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Compound 48/80 and calmodulin modify the interaction of ATP with the $(Ca^{2+} + Mg^{2+})$-ATPase of red cell membranes

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(1) Compound 48/80, an anti-calmodulin agent, reduces the maximum effect of ATP and does not affect the apparent affinity for ATP of the high-affinity site of the Ca^{2+}-ATPase from calmodulin-bound membranes of human red cells. (2) In the same preparation, 48/80 reduces more than 50-times the apparent affinity for ATP of the low-affinity site with little change in the maximum effect of the nucleotide at this site of the Ca^{2+}-ATPase. (3) The effects of compound 48/80 are independent of the concentration of Ca^{2+} between 30 and 200 μM. (4) The apparent affinity of the low-affinity site of the Ca^{2+}-ATPase for ATP is almost 100-fold less in calmodulin-stripped membranes than in calmodulin-bound membranes. In calmodulin-stripped membranes, exogenous calmodulin increases the apparent affinity for ATP up to the control values. (5) These results indicate that apart from increasing the apparent affinity of the transport site for Ca^{2+}, calmodulin also increases the apparent affinity of the regulatory site of the Ca^{2+}-ATPase for ATP. Since this effect is exerted within the physiological ranges of ATP concentrations, it may participate in the physiological regulation of Ca^{2+} pumping by calmodulin.

Introduction

It is known that the substrate curve of the Ca^{2+}-ATPase of red cell membranes is biphasic, with a component of high apparent affinity (K_m = 2–4 μM) and low velocity and a component of low apparent affinity (K_m = 140–300 μM) and high velocity [1,2]. Muallem and Karlish [3], provided evidence that such behavior is observed only in calmodulin-bound membranes while in calmodulin-stripped membranes the substrate curve is essentially hyperbolic. On the other hand, Scharff [4] reported that in calmodulin-deficient Ca^{2+}-ATPase, the biphasic response to ATP reappears at high Ca^{2+} concentrations. In 1983, Gietzen et al. [5] provided evidence that compound 48/80, a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde, is a highly specific inhibitor of the effects of calmodulin on the Ca^{2+}-ATPase.

We have studied the effects of 48/80 and calmodulin on the substrate curve of the Ca^{2+}-ATPase of human red cell membranes, finding that the main effect of calmodulin is to increase the Michaelis constant of the low-affinity site for ATP.

Materials and Methods

Materials. Fresh blood from hematologically normal adults collected on acid/citrate/dextrose solutions was always used. Red cell membranes

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

were prepared following the procedure of Gietzen et al. [6] which yields membranes devoid of endogenous calmodulin. Calmodulin was purified from bovine brain as described by Kakiuchi et al. [7]. [γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chapell [8] except that no unlabelled orthophosphate was added to the incubation media. [^{32}P]Orthophosphate was provided by the Comisión Nacional de Energía Atómica (Argentina). Compound 48/80, ATP, enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were obtained from Sigma (U.S.A.). Salts and reagents were of analytical reagent grade.

Methods. Except when otherwise indicated in Results, ATPase activity was measured at 37°C in media containing: 120 mM KCl, 4 mM $MgCl_2$, 1.0 mM EGTA, 1 mM ouabain, 1.2 mM $CaCl_2$, and various concentrations of ATP. When the concentration of ATP was 0.2 mM or more, the release of inorganic phophate for the nucleotide was estimated by a modification of the procedure of Fiske and SubbaRow [9]. When the concentration of ATP was less than 0.2 mM, [γ-^{32}P]ATP was used and the [^{32}P]P_i liberated was measured by the procedure described previously [1]. When the concentration of ATP was higher than 0.1 mM, the nucleotide was added with an equimolar amount of $MgCl_2$. The concentration of free Ca^{2+} was measured with an IS 561 Ca^{2+}-selective electrode [10] in media with the composition of those described above. Ca^{2+}-ATPase activity was considered to be the difference between the activity in the above-mentioned media and the activity in same media except that $CaCl_2$ was omitted. Before the reaction was started by the addition of ATP, the membranes were preincubated at 37°C first with 48/80 during 5 min and then calmodulin for an additional 5 min period. Throughout the assays, the concentration of calmodulin was 30 nM and the concentration of membrane protein was 50 μg/ml. Protein was estimated by the method of Lowry et al. [11].

