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LOW Ca^{2+} CONCENTRATIONS CONTROLLING TWO KINETIC STATES OF Ca^{2+}-ATPase FROM HUMAN ERYTHROCYTES

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Summary

The kinetics of Ca^{2+}-activation of (Ca^{2+} + Mg^{2+})-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) from human erythrocytes was influenced by the Ca^{2+} concentration during the membrane preparation.

When pCa (—log[Ca^{2+}]) during hemolysis, pCa_H, was above 7 the ATPase showed both positive and negative cooperativity (A-kinetics) but only positive cooperativity (B-kinetics) at pCa_H below 7.

Decreasing pCa_H from about 10 to 7, the Michaelis constant for Ca^{2+}, K_{Ca}, decreased from 46 μM to 0.8 μM. In the same range of pCa_H the Hill coefficient of Ca^{2+}-activation fluctuated around 1 and increased to 1.8 when pCa_H was decreased from 7 to 6.

High Ca^{2+} concentrations inhibited the ATPase. The optimum Ca^{2+} concentration activating the ATPase was 127 μM at pCa_H above 7 and 28 μM at pCa_H below 7.

These ATPase properties indicate that the Ca^{2+}-ATPase may occur in two different states which differ with respect to Ca^{2+} affinity, cooperativity between the Ca^{2+} sites, and kinetics of inhibition of high Ca^{2+} concentrations. The shift between the two kinetic states is mediated by Ca^{2+}.

When pCa_H was below 6, centrifugation of the membranes in a continuous flow device or treatment with ionophore A 23187 was necessary in order to make the ATPase sites accessible for Ca^{2+} and to obtain B-kinetics.

Introduction

It was shown previously [1] that (Ca^{2+} + Mg^{2+})-dependent ATPase showed positively cooperative behaviour and high affinity with respect to Ca^{2+} when the erythrocyte membranes were prepared in the presence of 0.7—500 μM Ca^{2+} during hemolysis, provided that the ATPase sites were made accessible by mechanical treatment of the membranes. An enzyme model with at least two

calcium-binding sites accounted for the kinetics [1], which is in accordance with the indication of two calcium-translocating sites of the calcium pump in human erythrocytes demonstrated by Ferreira and Lew [2].

When the erythrocyte membranes were prepared in the absence of Ca^{2+}, the (Ca^{2+} + Mg^{2+})-dependent ATPase showed reduced affinity for Ca^{2+} and a complicated kinetics which was fitted by an enzyme model with at least four calcium-binding sites [1].

The aim of the present investigation is to determine the range of Ca^{2+} concentrations in which the type of kinetics shifted. The calcium ionophore A 23187 is used to estimate the Ca^{2+} accessibility of the ATPase as a function of the Ca^{2+} concentration during hemolysis.

Methods

Preparation of erythrocyte membranes. Recently outdated bank blood was washed as described previously [1]. The washed erythroyctes were hemolyzed in 9 vol of a hemolyzing buffer and stored overnight. The ghosts were collected by centrifugation and washed twice in 9 vol of 10 mM Tris · HCl (pH 7.6 at 22°C). The temperature during preparation varied between 4 and 8°C. The membranes were stored at −25°C.

Hemolyzing buffers. By a set of buffers, pCa during hemolysis, pCa_H, was varied in the range of 4—9. The buffers contained 6.7 mM sodium phosphate, 1.0 mM calcium-chelating ligand, 0—0.9 mM $CaCl_2$, pH 7.4 (approx. 25 mOsM) leading to a pH between 7.7 and 7.9 during hemolysis. In the pCa_H range of 6.7—9.0 the calcium-chelating ligand used was ethyleneglycol bis(β-aminoethyl-ether)-*N,N'*-tetraacetic acid (EGTA). In the pCa_H range of 4.0—6.5 nitrilotriacetic acid was used.

