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Does calmodulin regulate the affinity of the human red cell Ca^{2+} pump for ATP?

Ariel J. Caride, Juan P.F.C. Rossi, Patricio J. Garrahan and Alcides F. Rega

Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires (Argentina)

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(1) We have reexamined the effects of calmodulin and of the calmodulin antagonist, compound 48/80 on the interaction of ATP at its low-affinity site in the Ca^{2+}-ATPase from human red cells. (2) At variance with our earlier proposal (Biochim. Biophys. Acta (1985) 816, 379–386) calmodulin increased the maximum effect of ATP without changing the apparent affinity for ATP at the low-affinity site. Accordingly, ATP increased the maximum activation by calmodulin without altering the apparent affinity of the Ca^{2+}-ATPase for calmodulin. (3) Confirming our previous observation (Biochim. Biophys. Acta (1985) 816, 379–386) compound 48/80 lowered the apparent affinity of the Ca^{2+}-ATPase for ATP at the low-affinity site. This has to be attributed to a direct effect of this compound on the enzyme rather than to its effect as calmodulin antagonist.

Introduction

The response of the Ca^{2+}-ATPase activity from plasma membranes to ATP can be described by the sum of two Michaelis-Menten equations, i.e.:

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

one with high apparent affinity and low maximal velocity ($K_{m1} = 2$–10 μM, $V_1 \approx 25\%$ of V_m and the other with low apparent affinity and high maximal velocity ($K_{m2} = 100$–500 μM, $V_2 \approx 75\%$ of V_m). This behavior was first detected in the Ca^{2+}-ATPase activity of red cell membranes [1], and afterwards found during active Ca^{2+} transport [2] and in purified preparations of the enzyme [3]. It is currently believed that the high-affinity component express the interaction of ATP with the catalytic site of the enzyme whereas the low-affinity component evinces an additional non-catalytic effect of the nucleotide as a non-essential activator (for references, see Ref. 4). Since it is as yet unknown whether the catalytic and regulatory effects of ATP are exerted at two different sites or at two states of the same site [4], it should be stressed that Eqn. 1 is only an empirical description of the substrate curve. In 1980 Muallem and Karlish [5] reported that the low-affinity component of the substrate curve was only apparent in the calmodulin-bound Ca^{2+}-ATPase. Based on experiments made with either fully calmodulin-bound or fully calmodulin-free enzyme in a previous paper we confirmed this and proposed that in the calmodulin-stripped Ca^{2+}-ATPase K_{m2} had a high value which returned to 100–500 μM upon addition of calmodulin [6]. This view seemed to gain support from the observation that in the calmodulin-bound enzyme the calmodulin antagonist compound 48/80 increased K_{m2} to about 15 mM [6]. After learning that calmodulin antagonist drugs can act directly on the ATPase rather than by blocking calmodulin [7,8], we have repeated in more detail some of the experiments we had made before and added new ones with the aim of confirming the hypothetical effects of calmodulin and compound 48/80 on K_{m2}. The results confirmed that 48/80 increases K_{m2}, but indicated that calmodulin acts increasing the turnover of the enzyme rather than the affinity for ATP at the regulatory site of the Ca^{2+}-ATPase.

In this paper we present this new data with the aim of minimizing possible confusion arising from incorrect interpretation of our earlier results.

Correspondence: A.J. Caride, Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina.

