0.1 μM Ca^{2+} may be higher than 25 μM if k_1 is lower than the value of $0.25 \cdot 10^6$ M^{-1} · min^{-1} which was determined at 0.7 μM Ca^{2+}.

From Ca^{2+}-binding studies [25,28] it appears that at 10^{-7} M Ca^{2+}, in the presence of Mg^{2+}, 98–99% of total calmodulin does not bind Ca^{2+}. Therefore, the apparent K_d value (25 μM or higher) at 0.1 μM Ca^{2+} indicates that the dissociation constant for the calcium-free enzyme-calmodulin complex (E_BZ^*, see Eqn. 2) is at least 25 μM. On the contrary, the K_d value of 2.5 nM at 20 μM Ca^{2+} indicates much lower dissociation constants for the complexes between Ca^{2+}-calmodulin and enzyme ($E_B(Ca_iZ^*)$, especially for i values of 3 and 4). Analogously, Wang et al. [25] found that the corresponding K_d values for the calmodulin-dependent phosphodiesterase also differ by a factor of at least 10^4 times.

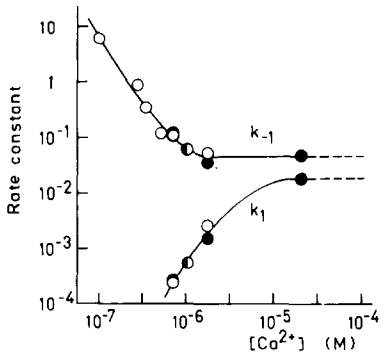

Fig. 6. Rate constants for association (k_1) and dissociation (k_{-1}) of calmodulin dependent on free Ca^{2+} concentration. The points represent mean ± S.E. of the experiments shown in Tables I (saponin-pretreated membranes), II and III. The units of ordinate are $nM^{-1} \cdot min^{-1}$ (k_1) and min^{-1} (k_{-1}). ●, activation experiment (A- to B-state shift); ○, dissociation experiment (B- to A-state shift).

As pointed out [25], this great effect of Ca^{2+} on the calmodulin-affinity is tantamount to a 10-fold increase of the Ca^{2+}-affinities, in average, of the four binding sites when calmodulin binds to the enzyme, and this seems to be a general effect on calmodulin that binds to another protein. For instance, Keller et al. [29] found that the binding of troponin I to calmodulin increased the apparent Ca^{2+} affinity about eight times.

The overall association of calmodulin to enzyme, characterized by the rate constant k_1, depends on the binding of the five different Ca_iZ^*-species ($0 \leq i \leq 4$), whereas the overall dissociation, characterized by k_{-1}, depends on the five $E_B(Ca_iZ^*)$ species (see Eqn. 2). The distribution of the different Ca^{2+}-calmodulin species depends on the Ca^{2+} concentration and the affinities of the Ca^{2+}-binding sites. If the Ca^{2+} affinities of the free calmodulin are lower than those of the calmodulin-enzyme complexes (cf. above), the distribution curves of the Ca_iZ^*-species will be displaced towards higher Ca^{2+} concentrations compared to those of the $E_B(Ca_iZ^*)$ species. This difference may be reflected in the Ca^{2+}-dependence of k_1 and k_{-1}.

Fig. 6 accordingly shows that the rate constant for association (k_1) increased in a range of Ca^{2+} concentration (10^{-6}–10^{-5} M) which is 10-times higher than the range (10^{-7}–10^{-6} M) in which the rate constant for dissociation (k_{-1}) increased. k_{-1} was nearly constant at Ca^{2+} concentrations higher than 10^{-6} M, and k_1 is probably near its maximum at 10^{-5} M Ca^{2+} because it has been shown [8] that $K_d = k_{-1}/k_1$ is nearly constant in the range of 10^{-5}–10^{-4} M Ca^{2+}.

Activation and deactivation of Ca^{2+}-pump enzyme in cells

The different Ca^{2+} dependences of the rate constants k_1 and k_{-1} (Fig. 6) suggest that the Ca^{2+}-pump enzyme in vivo would respond to an increased Ca^{2+}-influx (cf. Introduction) into the cell by binding calmodulin at first slowly, later on rapidly as the concentration of free Ca^{2+} is increasing to approx. 10^{-5} M. Possibly, due to the high capacity of the maximally activated Ca^{2+} pump, the pump-mediated Ca^{2+} efflux then exceeds the elevated Ca^{2+} influx, and the course of the k_{-1} curve in Fig. 6 now suggests that the Ca^{2+} pump remains in the active B-state because of the slow dissociation of calmodulin, until the Ca^{2+} concentration is decreased to 10^{-7}–10^{-6} M.