Determination of pCa_H. In the range of 4.0—7.4 pCa_H was measured in the hemolysates by a calcium ion selectrode (Radiometer F 2112 Ca). The selectrode was calibrated at 8°C and an ionic strength of 0.03 M with various calcium buffers which were modifications of the buffers used by Růžička et al. [3]. The calibration curve (mV vs. pCa) was a straight line in the pCa range of 2.0—7.4.

In addition, pCa_H was calculated using conditional stability constants (cf. Hansen et al., ref. 4) of the Ca · EGTA and Ca · nitrilotriacetic acid complexes. In the experimental conditions at pH 7.8 the conditional stability constants used were $10^{7.78}$ (Ca · EGTA) and $10^{4.46}$ (Ca · nitrilotriacetic acid) according to calculations (Scharff, O.) not yet published. The deviations between pCa(measured) and pCa(calculated) were in Ca · EGTA buffers −0.04 ± 0.05 (S.E. 22 experiments) and in Ca · nitrilotriacetic acid buffers 0.02 ± 0.02 (S.E. 10 experiments). Considering these insignificant deviations, the calculated values were used in the pCa range of 7.4—9.0.

Determination of ATPase activity. The Mg^{2+}-dependent activity was assayed [1] by measuring P_i liberated at 37°C in a basal medium of 3 mM Tris · ATP, 4 mM $MgCl_2$, 1 mM EGTA, 70 mM Tris · HCl, and 0.3—0.8 g dry membrane per l medium, pH 7.2. The (Ca^{2+} + Mg^{2+})-dependent activity was determined in the basal medium supplemented with $CaCl_2$ to obtain Ca^{2+} concentrations in the range of 0—10^{-6} M. In the range above 10^{-6} M Ca^{2+}, EGTA was replaced by

nitrilotriacetic acid. The Ca^{2+}-stimulated ATPase activity was determined as the difference between (Ca^{2+} + Mg^{2+})-dependent and Mg^{2+}-dependent activity and expressed as μmol · min^{-1} per g dry membrane exclusive of hemoglobin.

Methods of analysis. The determinations of pH, P_i, dry matter, protein, hemoglobin, ATP, calcium (atomic absorption), and Ca^{2+} (ion selectrode) were performed as previously described [1,5,6].

The ionophore A 23187 was a gift from Eli Lilly Co., Copenhagen.

Results

Determination of kinetics

In order to determine the range of Ca^{2+} concentrations in which the type of kinetics shifted, a number of erythrocyte specimens were hemolyzed at different Ca^{2+} concentrations, i.e. pCa_H was varied from about 10 to 3.3.

For every membrane preparation the kinetics of Ca^{2+} activation were determined by varying the Ca^{2+} concentration during the determination of the ATPase activity (cf. Methods). However, these various Ca^{2+} concentrations should not be confused with pCa_H which denotes the Ca^{2+} concentration during the hemolytic step of the membrane preparation in question.

Kinetic parameters. The ATPase activities (v) of every membrane preparation were plotted in a Lineweaver-Burk diagram vs. the Ca^{2+} concentrations during the ATPase determination (cf. Fig. 1). The maximum velocity (V) of the ATPase reaction was determined from the Lineweaver-Burk plot as the interception on the vertical axis. A Hill plot like those in Fig. 2 was made for every