Materials and Methods

Fresh blood from hematologically normal adults collected on acid/citrate/dextrose solutions was always used. Red cell membranes were prepared as described previously [9], except that 0.5 mM 2-mercaptoethanol was added to all solutions. Calmodulin was prepared from bovine brain as described by Kakiuchi et al. [10]. [γ-^{32}P]ATP was prepared by the procedure of Glynn and Chappell [11] except that no unlabeled orthophosphate was added to the incubation media. ATPase activity was estimated from the amount of [^{32}P]P_i liberated from [γ-^{32}P]ATP as described previously [1]. Incubations were carried out at 37 °C in media containing: 50 mM Tris-HCl (pH 7.4 at 37 °C), 100 mM KCl, 0.5 mM EGTA, 0.55 mM $CaCl_2$, 20 μg of membrane protein, enough $MgCl_2$ to obtain 2 mM free Mg^{2+} and the concentrations of [γ-^{32}P]ATP, calmodulin and compound 48/80 that are indicated in the legends of the figures. Ca^{2+}-ATPase activity was taken as the difference between the activity measured in the media described above and the activity measured in identical media but without $CaCl_2$. Membranes were preincubated during 10 min at 4 °C in the presence of calmodulin and 48/80 and the reaction was started by the addition of ATP. Activities were measured in duplicate. The individual measurements did not differ from the mean more than 5%. In each case, results shown are representative of three or more experiments. CDTA, EGTA, ATP, compound 48/80, Tris base and the enzymes and cofactors for [γ-^{32}P]ATP synthesis were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Equations were fitted to experimental data by a non-linear regression procedure based on the Gauss-Newton algorithm [12]. The concentration of ATP was adjusted for consumption according to Segel [13]. Protein concentration was estimated by the procedure of Lundahl [14].

Results and Discussion

Fig. 1 shows the results of an experiment in which Ca^{2+}-ATPase activity was measured as a function of ATP in a 100 to 2000 μM concentration range, in media with different concentrations of calmodulin. This range of ATP concentrations was chosen because it was the range in which the low-affinity component of the activation curve and the effect of calmodulin become manifest. Nevertheless similar results were obtained when the ATP concentration was varied from 0.002 to 4 mM (data not shown). At zero and at non-limiting concentrations of calmodulin (lower and upper curves in Fig. 1), as in our previous study [6], the experimental data were fitted by equations based on the assumption that calmodulin either increased V_2 (continuous lines and Eqn. 2) or lowered K_{m2} only (dashed lines and Eqn. 3). However, Fig. 1 makes clear that the data at intermediate concentrations of calmodulin 3 and 6 nM were fitted only by assuming that calmodulin acts solely on V_2. It seems therefore that calmodulin stimulated the Ca^{2+}-ATPase by increasing the turnover of the enzyme, and that activity vs. ATP concentration curves performed only on the fully calmodulin-bound and the

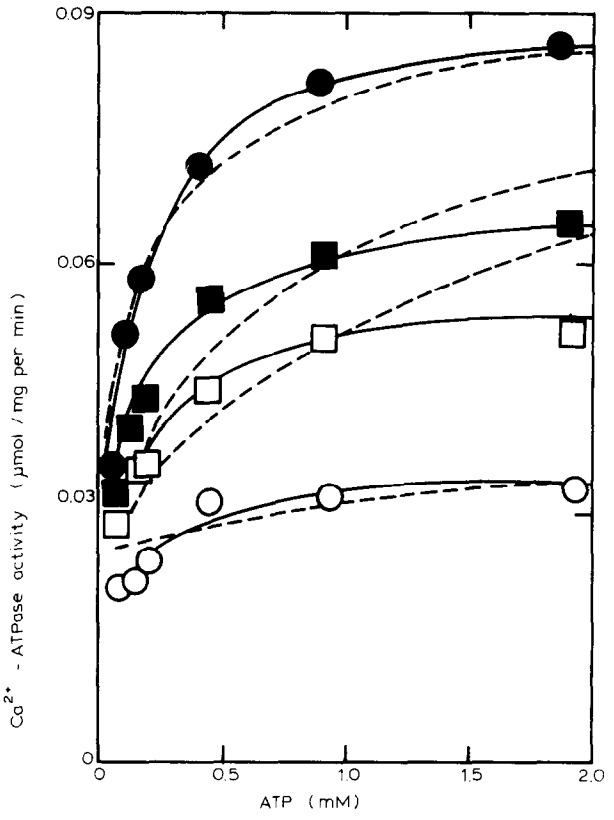

Fig. 1. Ca^{2+}-ATPase activity as a function of ATP concentration in media with 0 (○), 3 (□), 6 (■) and 60 nM (●) calmodulin.
The continuous lines represent:

$$v = V_1 + \frac{V_{2a} + \frac{V_{2c}}{1+(K_c/[\mathrm{CAM}])}}{1+(K_{m2}/[\mathrm{ATP}])} \quad (2)$$

where CAM is calmodulin, V_1 has the same meaning as in Eqn. 1 and K_c is an apparent dissociation constant for calmodulin. The value of V_2 goes from V_{2a} to $V_{2a} + V_{2c}$ as [CAM] goes from zero to infinity. The best-fitting values for each parameter (±S.E.) were: $V_1 = 0.0075 \pm 0.0041$ μmol/mg per min; $V_{2a} = 0.0283 \pm 0.0048$ μmol/mg per min; $K_c = 5.6 \pm 0.5$ nM; $V_{2c} = 0.0617 \pm 0.0125$ μmol/mg per min; $K_{m2} = 132 \pm 21$ μM.
The dashed lines represent:

$$v = V_1 + \frac{V_2}{1+\frac{K_{m2a}+(K_{m2c}/[1+([\mathrm{CAM}]/K_c)])}{[\mathrm{ATP}]}} \quad (3)$$

The value of K_{m2} goes from $K_{m2a} + K_{m2c}$ to K_{m2a} as [CAM] goes from zero to infinity, the rest of the parameters have the same meaning as in Eqn. 2.

fully calmodulin-free Ca^{2+}-ATPase could be misleading when used to measure kinetic parameters.

The view that calmodulin affects V_2 and not K_{m2} is further strengthened by results in Fig. 2 in which values of K_{m2} and of V_2 of the Ca^{2+}-ATPase were plotted as a function of calmodulin concentration. As the concentration of calmodulin was increased K_{m2} did not change significantly whereas V_2 increased along a curve that was half-maximum at about 6 nM calmodulin.

It can be predicted that if, as suggested by the data in Figs. 1 and 2, the only action of calmodulin were on V_2, ATP should have no effect on the apparent affinity for calmodulin as activator of the Ca^{2+}-ATPase. This was tested by measuring Ca^{2+}-ATPase activity as a function of calmodulin concentration in media containing either 0.1 or 2 mM ATP. Results in Fig. 3 show that the concentrations of calmodulin for half-maximal activation (±S.E.) were 9.7 ± 5.1 nM and 7.1 ± 3.1 nM in the presence of 0.1 and 2 mM ATP, respectively. The mean values of $K_{0.5}$ (±S.E.) from three independent experiments like that from Fig. 3 were 6.2 ± 2.4 nM and 7.6 ± 1.9 nM at 0.1 an 2 mM ATP, respectively. Hence, binding of ATP at the low affinity site had no effect on the apparent affinity of the Ca^{2+}-ATPase for calmodulin, a result that necessarily implies that calmodulin should not affect K_{m2}. Results in Fig. 3 also show that in the presence of 0.1 mM ATP calmodulin increased the activity about 70% whereas in the presence of 2 mM ATP activation by calmodulin was about 140%. This is in keeping with the idea that binding of ATP at the low-affinity site increases V_2 in a calmodulin-dependent fashion.

