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Similarities between the effects of dimethyl sulfoxide and calmodulin on the red blood cell Ca^{2+}-ATPase

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The Ca^{2+}-ATPase of the erythrocyte plasma membrane can be activated by calmodulin, acidic phospholipids, limited proteolysis and self-association. Recently, it has been shown that different organic solvents increase both the V_{max} and the Ca^{2+} affinity of the enzyme (Benaim, G. and De Meis, L. (1989) FEBS Lett. 244, 484–486). In this report the effects of calmodulin and dimethyl sulfoxide (20%, v/v) on the Ca^{2+}-ATPase are compared. Dimethyl sulfoxide also elicits the appearance of the low-affinity ATP binding site, which in this enzyme is strictly dependent on calmodulin. Dimethyl sulfoxide increases the Ca^{2+} affinity of the enzyme in a manner similar to that observed with the use of calmodulin and of acidic phospholipids. This was tested using both native and partially trypsinized ATPase. When activated by calmodulin the enzyme is inhibited by compound 48/80, trifluoperazine and calmidazolium. When activated by dimethyl sulfoxide the enzyme is still inhibited by calmidazolium but is no longer inhibited by either compound 48/80 or trifluoperazine. Activation of the ATPase promoted by either calmodulin or dimethyl sulfoxide is abolished when the Ca^{2+} concentration is raised from 10 μM to 2 mM. The effect of dimethyl sulfoxide is also abolished by 20 mM P_i. In the presence of 1 to 10 mM Ca^{2+} the ATPase catalyzes an ATP $\rightleftharpoons$ P_i exchange. The rate of exchange increases several fold when dimethyl sulfoxide is included in the assay medium.

Introduction

The calcium-pumping ATPase of the erythrocyte plasma membrane is responsible for calcium homeostasis in these cells [1] This enzyme can be activated by the calcium calmodulin complex [2,3], by acidic phospholipids and long-chain, polyunsaturated fatty acids [4,5], by limited proteolysis [6–9], and by self-association [10,11] Data from different laboratories [12–15] indicate that the binding of calmodulin to the regulatory domain of the enzyme is mediated by hydrophobic interactions Modulation of the enzyme by acidic phospholipids or by self-association also involves hydrophobic interactions [16] These findings led us to investigate the effect of organic solvents on the Ca^{2+}-ATPase from red blood cells [17] In a previous work it was shown that different organic solvents increase both the V_{max} and the Ca^{2+} affinity of the enzyme to values that are similar to those attained with calmodulin [17] The effect of calmodulin was best mimicked with the use of 20% (v/v) dimethyl sulfoxide In this report we compared the effects of dimethyl sulfoxide and calmodulin on the apparent affinity of the enzyme for ATP, on the trypsinized ATPase, on the action of calmodulin antagonists and on the ATP $\rightleftharpoons$ P_i exchange reaction

Methods

Calmodulin was isolated from bovine brain by phenyl-Sepharose chromatography [18,19] Human erythrocyte membranes deficient in calmodulin were prepared from recently outdated human blood [20]

The erythrocyte Ca^{2+}-ATPase was purified by affinity chromatography using a calmodulin affinity column [8] Routinely, 0 5–0 6 mg of ATPase was obtained from 500–600 mg of ghost protein. The purified ATPase was stored under N_2 at −173° C at a concentration of 100–200 μg/ml, in a buffer containing 0 04% Triton X-100, 130 mM KCl, 20 mM Hepes-KOH (pH 7 4), 2

Abbreviations Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, EGTA, (ethylenebis-(oxyethylenenitrilo))tetraacetic acid, Mops, 3-(*N*-morpholino)propanesulfonic acid