Activities were measured in triplicate. The individual measurements did not differ from the mean more than 15%. Theoretical equations were adjusted to the experimental results by least-squares non-linear regression using the procedure of Gauss-Newton with optional damping. The concentration variable was assumed to have negligible error and the velocity variable was assumed to be homoscedastic. The program was run on a microcomputer with 14 digit precision (Rossi, R.C. and Garrahan, P.J., unpublished data).

Results

In the experiment shown in Fig. 1, the effects of increasing concentrations of compound 48/80 were tested on the Ca^{2+}-ATPase activity of calmodulin-stripped membranes incubated in media with or without an excess of exogenous calmodulin. It can be seen that in media without 48/80, calmodulin increases almost 5-times the Ca^{2+}-ATPase activity of the membranes. Compound 48/80 is almost without effect on the activity in the absence of calmodulin, but as the concentration of 48/80 raises, the extra activity elicited by calmodulin decreases along a rectangular hy-

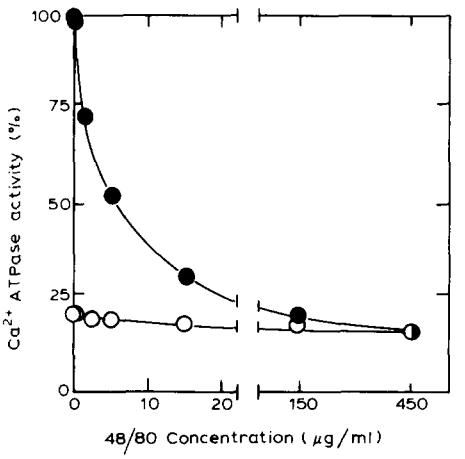

Fig. 1. Effects of increasing concentrations of compound 48/80 on Ca^{2+}-ATPase activity of red cell membranes stripped of their endogenous calmodulin and incubated in media containing 30 μM Ca^{2+} and either 0 (○) or 30 nM (●) calmodulin. The maximum activity (100%) was 0.0285 μmol/mg protein per min. The curve that fits the filled circles represents the equation:

$$v = V_0/(1+([I]/K_i))+V_r \quad (1)$$

where V_0 is the fraction of the activity that is sensitive to 48/80, V_r the activity that is insensitive to 48/80, [I] the concentration of 48/80 and K_i the concentration of 48/80 for half-maximal inhibition. The curve was drawn using the following values for the parameters: $V_0 = 84.5\%$, $V_r = 15.5\%$, $K_i = 3.37$ μg/ml.

perbola until it is fully abolished at the highest concentration of 48/80 tested. These results are in agreement with previous findings by Gietzen et al. [5]. In the experiment in Fig. 1, the value of K_i for 48/80 (see Eqn. 1 in the legend to Fig. 1) was 3.37 ± 0.33 μg/ml (mean ± S.E.).

The effect of compound 48/80 on the substrate curve of the Ca^{2+}-ATPase

Ca^{2+}-ATPase activity was measured as a function of ATP concentrations from 1 to 1300 μM in media with and without 50 μg/ml of 48/80 and in the presence of enough calmodulin for maximum activation (Fig. 2A). In the absence of 48/80, the activation shows its well-known biphasic response to the concentration of ATP with high-affinity low-velocity and low-affinity high-velocity components. As we have described in detail elsewhere [1,12], this curve can be adjusted by the sum of two Michaelis-Menten equations, i.e.

$$V = \frac{V_1[\text{ATP}]}{[\text{ATP}] + K_{m1}} + \frac{V_2[\text{ATP}]}{[\text{ATP}] + K_{m2}} \quad (2)$$

Where V_1 and V_2 are the maximum velocities and K_{m1} and K_{m2} are the Michaelis constants of the high- and low-affinity components, respectively.