According to this picture, the Ca^{2+} pump tends towards working as an all-or-none device, ensuring a Ca^{2+} concentration of 10^{-7} M or lower in the resting cell and allowing transient Ca^{2+} signals in the cytoplasm with amplitudes of approx. 10^{-5} M Ca^{2+}. The duration of transient Ca^{2+} signals can be estimated from the rate constants for the calmodulin-binding in the Ca^{2+}-ATPase reaction, by assuming that the rate constants are also valid for the Ca^{2+} pump in the intact cell.

In human erythrocytes the calmodulin concentration seems to be approx. 4 μmol/l cells [8,30,31]. However, the concentration of Ca^{2+}-calmodulin available for the Ca^{2+} pump may be less because spectrin [32] or other erythrocyte proteins [30,33,34] binds calmodulin in the presence of Ca^{2+}.

If the available calmodulin concentration amounts to 2 μmol/l cells, Table IV shows that the estimated time needed for half maximum activation (50% shift in state, A to B) of the Ca^{2+}-pump enzyme in erythrocytes decreases from 95 to 1.2 seconds when the cellular concentration of free Ca^{2+} increases from 0.7 to 20 μM. The shift in state is important for the activation of the Ca^{2+}-ATPase because, at 10^{-5} M free Ca^{2+}, the activity of the A-state is only 10% of that of the B-state [8].

The half times for dissociation of calmodulin

TABLE IV

ESTIMATES OF TIME PERIODS REQUIRED FOR 50% STATE SHIFT (A TO B) OF Ca^{2+}-PUMP ENZYME IN ERYTHROCYTES

The time values were calculated from Eqn. 6 (see Appendix) at various concentrations of free Ca^{2+} and calmodulin. The enzyme concentration was assumed to be 70 nmol/l cells. The k_1-values used for calculation were taken from Table I (saponin-pretreated membranes) and Table II, and the K_d values were obtained from the experiments in Fig. 2B.

Ca^{2+} concn. (μM)	Time period (s) Calmodulin concentration (μM)		
	1	2	4
0.7	227	95	33
1.0	82	39	19
1.8	21	11	5
21	2.5	1.2	0.6

from the activated Ca^{2+}-pump enzyme appear from Table III. For instance, the half time is approx. 7 s when the concentration of free Ca^{2+} is 10^{-7} M. At 10^{-8} M free Ca^{2+} the half time seems to be even lower [18]. Possibly the half times in erythrocytes are higher than those in Table III, especially at the highest Ca^{2+} concentrations, because re-binding of calmodulin may occur at the cellular calmodulin concentration which is much higher than that in the experiments from Table III. According to this, the shift from B to A state is a relatively slow reaction, even at 10^{-7} M free Ca^{2+}. However, apart from the shift in state, the activity of the Ca^{2+}-pump enzyme decreases strongly when the Ca^{2+} concentration approaches 10^{-7} M [8].

Experiments with intact cells are needed to test the validity of the results above for the Ca^{2+} pump. Recently, Vestergaard-Bogind and Bennekov [35] demonstrated repeated transient activations (oscillations with periods of 3–4 min) of the Ca^{2+}-sensitive potassium channel in human erythrocytes, the Ca^{2+}-influx being mediated by A23187. The oscillations only occurred in the presence of ATP, and analysis of the oscillating parameters indicated that activation and deactivation of the Ca^{2+} pump took place with marked delays.

As mentioned above, the rate constants for the calmodulin-binding in the phosphodiesterase reaction have been reported [25] to be higher than the rate constants in the Ca^{2+}-ATPase reaction. If this difference between the rate constants is valid for the two enzymes in different cell types, the phosphodiesterase may generally be able to respond more rapidly to an increase of the cytoplasmic Ca^{2+} concentration than the Ca^{2+}-pump enzyme. This means that the phosphodiesterase reaction, and possibly other enzyme reactions dependent on the presence of Ca^{2+}-calmodulin complexes, may proceed before the level of cytoplasmic Ca^{2+} is reduced by the activated Ca^{2+} pump.

