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Calcium-dependent inhibition of the erythrocyte Ca^{2+} translocating ATPase by carbodiimides

Antonio Villalobo *, James W. Harris and Basil D. Roufogalis

The Laboratory of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, B.C., V6T 1W5 (Canada)

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The ATP hydrolytic activity of the solubilized and purified Ca^{2+}-translocating ATPase from human erythrocyte plasma membrane was strongly inhibited by the nonpolar compound, *N,N'*-dicyclohexylcarbodiimide, both in the presence and in the absence of calmodulin. However, the more water-soluble carbodiimides, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide had little inhibitory effect on the enzyme. The inhibitory effect of *N,N'*-dicyclohexylcarbodiimide was most pronounced at acid pH, and declined sharply at alkaline pH values. In addition, the optimum pH for the enzyme activity also shifted to more alkaline values in the presence of the carbodiimide. Calcium ion appears to favor the inhibition induced by the carbodiimide, in contrast to the observed protection by Ca^{2+} in the sarcoplasmic reticulum Ca^{2+}-translocating ATPase. *N,N'*-Dicyclohexylcarbodiimide also dramatically decreased the stimulatory effect of calmodulin on the activity of the enzyme.

Introduction

The chemical modification of different amino acid residues on enzymes has been of great help in understanding critical cell functions and the mechanism of action of a great variety of enzymes. Several *N,N'*-disubstituted carbodiimides have been widely used as protein carboxyl group(s) modifying reagents, in order to inhibit H^+ translocation coupled to the function of several energy transducing enzymatic complexes (see Ref. 1 for review). In addition, carbodiimides have also been reported to inhibit the Ca^{2+}-translocating ATPase from sarcoplasmic reticulum [1–6]. However, to the best of our knowledge, no information has been published to date on the action of these compounds on the plasma membrane Ca^{2+}-translocating ATPase. The present paper attempts to fill that gap, and reports for the first time such a study on the solubilized and purified Ca^{2+}-translocating ATPase from human erythrocytes. Emphasis will be placed on the similarities and differences between the inhibitory effect of *N,N'*-dicyclohexylcarbodiimide on the two calcium ion translocating ATPases.

Materials and Methods

Chemicals

Bovine brain calmodulin was obtained from

* To whom correspondence should be addressed.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CMCD, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; EEDQ, *N*-ethoxycarbonyl-3-ethoxy-1,2-dihydroquinoline.

Calbiochem (La Jolla, CA). Bovine brain phosphodiesterase 3',5'-cyclic nucleotide activator-Agarose gel, bovine serum albumin (free fatty acid), Triton X-100, dithiothreitol, DCCD, EDAC, CMCD, Hepes, ATP (disodium salt), and EGTA were purchased from Sigma Chem. Co. (St. Louis, MO). Asolectin was purchased from MCB Manufacturing Chemical Inc. (Cincinnati, Ohio), and EDTA from BDH (Toronto). EEDQ was a gift from Professor B. Belleau, McGill University, Montreal. All other chemicals used in this work were of the highest purity available.

Preparation of calmodulin-depleted erythrocyte membranes

The calmodulin-depleted erythrocyte membranes were prepared at 4°C in the presence of 0.1 mM PMSF using a Millipore Pellicon system equipped with filters of 0.5 μm diameter pore size, following the method described in [7,8], modified as follows. 2 units (approx. 500 g) of human packed red cells (4–5 days old), suspended in a citrate-phosphate-dextrose medium containing adenine, were obtained from the Canadian Red Cross and washed three times in isotonic 150 mM KCl/20 mM Tris-HCl (pH 7.4). The washed cells were haemolyzed by vigorous agitation in 11 litres of 10 mM Tris-HCl/2 mM EDTA (pH 7.6). The haemolyzate was passed through the cassette system, operating in the concentration mode, until the volume was reduced to approx. 400 ml. The concentrated membranes were washed with 7–8 litres of 50 mM KCl/10 mM ascorbic acid/5 mM EDTA/5 mM Hepes (pH 7.6), with the casette system operating in the constant volume mode to remove haemoglobin, calmodulin and other soluble proteins. The membranes were further washed, also in constant volume mode, with 5–6 litres of 50 mM KCl/10 mM ascorbic acid/5 mM Hepes (pH 7.6) in order to remove the EDTA. Finally, they were washed with 2 litres of 130 mM KCl/0.5 mM $MgCl_2$/50 μM $CaCl_2$/2 mM dithiothreitol/20 mM Hepes (pH 7.4) and concentrated by centrifugation at $20\,000 \times g_{max}$ for 20 min with the centrifuge brake off. The concentrated membranes (90 ml, containing approx. 800 mg total protein) were quick-frozen in liquid nitrogen (−196°C) and stored at −70°C until use.