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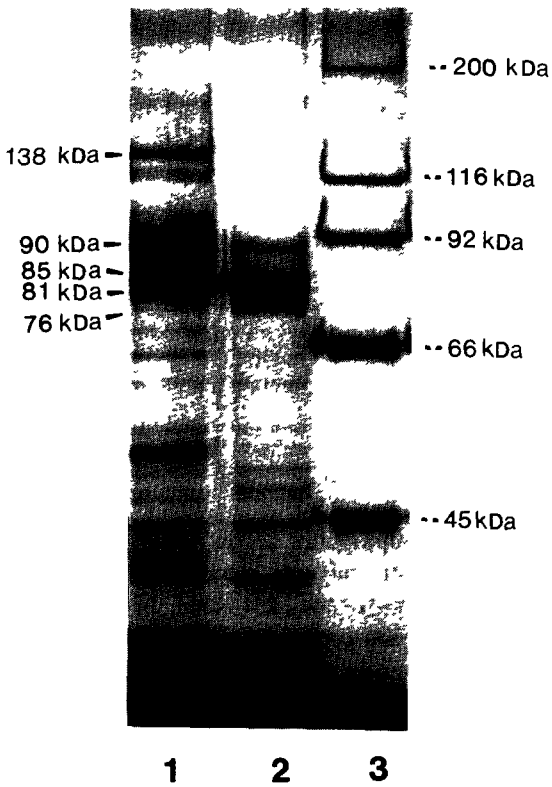

Fig 1 Electrophoresis of the trypsinized Ca^{2+}-ATPase The purified ATPase (100–200 μg/ml) was treated with trypsin (50 μg/ml) at 4°C The digestion was arrested by the addition of 10-fold excess soybean trypsin inhibitor after 10 min (lane 1) and 30 min (lane 2) Lane 3 are standards myosin (200 kDa), β-galactosidase (116 2 kDa), phosphorylase *b* (92 5 kDa), bovine serum albumin (66 2 kDa) and ovalbumin (45 kDa)

mM EDTA, 2 mM $MgCl_2$, 50 μM $CaCl_2$, 2 mM dithiothreitol, 5% glycerol (v/v) and 0 5 mg/ml phosphatidylcholine

$^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and purified as previously described [21]

[γ-^{32}P]ATP was prepared according to the method of Glynn and Chappell [22] ATPase activity was assayed by measuring the release of P_i from [γ-^{32}P]ATP at 35°C The reaction was quenched with two vol of a suspension of activated charcoal in 0 1 M HCl [23] After centrifugation, aliquots of the supernatant containing [^{32}P]P_i were counted in a liquid scintillation counter ATP ⇌ P_i exchange was determined by measuring the incorporation of [^{32}P]P_i into ATP [21] Free calcium concentrations were calculated as described by Fabiato and Fabiato [24], taking into account the concentration of ATP and $MgCl_2$ in the media and using the dissociation constant reported by Schwartzenbach et al [25] for the Ca-EGTA complex

Controlled trypsin proteolysis of the purified enzyme and SDS-electrophoresis were performed as previously described [8,26] The digestions were carried out at 4°C for 30 min Trypsin (50 μg/ml) was added to aliquots containing 100–200 μg/ml of purified enzyme suspended in the same buffer solution in which the enzyme was stored The digestion was arrested by addition of 10-fold excess soybean trypsin inhibitor After 10 min digestion (Fig 1, lane 1) a small part of the ATPase was not digested (138 kDa) and a 90 kDa part was the dominant fragment After 30 min digestion (Fig 1 lane 2), the main polypeptides of high molecular mass were those of 85 and 81 kDa which are not stimulated by calmodulin [8,26] The 90 kDa fragment which still can be activated by calmodulin and the 76 kDa peptide were still visible as faint bands Trypsin and soybean trypsin inhibitor were purchased from Sigma Chemical Co The protein concentration was determined by the method of Lowry et al [27,28], using bovine serum albumin as standard

Results

ATP dependence

Previous reports have shown that calmodulin increases the apparent affinity of the enzyme for ATP and also elicits the appearance of a second, low-affinity (regulatory) binding site for ATP [29,30] We now show that both of these effects can also be induced by dimethyl sulfoxide (Fig 2) The concentration of organic solvent selected was that shown to maximally activate the enzyme in a previous work [17] The effects of calmodulin and dimethyl sulfoxide are not additive, since the same ATP dependence was observed in the presence of either calmodulin, dimethyl sulfoxide or calmodulin plus dimethyl sulfoxide

The degree of stimulation by calmodulin and dimethyl sulfoxide varied among the different enzyme