In the media with 48/80, Ca^{2+}-ATPase activity is lower at all the concentrations of ATP tested, and the substrate curve instead of saturating, tends to a straight line of positive slope at high ATP concentrations. This suggests that in the presence of 48/80, K_{m2} becomes much larger than the highest concentration of ATP tested so that the second term in the right-hand side of Eqn. 2 approaches a straight line of slope V_2/K_{m2}. On the basis of this reasoning, the sum of a Michaelis-Menten equation, representing the high-affinity component, and a linear term, representing the low-affinity component was adjusted to the experimental points. Comparison of the best-fitting values of K_{m1} and V_1 obtained in this way with those obtained for the control curve (see legend to Fig. 2A) suggests that 48/80 decreases V_1 and has no significant effect on K_{m1}.

The concentration of free Ca^{2+} in the incubation media used in the experiment in Fig. 2A was 27–35 μM. Since it has been reported [4] that the shape of the substrate curve of the ATPase depends on the concentration of Ca^{2+}, an experiment like that in Fig. 2A was repeated in media containing 170–205 μM free Ca^{2+}. Results in Fig. 2B show that under these conditions, 48/80 is still an inhibitor of Ca^{2+}-ATPase activity and that the substrate curve has the same shape as that ob-

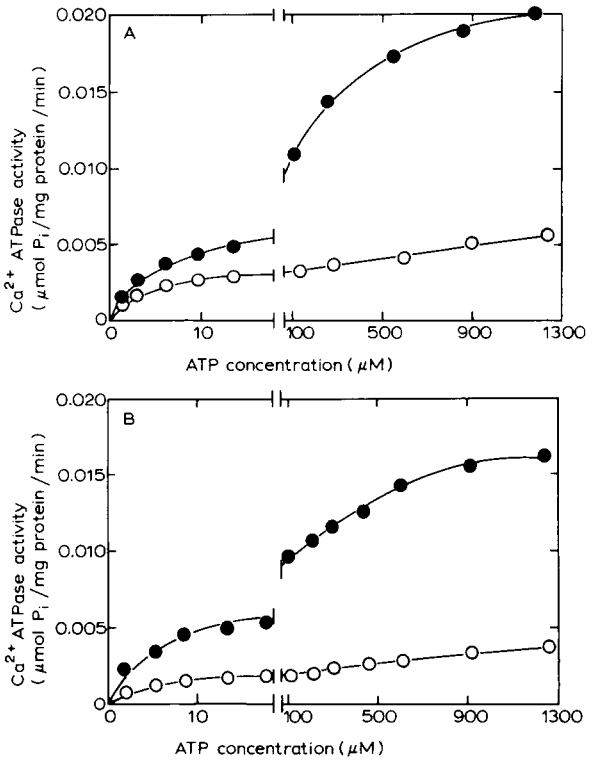

Fig. 2. (A) The substrate curve of the Ca^{2+}-ATPase in the presence (○) and in the absence (●) of 50 μg/ml of compound 48/80. The actual concentration of Ca^{2+} was measured in each of the media and ranged from 27 to 35 μM. The curve that fits the filled circles represents Eqn. 2. in Results with $K_{m1} = 2.04$ μM, $K_{m2} = 176$ μM, $V_1 = 0.0030$ μmol P_i/mg protein per min and $V_2 = 0.0194$ μmol P_i/mg protein per min. The curve that fits the open circles represents a modification of Eqn. 2 in which the second term of its right-hand side has been replaced by: $V_2/K_{m2} \times [\text{ATP}]$ (see text for justification) with $K_{m1} = 2.22$ μM, $V_1 = 0.0015$ μmol P_i/mg protein per min and $V_2/K_{m2} = 1.967 \cdot 10^{-6}$ l/(min/mg protein). (B) An experiment identical to that in (A) except that the media contained from 170–205 μM Ca^{2+}. For the curve that fits the filled circles $K_{m1} = 3.29$ μM, $K_{m2} = 379$ μM, $V_1 = 0.0041$ μmol P_i/mg protein per min and $V_2 = 0.0157$ μmol P_i/mg protein per min. For the curve that fits the open circles, $K_{m1} = 2.62$ μM, $V_1 = 0.0021$ μmol P_i/mg protein per min and $V_2/K_{m2} = 1.187 \cdot 10^{-6}$ l/(min/mg protein).

