Effect of cyclic AMP-dependent phosphorylation on the reactivity of sulphydryl groups in cardiac sarcoplasmic reticulum

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Phosphorylation of cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase results in enhanced Ca^{2+} transport even though Ca^{2+} -dependent ATPase is not a substrate for the kinase. The mechanisms involved in this enhancement are not clear. In the present study, we used the reactivity of sulphydryl groups in the Ca^{2+} -dependent ATPase as an index of conformational change during the Ca^{2+} transport cycle and examined the effects of protein kinase-catalysed phosphorylation. *N*-Ethylmaleimide alkylation allowed the distinction of several thiol groups with variable functional significance for the ATPase. A sulphydryl group involved in the formation of the phosphorylated intermediate (EP) of the Ca^{2+} -dependent ATPase was protected by adenosine 5'-[β , γ -imido]triphosphate. Reactivity of an additional thiol group was related to EP dephosphorylation and was dependent on Ca^{2+} . The Ca^{2+} concentration for change in the reactivity of this sulphydryl group and ATPase inhibition occurred within the range for Ca^{2+} binding to the high-affinity sites. Phosphorylation of cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase resulted in decreased *N*-ethyl[1- ^{14}C]maleimide binding and ATPase inhibition; the thiol groups involved in EP dephosphorylation were selectively protected. The results indicate that protein kinase-catalysed phosphorylation results in conformational changes of the ATPase, which renders certain thiol groups inaccessible to *N*-ethylmaleimide. This conformational change may facilitate functional movements involved in Ca^{2+} transport.

Phosphorylation of a 22000-mol.wt. protein (phospholamban) of cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase results in enhanced ATP hydrolysis and Ca^{2+} transport (LaRaia & Morkin, 1974; Kirchberger *et al.*, 1974). The mechanisms by which phospholamban phosphorylation effects Ca^{2+} movements across the sarcoplasmic-reticulum membrane are not clear. Although the Ca^{2+} -transport ATPase is not a substrate for the protein kinase, elementary steps of the ATP hydrolysis reaction are indirectly affected by phospholamban phosphorylation. The most prominent effect is the increased decomposition rate of the phosphorylated intermediate (EP) of the ATPase, the rate-limiting step in the reaction sequence. In addition, a step associated with Ca^{2+} binding to the ATPase and the rate at which EP is formed are enhanced, resulting in an increased rate of Ca^{2+} translocation across the microsomal mem-

brane (Tada *et al.*, 1980). These effects make it likely that phospholamban phosphorylation is an important regulator of Ca^{2+} transport in the myocardium and a determinant of the positive inotropic effects of catecholamines.

The mechanisms through which Ca^{2+} -dependent ATPase function is affected by phospholamban are not known. In the present study, we examine the possibility that phospholamban influences the structural changes in the enzyme molecule during the Ca^{2+} transport cycle. These changes involve interactions between ions, nucleotides and the micro-environment of the ATPase and result in conformational changes of the enzyme, which facilitate Ca^{2+} translocation. We have used the reactivity of sulphydryl groups with *N*-ethylmaleimide as an index of conformational changes in the ATPase and report significant differences between control and phosphorylated microsomal fraction ('microsomes'). These differences suggest that phospholamban phosphorylation protects from *N*-ethylmaleimide alkylation sulphydryl groups related to

Abbreviations used: EP, phosphorylated intermediate of Ca^{2+} -dependent ATPase; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate.

Ca^{2+} binding to the high-affinity sites and to EP dephosphorylation.

Materials and methods

Experiments were carried out on adult male Sprague-Dawley rats weighing 350–400 g.

Cardiac sarcoplasmic reticulum

This was isolated essentially as described by Sumida *et al.* (1978). Briefly, rat hearts were homogenized in 10 mM-Tris/maleate (pH 6.8)/5 mM- NaN_3 with a Polytron PT-20 homogenizer at a setting of 5 for 15 s. The homogenate was centrifuged at 4000 g for 10 min. The resultant supernatant was passed through four layers of cheesecloth and the filtrate was centrifuged at 15 000 g for 20 min. The supernatant was again passed through four layers of cheesecloth and centrifuged at 143 000 g for 30 min. The pellet was resuspended in the homogenization buffer containing 0.6 M-KCl and centrifuged at 143 000 g for 45 min. The final pellet was resuspended in 10 mM-Tris/maleate (pH 6.8)/0.1 M-KCl and used on the day of preparation.