Calmodulin-dependent Ca^{2+}-pump enzymes have been found in plasma membranes from, for instance, brain cortex [36], neurohypophysis [37], pancreatic islet [38], and kidney cortex [39]. The Ca^{2+}-pump enzyme in these cells may have rate constants for the calmodulin-dependent activation and deactivation similar to those of the erythrocyte enzyme. However, as shown in Table IV, the rate of activation is also dependent on the free calmodulin concentration which may be higher in the cells mentioned, because the content of total calmodulin in these cells has been reported [40,41] to be from 20 to 40 μmol/kg tissue, i.e. 5–10-times higher than in erythrocytes.

Increases in the cellular content of calmodulin, reported for transformed cells [42], hepatoma cells [43], and cystic fibrosis fibroblasts [44], or changes in the ratio of membrane-bound to soluble calmodulin [42] will probably change the duration of transient Ca^{2+} signals in the cells.

Appendix

A shift between A-state and B-state of Ca^{2+}-ATPase (E) dependent on activated calcium-calmodulin complex (Ca_iZ^*) can be described by Eqn. 2 (see Enzyme model above). The rate constants for association of E_A and Ca_iZ^*, k_1, and dissociation of $E_B(Ca_iZ^*)$, k_{-1}, can be derived from

$$\frac{dx}{dt} = k_1(E_t - x)(Z_t - x) - k_{-1}x \qquad (3)$$

where $x = [E_B(Ca_iZ^*)]$ is the concentration of calmodulin-associated enzyme, dx/dt is the rate of shift from A to B state, E_t and Z_t refer to total

concentrations of enzyme and calmodulin. Rearrangement of Eqn. 3 and insertion of $K_d = k_{-1}/k_1$ lead to

$$\frac{dx}{x^2 - (E_t + Z_t + K_d)x + E_t Z_t} = k_1\, dt \tag{4}$$

which by integration gives

$$\left(\frac{1}{\sqrt{D}} \ln \frac{2x - (E_t + Z_t + K_d) - \sqrt{D}}{2x - (E_t + Z_t + K_d) + \sqrt{D}} \right)_{x_1}^{x_2} = (k_1 t)_0^t \tag{5}$$

where $D = (E_t + Z_t + K_d)^2 - 4E_t Z_t$. When the enzyme shifts from A to B state the time interval (0 to t) corresponds to $x_1 = 0$ and $x_2 = x$, which inserted in Eqn. 5 lead to

$$Y_1 = \frac{1}{\sqrt{D}} \ln \left(\frac{\left(2x - (E_t + Z_t + K_d) - \sqrt{D}\right)}{\left(2x - (E_t + Z_t + K_d) + \sqrt{D}\right)} \times \frac{\left(E_t + Z_t + K_d - \sqrt{D}\right)}{\left(E_t + Z_t + K_d + \sqrt{D}\right)} \right) = k_1 t \tag{6}$$

When the enzyme shifts into the opposite direction, i.e. from B to A state the time interval (0 to t) corresponds to $x_1 = E_t$ and $x_2 = x$, which inserted into Eqn. 5 leads to

$$Y_{-1} = \frac{1}{\sqrt{D}} \ln \left(\frac{\left(2x - (E_t + Z_t + K_d) + \sqrt{D}\right)}{\left(2x - (E_t + Z_t + K_d) - \sqrt{D}\right)} \times \frac{\left(E_t - Z_t - K_d - \sqrt{D}\right)}{\left(E_t - Z_t - K_d + \sqrt{D}\right)} \right) = -k_1 t \tag{7}$$

According to the most simplified enzyme model [8], $\Delta v/\Delta V = x/E_t$. The time-dependent linear functions Y_1 and Y_{-1} can now be calculated from Eqns. 6 and 7 by inserting $x = E_t \cdot \Delta v/\Delta V$. It appears from Eqns. 6 and 7 that the rate constant k_1 is the slope of a straight line both when the enzyme shifts from A to B state (Y_1 vs. t) and in case of shift from B to A state (Y_{-1} vs. t). k_{-1} can be calculated from the dissociation constant (K_d), as $k_{-1} = K_d k_1$ (cf. above).

If the enzyme is not entirely saturated with calmodulin at the start of experiments which show shift of enzyme from B to A state, then, in Eqn.5, zero time ($t = 0$) corresponds to $x_1 = \alpha \cdot E_t$, where $\alpha < 1$. This implies that Eqn. 7 is changed to $Y_{-1} = -k_1 t + C$, where C is a negative constant. This means that, in case of $\alpha < 1$, the intercept of the line Y_{-1} vs. t will be below zero, whereas the slope ($-k_1$) of the line will be unchanged. Therefore, this phenomenon has no consequences for the determination of k_1 and k_{-1}.

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