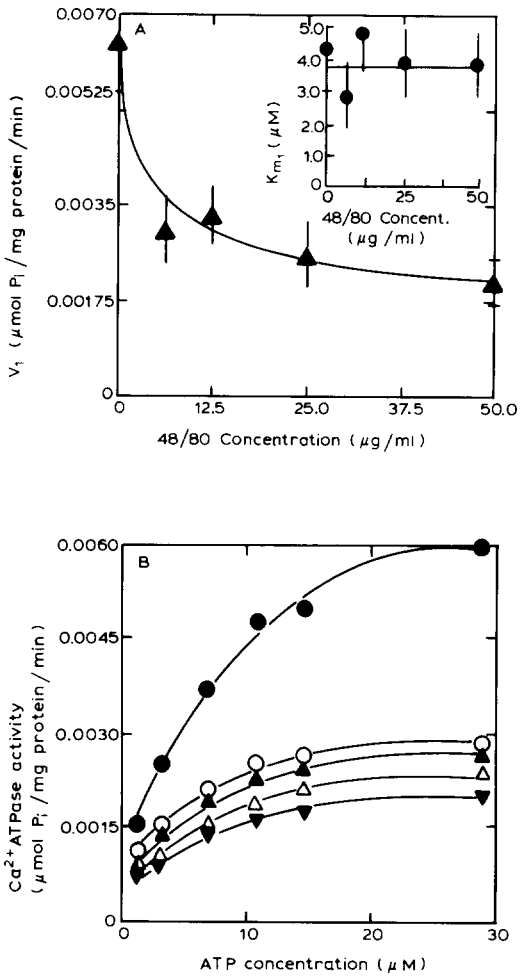

Fig. 3. (A) A plot of V_1 (see Eqn. 2 in the text) as a function of the concentration of 48/80. Inset: a plot of K_{m1} as a function of the concentration of 48/80. Experiments were made in the presence of 30 nM calmodulin. V_1 and K_{m1} ± their standard errors (vertical bars in the graph) were estimated by non-linear regression as described in Results. (B). Ca^{2+}-ATPase activity as a function of ATP concentrations in media with 30 nM calmodulin and 0 (●), 6.25 (○), 12.5 (▲), 25 (△) and 50 (▼) μg/ml of compound 48/80. The continuous curves are solutions of the equation:

$$v = \frac{(V_1/(1+[I]/K_i)+V_r)}{(1+(K_{m1}/[ATP])+(V_2/K_{m2})[ATP]/(1+[I]/K_i))} \quad (3)$$

where the meaning of the parameters is the same as in Figs. 1 and 2A and Eqn. 2 in the text. The best-fitting values of the parameters were: $K_i = 3.09$ μg/ml, $K_{m1} = 3.68$ μM, $V_1 = 0.00245$ μmol P_i/mg protein per min, $V_r = 0.00131$ μmol P_i/mg protein per min and $V_2/K_{m2} = 1.68 \cdot 10^{-5}$ l/(min/mg protein).

served at lower Ca^{2+} concentrations. It seems therefore that the effects of 48/80 on the substrate curve of the Ca^{2+}-ATPase do not depend on the concentration of Ca^{2+}.

Effect of compound 48/80 at low ATP concentrations

The effects of 48/80 on the high-affinity component of the substrate curve of the Ca^{2+}-ATPase were studied measuring activity as a function of the concentration of ATP from 1 to 30 μM, at different concentrations of 48/80 and in media with excess calmodulin. Since the highest concentration of ATP used in these experiments was about 6-times less than K_{m2}, the second term of Eqn. 2 was replaced by a linear function of ATP concentration. This simplified expression was adjusted by non-linear regression to the experimental data of activity vs. ATP concentration at each of the concentrations of 48/80 tested. The best-fitting values of V_1 and K_{m1} obtained in this way are plotted in Fig. 3A as a function of the concentration of 48/80. It can be seen that as 48/80 increases, V_1 decreases tending to a value about 30% of the control, whereas K_{m1} (inset to Fig. 3A) is not significantly affected.