Microsomal phosphorylation by cyclic AMP-dependent protein kinase

Cardiac sarcoplasmic reticulum (4 mg/ml) was incubated at 25°C in a medium containing 10 mM-Tris/maleate (pH 6.8)/0.1 M-KCl and 2 mg of cyclic AMP-dependent protein kinase/ml (Sigma Chemical Co.), 1 μM -cyclic AMP, 1 mM-ATP, 2 mM- MgCl_2 in a total volume of 3 ml. After 10 min incubation, 4 vol. of ice-cold 10 mM-Tris/maleate buffer (pH 6.8) was added and the mixture was applied to a column (1.3 cm $\times$ 2 cm) of Dowex 1 (X8) pre-equilibrated with the same buffer at 0°C. Sarcoplasmic reticulum pre-incubated in the absence of cyclic AMP and protein kinase (control) and phosphorylated microsomes were eluted from the column at a flow rate of 1 ml/min at 0°C and were used immediately for ATPase assays or *N*-ethylmaleimide modification. Under the conditions of the experiment, phospholamban phosphorylation determined as previously described (Limas, 1978) was 123 ± 7 pmol/mg of microsomal protein.

Ca^{2+} -dependent ATPase activity

This was determined as previously described (Limas, 1978). The reaction mixture contained 40 mM-Tris/maleate buffer (pH 6.8), 5 mM- MgCl_2 , 5 mM-ATP, 0.1 M-KCl, 5 mM- NaN_3 , 5 mM-sodium oxalate, Ca^{2+} -EGTA buffer containing free 1 μM - Ca^{2+} and 50–80 μg of microsomal protein/ml, in a total volume of 0.5 ml. Assay mixtures were pre-incubated at 25°C for 5 min, after which the reaction was started by the addition of microsomal protein. To determine 'basal' (Mg^{2+} -dependent ATPase)

activity, reactions were carried out in the presence of 0.2 mM-EGTA instead of the Ca^{2+} -EGTA buffer. At various intervals after the start of the reaction, 100 μl portions were added to tubes (5 $\times$ 50 mm) containing 5 ml of 25% (w/v) trichloroacetic acid and 125 μM - P_i . After centrifugation at 3000 g for 10 min, P_i was determined in the supernatant (Martin & Doty, 1949).

Formation of the phosphoprotein intermediate (EP) of the Ca^{2+} -transport ATPase

This was followed in a medium containing 40 mM-Tris/maleate (pH 6.8), 0.1 M-KCl, 5 mM- MgCl_2 , 50 μM - CaCl_2 , 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 10 Ci/mmol; from Amersham/Searle, Arlington Heights, IL, U.S.A., with sp. radioactivity 15 Ci/mmol) and 1 mg of sarcoplasmic-reticulum protein/ml. Reactions were carried out at 0°C for 20 s and were stopped with the addition of 5 ml of ice-cold 5% trichloroacetic acid containing 20 mM- P_i and 2 mM-ATP. The pellet was collected by centrifugation at 10 000 g for 15 min and was washed once in 5% trichloroacetic acid/20 mM- P_i /2 mM-ATP, twice with 5% trichloroacetic acid/20 μM - P_i and once with ice-cold water. The washed pellet was resuspended in 1.0 ml of 0.1 M-NaOH and heated in boiling water for 15 min; portions were assayed for radioactivity in Bray's solution.

**N*-Ethyl[1- ^{14}C]maleimide binding*

The reaction for *N*-ethylmaleimide binding was started by adding *N*-ethyl[^{14}C]maleimide (sp. radioactivity 100 Ci/mol; New England Nuclear Corp.) to a solution containing 2 mg of sarcoplasmic-reticulum protein/ml, 0.1 M-Tris/maleate (pH 7.0), 5 mM- MgCl_2 , 0.1 M-KCl, 2 mM-EGTA with or without various amounts of CaCl_2 . Incubation was carried out at 25°C for 1–60 min and was stopped with the addition of 1.5 ml of 5% HClO_4 to a 50 μl portion. The precipitate was collected in Whatman GF/C glass-fibre filters, washed twice with 0.5% HClO_4 , once with 95% ethanol, dried and counted for radioactivity.