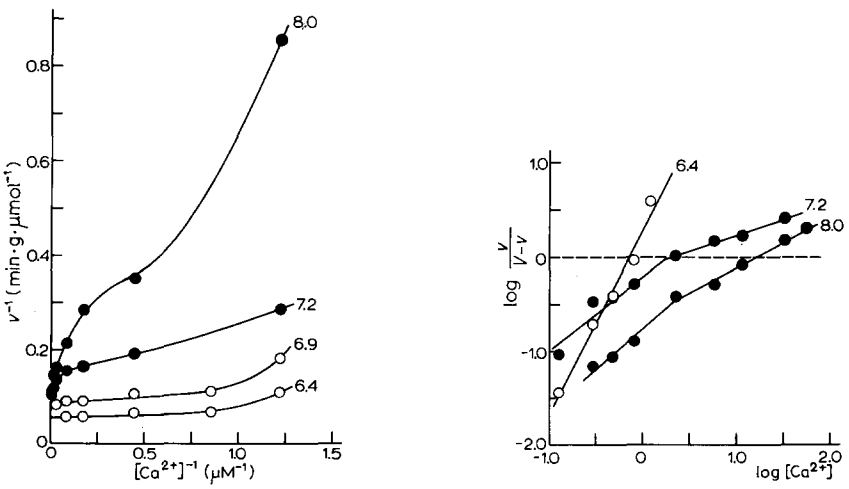

Fig. 1. Lineweaver-Burk plot of Ca^{2+}-stimulated ATPase activity (v) vs. Ca^{2+} concentration during ATPase determination. Four membrane preparations from a single blood specimen hemolyzed at the different pCa values, pCa_H, which mark the curves. Continuous flow centrifugation. ATPase medium, 3 mM Tris · ATP, 4 mM $MgCl_2$, 1 mM EGTA of nitrilotriacetic acid, 70 mM Tris · HCl, 0.3—0.8 g dry membrane per 1 medium, various concentrations of $CaCl_2$, pH 7.2. ●, A-kinetics; ○, B-kinetics (see text).

Fig. 2. Hill plot of three experiments from Fig. 1. $\log(v/(V-v))$ vs. $\log[Ca^{2+}](\mu M)$. ●, A-kinetics; ○, B-kinetics. pCa_H 8.0, K_{Ca} = 15.8 μM, n_H = 0.86. pCa_H 7.2, K_{Ca} = 2.00 μM, n_H = 0.79. pCa_H 6.4, K_{Ca} = 0.70 μM, n_H = 1.97. (Cf. Figs. 3 and 4).

membrane preparation. The maximum slope (n_H) was determined from the steepest regression line of every preparation. The Michaelis constant for Ca^{2+} (K_{Ca}) was determined from the regression line which passes log $(v/(V-v)) = 0$, the abscissa of the intersection point being log K_{Ca}.

A-kinetics. Showing both positive and negative cooperativity, the kinetics of Ca^{2+} activation of membrane bound ATPase were referred to as A-kinetics. This was indicated by a Lineweaver-Burk plot showing two curvatures, one concave up and one concave down (cf. Fig. 1, pCa_H 8.0).

B-kinetics. Showing only positive cooperativity, the kinetics of Ca^{2+} activation were referred to as B-kinetics which were indicated by a Lineweaver-Burk plot with one curvature being concave up (cf. Fig. 1, pCa_H 6.4 and 6.9).

Kinetics dependent on centrifugation. It was shown previously [1] that at pCa_H below 6 the B-kinetics were achieved only when the membranes were subjected to centrifugation in a continuous-flow device which involves a shear stress on the membranes. Using ordinary centrifugation in capped tubes, the Ca^{2+}-ATPase showed only A-kinetics [1].

In order to separate the effect of centrifugation, the present experiments were divided into two series, the first based on membranes prepared by continuous-flow centrifugation (cf. Figs. 1—4), and the second based on membranes centrifuged in capped tubes during preparation (cf. Figs. 5 and 6).

Kinetics of ATPase in membranes prepared by continuous-flow centrifugation

Fig. 1 shows Lineweaver-Burk plots of four different membrane preparations which were exposed to pCa_H 8.0, 7.2, 6,9, and 6.4, respectively. With decreasing pCa_H the kinetics shifted from type A to B. Showing a curvature concave down in the region of $[Ca^{2+}]^{-1}$ below 0.2 μM^{-1}, the membranes prepared at pCa_H 7.2 were grouped with A-kinetics. In this particular blood specimen a small change of pCa_H to 6.9 resulted in B-kinetics. Including preparations from other blood specimens, the shift of kinetics occurred more randomly around pCa_H 7 (cf. Fig. 3).

