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Mg^{2+}-ADP PROTECTS AGAINST INACTIVATION OF SARCOPLASMIC RETICULUM Ca^{2+},Mg^{2+}-ATPase BY N-CYCLOHEXYL-N'-(4-DIMETHYLAMINO-α-NAPHTHYL) CARBODIIMIDE

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SUMMARY: N-cyclohexyl-N'-(4-dimethylamino-α-naphthyl) carbodiimide (NCD-4) inactivates the sarcoplasmic reticulum Ca^{2+}-ATPase by covalent labelling at or near the high affinity (transport) Ca^{2+} sites. Mg^{2+}-ADP protects against the inactivation of the Ca^{2+}-ATPase produced by NCD-4, with a $K_{0.5}$ of Mg^{2+}-ADP of 28 ± 6 μM for purified Ca^{2+}-ATPase. With native and solubilized sarcoplasmic reticulum membranes millimolar Mg^{2+}-ADP concentrations are needed to produce an effective protection of the Ca^{2+}-ATPase against inactivation by NCD-4. These results suggest a tight structural interconnection between catalytic and transport Ca^{2+} sites in the Ca^{2+}-ATPase, modulated by protein-protein interactions in the SR membrane.

The Ca^{2+}-ATPase from sarcoplasmic reticulum (SR) couples the transport of two Ca^{2+} ions to the hydrolysis of one molecule of ATP (1). Binding of Ca^{2+} to high affinity (transport sites) in the Ca^{2+}-ATPase largely stimulates ATP hydrolysis, which is negligible in the absence of Ca^{2+} (1). Fluorescence studies using different probes have located some of the functional sites in the structure of the protein in the SR membrane (2,3).

Micromolar Ca^{2+} concentrations protect purified Ca^{2+}-ATPase against inactivation by N-cyclohexyl-N'-(4-dimethylamino-α-naphthyl) carbodiimide (NCD-4), suggesting that the probe labels at or near Ca^{2+} binding sites in the Ca^{2+}-ATPase (4-7). Work from several laboratories (4-7) has shown that NCD-4 labels two Ca^{2+}-protectable sites and one or more sites poorly defined, which are not protected by up to 0.25 mM free Ca^{2+}. The NCD-4 labelling of Ca^{2+}-protectable sites was located in a segment of tryptic fragment A1, between

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Abbreviations: Ca^{2+}-ATPase, Ca^{2+} and Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.38); $C_{12}E_8$, octaethylene glycol dodecyl ether; cmc, critical micelle concentration; DSC, differential scanning calorimetry; FITC, fluorescein isothiocyanate; IU, amount of enzyme which releases 1 μmol product per min; K_{sv}, Stern-Volmer constant; NCD-4, N-cyclohexyl-N'-(4-dimethylamino-α-naphthyl) carbodiimide; SR, sarcoplasmic reticulum.

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Glu-231 y Glu-309, that includes two transmembrane helices (7). Fluorescence energy transfer studies have located NCD-4 bound at or near the Ca^{2+} sites 2 nm from the lipid-water interface (6), and at less than 2 nm from the Trp residues of the Ca^{2+}-ATPase (7). Since 12 out of the 13 Trp of the Ca^{2+}-ATPase are located in the transmembrane domain of the protein (8), it has been proposed that the Ca^{2+}-protectable sites labelled by NCD-4 are located in the transmembrane domain approximately 2 nm under the lipid-water interface (7). Consistently, the lack of significant fluorescence energy transfer between FITC covalently bound at or near the ATP site and NCD-4 bound at the Ca^{2+} sites (5,7) indicated that Ca^{2+} sites are located far from the catalytic centre, which has been located 5-6 nm above the lipid-water interface (2).

In this communication we show that Mg^{2+}-ADP, which binds to the catalytic site of the Ca^{2+}-ATPase (9), blocks the reaction of NCD-4 with the high affinity (transport) Ca^{2+} sites, thus, probing that there is an intimate structural connection between Ca^{2+} transport sites and the catalytic centre in the SR Ca^{2+}-ATPase.

MATERIALS AND METHODS

SR vesicles were prepared as previously described (10). Purified Ca^{2+}-ATPase was prepared according to MacLennan (11). Protein concentration was measured following the method of Lowry et al. (12), with bovine serum albumin as standard, and also with the following extinction coefficients at 280 nm: 1 and 0.75 OD per mg/ml of SR and of purified Ca^{2+}-ATPase, respectively. Ca^{2+}-ATPase activity was measured as in (13), with the following assay medium: 0.1 M KCl, 0.1 M Tes/KOH (pH 7.45), 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 2.5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, 7.5 IU pyruvate kinase and 18 IU lactate dehydrogenase. Purified Ca^{2+}-ATPase showed negligible Ca^{2+}-independent ATPase activity.