Total sulphhydryl content in sarcoplasmic-reticulum suspension was determined by the method of Boyne & Ellman (1972). Protein was determined by the method of Lowry *et al.* (1951). For electrophoretic analysis of the *N*-ethyl[1- ^{14}C]maleimide-labelled proteins, sarcoplasmic reticulum was incubated with 1.0 mM-*N*-ethyl[1- ^{14}C]maleimide for 45 min. The labelled proteins were precipitated with 5% HClO_4 , washed once with 0.5% HClO_4 , once with diethyl ether and were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). After electrophoresis, unstained gels were sliced, each slice was extracted overnight at 37°C with 0.3 ml of 0.5% sodium dodecyl sulphate and then mixed with 5.0 ml

of a toluene/Triton X-100 scintillation fluid and counted for radioactivity.

Results

Titration of sarcoplasmic-reticulum membranes with 5,5'-dithiobis-(2-nitrobenzoate) by the method of Boyne & Ellman (1972) revealed the presence of 18–20 thiol groups/mg of protein. A small number of these groups could be alkylated by *N*-ethylmaleimide. The time course of thiol-group modification by 1.5 mM-*N*-ethylmaleimide is shown in Fig. 1; binding of 13 nmol/mg of protein at 40 min corresponds to alkylation of 6 mol of thiol group/mol of ATPase based on 25% ATPase content in cardiac sarcoplasmic reticulum (C. J. Limas, unpublished work). *N*-Ethyl[14 C]maleimide binding to microsomes phosphorylated by cyclic AMP-dependent protein kinase is significantly lower than to unphosphorylated controls. Incubation of microsomes in the presence of protein kinase inhibitor (4 mg/ml) in addition to protein kinase (2 mg/ml) increased *N*-ethyl[14 C]maleimide binding to near control values (results not shown).

The dependence of *N*-ethyl[1- 14 C]maleimide binding and Ca^{2+} -transport ATPase inhibition on the concentration of *N*-ethylmaleimide in the assay medium is shown in Fig. 2; at *N*-ethylmaleimide < 0.5 mM, no ATPase inhibition occurred and the extent of *N*-ethyl[1- 14 C]maleimide binding was not different in phosphorylated or control

microsomes; this binding, therefore, reflected modification of two thiol groups non-essential for ATPase activity. Higher amounts of *N*-ethylmaleimide resulted in a sharp decline in ATPase activity; at all *N*-ethylmaleimide concentrations higher than 1 mM, the percentage decline in enzymic activity was lower in the phosphorylated microsomes fraction. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3) showed that *N*-ethyl[1- 14 C]-

Fig. 2. Dependence of *N*-ethyl[14 C]maleimide binding (●, ○) and ATPase inhibition (■, □) in control (●, ■) and phosphorylated (○, □) microsomes on the concentration of *N*-ethylmaleimide in the assay mixture

Reactions were carried out at 25°C for 10 min as described in the text. ATPase activity in the absence of *N*-ethylmaleimide (40 nmol of P_i /mg per min in control and 63 nmol/mg per min in phosphorylated microsomes) was taken as 100%.

Fig. 1. Time course of *N*-ethyl[14 C]maleimide binding to control (●) and phosphorylated (○) microsomal fraction

Cyclic AMP-dependent phosphorylation was carried out at 25°C for 10 min in the presence of 2 mg of protein kinase/ml and 4 mg of cardiac sarcoplasmic reticulum/ml. *N*-Ethyl[14 C]maleimide binding was assayed in the presence of 1.4 mM-*N*-ethylmaleimide, 50 μM - CaCl_2 and 2 mg of protein/ml as described in the text. Results represent means $\pm$ S.E.M. for seven comparisons.

Fig. 3. Labelling of cardiac sarcoplasmic-reticulum proteins with *N*-ethyl[14 C]maleimide

Reaction was carried out at 25°C for 45 min with 1.5 mM-*N*-ethylmaleimide and 1 mg of protein/ml.

maleimide was incorporated into the 100000-mol.wt. polypeptide corresponding to the Ca^{2+} -dependent ATPase (Limas, 1978) in both control and phosphorylated microsomal fractions.

