IMMUNOCHEMICAL EVIDENCE OF THE INDEPENDENCE OF THE Ca^{2+}/Na^{2+} ANTIPORTER AND ELECTROPHORETIC Ca^{2+} UNIPORTER IN HEART MITOCHONDRIA

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1. Introduction

A general feature of animal mitochondria is their ability to accumulate Ca^{2+} in response to either substrate oxidation or ATP hydrolysis. This process is considered to be dependent on a negative inside membrane potential created either by the operation of the respiratory chain or by ATP hydrolysis. The system is considered therefore to be an electrophoretic Ca^{2+} uniport. The molecular aspects of this process have been to a certain extent elucidated. A necessary component of the system is the Ca^{2+}-binding glycoprotein isolated by us in 1971 [1,2]. The most convincing evidence concerning the involvement of the glycoprotein in mitochondrial Ca^{2+} transport is based on the results obtained using specific affinity chromatography-purified, antiglycoprotein antibodies which were shown to inhibit both respiratory chain-driven Ca^{2+} uptake [3] and uncoupler-induced Ca^{2+} release [4,5] in rat liver mitochondria and mitoplasts. Other Ca^{2+} movements such as valinomycin–K^+-driven Ca^{2+} uptake and EDTA-driven Ca^{2+} extrusion are also inhibited by the addition of antibodies (unpublished). In [6] we showed that the Ca^{2+} efflux induced by oxidation of intramitochondrial pyridine nucleotides [7,8] is also sensitive to the inhibitory action of the antibodies, indicating that also this phenomenon results from the operation of a system including the glycoprotein. Even more compelling evidence concerning the necessary involvement of the glycoprotein in mitochondrial Ca^{2+} transport is provided by reconstitution experiments with glycoprotein-depleted mitochondria and pure glycoprotein [9].

Mitochondria from heart and some other tissues contain, in addition to the Ca^{2+} uniporter, a Na^+–Ca^{2+} antiporter, which catalyses the active extrusion of Ca^{2+} [10–15]. The uniporter and antiporter have been distinguished on the basis of their differing sensitivities to ruthenium red, Na^+, lanthanides and K^+ [16,17]. The question arises, therefore, whether the glycoprotein participates specifically in the uniporter reaction of heart mitochondria, or whether it is involved in the physiological routes of both influx and efflux. This report answers this question by investigating the effect of antiglycoprotein antibody on the activities of the uniporter and Na^+–Ca^{2+} antiporter.

2. Materials and methods

Rat heart mitochondria were prepared in a medium containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl (pH 7.4) and 0.1 mM EDTA as in [10]. Ca^{2+} uptake and release were measured spectrophotometrically at 685–665 nm using arsenazo III, purified as in [18]. An important modification was introduced in the purification procedure using potassium acetate instead of sodium acetate. The product of the original procedure contains, in fact, substantial amounts of sodium acetate, and cannot be used to show the Na^+-dependent Ca^{2+} extrusion. Ruthenium red was purified according to [19]. Antiglycoprotein antibodies were raised and affinity chromatography-purified as in [3]. The antibody was finally dissolved in 50 mM triethanolamine–HCl

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid; arsenazo III, 2,2'-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-bisazo) bis-(benzenarsonic acid)

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(pH 7.8) containing 100 mM HCl. Further technical details are given in the legends to the figures.

3. Results and discussion

Fig.1 reports the effect of antiglycoprotein antibody on the uptake and Na^+-induced release of Ca^{2+} from cardiac mitochondria. Uptake of Ca^{2+} was begun by the addition of Ca^{2+} to respiring mitochondria and terminated by the addition of ruthenium red, a specific inhibitor of the Ca^{2+} uniporter. Na^+-induced efflux was started by the addition of Na^+ 1 min after ruthenium red. The ability of antibody to inhibit these fluxes was investigated by preincubating the mitochondria with antibody for 1 h at 0°C and comparing the activities of these mitochondria with the activities of control mitochondria, which were preincubated similarly with the buffer used to dissolve the antibody.

A preincubation period of 1 h was used routinely since penetration of the antibody across the outer membrane into the intermembrane space would be predicted to be slow. In fact, it was observed that the magnitude of the effects of the antibody decreased with shorter preincubation periods and increased when the preincubation period was prolonged.