K_{Ca} dependent on pCa_H. In Fig. 3 log K_{Ca} of all membrane preparations cen-

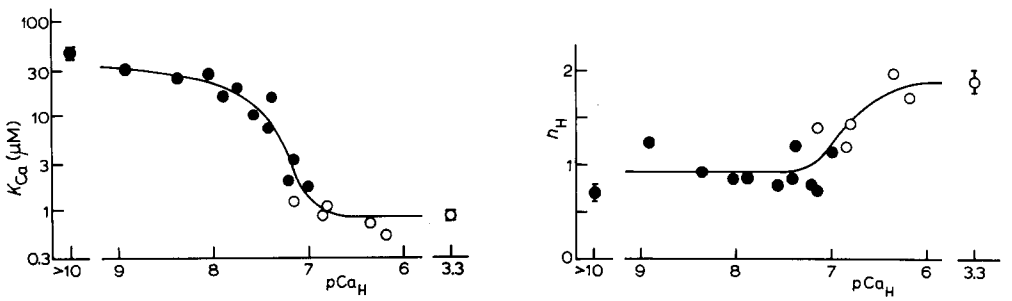

Fig. 3. Dependence of log K_{Ca} on pCa_H. K_{Ca} (μM) refers to the Michaelis constant for Ca^{2+} of the ATPase reaction. $pCa_H = -\log[Ca^{2+}]_H$ where $[Ca^{2+}]_H$ designates the Ca^{2+} concentration (M) during the hemolysis-step of the involved membrane preparations. Single preparations except for two points (±S.E., 8 preparations) representing hemolysis in 1 mM EGTA ($pCa_H > 10$) and in 0.5 mM Ca^{2+} ($pCa_H = 3.3$), respectively (cf. ref. 1). Continuous-flow centrifugation. ●, A-kinetics; ○, B-kinetics.

Fig. 4. Dependence of the Hill coefficient (n_H) of Ca^{2+} activation on pCa_H. Same experiments as in Fig. 3. ●, A-kinetics; ○, B-kinetics.

trifuged in the continuous-flow device are plotted vs. pCa_H. It appears that K_{Ca} decreased with decreasing pCa_H. When pCa_H reached 7 the kinetics shifted from type A to B and K_{Ca} became constant about 1 μM. This value agrees with K_{Ca} obtained by others for both Ca^{2+}-stimulated ATPase and calcium pump when the sites of the transport enzyme were exposed to Ca^{2+} concentrations in the micromolar range during the preparation of ATPase or during the pumping activity [2,7,8].

n_H dependent on pCa_H. In Fig. 4 n_H of all membrane preparations centrifuged in the continuous-flow device are plotted vs. pCa_H. n_H fluctuated around 1 when pCa_H was above 7. When pCa_H decreased below 7, n_H increased, which indicates increasing cooperativity between the Ca^{2+}-activating sites of the ATPase. n_H reached a maximum near two at pCa_H about 6. Assuming the existence of two Ca^{2+}-activating sites per mol ATPase, a value of n_H near two implies full cooperativity between the sites, which is in agreement with the view of Ferreira and Lew [2]. However, Wolf et al. [9] found that n_H was only 1.25. This discrepancy may be due to differences in membrane preparation. For instance, n_H is higher in membranes prepared by continuous-flow centrifugation than in membranes centrifuged in capped tubes (cf. below). The high n_H in the present investigation seems not to be due to an artifact caused by lack of stirring during the ATPase determination because vigorous stirring did not change the kinetic parameters.