Solubilization and purification of the Ca^{2+}-translocating ATPase

The solubilization and purification of the Ca^{2+}-translocating ATPase was performed as previously described [9], except that the calmodulin-Agarose gel column used was larger (17 cm high × 1.4 cm diameter, containing 28 mg covalently-bound calmodulin), and 0.1 mM PMSF was systematically added to the solubilization buffer.

Analytical procedures

The inorganic phosphate liberated to the medium was determined colorimetrically [10]. Protein concentration was determined according to a modification of the method of Lowry et al. [11] after treatment of the sample with 0.05% (w/v) deoxycholic acid and precipitation in a final concentration of 10% (w/v) trichloracetic acid at room temperature, as in Ref. 12, using bovine serum albumin as a standard. Calculation of the concentration of free calcium ion was done by the aid of computer programs as outlined in Ref. 9. Carbodiimide stock solutions were prepared in *N,N'*-dimethylformamide. The solvent, at 1% (v/v) final concentration, was systematically added to the assay system when the carbodiimide was not present, as a control. No inhibitory effect of the solvent by itself was found.

Results

Effects of different carbodiimides on the ATP hydrolytic activity and on the stimulatory effect induced by calmodulin

Three different carbodiimides of different degrees of hydrophobicity and molecular bulk were employed in these experiments. Fig. 1A shows the inhibition of the ATP hydrolytic activity of the enzyme by increasing concentrations of carbodiimide. The strongly hydrophobic carbodiimide, DCCD, inhibited the Ca^{2+}-ATPase activity with a half maximum concentration between 0.1 and 0.2 mM, both in the presence and in the absence of calmodulin. However, little inhibition was found when the moderately hydrophobic CMCD was employed in the absence of calmodulin, and the inhibition was only slightly increased in the presence of calmodulin. Even less inhibition was found with the water-soluble EDAC (see Fig. 1A). The

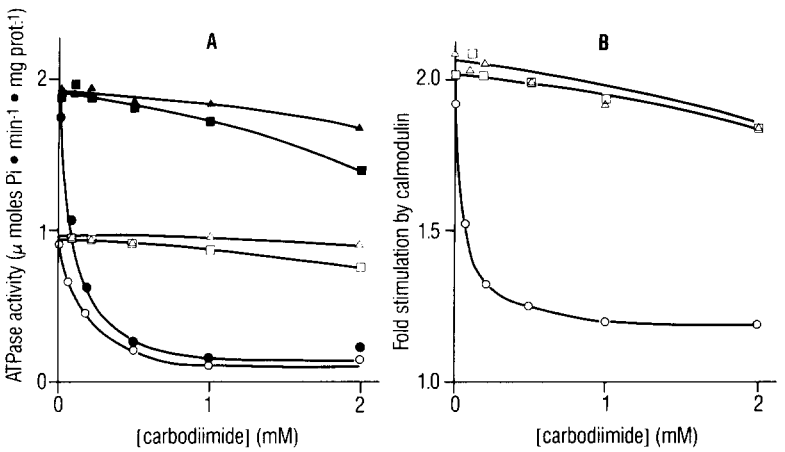

Fig. 1. Effects of carbodiimides of various hydrophobicity on Ca^{2+}-ATPase activity. (A) The purified enzyme (2.8 μg protein) was assayed at 37°C for 1 h in a total volume of 1 ml of the following medium: 124 mM KCl, 100 mM Tris, 100 mM Hepes pH 7.4, 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM ATP, 5 mM $MgCl_2$, 160 μM total $CaCl_2$ (0.22 μM free Ca^{2+}), 160 μM EDTA, 240 μM EGTA, in the absence (open symbols) and in the presence (filled symbols) of 120 nM calmodulin, and the indicated concentrations of DCCD (circles), CMCD (squares) and EDAC (triangles). All the assays contained 1% (v/v) *N,N'*-dimethylformamide. The inorganic phosphate released was assayed as described in Materials and Methods. (B) The fold stimulation induced by calmodulin was determined by calculating the ratio of the specific activity of the enzyme in the presence and in the absence of calmodulin, under the conditions indicated in (A). DCCD (circles), CMCD (squares) and EDAC (triangles).