In the presence of 1 mM-p[NH]ppA, steady-state concentrations of the phosphoprotein intermediate EP of the Ca^{2+} -dependent ATPase were essentially unchanged during *N*-ethyl[^{14}C]maleimide binding (Fig. 4), even though the extent of ATPase inhibition was not modified. This suggests that p[NH]ppA protects thiol groups essential for EP formation without affecting EP dephosphorylation. Since *N*-ethyl[^{14}C]maleimide binding and ATPase inhibition were lower in phosphorylated sarcoplasmic reticulum even in the presence of p[NH]ppA, cyclic AMP-dependent protein kinase may have protected the same thiol groups as P[NH]ppA or affected EP dephosphorylation selectively. The latter possibility is supported by the results in Fig. 5, in which decay of EP after an unlabelled ATP 'chase' is compared in control and phosphorylated microsomal fraction. Addition of *N*-ethylmaleimide in the presence of p[NH]ppA results in a decreased rate of EP dephosphorylation compared with preparations without *N*-ethylmaleimide; EP dephosphorylation is, however, more rapid in cyclic AMP-dependent protein kinase phosphorylated rather than control microsomes (not pre-incubated with protein kinase). This indicates protection by cyclic AMP-dependent protein kinase of thiol groups essential for EP dephosphorylation.

Both extent of *N*-ethyl[^{14}C]maleimide binding and ATPase inhibition in the presence of p[NH]ppA are

Fig. 4. Effect of p[NH]ppA on steady-state EP concentrations ($\square$, $\bullet$) and ATPase activity ($\circ$, $\bullet$, Δ , $\blacktriangle$) during alkylation with *N*-ethylmaleimide

Control ($\bullet$, $\bullet$, $\blacktriangle$) and phosphorylated ($\circ$, $\bullet$, Δ) microsomes were incubated with 1.5 mM-*N*-ethylmaleimide for the indicated lengths of time in the presence ($\square$, $\bullet$, $\circ$, $\bullet$) or absence (Δ , $\blacktriangle$) of 1 mM-p[NH]ppA. Ca^{2+} -dependent ATPase activity in the absence of *N*-ethylmaleimide (38 nmol/mg per min in control and 59 nmol/mg per min in phosphorylated microsomes) was taken as 100%.

dependent on the Ca^{2+} concentration in the assay medium (Fig. 6). For both experimental preparations, ATPase inhibition is more extensive at the

Fig. 5. Effect of phospholamban phosphorylation on the rate of EP decomposition

Control ($\bullet$, $\blacktriangle$) and phosphorylated ($\circ$, Δ) microsomes were first incubated with [γ - ^{32}P]ATP for 20 s and, at zero time, unlabelled 1.0 mM-ATP was added. The decline of EP concentrations was measured in microsomes pre-incubated for 10 min with 1 mM-p[NH]ppA + 1.5 mM-*N*-ethylmaleimide ($\blacktriangle$, Δ) or p[NH]ppA alone ($\bullet$, $\circ$).

Fig. 6. Effect of Ca^{2+} on the extent of Ca^{2+} -transport ATPase inhibition during alkylation by *N*-ethylmaleimide

Control ($\bullet$, $\blacktriangle$) or phosphorylated ($\circ$, Δ) microsomes were incubated with 1.5 mM *N*-ethylmaleimide for the indicated lengths of time in the presence of 50 μM Ca^{++} ($\circ$, $\bullet$) or 20 nM Ca^{++} (Δ , $\blacktriangle$).

Fig. 7. Ca^{2+} dependence of ATPase inhibition by 1.5 mM-*N*-ethylmaleimide in control (●) and phosphorylated (○) microsomes

Reactions were carried out for 10 min as described in the text.

Fig. 8. Correlation between *N*-ethyl[¹⁴C]maleimide binding and ATPase inhibition in control (●) and phosphorylated (○) microsomes

Reactions were carried out with 1.5 mM-*N*-ethylmaleimide and 50 μM - CaCl_2 for various lengths of time.

groups is not accompanied by loss of ATPase activity, the results suggest the presence of two essential thiol groups in both control and phosphorylated microsomes. Modification of two thiol groups also appears to abolish EP formation. Since *N*-ethyl[1-¹⁴C]maleimide binding takes place at low Ca^{2+} concentration even after ATPase inhibition has reached a plateau, an additional undetermined number of thiol groups, non-essential for ATPase activity, appear to be modified after prolonged incubation with *N*-ethylmaleimide.