Labelling of the Ca^{2+}-ATPase with NCD-4. Labelling with NCD-4 was carried out with 1 mg protein/ml at 25°C in a medium containing 0.25 M sucrose, 0.1 M KCl and 0.05 M Mes/KOH (pH 6.15). EGTA, Ca^{2+} and Mg^{2+}-ADP were added as needed and when indicated in the legend of the Figures. The pH did not significantly change (less than 0.1 unit) during these assays. The probe NCD-4 was dissolved in ethanol and the amount added was less than 1% of the total volume. The initial molar ratio NCD-4:Ca^{2+}-ATPase was 10:1. To obtain a total inhibition of Ca^{2+}-ATPase activity, 3 and 6 hours of incubation were needed for purified Ca^{2+}-ATPase and SR membranes, respectively. The labelling ratio of NCD-4:Ca^{2+}-ATPase (mol/mol) was 3:1, with an extinction coefficient for NCD-4 at 330 nm of 7500 OD. M^{-1} cm^{-1} (measured in 1% w/w SDS in water). The K_d for Mg^{2+}-ADP at pH 6.15 was taken as 1 mM (14).

Fluorescence. Fluorescence measurements were carried out at 25°C with a Perkin-Elmer 650-40 spectrofluorimeter, operated in ratio mode. Excitation and emission wavelengths were: (Trp) 280 and 330 nm, and (NCD-4) 332 and 420 nm, respectively. Potassium iodide solutions were prepared immediately before use. Fluorescence energy transfer was analyzed as described in earlier papers from this laboratory (2).

Chemicals. NCD-4 was purchased from Molecular Probes. Pyruvate kinase, lactate dehydrogenase, phospho*enol*pyruvate, calcimycin, NADH, ADP and ATP were obtained from Boehringer Mannheim. All the other chemicals were obtained from Merck.

RESULTS AND DISCUSSION

Incubation of purified Ca^{2+}-ATPase and SR membranes (at a concentration of 1 mg/ml) with 150 μM NCD-4 in the presence of 1 mM EGTA [free Ca^{2+} concentration 0.4 μM, calculated with a K_d Ca^{2+}-EGTA of 3.85 . 10^{-5} M at pH 6.0, (14)] produces a slow inactivation of the Ca^{2+}-ATPase activity. Ca^{2+}, but not Mg^{2+}, fully protects against this inactivation (Figures 1A and 1B), in parallel to protection against labelling of the high affinity Ca^{2+} sites in the Ca^{2+}-ATPase (4; and results not shown). Figure 1A also shows that the rate of inactivation of the Ca^{2+}-ATPase activity is slower in native SR than in purified Ca^{2+}-ATPase. This indicates that the accessibility of the high affinity Ca^{2+} sites of the Ca^{2+}-ATPase to NCD-4 is lower in the SR membrane than in the purified enzyme. It is to be noted here that DSC studies also show that the structure of purified Ca^{2+}-ATPase is somewhat different to that of the enzyme in the SR membrane (15, 16; and unpublished results).

Surprisingly, 3 mM Mg^{2+}-ADP in the presence of 1 mM EGTA also protects against inactivation by NCD-4 of purified Ca^{2+}-ATPase (Figure 1B), and decreases the extent of labelling from 3-4 to approximately 1-2 mol NCD-4 per mol of Ca^{2+}-ATPase. However, only a weak protection by millimolar concentrations of Mg^{2+}-ADP is obtained with the native SR membrane (see Table I). However, when the membrane was disrupted by the detergent $C_{12}E_8$ (50 μg/ml, 1 cmc) the protection afforded by Mg^{2+}-ADP against inactivation of the Ca^{2+}-ATPase by NCD-4 became stronger. The possibility that the effect of $C_{12}E_8$ is due to a conformational change in the Ca^{2+}-ATPase associated with the disruption of the Ca^{2+} gradient

