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Biochimica et Biophysica Acta 1240 (1995) 115–117

BBA Biochimica et Biophysica Acta

Rapid report

Alteration by EGTA of the human red cell Ca^{2+}-ATPase

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Received 28 July 1995; accepted 4 August 1995

Abstract

The effect of EGTA on Ca^{2+}-ATPase activity was studied in fragmented membranes and solubilized preparations from human red cells. A dual action was found. At low concentrations (0.1–1 mM), EGTA increased Ca^{2+} affinity without affecting V_{max}. By contrast, at high concentrations (5–10 mM), EGTA was inhibitory. Both effects were partially reversible.

Keywords: ATPase, Ca^{2+}-; Modulation by EGTA; Activation by EGTA; EGTA; Erythrocyte; (Human)

The ATPase activity of Ca^{2+} pumps is routinely assayed in the presence of EGTA. This compound, however, seems to modify some kinetic parameters of the Ca^{2+}-ATPase. Thus, the affinity for Ca^{2+} of the human red cell enzyme is raised by several orders of magnitude when measured in the presence of EGTA [1–4]. A similar effect also has been described for the sarcoplasmic reticulum Ca^{2+}-ATPase [5].

With the interest of confirming and to extend above findings, the action of EGTA on the Ca^{2+}-ATPase activity of human red cells was studied over a wide concentration range.

Two enzyme preparations were used in the present work. First, a membrane-bound enzyme, freed of CaM. This was prepared from fresh blood (mainly O(+) group) by fragmentation of erythrocyte ghosts after two cycles of freezing and thawing, as described earlier [6]. Second, a high-Ca^{2+} affinity enzyme, which was obtained in solubilized form from outdated red cells (3–4 weeks bank-stored blood, mainly O(+) group). It was assumed that the Ca^{2+}-ATPase will not bind to a CaM-affinity column if lacking the CaM-binding domain. As the red cell ages in vitro the total Ca^{2+} content should rise to levels capable of activating endogenous calpain. This in turn would cleave its primary target, the Ca^{2+}-ATPase, and remove the CaM-binding domain [7]. Alternatively, the enzyme may be unable to bind to the affinity column if already possessing tightly-bound CaM.

Accordingly, red cell ghosts (about 5 mg protein/ml) were extensively washed with EDTA to remove bound CaM, and then solubilized (about 5 mg protein/ml) with 0.5% Triton X-100 in a medium containing (mM): KCl, 130; $MgCl_2$, 0.5; $CaCl_2$, 0.05; Hepes (pH 7.4), 20; to which 0.5 mg/ml phosphatidylcholine (PC) was also added [8]. The solubilized material was consecutively passed through two CaM-sepharose columns, and the enzyme in the eluent, incapable of binding to the CaM-column, was reconstituted into mixed PC micelles by removing Triton with SM-2 beads (2 g/ml) [9].

Both enzyme preparations were exposed to EGTA only during Ca^{2+}-ATPase assays, unless stated otherwise. Ca^{2+}-ATPase was determined after 2 h incubation at 37°C, in a medium containing (mM): KCl, 130; $MgCl_2$, 2; ATP-Tris, 2; ouabain, 0.1; Tris-HCl (pH 7.55 at room temperature), 10; in the presence of 0.1–10 mM EGTA and sufficient $CaCl_2$ to give 0.05–10 μM free Ca^{2+} levels. ATP hydrolysis was linear with time for up to 2 h. Ca^{2+}-ATPase activity was obtained by subtracting from the activity in the presence of Ca^{2+} that in its absence. It was related to the total amount of protein contained in either membrane fragments or solubilized Triton extracts and expressed as μmol P_i/mg protein per h. Protein was determined by the method of Lowry et al. [10], using bovine serum albumin as standard. Free Ca^{2+} levels were calculated from a computer based program [11]. The re-

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SSDI 0005-2736(95)00206-5

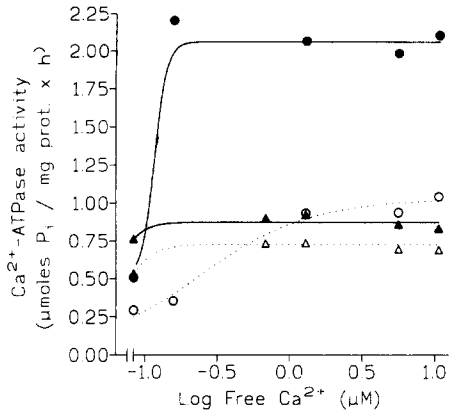

Fig. 1. A high-Ca^{2+} affinity ATPase from outdated red cells. Both membrane-bound Ca^{2+}-ATPase, obtained from fresh human blood, and solubilized enzyme, prepared from outdated bank blood (see the text) were assayed for ATPase activity in the presence of 1 mM EGTA. Ca^{2+}-ATPase was determined after 2 h incubation at 37°C, in a medium containing (mM): KCl, 130; $MgCl_2$, 2; ATP-Tris, 2; ouabain, 0.1 mM; Tris-HCl (pH 7.55 at room temperature), 10; and sufficient $CaCl_2$ to give 0.05–10 μM free Ca^{2+} levels. The graph shows collected results from two experiments done on fragmented membranes (circles) and solubilized preparations (triangles), in the absence (empty symbols) and presence of 1 μg/ml CaM (filled symbols).