Kinetics of ATPase in membranes prepared by centrifugation in capped tubes

K_{Ca} dependent on pCa_H. Fig. 5 shows the effect of pCa_H in the range of 4—8 on membranes which were prepared by centrifugation in capped tubes. Above

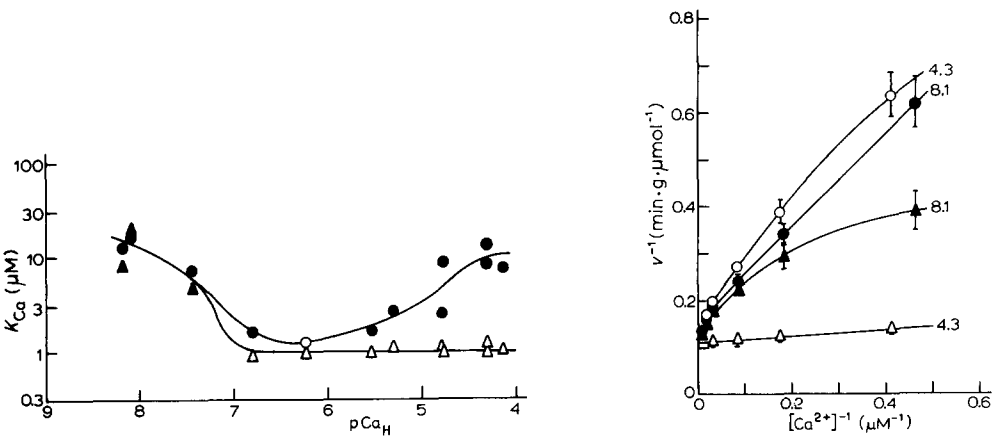

Fig. 5. Dependence of log K_{Ca} on pCa_H without and with ionophore A 23187. Single preparations. Centrifuged in capped tubes. ATPase determination as in Fig. 1. Ionophore treatment by adding 1 mg A 23187 per ml ethanol to membrane suspension, stirring continuously, 15 min before ATPase determination. 7—10 μmol A 23187 per g dry membrane corresponding to about 100 μmol per l cells. • and ○, without ionophore; A- and B-kinetics, respectively. ▲ and △, with ionophore; A- and B-kinetics, respectively.

Fig. 6. Lineweaver-Burk plot of Ca^{2+}-stimulated ATPase activity (v) vs. Ca^{2+} concentration during ATPase determination without and with ionophore A 23187. Mean of two experiments (±S.E.). The pCa_H values used mark the curves. Centrifugation in capped tubes. • and ○, without ionophore; pCa_H 8.1 and 4.3, respectively. ▲ and △, with ionophore (see Fig. 5); pCa_H 8.1 and 4.3, respectively.

pCa_H 6 the effect of pCa_H on K_{Ca} was quite similar to the effect obtained with membranes centrifuged in the continuous-flow device (cf. Fig. 3). However, decreasing pCa_H below 6, K_{Ca} increased and the kinetics shifted again from B to A.

Effect of ionophore A 23187 on K_{Ca} and on kinetics. In order to investigate the relationship between K_{Ca} and the Ca^{2+}-accessibility of the membrane-bound ATPase, the Ca^{2+} conducting ionophore A 23187 was added to the membranes 15 min before the ATPase determination. Fig. 6 shows that the ionophore treatment changed the kinetics from type A to B at pCa_H 4.3. At pCa_H 8.1 the ionophore affected the ATPase reaction (v) only at the lowest Ca^{2+} concentrations and the A-kinetics was preserved.

When pCa_H was above 6 the ionophore affected K_{Ca} only a little (Fig. 5). At pCa_H 6.8 the ionophore changed the kinetics from A to B. This change was not observed at pCa_H above 7. When pCa_H decreased below 6 the effect of ionophore was more pronounced. A-kinetics was changed to B-kinetics and K_{Ca} was reduced to about 1 μM.