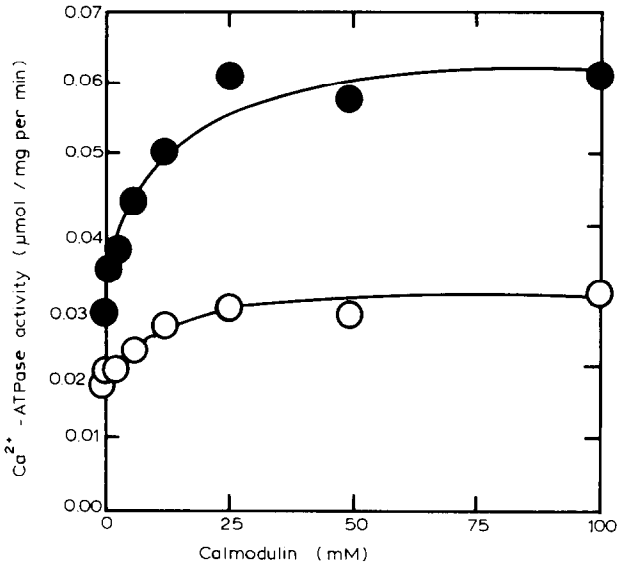

Fig. 3. Ca^{2+}-ATPase activity as a function of calmodulin concentration in media with 0.1 (○) or 2 (●) mM ATP. The continuous lines represent the following equation:

$$v = V_0 + \frac{V_c}{1 + (K_c/[\mathrm{CAM}])} \quad (4)$$

where V_0 is the activity in the absence of calmodulin, V_c is the maximum activating effect of calmodulin and the other parameters and symbols have the meaning given in Eqn. 2. The best-fitting value of the parameters (±S.E.) was: $V_0 = 0.018 \pm 0.001$ μmol/mg per min; $V_1 = 0.013 \pm 0.002$ μmol/mg per min; $K_c = 9.7 \pm 5.1$ nM for 0.1 mM ATP and $V_0 = 0.027 \pm 0.003$ μmol/mg per min; $V_1 = 0.037 \pm 0.004$ μmol/mg per min; $K_c = 7.1 \pm 3.1$ nM for 2 mM ATP.

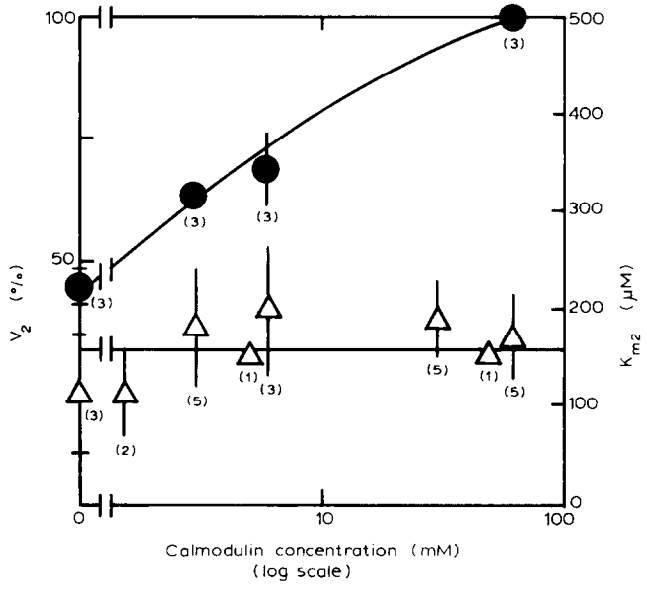

Fig. 2. Values of K_{m2} (±S.E.) (△) and V_2 (±S.E.) (●) as a function of calmodulin concentration. The values of K_{m2} and V_2 were estimated from activity vs. ATP concentration curves at different calmodulin concentrations using Eqn. 1. Numbers in parenthesis are the number of experiments. The value of V_2 considered to be 100% was 0.043 ± 0.005 μmol/mg per min.

After these findings we reexamined the effects of compound 48/80 on the activation of the enzyme by ATP. For this purpose the values of K_{m2} and V_2 were estimated measuring Ca^{2+}-ATPase activity as a function of ATP concentration from 0.1 to 4 mM in the absence and in the presence of 14.2 μg/ml of compound 48/80. All media contained 60 nM calmodulin. Results showed that upon the addition of 48/80, K_{m2} increased from 141 to 2949 μM whereas V_2 decreased from 0.075 to 0.050 μmol/mg protein per min only. The fact that, in contrast with what was already shown for calmodulin, 48/80 was much more effective on K_{m2} than on V_2 is in keeping with our earlier results and suggests that 48/80 modifies Ca^{2+}-ATPase activity acting directly on the enzyme molecule (see also Ref. 7).

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