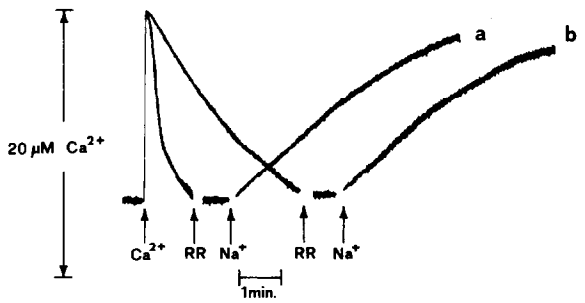

Fig.1. Ca^{2+} movements in the absence (a) and presence (b) of specific antiglycoprotein antibodies. Experimental conditions: Phoenix dual wavelength recording spectrophotometer. The assay solution (3 ml) contained 2.7 mg mitochondrial proteins, 120 mM KCl, 10 mM Hepes (K^+ salt, pH 7), 15 μM rotenone, 0.1 mM purified arsenazo III and 6.5 mM succinate. The reaction was started by the addition of 60 nmol Ca^{2+}. Purified ruthenium red (RR) (0.48 nmol) and 50 μmol NaCl were added where indicated. System temp. 25°C. Treatment with antibodies was carried out in 100 μl final vol. at 0°C for ⩾1 h. Samples were then transferred in the measuring vessel and diluted with the medium to the final volume. Controls were also pre-incubated under the same conditions without antibody.

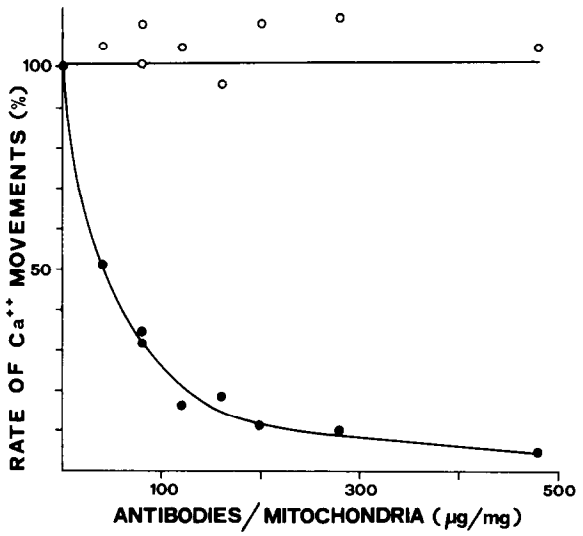

Fig.2. The dependence of the effect of specific antiglycoprotein antibodies on Ca^{2+} influx and Na^+-induced Ca^{2+} efflux on the quantity of antibody. Experimental conditions: as in fig.1; (•) Ca^{2+} influx; (○) Na^+-induced Ca^{2+} extrusion.

The traces show clearly that the rate of Ca^{2+} uptake is strongly depressed by the presence of antibody whereas the rate of Na^+-induced efflux is not changed.

Fig.2 reports the degree of inhibition of uniporter activity by different amounts of antibody. The relation between the amount of antibody and degree of inhibition is hyperbolic, and 50% inhibition is achieved with ~40 μg antibody/mg protein. The amount of antibody required for 50% inhibition of uniporter activity varied with different mitochondrial preparations, which presumably reflects the different degrees to which the outer membrane is intact in different preparations, since this would influence the accessibility of the glycoprotein in the intermembrane space to externally-added antibody. The important point, however, is that the rate of Na^+-induced efflux is not changed even by amounts of antibody that yield >90% inhibition of uniporter activity.

In summary, these data indicate that, in contrast to respiratory chain-driven Ca^{2+} uptake, the Na^+-induced Ca^{2+} extrusion is completely insensitive to antiglycoprotein antibodies, which allows 2 important conclusions to be drawn:

1. The Na^+-dependent Ca^{2+} flux proceeds by a pathway that is quite separate from the electrophoretic

uniporter that catalyses net Ca^{2+} influx, in agreement with previous evidence that the Na^+-dependent flux is catalysed by a distinct Na^+–Ca^{2+} antiporter. The proposal [10–14,16,20,21] that these 2 carriers provide the fundamental mechanism for physiological recycling of Ca^{2+} across the inner membrane is thereby substantiated. This conclusion should be considered in relation to the fact that in isolated liver mitochondria, which exhibit little or no Na^+–Ca^{2+} antiporter activity, all Ca^{2+} fluxes so far investigated are sensitive to antiglycoprotein antibody (see section 1).

2. The data strongly indicate that the glycoprotein is a specific component of the uniport system, and that the antiglycoprotein antibody binds selectively to this system without causing general changes in the permeability properties of the inner membrane. This conclusion is supported by the observation that P/O ratios, respiratory control ratios and electron flux are not changed by antibodies sufficient to cause 50% inhibition of uniporter activity [3].

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