Using the information in Fig. 3A, multiple non-linear regression was employed to adjust Eqn. 3 in the legend to Fig. 3B, to all the experimental data of velocity vs. ATP concentration. Eqn. 3 is a modification of Eqn. 2 and is based on the following assumptions: (i) at low ATP concentrations, the substrate curve can be described by the sum of a hyperbolic and a linear term (see comments to Fig. 3A), (ii) both V_1 and the slope of the linear term decrease hyperbolically as 48/80 is increased, (iii) 48/80 is a partial inhibitor of the hyperbolic term and, (iv) the value of K_i for the effects of 48/80 on the high- and low-affinity components is the same. The continuous lines in Fig. 3B are the solutions of this equation for the best-fitting values (see legend to Fig. 3B) of the parameters. It is clear that Eqn. 3 gives a reasonable description of the experimental points. It can be concluded therefore that the results in Fig. 3A and B are consistent with the idea that 48/80 acts as a partial non-competitive inhibitor of the effects of ATP at the high-affinity component of the substrate curve of the Ca^{2+}-ATPase.

Effects of compound 48/80 at high ATP concentration

The effects of 48/80 on the low-affinity component of the substrate curve of the Ca^{2+}-ATPase

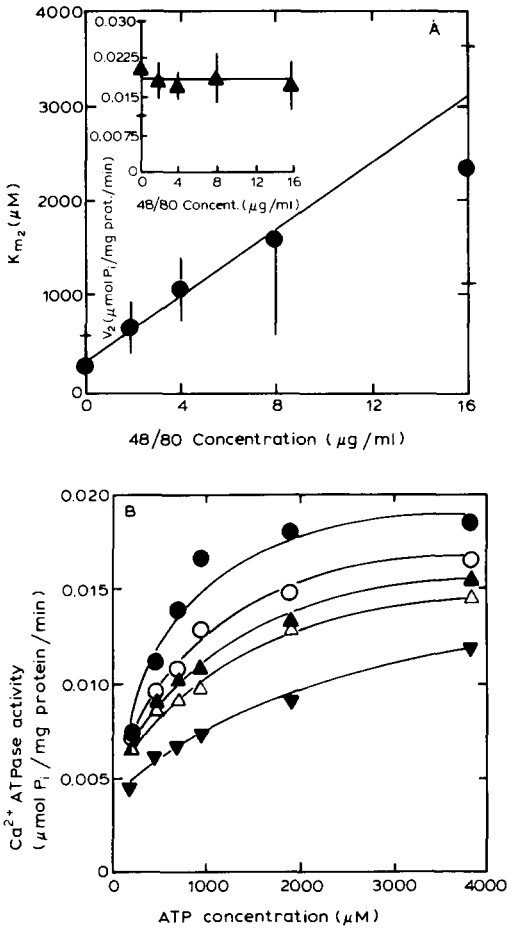

Fig. 4. (A) A plot of K_{m2} as a function of the concentration of 48/80. The inset is a plot of V_2 as a function of 48/80. V_2 and $K_{m2} \pm$ their standard errors (vertical bars in the graphs) were estimated by non-linear regression as described in Results. (B) Ca^{2+}-ATPase activity as a function of ATP concentrations in media with 30 nM calmodulin and 0 (●), 2.0 (○), 4.2 (▲), 8.0 (△) and 16.0 (▼) μg/ml of compound 48/80. The continuous curves are solutions of the equation:

$$v = V_1/(1+[I]/K_i) + V_r + V_2$$
$$/(1+(K_{m2}/[ATP])(1+[I]/K_i)) \quad (4)$$

where the meaning of the parameters is the same as in Fig. 3A and Eqn. 2. The best-fitting values of the parameters were: V_1 = 0.00403 μmol P_i/mg protein per min, V_r = 0.00098 μmol P_i/mg protein per min, V_2 = 0.01552 μmol P_i/mg protein per min, K_i = 3.25 μg/ml and K_{m2} = 580 μM.