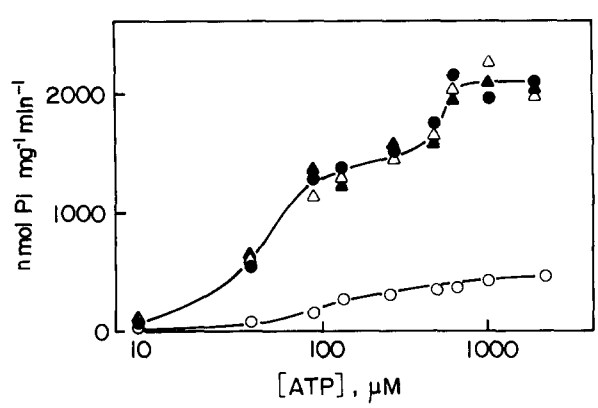

Fig 2 Effect of dimethyl sulfoxide on the enzyme affinity for ATP The assay medium contained 50 mM Mops-Tris buffer (pH 7 4), 100 mM KCl, 10 mM $MgCl_2$ 1 mM EGTA and 1 05 mM $CaCl_2$ The calculated free Ca^{2+} concentration was 10 μM The reaction was started by the addition of enzyme to a final concentration of 1–2 μg per ml and quenched after 30 min at 35°C ○, no additions, ●, 4 μg/ml calmodulin, △, 20% (v/v) dimethyl sulfoxide, ▲, 4 μg/ml calmodulin plus 20% (v/v) dimethyl sulfoxide

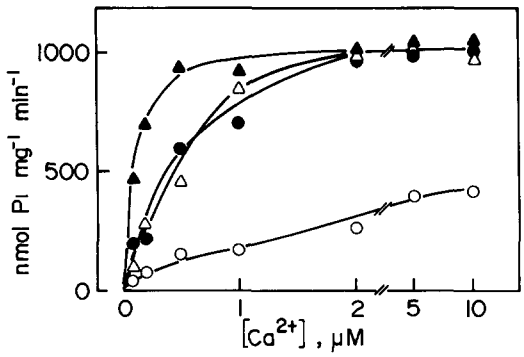

Fig 3 Effects of dimethyl sulfoxide and trypsin proteolysis on the enzyme affinity for Ca^{2+} Proteolysis was carried out for 30 min as described in Methods and shown in Fig 1 lane 2 After proteolysis the enzyme was no longer activated by calmodulin (data not shown) Conditions and assay media composition were as in Fig 2 except that the ATP concentration was 200 μM and different $CaCl_2$ concentrations were added to obtain the free Ca^{2+} concentrations shown in the figure ○, untreated enzyme, ●, trypsinized enzyme, △, untreated enzyme plus 20% (v/v) dimethyl sulfoxides, ▲, trypsinized enzyme plus 20% (v/v) dimethyl sulfoxide

preparations tested However, in each preparation the increment in V_{max} elicited by calmodulin was the same as that elicited by dimethyl sulfoxide

Ca^{2+} affinity

Calmodulin increases the enzyme affinity for Ca^{2+} [29] An increase in affinity is also observed after controlled proteolysis of the ATPase [31] In agreement with previous reports [8,31,32] it was found that after 30 min proteolysis (Fig 1, lane 2) the enzyme is not further stimulated by calmodulin (data not shown) The effects of calmodulin and proteolysis can be mimicked by addition of dimethyl sulfoxide to the medium (Fig 3) In a previous report [17] it was shown that the effects of calmodulin and dimethyl sulfoxide on the calcium affinity of the enzyme are additive We now show that an additive effect of the solvent is also observed with the trypsinized enzyme (Fig 3) dimethyl sulfoxide, but not calmodulin [31,33], further increases the Ca^{2+} affinity of the trypsinized ATPase

Anti-calmodulin drugs

Trifluoperazine and calmidazolium inhibit the plasma membrane Ca^{2+}-ATPase both in the absence (basal activity) and in the presence of calmodulin. These drugs also inhibit the ATPase activated by either proteolysis or acidic phospholipids [35,36] Compound 48/80 impairs only the activation promoted by calmodulin, it has no effect on the basal activity [37,38]