inhibition observed with DCCD was slightly increased when the carbodiimide was preincubated with the enzyme at 37°C for 30 min prior to the assay of the enzymatic activity (results not shown). However, since the difference was not pronounced, we decided not to pursue the preincubation experiments, since the enzyme alone was shown to be very sensitive to mild thermal treatment under the preincubation conditions described above and it was difficult to remove DCCD by subsequent dilution of the preincubation mixture in the assay medium. We also observed that DCCD strongly diminished the stimulation of the ATP hydrolytic activity induced by calmodulin, with a similar potency to its inhibition of basal ATP hydrolytic activity (Fig. 1B). However, little effect on the stimulation induced by calmodulin was observed when either CMCD or EDAC was used.

pH dependency of the inhibitory effect of carbodiimide

Fig. 2 shows the Ca^{2+}-ATPase activity as a function of pH in the assay medium at different concentrations of DCCD, at a constant Ca^{2+} concentration. It was observed that progressively increasing concentrations of DCCD, up to 1 mM, induced a significant shift of the optimum pH of the enzyme, from around pH 7.0 to pH 8.0, at the same time that progressively more enzyme was inhibited (Fig. 2A). Furthermore, it was observed (Fig. 2B) that the inhibitory effect of DCCD was more marked in acid conditions (pH 6) than in alkaline conditions (pH 8). Similar results were obtained in the presence of calmodulin (results not shown).

Calcium ion favors the inhibitory effect of carbodiimide

We subsequently studied the effect of different concentrations of free calcium ion on the inhibitory effect induced by DCCD on the Ca^{2+}-ATPase activity. Fig. 3A shows a semilogarithmic plot of the Ca^{2+}-ATPase activity (both in the presence and in the absence of DCCD) as a function of the concentration of free calcium ion in the assay system, in the presence and absence of calmodulin. The replot in Fig. 3B makes it most apparent that at very low concentrations of free calcium ion in the assay system, the percent of inhibition induced by DCCD is smaller than at progressively higher concentrations of the ion. Moreover,

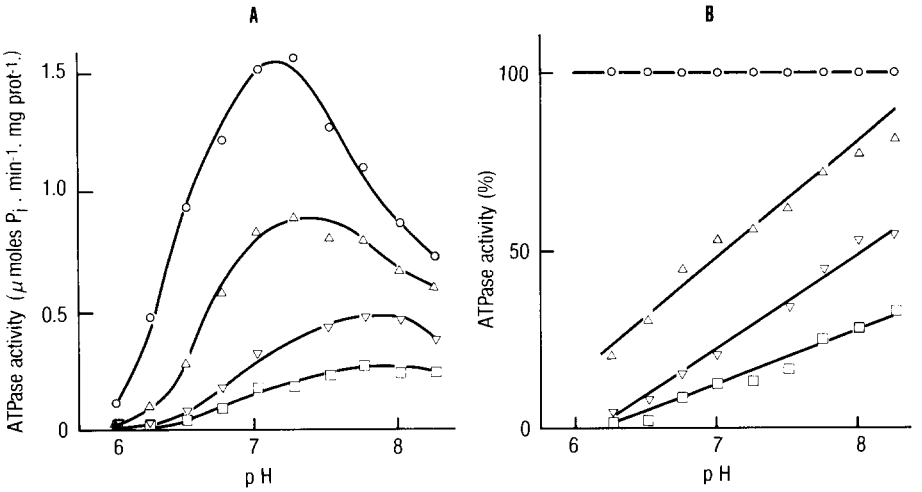

Fig. 2. Effect of DCCD on the pH dependence of the Ca^{2+}-ATPase. (A) The purified enzyme (5.7 μg protein) was assayed at 37°C for 1 h in a total volume of 1 ml of the following medium: 124 mM KCl, 100 mM Tris, 100 mM Hepes at the indicated pH, 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM ATP, 5 mM $MgCl_2$, 160 μM total $CaCl_2$ (the free calcium ion concentration was calculated to change only from 80.4 μM at pH 6 to 78.5 μM at pH 8.25 under these conditions), 160 μM EDTA, in the absence of calmodulin, and the following concentrations of DCCD: none (○), 0.2 mM (△), 0.5 mM (▽) and 1 mM (□). All the assays contained 1% (v/v) *N,N'*-dimethylformamide. The inorganic phosphate released was assayed as described in Material and Methods. (B) Ca^{2+}-ATPase activity was calculated as a percentage of the control in the absence of DCCD as a function of pH.

calmodulin appears to favor further the inhibitory effect induced by DCCD. The DCCD-induced decrease in stimulation of the Ca^{2+}-ATPase by calmodulin was observed at all the concentrations of free calcium ion tested (Fig. 3C), although as previously reported [9], the stimulatory effect of calmodulin is most apparent at low concentrations of free calcium ion. However, it was demonstrated

