THE TRANSPORT OF Ca^{2+} IN A PURIFIED POPULATION OF INSIDE-OUT VESICLES FROM RAT LIVER MITOCHONDRIA

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1. Introduction

The energy-linked uptake of Ca^{2+} in sonic vesicles (SMP) from heart mitochondria has been described in [1]. The reaction required both ATP and a respiratory substrate, and was described only in the presence of inorganic phosphate. Since sonic SMP are assumed to have a predominant inside-out-polarity [2,3] and the energy-linked uptake of Ca^{2+} in mitochondria is assumed to be driven by the negative inside potential [4], the observations in [1] were difficult to rationalize.

One general problem in studies with SMP is their heterogeneous membrane polarity. This problem has probably been responsible for some of the conflicting results reported on the energy-linked uptake of Ca^{2+} by sonic (and other) SMP [5–8]. Clearly, a 'pure' population, consisting entirely of inside-out SMP would be helpful.

From the studies reported on Ca^{2+} transport in (inverted) SMP, one conclusion, however, has in general emerged, namely, the requirement for inorganic phosphate. This has been interpreted with an energy-linked uptake of phosphate, (which would correspond to its active extrusion in intact mitochondria) on one of the two known phosphate carriers [9–12], or on a new one [13]. Thus, the uptake of Ca^{2+} in inverted SMP, might be secondary to the uptake of phosphate.

Recently, it has become clear that mitochondria use separate pathways for Ca^{2+} uptake and release [14,15]. In heart and other mitochondria, release occurs via a ruthenium red (RR)-insensitive Na^+/Ca^{2+} exchange [16]. In other mitochondria (liver, kidney, lung) the release pathway is still unidentified [17,18].

In this article, Ca^{2+} transport has been studied on a homogenous population of inside-out SMP, obtained by an affinity chromatography method, based on the location of the cytochrome *c* binding site on the outer face of the inner membrane. This method has already been used [19], to eliminate inside-out vesicles from mitoplasts. The results indicate that the uptake of phosphate in inverted SMP may reflect the system by which Ca^{2+} is normally released from liver mitochondria.

2. Materials and methods

2.1. *Isolation of mitochondria*

Rat liver mitochondria were prepared in 220 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM Na-ethylendiamine tetraacetic acid (EDTA). The pellet was washed twice, the second time without EDTA.

2.2. *Preparation of 'standard' SMP*

The mitochondrial pellet (20 mg protein/ml) was sonified in 2 mM EDTA (pH 8.5) for 2 min, at 0°C, under N_2. The output of the Branson sonifier was set at 80–100 W, with 50% interruption. After a first centrifugation at 10 000 × *g* for 10 min, SMP were collected at 10 000 × *g* for 30 min and washed with

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220 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl (pH 7.4). For the experiments on Ca^{2+} transport, sonication was performed in 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–Cl (pH 7.4), 0.05 mg bovine serum albumin (BSA)/mg protein, 0.1 mg asolectin/mg protein.

2.3. *Binding of cytochrome* c *to Sepharose 4B*

After swelling and washing with 1 mM HCl, CNBr-activated Sepharose 4B was washed twice with 0.1 M bicarbonate in 0.4 M NaCl (pH 8.5) and suspended to 1 g dry gel/10 ml. Cytochrome *c* (20 mg/g dry gel) was added, and the mixture was incubated 16 h at 4°C with gentle shaking. After washing with 0.1 M bicarbonate in 0.4 M NaCl (pH 8.5) and after incubating for 1 h in ethanolamine (30 ml/g dry gel) at 4°C, the product was washed 3 times, alternatively with 0.1 M acetate in 1 M NaCl (pH 4.0) and 0.1 M bicarbonate in 1 M NaCl (pH 8.5).