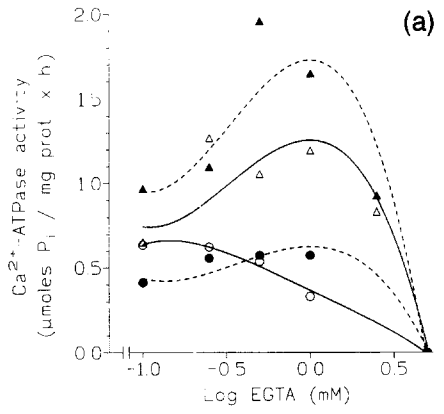

Fig. 3. Inhibitory action of EGTA. (a) The Ca^{2+}-ATPase activity of fragmented membranes (triangles) and solubilized preparations (circles) was systematically studied over 0.1–10 mM EGTA concentration range, keeping the free Ca^{2+} level constant either at 1 μM (empty symbols, solid lines) or 5 μM (filled symbols, dashed lines). Collected results from two experiments are shown. (b) The action of EGTA on the V_{max} of both membrane-bound enzyme (squares) or solubilized preparations assayed either in the presence (triangles) or absence of 1 μg/ml CaM (circles). Collected results from at least four experiments are presented. Vertical bars represent ± 1 S.D. of mean.

sults presented are the average value of two to four experiments ± 1 S.D. Kinetic parameters were obtained from linear regression analyses of Eadie-Hofstee plots, using a GPAD INPLOT software. The curves shown were drawn by non-linear regression fitting of experimental data using the above software. A preliminary account of this work was previously presented [12].

Fig. 1 shows data taken from assays done with 1 mM EGTA on fragmented membranes and solubilized preparations, in the absence and presence of 1 μg/ml CaM. As expected, the solubilized enzyme showed a high-Ca^{2+} affinity (the apparent $K_m(Ca^{2+})$ measured in three experiments being 0.04 ± 0.03 μM), after passing through CaM-affinity columns. Addition of CaM had very little effect on this preparation, being only about 20% stimulatory at any Ca^{2+} concentration tested, thus indicating that activity was maximal. By contrast, CaM stimulated the membrane-bound enzyme by at least 120% at saturating Ca^{2+} levels and decreased the apparent $K_m(Ca^{2+})$. The latter reached a value of 0.25 ± 0.13 μM in three similar experiments. These findings demonstrate that the solubilized enzyme is in a high-Ca^{2+} affinity state.

In contrast with published findings [1,4], addition of EGTA to membrane fragments has two effects. First, it increases Ca^{2+} affinity. Thus, the apparent $K_m(Ca^{2+})$ being 0.56, 0.35 and 0.03 μM after incubating with 0.5, 1 and 2.5 mM EGTA, respectively (Fig. 2). Secondly, V_{max} is inhibited by about 40% by raising EGTA concentration to 2.5 mM. Essentially similar results were found with the solubilized enzyme, regardless the presence of CaM (see

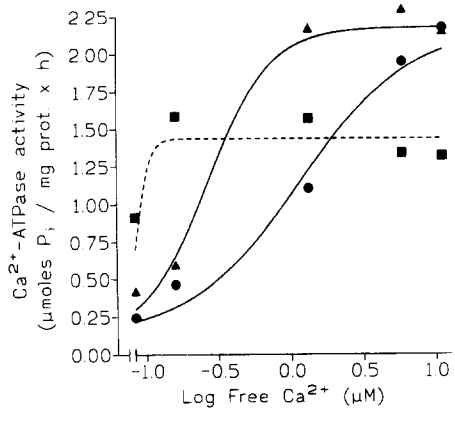

Fig. 2. The dual action of EGTA. The activity of membrane bound Ca^{2+}-ATPase was measured as indicated in legend to Fig. 1, but in the presence of 0.5 (circles); 1 (triangles) and 2.5 mM EGTA (squares). Notice a shift to the left of the activity-vs.-concentration curve and the decrease in V_{max} by raising EGTA to 2.5 mM. Results from typical experiments are shown.

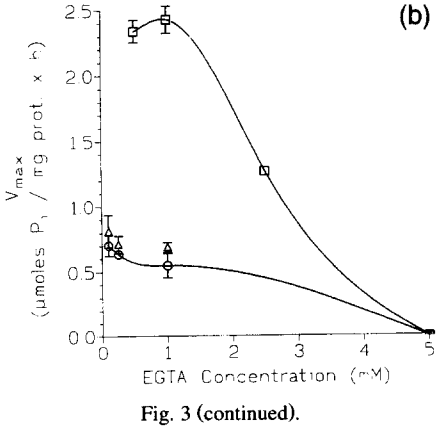

Fig. 3 (continued).

below). Thus, by raising EGTA from 0.1 to 1 mM, the apparent $K_m(Ca^{2+})$ decreased from 130 to 40 nM (results not shown) and V_{max} was reduced by about 25% (see below).