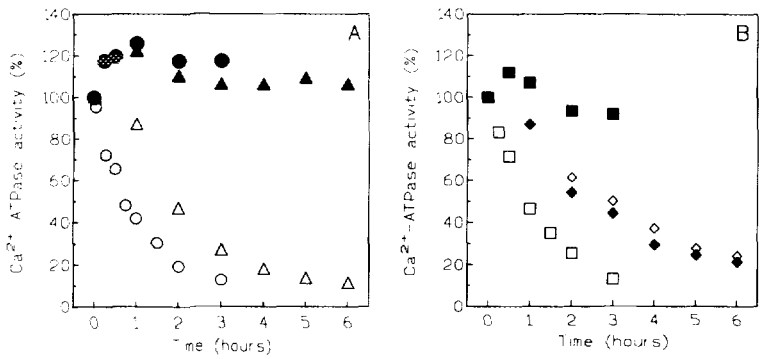

Figure 1. Time dependence of the Ca^{2+}-ATPase activity of purified Ca^{2+}-ATPase (circles and squares) and of SR membranes (triangles and diamonds) after addition of 150 μM NCD-4. Protein concentration in the incubation mixture: 1 mg/ml. Other experimental conditions are as indicated in the Materials and Methods section. The 100% activity (in μmols ATP hydrolyzed per min per mg protein) were 2.2 ± 0.5 and 5.7 ± 1.0 for SR membranes and for purified Ca^{2+}-ATPase, respectively. Additions to the buffer: Panel A, (○ , △) 1 mM EGTA; (● , ▲) 1 mM EGTA and 1.25 mM $CaCl_2$; Panel B, (□ , ◊) 1 mM EGTA and 5 mM $MgCl_2$; (■ , ♦) 1 mM EGTA, 5 mM $MgCl_2$ and 5 mM ADP.

Table I. Protection by Mg^{2+}-ADP against NCD-4 inactivation of the Ca^{2+}-ATPase activity in the presence of 1 mM EGTA and under different conditions. Purified Ca^{2+}-ATPase and SR membranes (1 mg protein/ml) were incubated in the presence of 1 mM EGTA with 150 μM NCD-4 during 3 and 6 hours, respectively, in 50 mM Mes/KOH (pH 6.15), 0.1 M KCl and 0.25 M sucrose, and the additions indicated in the Table. Aliquots were pooled for Ca^{2+}-ATPase activity measurements in the assay medium indicated in the Materials and Methods. The data shown are the average of, at least, triplicate measurements.

Additions to the incubation medium	Inhibition of ATPase activity (%)
Purified Ca^{2+}-ATPase	
none	87 ± 5
3 mM Mg^{2+}-ADP	13 ± 4
SR membranes	
none	90 ± 4
3 mM Mg^{2+}-ADP	79 ± 4
3 mM Mg^{2+}-ADP + 50 μg $C_{12}E_8$/ml	42 ± 6
7 mM Mg^{2+}-ADP	62 ± 5
7 mM Mg^{2+}-ADP + 0.04 μg calcimycin/μg protein	51 ± 5
7 mM Mg^{2+}-ADP + 50 μg $C_{12}E_8$/ml	22 ± 5

can be excluded, because addition of the ionophore calcymicin (0.04 μg/μg of SR protein) only produces, at most, a slight enhancement of the protection by Mg^{2+}-ADP against inactivation of the Ca^{2+}-ATPase by NCD-4 (Table I). Thus, solubilization of the SR membrane largely enhances the protection afforded by Mg^{2+}-ADP against inactivation by NCD-4.

The protection by Mg^{2+}-ADP against inactivation by NCD-4 at different Mg^{2-}-ADP concentrations is shown in the Figure 2. These results show that the $K_{0.5}$ of protection by Mg^{2+}-ADP are 28 ± 6 μM and 2.8 ± 0.6 mM for purified Ca^{2+}-ATPase and for SR membranes solubilized with 1 cmc of $C_{12}E_8$, respectively. It is worthnoting here that the $K_{0.5}$ value obtained for the Ca^{2+}-ATPase is close to the K_d of Mg^{2+}-ADP from the catalytic site at pH 6 (9), the pH of incubation of NCD-4 with the ATPase. On the contrary, for the SR membrane solubilized with 1 cmc of $C_{12}E_8$ the $K_{0.5}$ value obtained is close to that obtained from kinetic studies for the regulatory site of Mg^{2+}-ATP, in the millimolar range (9,17). Due to the proximity between catalytic centres in the Ca^{2+}-ATPase oligomer (13), the simplest hypothesis to rationalize these different $K_{0.5}$ values of protection by Mg^{2+}-ADP is that protein-protein interactions modulate the binding of Mg^{2+}-ADP to the catalytic centre. The full protection afforded by 3 mM Mg^{2+}-ADP against inactivation by NCD-4 of monomeric and delipidated Ca^{2+}-ATPase solubilized in $C_{12}E_8$, prepared as in (18), gives additional support to this