Here we show that when activated by dimethyl sulfoxide, the enzyme is no longer inhibited by either trifluoperazine or by compound 48/80 (Figs 4 and 5) With the use of trifluoperazine a small but significant activation, compared to the control containing dimethyl sulfoxide but not trifluoperazine, was observed when

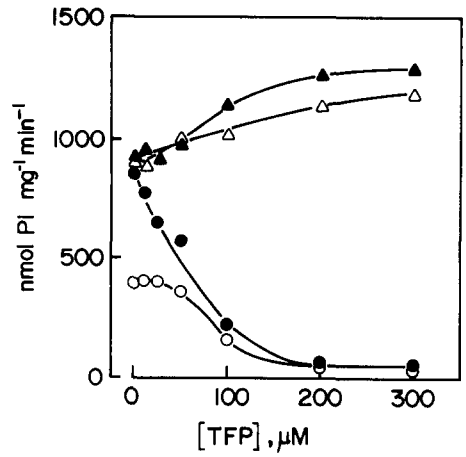

Fig 4 Inhibition by trifluoperazine (TFP) The assay media composition and experimental conditions were as in Fig 2 The ATP concentration was 200 μM ○, no addition, ●, 4 μg/ml calmodulin, △, 20% (v/v) dimethyl sulfoxide, ▲, 4 μg/ml calmodulin plus 20% (v/v) dimethyl sulfoxide

dimethyl sulfoxide was included in the assay medium (Fig 4) Contrasting with these findings, the inhibitory activity of calmidazolium was not suppressed in the presence of dimethyl sulfoxide (Fig 6)

The data of Figs 4 to 6 indicate that the drugs tested interact with different regions of the enzyme molecule

Effects of P_i and high Ca^{2+} concentration

The activation of the ATPase activity promoted by dimethyl sulfoxide is antagonized by P_i (Fig 7) and by high Ca^{2+} concentrations (Fig 8) An inhibition of the ATPase activity is observed when the Ca^{2+} concentration of the medium is raised to the millimolar range [29] In the presence of calmodulin, the inhibition curve is shifted to lower Ca^{2+} concentrations [29,39] A similar effect is observed when the enzyme is activated by

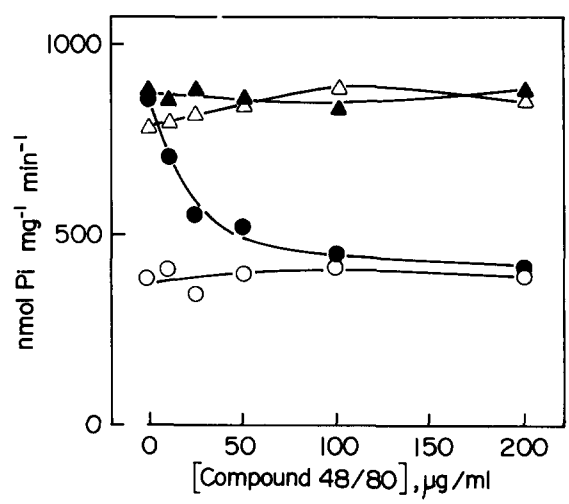

Fig 5 Inhibition by compound 48/80 The assay media composition and experimental conditions were as in Fig 2 but with 200 μM ATP ○, no additions, ●, 4 μg/ml calmodulin, △, 20% (v/v) dimethyl sulfoxide, ▲, 4 μg/ml calmodulin plus 20% (v/v) dimethyl sulfoxide

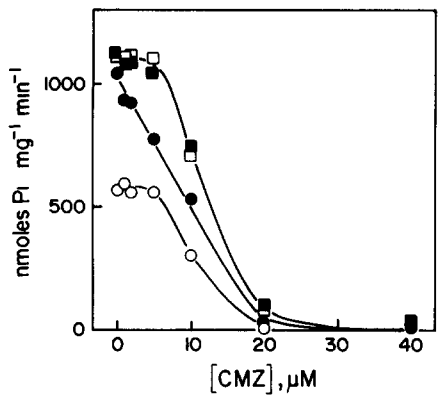

Fig 6 Inhibition by calmidazolium (CMZ) The assay media composition and experimental conditions were as in Fig 2 but with 200 μM ATP ○, no additions, ●, 4 μg/ml calmodulin, □, 20% (v/v) dimethyl sulfoxide, ■, 4 μg/ml calmodulin plus 20% (v/v) dimethyl sulfoxide

dimethyl sulfoxide Under the conditions of Fig 8, the calcium concentration needed for half-maximal inhibition of the ATPase activity in the absence of calmodulin was 7 0 mM This value decreased to 1 6 mM on addition of calmodulin and to 1 0 mM when 20% (v/v) dimethyl sulfoxide was present in the medium