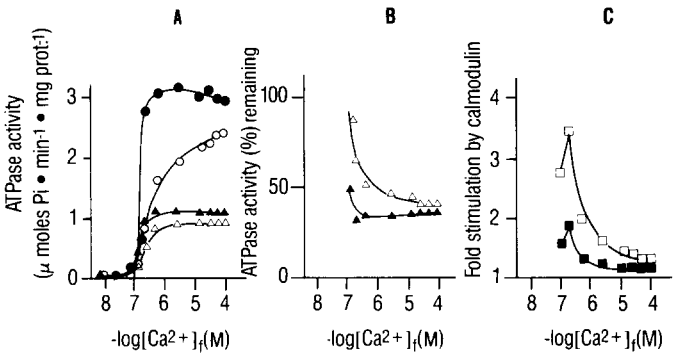

Fig. 3. Ca^{2+} dependency of the inhibition of Ca^{2+}-ATPase by DCCD. (A) The purified enzyme (4.2 μg protein) was assayed at 37°C for 1 h in a total volume of 1 ml of the following medium: 124 mM KCl, 25 mM K-Hepes at pH 7.4, 2 mM dithiothreitol, 0.054% (w/v) Triton X-100, 0.1% (w/v) sonicated asolectin, 2 mM ATP, 5 mM $MgCl_2$, 160 μM $CaCl_2$, 160 μM EDTA and from 0 to 2 mM EGTA to obtain the indicated concentration of free calcium ion in the presence (filled symbols) and in the absence (open symbols) of 120 nM calmodulin, and in the presence (triangles) and in the absence (circles) of 0.5 mM DCCD. All the assays contained 1% (v/v) *N,N'*-dimethylformamide. The inorganic phosphate released was assayed as described in Materials and Methods. (B) Ca^{2+}-ATPase activity calculated as a percentage of the control activity in the absence of DCCD was replotted as a function of the concentration of free calcium ion in the presence (filled triangles) and in the absence (open triangles) of 120 nM calmodulin. (C) The fold stimulation induced by calmodulin was replotted as a function of the concentration of free calcium ion in the presence (filled squares) and in the absence (open squares) of 0.5 mM DCCD.

(results not shown) that DCCD does not modify the degree of cooperativity of the enzyme induced by calcium ion. The Hill coefficient at pH 7.4 remains near 4 in the presence of calmodulin and near 2 in its absence, as previously reported [9], despite the partial inhibition induced by the presence of 0.5 mM DCCD in the assay system.

Discussion

The earliest studies [2] of the inhibition by carbodiimides of the sarcoplasmic reticulum Ca^{2+}-translocating ATPase indicated that DCCD was a strong inhibitor of the enzyme. However, the water-soluble carbodiimide, EDAC, was ineffective. Other hydrophobic carbodiimides were also found to be good inhibitors of this enzyme [4,5]. Our results show (Fig. 1) that the plasma membrane Ca^{2+}-translocating ATPase is also inhibited efficiently only by hydrophobic carbodiimides, suggesting that the DCCD-binding residue(s) is located in a very hydrophobic domain of the enzyme. However, Chadwick and Thomas [4] have recently shown that the molecular bulk of seven hydrophobic carbodiimides is also of great importance in determining their capacity to inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase. They found no correlation between the inhibitory capacities and the chemical reactivities of the carbodiimides, but rather, they found more inhibitory capacity when less bulky carbodiimides were employed.

A difference with respect to the inhibition observed in the sarcoplsmic reticulum Ca^{2+}-ATPase is that the plasma membrane Ca^{2+}-ATPase required higher concentrations of the inhibitor than used by Pick and Racker [2] for the sarcoplasmic reticulum ATPase, although higher concentrations (around 0.15 mM) were used in subsequent studies on the sarcoplasmic reticulum enzyme [3–6]. As reviewed in Ref. 1, carbodiimides are able to interact not only with carboxylic groups, but most significantly with sulfhydryl groups and, less efficiently, with tyrosine and serine residues. We concluded that, since the inactivation by DCCD is most pronounced in acid conditions, it is most likely that the affected group(s) have to be protonated for the inhibition to take place, indicating that the most probable candidates are carboxyl group(s). The presence of carboxyl group(s) essential to the ATP hydrolytic activity of the enzyme was further supported by an experiment showing that the enzyme activity was also blocked by the selective carboxyl group reactive agent, EEDQ (results not shown). Furthermore, the requirement for a hydrophobic carbodiimide suggests that the reactive group is buried in a hydrophobic environment, either free or possibly bonded to a basic group. Moreover, the change of optimum pH of the enzyme following inhibition by DCCD to higher values could indicate that the dissociation of H^+ from the carboxyl group(s) during the enzymatic cycle could be a feature of great relevance in this enzyme. DCCD has been shown to inhibit H^+ translocation by the F_1F_0-ATPases of different origins [13–15], the mitochondrial NADPH-NAD^+-transhydrogenase [16,17], the cytochrome *c* oxidase [18,19], and the cytochrome bc_1 complex [20–22]. Furthermore, Ca^{2+} transport by the plasma membrane Ca^{2+}-translocating ATPase from erythrocytes has been considered to involve the translocation of H^+ in the opposite direction to the calcium ion [23–25]. It will be of great interest to try to determine in future work whether the translocated H^+ comes from the carboxyl group(s) that appears to be blocked by the DCCD.