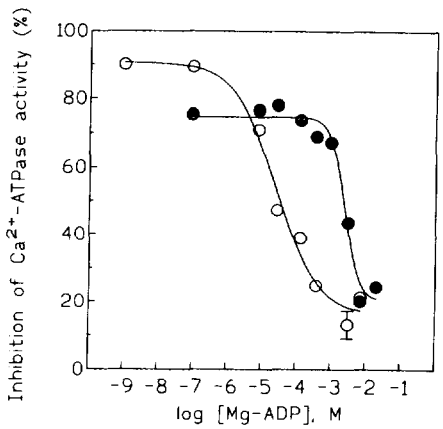

Figure 2. Dependence upon the concentration of Mg^{2+}-ADP of the inhibition by NCD-4 in the presence of 1 mM EGTA of the Ca^{2+}-ATPase activity of purified Ca^{2+}-ATPase (○) and of SR membranes (●). Purified Ca^{2+}-ATPase and SR membranes were incubated with 150 μM NCD-4 at 25°C for 3 and 6 h, respectively. Protein concentration in the incubation mixture: 1 mg/ml. For SR membranes, 50 μg $C_{12}E_8$/ml was added to the incubation medium. Other experimental conditions are as indicated in the Materials and Methods section. The 100% activity (in μmols ATP hydrolyzed per min per mg protein) was 2.2 ± 0.5 and 5.7 ± 1.0 for SR membranes and for purified Ca^{2+}-ATPase, respectively.

hypothesis (data not shown). Due to the high unstability of monomeric Ca^{2+}-ATPase in the presence of EGTA (19) the titration with lower Mg^{2+}-ADP concentrations could not be done.

The efficiency of fluorescence energy transfer from Trp to NCD-4 in our samples of purified Ca^{2+}-ATPase labelled with NCD-4 in the presence of 1 mM EGTA is 80-85 % (results not shown), which is consistent with the data reported by Sumbilla et al. (7) and with the location of the Ca^{2+} sites embedded in the transmembrane domain. In addition, the analysis of the quenching of the fluorescence of NCD-4 labelled Ca^{2+}-ATPase by KI, a water soluble quencher, yields linear modified Stern-Volmer plots (Figure 3), from which we obtain a value of 0.3 for f_a, the fraction of the initial fluorescence accessible to the quencher (20). This value is consistent with two sites of labelling not accessible to a water soluble quencher (as expected for the Ca^{2+} transport sites embedded in the transmembrane domain), and one site accessible to the water soluble quencher.

CONCLUSION

The covalent binding of NCD-4, which leads to inactivation of the Ca^{2+}-ATPase by blockade of Ca^{2+} binding to the high affinity sites takes place in carboxyl residues of the transmembrane domain of the protein, when it is in the presence of EGTA (E2 state) (7). Binding of Mg^{2+}-ADP largely decreases the accessibility of these carboxyl residues to NCD-4. Because of the Ca^{2+} sites labelled with NCD-4 are located far from the catalytic site (approx.

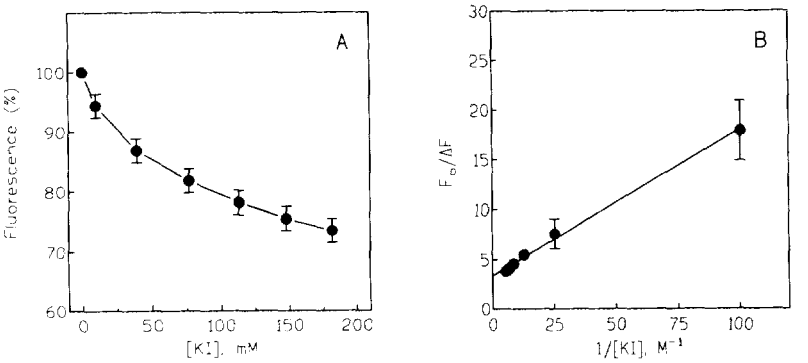

Figure 3. Quenching by KI of the fluorescence of purified Ca^{2+}-ATPase labelled with NCD-4. Panel A. Plot of F/F_0 versus the concentration of KI. Buffer: KCl 0.1 M and Tes/KOH 0.1 M (pH 8.0). Protein concentration: 50 μg purified ATPase/ml. Panel B. Modified Stern-Volmer plot of the data shown in the Panel A. ΔF is (F_0 - F), where F_0 and F are the fluorescence in the absence and in the presence of each quencher concentration, respectively. From the slope of the straight line fit by linear regression, a K_{sv} value of 20 M^{-1} is obtained for the quenching by I^- of the accessible NCD-4.

6-7 nm) this is an allosteric effect of Mg^{2+}-ADP, and points out an intimate structural connection between Ca^{2+} transport sites and the catalytic centre. Since extensive X-rays diffraction (21) and circular dichroism (22) studies have shown that the E1 to E2 conformational change only produces a small redistribution of the protein mass, these results give support to a three-dimensional structure of the Ca^{2+}-ATPase in which the catalytic centre is located near the entrance of a Ca^{2+} channel structure within the ATPase, as proposed in (13).

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