were studied measuring activity as a function of ATP concentrations from 0.25 to 4.0 mM in media with an excess of calmodulin and different concentrations of 48/80. The experimental values of activity vs. ATP concentration at each of the concentrations of 48/80 tested were adjusted to the sum of a constant term (V_1) describing the high-affinity component and a hyperbolic term describing the low-affinity component. The use of a constant term for the high-affinity component is justified because the concentrations of ATP tested were at least 50–100-times higher than K_{m1}. The best-fitting values of V_2 and K_{m2} are plotted in Fig. 4A as a function of the concentration of 48/80. It is clear that as the concentration of the inhibitor increases, K_{m2} raises in an approximately linear fashion while V_2 (inset to Fig. 4A) is not significantly affected.

Using the information provided by the plots in Fig. 4A, Eqn. 4 in the legend to Fig. 4B was adjusted by multiple non-linear regression to all the experimental data. Eqn. 4 is a modification of Eqn. 2 based on the following assumptions: (i) the high-affinity component is fully saturated, (ii) V_1 decreases hyperbolically with 48/80 leaving a residual non-inhibitable activity, (iii) K_{m2} increases linearly with 48/80, but V_2 is not affected and, (iv) K_i for 48/80 has the same value for both high- and low-affinity components. The continuous curves in Fig. 4A are the solutions of Eqn. 4 for the best-fitting values of the parameters involved (see legend to Fig. 4B). It can be seen that there is a reasonable agreement between theory and experiment.

It can be concluded therefore that results in Fig. 4A and B, are consistent with the idea that the main effect of 48/80 on the low-affinity component of the substrate curve of the Ca^{2+}-ATPase is to decrease the apparent affinity for ATP.

Effect of calmodulin on the substrate curve

If the only action of 48/80 were to block activation by calmodulin, the results described insofar would indicate that at saturating concentrations of Ca^{2+} the main effects of calmodulin on the Ca^{2+}-ATPase is to increase the turnover at the high-affinity site for ATP and the apparent affinity at the low-affinity site for ATP. To test this hypothesis, Ca^{2+}-ATPase activity was measured as

a function of ATP in a 1–4000 μM concentration range in membranes stripped of their endogenous calmodulin and incubated in media with and without 30 nM calmodulin. Results are plotted in Fig. 5 together with the solutions of the same equations that were used to fit the data of the experiment in Fig. 2A and B. It can be seen that in calmodulin-depleted membranes, activity is lower at all ATP concentrations tested and that at high ATP concentrations the activity instead of saturating, increases along a straight line. Moreover, the values given in the legend to Fig. 5 show that calmodulin has little effect on the kinetic parameters of the high-affinity component of the substrate curve. The effects of calmodulin depletion on the substrate curve of the Ca^{2+}-ATPase are therefore very similar to those of 48/80. This makes it reasonable to consider that the main mechanism for inhibition by compound 48/80 is the blockage of the effects of calmodulin. This view implies that at a given concentration of ATP, the degree of occupation of the low-affinity site for the nucleotide of the Ca^{2+}-ATPase will depend largely on the concentration of calmodulin.