Discussion

Sulphydryl groups are essential for the activity of Ca^{2+} -transport ATPase of sarcoplasmic reticulum since reagents that alkylate these groups inhibit both ATP hydrolysis and Ca^{2+} transport (Hasselbach & Seraydarian, 1966; Panet & Selinger, 1970; Champeil *et al.*, 1976; Thorley-Lawson & Green, 1977; Andersen & Moller, 1977; Yamada & Ikemoto, 1978; Murphy, 1978; Kawakita *et al.*, 1980). The use of sulphydryl reagents such as *N*-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoate) and iodoacetamide has permitted the identification of several, kinetically distinguishable, thiol groups, which may be involved in different steps of the Ca^{2+} transport cycle. Conformational changes in the ATPase molecule induced by the binding of ions and nucleotides are reflected in altered reactivity of thiol group subsets. Thus they provide evidence for structural changes during Ca^{2+} translocation and are useful for monitoring the effects of agents influencing Ca^{2+} ATPase. The present study extends these observations to the cardiac-muscle ATPase. In general, the pattern of sulphydryl-group reactivity was similar to that previously described for skeletal muscle (Yamada & Ikemoto, 1978; Kawakita *et al.*, 1980). In the presence of Ca^{2+} and increasing amounts of *N*-ethylmaleimide, alkylation of approx. 2 mol of thiol groups/mol of ATPase did not affect Ca^{2+} -dependent ATPase activity (or Ca^{2+} uptake; results not shown). Millimolar concentrations of *N*-ethylmaleimide and more prolonged incubation resulted in modification of at least six additional thiol groups/mol of ATPase. We confirmed the previous observation by Kawakita *et al.* (1980) that p[NH]ppA protected thiol groups essential for the formation of the phosphoprotein intermediate EP of the Ca^{2+} -transport ATPase, perhaps related to the nucleotide binding site. In addition, alkylation of a thiol not by p[NH]ppA decreased the rate of EP dephosphorylation and ATP hydrolysis. Reactivity of this thiol depends on the presence of Ca^{2+} ; in view of the fact that the Ca^{2+} concentration for change in thiol-group reactivity and ATPase inhibition occurred within the range for Ca^{2+} binding to the high-affinity sites (Ikemoto, 1974), changes in

higher $[\text{Ca}^{2+}]$. The change in reactivity of thiol groups involved in EP dephosphorylation occurs at $[\text{Ca}^{2+}] > 1 \mu\text{M}$ (Fig. 7). In contrast, modification of thiol groups by p[NH]ppA is not dependent on the Ca^{2+} concentration (results not shown). A plot of the extent of ATPase inhibition as a function of *N*-ethylmaleimide binding (Fig. 8) reveals that 90% of the activity is lost when four thiol groups are modified. Since alkylation of the first two thiol

reactivity of this thiol group may reflect conformational changes in the enzyme associated with Ca^{2+} binding. Finally, continued *N*-ethyl[1- ^{14}C]-maleimide binding after prolonged incubation without further change in ATPase may reflect binding to non-essential thiols or, perhaps, other chemical groups in the sarcoplasmic reticulum.

Phosphorylation of cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase decreased the reactivity of sulphhydryl groups, particularly that involved in EP dephosphorylation. Phospholamban phosphorylation thus protected the ATPase from inactivation probably because it induced conformational changes that rendered some of the thiol groups inaccessible to *N*-ethylmaleimide. Reactivity of sulphhydryl groups reflects their microenvironment in the Ca^{2+} -dependent ATPase molecule. By using *N*-ethylmaleimide, it is possible to identify thiols involved in different steps of the ATPase reaction sequence. The ATPase itself is not a substrate for cyclic AMP-dependent protein kinase, but both the formation and decomposition of EP are secondarily affected (Sumida *et al.*, 1978; Tada *et al.*, 1980). In addition, the Ca^{2+} dependency of the transport process is changed so that the positive co-operativity between the two Ca^{2+} -binding sites of the Ca^{2+} pump is decreased (Hicks *et al.*, 1979). These effects may be partly dependent on conformational changes in the enzyme brought about by phospholamban phosphorylation. Supportive evidence for this concept is offered by the observation that phospholamban phosphorylation protects against the ATPase inhibition induced by borate ions (Will *et al.*, 1976), which are thought to modify essential histidine residues in the enzyme (Yu *et al.*, 1974). Changes in the temperature dependency of cardiac ATPase after phosphorylation (Chiesi, 1979) also indicate alteration in the fluidity of the hydrophobic microenvironment of the ATPase that, in turn, would facilitate functional movements involved in the transport process.

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