ATP ⇌ P_i exchange

In previous reports [10,40] it has been shown that the Ca^{2+}-ATPase catalyzes a rapid ATP ⇌ P_i exchange when the Ca^{2+} concentration is raised to the millimolar range During this exchange, the enzyme catalyzes simultaneously the hydrolysis of ATP and its synthesis from ADP and P_i We now show that the rate of ATP synthesis increases when dimethyl sulfoxide is included in the assay medium (Figs 8B and 9B) The concentrations of Ca^{2+} and P_i used were not sufficient to reach saturation Higher concentrations of these ions could not be tested due to the formation of calcium phosphate precipitate Thus, from the data of Figs 8 and 9 it is not possible to ascertain whether dimethyl sulfoxide in-

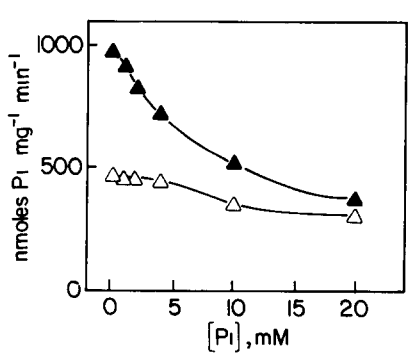

Fig 7 Effect of P_i The assay media composition and experimental conditions were as in Fig 2 but with 200 μM ATP △, No additions, ▲, 20% (v/v) dimethyl sulfoxide

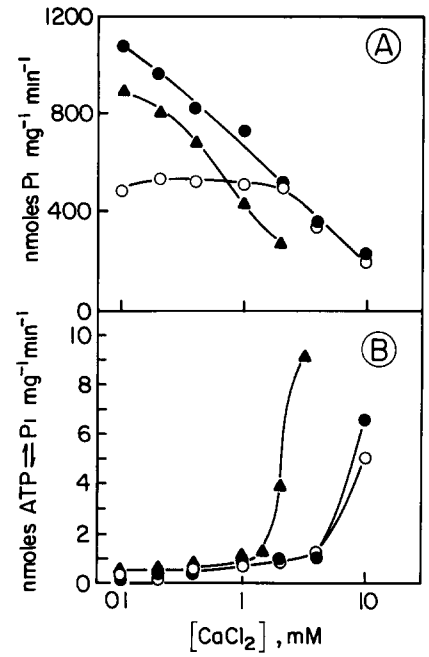

Fig 8 Effect of high calcium concentrations on the rates of ATP hydrolysis and of ATP ⇌ P_i exchange The assay media contained 50 mM Mops-Tris buffer (pH 7 0), 100 mM KCl, 2 mM $MgCl_2$, 50 μM ADP, 2 mM P_i, 200 μM ATP, and different $CaCl_2$ concentrations The reaction was started by the addition of ATPase to a final concentration of 2–4 μg/ml and arrested after 10 min incubation at 35 °C (A) ATPase activity [γ-^{32}P]ATP and non-radioactive P_i were used The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 8% (w/v) (B) ATP ⇌ P_i exchange [^{32}P]P_i and non-radioactive ATP were used and the reaction arrested with a suspension of activated charcoal in 0 1 M HCl ○, no additions, ■, 4 μg/ml calmodulin, ▲, 20% (v/v) dimethyl sulfoxide