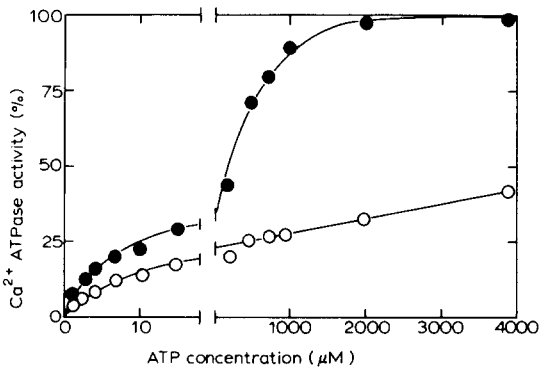

Fig. 5. The substrate curve of the Ca^{2+}-ATPase of membranes stripped of their calmodulin and incubated in media with (●) and without (○) 30 nM calmodulin. The continuous curves were drawn following the procedure described in the legend to Fig. 2A with the following values for the parameters: K_{m1} = 3.60 μM, K_{m2} = 503 μM, V_1 = 27.0% and V_2 = 83.9% for the curve in the presence of calmodulin and K_{m1} = 2.22 μM, V_1 = 21.5% and V_2/K_{m2} = 0.00559%/μM for the curve in the absence of calmodulin. The activity corresponding to 100% was 0.066 μmol P_i/mg protein per min.

Discussion

Results in this paper show that at non-limiting concentrations of Ca^{2+} and in the presence of calmodulin, compound 48/80 induces a marked change in the response of the Ca^{2+}-ATPase to the concentration of ATP. Compound 48/80 is a relatively poor inhibitor at concentrations of ATP in which the high-affinity component of the substrate curve predominates in the overall activity, but it is a potent inhibitor of the low-affinity component of the substrate curve. As a consequence of this, when applied to red cell membranes suspended in media with high concentrations of ATP (more than 100 μm) compound 48/80 lowers Ca^{2+}-ATPase activity to a constant level which is close to that predictable from the activity of the high-affinity component only.

The concentration of 48/80 for half-maximal inhibition found in the experiments reported in this paper (3.4 μg/ml) is about 4-times higher than that mentioned by Gietzen et al. in their initial report [5]. More recently [13], these authors found a value of 3.3 μg/ml for half-maximal inhibition when 48/80 is tested in media containing more calmodulin and more membrane protein than those used in their initial studies. Therefore, the apparent affinity for inhibition by 48/80 seems to depend on the concentrations of calmodulin and of membrane protein. This is not surprising in view of the known antagonism between calmodulin and 48/80 and of the fact that 48/80 binds to proteins.

Analysis of the effects of 48/80 at low concentrations of ATP shows that the compound does not alter the K_{m1} and reduces partially the maximum effect of ATP at the high-affinity component of the substrate curve.

Kinetic analysis of the activity at high concentrations of ATP shows that 48/80 does not modify the turnover but lowers drastically the apparent affinity for ATP of the low-affinity component of the substrate curve. The effect on apparent affinity seems to be the main cause of the inhibitory effect of 48/80 on Ca^{2+}-ATPase activity. On this view, it can be predicted that at any fixed concentration of 48/80 and at non-limiting concentrations of ATP, Ca^{2+}-ATPase should exhibit its full catalytic capacity. The kinetic equa-

tions used in this paper are based on the assumption that the K_m of the low-affinity site is a linear function of the concentration of 48/80. This implies that K_{m2} will increase without bounds as the concentration of 48/80 is raised. However, experimental results show that low-affinity activation by ATP persists at concentrations of 48/80 that are fully inhibitory. It seems therefore more likely that as the concentration of the inhibitor increases, K_{m2} instead of increasing indefinitely goes from its physiological value to a value that is well above the highest concentration of ATP tested in the experiments reported in this paper. A view like this would be compatible with the observed linear response of K_{m2} to 48/80 without implying that the low-affinity site completely loses its capacity to bind ATP at non-limiting concentrations of 48/80. If the curves in the presence of 50 μg/ml of 48/80 (Fig. 2A and B) are taken as representative of the substrate kinetics at fully saturating concentrations of 48/80, an estimate of the value of K_{m2} under these conditions can be obtained from the ratio of the control value of V_2 to the slope of the linear part of the substrate curve in the presence of 48/80. The values for K_{m2} calculated in this way are 10.0 and 13.2 mM for the experiments in Fig. 2A and B, respectively.