Comparison of kinetic parameters in different membrane preparations

K_{Ca} and n_H. The comparison of Fig. 5 with Fig. 3 shows that the type of centrifugation influenced the dependence of K_{Ca} on pCa_H. However, when the membranes which were prepared by centrifugation in capped tubes were treated with ionophore A 23187 (Fig. 5) the two types of membranes responded similarly to variations in pCa_H (cf. Fig. 3), and at pCa_H below 7 the two corresponding values of K_{Ca} did not differ (Table I).

This effect of ionophore indicates that in the case of membranes centrifuged in capped tubes the effect of pCa_H below 6 may be due to a decreased accessibility of the ATPase sites and not to a genuine change of the kinetic state of the ATPase. The effect may be similar to the effect reported by Porzig [10] that an intracellular Ca^{2+} concentration of about 10^{-5} M caused the membrane of erythrocyte ghosts to become impermeable to extracellular Ca^{2+}.

However, in spite of the presence of ionophore the Hill coefficient n_H was lower in the membranes centrifuged in capped tubes (Table I).

The ionophore had no effect on membranes prepared by continuous flow centrifugation, in the case of neither A-kinetics nor B-kinetics.

Maximum activity, V. At pCa_H below 6 the maximum activity was lower in

TABLE I

THE EFFECT OF pCa_H ON K_{Ca} AND n_H FOR Ca^{2+}-STIMULATED ATPase

Membranes were centrifuged in continuous-flow device (5—7 experiments) or in capped tubes (9 experiments). ATPase was determined as in Fig. 1. Ionophore treatment (A 23187) as in Fig. 5. Mean ± S.E. $pCa_H > 10$ vs. $pCa_H < 7$ (continuous flow) and continuous flow vs. capped tubes ($pCa_H < 7$) were tested by Student's t test.

	Continuous flow			Capped tubes, A 23187 present	
	$pCa_H > 10$	$pCa_H < 7$	Significance	$pCa_H < 7$	Significance
K_{Ca} (μM):	46.3 ± 4.8	0.82 ± 0.12	$P < 0.001$	1.00 ± 0.03	$P > 0.1$
n_H:	0.79 ± 0.12	1.83 ± 0.18	$P < 0.002$	1.39 ± 0.07	$P < 0.05$

TABLE II

EFFECT OF IONOPHORE A 23187 ON OPTIMUM Ca^{2+} CONCENTRATION DURING ATPase DETERMINATION

Membranes prepared by two types of centrifugation. ATPase determined as in Fig. 1. Mean ± S.E. (*n* observations), tested by Student's *t* test. Ionophore treatment (A 23187) as in Fig. 5.

	Type of kinetics	Optimum Ca^{2+} conc. (μM)		Significance
		A 23187 absent	A 23187 present	
$pCa_H > 7$				
Capped tubes	A	111 ± 19 (3)	135 ± 24(3)	$P > 0.4$
Continuous flow	A	114 ± 9 (13)	158 ± 24 (3)	$P > 0.05$
$pCa_H < 7$				
Capped tubes	A or B	56 ± 12 (6)	25 ± 8 (6)	$P < 0.01$
Continuous flow	B	31 ± 10 (4)	22 ± 10 (2)	$P > 0.5$

membranes prepared by centrifugation in capped tubes than in membranes which were centrifuged in the continuous-flow device in accordance with previous results [1,6]. The ionophore could not raise V in membranes from capped tubes even if the ionophore concentration was increased to about 30 μmol per g dry membrane.

Optimum Ca^{2+} concentration during ATPase reaction. It was shown previously [5] that high Ca^{2+} concentrations during the ATPase reaction inhibited the enzyme activity and that the ATPase activity was optimum in a rather narrow range of Ca^{2+} concentrations. Table II shows that the ionophore reduced the optimum Ca^{2+} concentration for membranes centrifuged in capped tubes at pCa_H below 7. However, at higher pCa_H there was no effect of ionophore. Membranes prepared by continuous flow centrifugation were not affected by the ionophore treatment.