It has been shown that Ca^{2+} protects against inactivation induced by DCCD [2,3] and other carbodiimides [4] of the Ca^{2+}-translocating ATPase from sarcoplasmic reticulum. However, further work [5] has indicated that protection by Ca^{2+} could not be attained when *N*-methyl-*N'*-phenylcarbodiimide or *N*-methyl-*N'*-(4-trifluoromethyl-1-phenyl)carbodiimide were used. These studies concluded that the calcium ion binding site(s) were the site of inhibition of DCCD, which appears to be located in a hydrophobic region of the protein [2–4]. In addition, it was shown that under some conditions, Ca^{2+} protects against DCCD inhibition of the phosphorylation of the enzyme by inorganic phosphate, and its reversal, rather than against the interaction of DCCD at the site of Ca^{2+} binding or phosphorylation by ATP [6]. In contrast, the plasma membrane Ca^{2+}-translocating ATPase examined in the present study was inactivated by DCCD to a greater extent in the presence of increasing concentrations of calcium ion (Fig. 3B). Furthermore, it appears

that binding of calmodulin to the enzyme further favors the Ca^{2+}-dependent inactivation induced by DCCD (Fig. 3B). In addition, the inhibition by DCCD occurs primarily by decreasing the V_{max} of the enzyme, rather than by a significant change in the $K_{0.5}(Ca^{2+})$, indicating that DCCD does not interact with residues directly involved in Ca^{2+} binding.

Calmodulin contains a large number of carboxyl groups and it is possible that when calmodulin was present in the assay mixture, it was also inactivated to some extent by DCCD. However, in contrast to the results we obtained (Fig. 1), the inactivation of calmodulin should also have been observed with EDAC, since this carbodiimide has been shown to promote amidation of carboxyl groups on calmodulin and inactivation of its activity [26]. The lack of inhibition by DCCD may be due to rapid hydrolysis of the activated carboxyl groups in the absence of added or proximal endogenous nucleophiles. To check this point directly, calmodulin was preincubated with 1.6 mM DCCD, in the absence and presence of calcium ion, for 1 h at 37°C. After this treatment, calmodulin stimulated the Ca^{2+}-ATPase activity to within 90% of the stimulation obtained in identical control experiments (in the absence of DCCD) (results not shown). Therefore, the inhibition of calmodulin-activation of the Ca^{2+}-ATPase by DCCD appears to be due mainly to modification by DCCD of either calmodulin binding to the enzyme or of a step in the reaction sequence associated with calmodulin activation, rather than by direct inactivation of the calmodulin molecule itself. The Ca^{2+}-ATPase in this study was purified and assayed in the presence of asolectin that presumably contains acidic phospholipids. Consequently, the stimulation of the enzyme by calmodulin was most marked only at low Ca^{2+} concentrations, in contrast to the larger stimulation found by others with enzyme purified and reconstituted in L-α-phosphatidylcholine vesicles, in which stimulation was found by calmodulin also at high concentrations of calcium ion [27]. We have confirmed that the stimulation by calmodulin is higher, and over a broader Ca^{2+} concentration range, when the enzyme was purified and assayed with L-α-phosphatidylcholine at a low free Mg^{2+} concentration in the assay medium (Villalobo, A. and Roufogalis, B.D., unpublished data).

In closing, we would like to remark that the use of these carbodiimide chemical probes on the plasma membrane Ca^{2+}-translocating ATPase will help us to understand more clearly the catalytic cycle of the enzyme, as in the case of the sarcoplasmic reticulum enzyme [6], where a specific step of the catalytic cycle has been implicated as being inhibited by DCCD. The extension of the action of DCCD to the transport function(s) of this enzyme will undoubtedly be of great help in the overall understanding of these ion translocating enzymes.

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