The inhibitory action of EGTA was systematically studied over a wide concentration range (0.1 to 10 mM), keeping the free Ca^{2+} level constant either at 1 or 5 μM. Both membrane-bound and solubilized enzymes were fully inhibited by increasing EGTA at or above 5 mM (Fig. 3a). Such an inhibition appears to be overcome to some extent by increasing free Ca^{2+}, thus suggesting a competition between Ca^{2+} and EGTA for their actions.

The results of EGTA on V_{max} of membrane-bound and solubilized Ca^{2+}-ATPase with and without CaM are summarized in Fig. 3b. EGTA is equally effective on both preparations: half-maximal reduction being attained with about 2.5 mM. This effect of EGTA is in striking contrast with its action on Ca^{2+} affinity, which appeared more marked on the solubilized preparation. Thus, the apparent $K_m(Ca^{2+})$ of the membrane-bound enzyme was half-maximally reduced by about 0.7 mM EGTA whilst a similar change was attained on the solubilized preparation with only 0.15 mM EGTA (results not shown).

The inhibitory effect of EGTA was partially reversible. Preincubation of membrane fragments with 5 mM EGTA for 1 h at 37°C, followed by extensive washing with EGTA-free solutions, rendered an enzyme preparation with nearly half activity of control. Thus, in four experiments using 0.5 mM EGTA in the assay medium, the Ca^{2+}-ATPase activity (in μmol P_i/mg protein per h) of control preparations (without preincubation but extensively washed) was 0.70 ± 0.077 and 0.71 ± 0.17 at 1 and 5 μM free Ca^{2+}, whilst the corresponding activity for EGTA-treated fragments was 0.39 ± 0.077 and 0.40 ± 0.065, respectively.

On the other hand, the effect of EGTA on Ca^{2+} affinity was also partially reversible. Thus, when membrane fragments were preincubated with 1 mM EGTA for 1 h at 37°C and then extensively washed, the apparent $K_m(Ca^{2+})$ was nearly a half of control values when assessed under similar conditions as above (results not shown).

The preceding results show that EGTA alters Ca^{2+} affinity and V_{max} of both membrane-bound and solubilized enzymes in a concentration dependent way. At low concentrations (0.1–1 mM), EGTA increased Ca^{2+} affinity without affecting V_{max} whilst it was markedly inhibitory at or above 5 mM. It seems possible that rather than eliciting two distinct effects, EGTA may increase the affinity for Ca^{2+} of the pump binding sites. Binding of Ca^{2+} to the low-affinity site would inhibit the ATPase reaction by facilitating reversal, as demonstrated for the action of DMSO on IOV's active Ca^{2+} transport [13].

It is well known that the Ca^{2+}-ATPase undergoes alternate E_1-E_2 conformational changes during catalysis. It is tempting to speculate that freezing of the E_2 conformation may be the basis of the inhibitory action of EGTA.

Finally, the effect of EGTA on both V_{max} and $K_m(Ca^{2+})$ of the solubilized enzyme was independent of the presence of CaM, showing that the CaM binding domain is not involved in these effects. This is in sharp contrast with previous findings on the Ca^{2+}-ATPase of human red cells [2,3].

This work was supported by Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICIT (Grant S1-1899) and CDCH of Universidad Central de Venezuela (Grant 030-10-0029-93).

References

[1] Schatzmann, H.J. (1973) J. Physiol. 235, 551–569.
[2] Sarkadi, B., Schubert, A. and Gárdos, G. (1979) Experientia 35, 1045–1047.
[3] Al-Jobore, A. and Roufogalis, B.D. (1981) Biochim. Biophys. Acta 645, 1–9.
[4] Rossi, J.P.F.C. and Rega, A. (1989) Biochim. Biophys. Acta 996, 153–159.
[5] Berman, M.C. (1982) J. Biol. Chem. 257, 1953–1957.
[6] Romero, P.J. and Weitzman, C. (1991) Biochem. Biophys. Res. Commun. 181, 208–212.
[7] Molinari, M., Anagli, J. and Carafoli, E. (1994) J. Biol. Chem. 269, 27992–27995.
[8] Niggli, V., Penniston, J.T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958.
[9] Lévy, D., Bluzat, A., Seigneuret, M. and Rigaud, J.-L. (1990) Biochim. Biophys. Acta 1025, 179–190.
[10] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[11] Fabiato, A. and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505.
[12] Romero, P.J. and Salas, V. (1993) J. Physiol. (Lond) 473, 225P.
[13] Romero, P.J. (1992) Cell Calcium 13, 659–667.