The optimum Ca^{2+} concentration for membranes with A-kinetics and B-kinetics were 127 ± 6 μM (S.E., 21 experiments) and 28 ± 4 μM (S.E., 15 experiments), respectively. This difference indicates that the kinetics of Ca^{2+} inhibition of the ATPase depends on pCa_H. The value of B-kinetics agrees with the value reported by Wolf et al. [9].

Conclusion of ionophore experiments. The sensitivity to treatment with ionophore A 23187 was restricted to the membranes which were prepared at pCa_H below 7 by centrifugation in capped tubes.

In membranes prepared at pCa_H above 7 the A-kinetics, K_{Ca}, and optimum Ca^{2+} concentration were not changed by the ionophore treatment, irrespective of the type of centrifugation. Furthermore, the reduced V of the ATPase in membranes centrifuged in capped tubes was not increased by the ionophore treatment. These phenomena may therefore not be due to reduced access of Ca^{2+} to the ATPase sites.

Discussion

Kinetics dependent on pCa_H

Two different types of ATPase kinetics, A and B, could be detected in mem-

branes prepared at different values of pCa_H (cf. Figs. 1—4). At high pCa_H both Ca^{2+} sensitivity of the ATPase and cooperativity between the Ca^{2+} sites were low. With decreasing pCa_H both Ca^{2+} sensitivity and cooperativity increased and reached maximum at pCa_H 6—7. Generally, these results imply that the kinetic properties induced during the membrane preparation were preserved to such an extent that they could be detected. Especially it is noticed that the kinetic state corresponding to type A, induced at pCa_H above 7, was not changed during the ATPase reaction, in spite of the presence of high Ca^{2+} concentrations.

However, the preservation in vitro of the kinetic state does not exclude the possibility that the state of enzyme could be changed reversibly by Ca^{2+} in vivo. The Ca^{2+}-ATPase may be a hysteretic enzyme (as defined by Frieden [11]) and it may shift between two conformational states, corresponding to reduced and high Ca^{2+}-pumping activity, respectively, as suggested previously [1].

The maximum change of kinetics was observed in the region of pCa_H about 7 (cf. Figs. 3 and 4), which indicates the existence of a Ca^{2+} binding site with an apparent dissociation constant of about 10^{-7} M. Considering that pCa_H was varied at lower temperature and lower ionic strength than used during the ATPase determination, the possibility cannot be excluded that the Ca^{2+}-binding site(s) responsible for the change of kinetic state are identical with the sites involved in Ca^{2+} activation of the ATPase which showed an apparent dissociation constant (K_{Ca}) of about 10^{-6} M (cf. Table I). However, it is also possible that the kinetic state is controlled by a specific Ca^{2+} site.

Regulation of intracellular Ca^{2+}

Lew and Beaugé [12] have emphasized that the Ca^{2+}-permeability of the intact erythrocyte membrane is very low and suggested this to be a permanent condition throughout the life of the erythrocyte. However, Rasmussen et al. [13] have demonstrated an oscillating ^{45}Ca pool in erythrocytes, which was influenced by isoproterenol and prostaglandin E_2. This suggests that the Ca^{2+}-permeability or the Ca^{2+} pump activity, or both, may undergo periodic changes.

According to Fig. 5 the access of Ca^{2+} to the ATPase sites seems to decrease when pCa_H is changed from 6 to 4. This suggests that the Ca^{2+}-permeability of the membrane is minimum at high intracellular Ca^{2+} concentration. However, the high Ca^{2+}-permeability of erythrocyte ghosts at low Ca^{2+} concentrations (cf. Fig. 5 and refs. 8,10) may indicate that the low Ca^{2+}-permeability of intact erythrocytes [12] is not permanent but can be increased provided the intracellular Ca^{2+} concentration is low.

It seems likely that both a shift between two states of the Ca^{2+} pump and an increase of Ca^{2+}-permeability triggered by some stimulus may occur in the intact cell, and that both phenomena may depend on the intracellular Ca^{2+}-level.

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