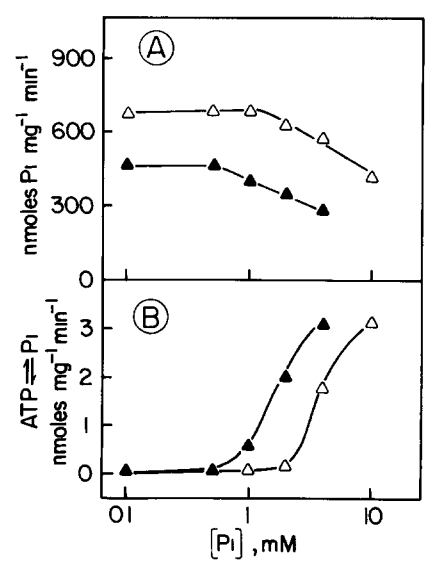

Fig 9 Effect of P_i on the rates of ATP hydrolysis and of ATP ⇌ P_i exchange The assay media contained 50 mM Mops-Tris buffer (pH 7 0), 100 mM KCl, 2 mM $MgCl_2$, 50 μM ADP, 2 mM $CaCl_2$, 200 μM ATP and the P_i concentrations shown in the figure Other conditions were as in Fig 8 (A) ATPase activity, (B) ATP ⇌ P_i exchange △, no additions, ▲, 20% (v/v) dimethyl sulfoxide

creases the V_{max} of exchange or decreases the concentrations of Ca^{2+} and P_i needed for half-maximal exchange

Discussion

Two different K_m values for ATP are found in both the Ca^{2+}-ATPase of sarcoplasmic reticulum [41] and the $(Na^+ + K^+)$ ATPase of plasma membranes [42] The first K_m refers to the binding of ATP to the catalytic site of these enzymes The second K_m, detected at higher ATP concentration (0 05 to 0 20 mM), promotes an increase in the V_{max} and is thought to reflect the binding of ATP to a regulatory site of these enzymes For the erythrocyte calcium pump the second K_m for ATP previously has only been detected in the presence of calmodulin [29,30] In this work, using purified enzyme, we show that addition of dimethyl sulfoxide also promotes the appearance of the second K_m for ATP This finding supports the proposal that organic solvents can mimic the activation by calmodulin of the plasma membrane Ca^{2+}-ATPase

Peptides with distinct affinities for Ca^{2+} can be obtained depending on the conditions used for proteolysis of the ATPase [31] One of the peptides (81 kDa) has a higher affinity for Ca^{2+} than the native enzyme and is not activated by calmodulin, but its affinity for Ca^{2+} can be further increased by acidic phospholipids The 76 kDa tryptic fragment has a higher Ca^{2+} affinity than either the native enzyme or the 81 kDa fragment, and is no longer activated by either calmodulin or acidic phospholipids The dominant peptides attained in the proteolysis conditions used were those with molecular weights of 81 and 85 kDa (Figs 1 and 3) Dimethyl sulfoxide can further increase the Ca^{2+} affinity of the trypsinized ATPase to a level similar to that attained with acidic phospholipids [31,33] Thus, it seems that organic solvent interacts with different domains of the protein and is able to simulate the effects of both calmodulin and of acidic phospholipids The mechanism by which dimethyl sulfoxide may abolish the inhibitory effects of hydrophobic molecules such as trifluoperazine and compound 48/80 has been discussed in detail in previous reports [43,44] Recently [32,45], it has been shown that phenothiazines bind to the same 9 kDa peptide of the enzyme that interacts with calmodulin [8,26,34]

At present, we do not know why P_i (Fig 7) and high Ca^{2+} concentrations (Fig 8) impair the effect of dimethyl sulfoxide Perhaps this antagonism is related to the reversal of the catalytic cyle of the enzyme In fact, dimethyl sulfoxide was found to favor the synthesis of ATP observed during the ATP $\rightleftharpoons$ P_i exchange reaction (Figs 8 and 9) Reversal of the catalytic cycle of the Ca^{2+}-ATPase from either erythrocytes [46] or sarcoplasmic reticulum [44] is only observed after the enzyme has been phosphorylated by P_i and Ca^{2+} has bound to a low-affinity site on the enzyme (K_m, 1–2 mM) Phosphorylation of both Ca^{2+} transport enzymes by P_i is greatly facilitated by organic solvents such as dimethyl sulfoxide [44,46]

Acknowledgements

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