Results in this paper also show that most of the effects of 48/80 can be reproduced by removing calmodulin from the red cell membranes, a view which is in agreement with the fact that calmodulin-depleted membranes are practically insensitive to 48/80. A value of K_{m2} in calmodulin-depleted membranes can be estimated from the plot in Fig. 5, following the procedure outlined in the preceding paragraph. These findings provide additional evidence to the proposal of Gietzen et al. [5] that under appropriate conditions 48/80 is a specific antagonist, without side-effects, of the activation of the Ca^{2+}-ATPase by calmodulin. If this is taken for granted, the effects of 48/80 reported in this paper are useful to further understand the dependence on calmodulin of the regulatory site for ATP of the Ca^{2+}-ATPase. In fact, Muallem and Karlish [3] have provided evidence suggesting that biphasic activation of Ca^{2+}-ATPase by ATP is only seen in calmodulin-bound membranes, and that in the absence of calmodulin the substrate curve is essentially hyperbolic with kinetic parameters close to those of the high-affinity component. Data in this paper indicate that the dependence of the low-affinity site on calmodulin results from the ability of calmodulin to increase the apparent affinity for ATP of this site, without modifying the maximum effect of the nucleotide. In this view, therefore, the disappearance of the second component of the substrate curve in the absence of calmodulin proposed by Muallem and Karlish is only apparent since, in the absence of calmodulin, full expression of this component would be attainable at concentrations of ATP higher than those usually employed in ATPase assays.

Scharff [4] has reported that the effect of calmodulin depletion on the shape of the substrate curve is only apparent at the low (approx. 30 μM) Ca^{2+} concentrations used by Muallem and Karlish and that biphasic activation is apparent both in calmodulin-stripped and in calmodulin-bound membranes if Ca^{2+}-ATPase is tested in media with higher (approx. 150 μM) Ca^{2+} concentrations. If this were so, 40/80 should be without effect when the substrate curve of the Ca^{2+}-ATPase is measured in media with high concentrations of Ca^{2+}. Experiments in this paper do not confirm this prediction since they clearly show that the effect of 48/80 on the shape of the substrate curve in media with 170–205 μM Ca^{2+} is practically undistinguishable to that in media with about 30 μM Ca^{2+}.

For the effects of calmodulin on the regulation of the Ca^{2+}-ATPase by ATP to be of physiological significance, they have to be exerted within the range of physiological concentrations of ATP. Results in this paper show that K_{m2} goes from 0.18 mM in calmodulin-bound membranes to about 10–15 mM when the effects of calmodulin were blocked. These values are about 10-times less and 10-times more, respectively, than the physiological cytosolic concentration of ATP. In view of this, it is reasonable to propose that under physiological conditions calmodulin regulates the rate of active transport of Ca^{2+} not only via its well-known effect on the affinity for Ca^{2+} but also through the increase in the affinity of the regulatory site for ATP, so that activation of the Ca^{2+}-ATPase by calmodulin will be larger than that predictable solely from the higher occupancy of the transport site by Ca^{2+}.

It could be argued that the increase in affinity for Ca^{2+} induced by calmodulin is the primary event that leads to the increase in affinity for ATP. However, no experimental evidences in favor of the existence of positive interactions between the apparent affinities of Ca^{2+} and ATP are available. In fact, on the basis of the effects of *N*-ethylmaleimide [9] and of pH [14] on the Ca^{2+}-ATPase activity, it has been concluded that Ca^{2+} combines to its site with high affinity in media without ATP and that in the absence of Ca^{2+}, an ATP-enzyme complex is formed whose apparent dissociation constant does not differ from the K_m of the Ca^{2+}-ATPase.

It is known that the effect of calmodulin on the affinity for Ca^{2+} of the Ca^{2+} pump is mimicked by partial proteolysis [15], acidic lipids [16], organic polyanions [17] and EGTA [18]. It would be interesting to see if these substances and treatments also increase the affinity of the system for ATP at its regulatory site.

Note added in proof: (Received May 21st, 1985)

Since the submission of this paper, it came to our attention that Scharff and Foder [19] had discussed the possibility that the effects of 48/80 were caused by either a reduction of free calmodulin due to the formation of drug-calmodulin complexes or by decreasing the association of calmodulin to the Ca^{2+}-ATPase after binding to the enzyme. Results in this paper do not permit to see the difference between these two possible mechanisms of action.

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