2.4. *Cytochrome oxidase assay*

Cytochrome oxidase activity was measured polarographically, as in [20]. The medium contained 30 mM K-phosphate, 50 μM cytochrome *c*, 10 mM Na-ascorbate, 0.36 mM $AlCl_3$, 1 μg/ml valinomycin, 1 μM carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone and, where indicated, 1 mg/ml lubrol. Volume, 0.75 ml.

2.5. *Succinate dehydrogenase (SDH)*

SDH was measured at 25°C, in 220 mM mannitol, 70 mM sucrose, 10 mM Tris–Cl (pH 7.4), 40 mM Na-succinate, 0.1% BSA, 1.5 mM KCN, 5 mM K-phosphate, 1.5 mM K-ferricyanide and, where indicated, 1 μg antimycin A. Volume, 2 ml. The reduction of ferricyanide was followed at 420 nm.

2.6. *Ca^{2+}-transport measurements*

Ca^{2+}-uptake was measured isotopically. The medium (25°C) contained 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–Cl (pH 7.4), 0.1 mg/ml BSA, 5 mM phosphate, 5 mM Na-succinate, 2.5 mM Na-ATP, 1 mM $^{45}CaCl_2$, 1–2 mg protein and, where indicated, 1 μM RR. Volume, 1 ml. Aliquots (75 μl) were removed rapidly and filtered through Millipore filters (0.22 μm pore size). The filters were rinsed with 1.5 ml ice-cold 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–Cl (pH 7.4).

3. Results and discussion

Figure 1 shows the elution profile of SMP from a sepharose 4B-cytochrome *c* column. Two populations of vesicles, which will be termed 'fraction 1' and 'fraction 2', are visible. Fraction 1 (70–80% of the total SMP) is eluted at low ionic strength (100 mM KCl). Fraction 2 (20–30% of the total SMP) is eluted at higher ionic strength. On the 2 fractions, two membrane-polarity tests were performed, cytochrome *c* oxidation, and the antimycin A-sensitive reduction of ferricyanide by succinate [21]. In the first test, the stimulation of added cytochrome *c* oxidation by detergents was studied [20]. In mitochondria, and in right-side out SMP, no stimulation is to be expected, since the cytochrome *c* side is exposed. Stimulation is expected in inverted vesicles, since the detergent makes the cytochrome *c*-site available to added cytochrome *c*. As expected, the oxidation of cytochrome *c* was not stimulated by lubrol in mitochondria, stimulated 1.89-times in standard SMP and 2.49-times in fraction 1, (table 1). Fraction 1 is thus enriched in inside-out SMP, and fraction 2 in right-side out SMP. However, fraction 1 still oxidizes added cytochrome *c* without lubrol, and fraction 2 is stimu-

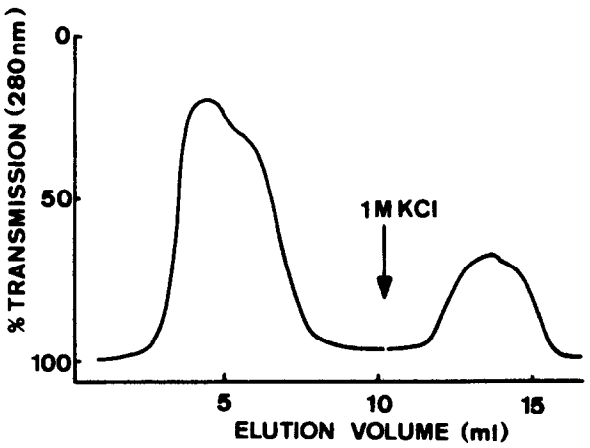

Fig.1. Separation of right-side out and inside-out vesicles from a preparation of sonified submitochondrial fragments. The preparation of liver mitochondria, the sonication procedure, and the preparation of the Sepharose 4B-cytochrome *c* column are described in section 2. The dimensions of the column were 17 × 0.9 cm. The column was loaded with about 40 mg of SMP protein.

Table 1
Membrane polarity tests in purified and unpurified SMP

Preparation	Cytochrome oxidase		
	–lubrol (*a*)	+lubrol (*b*)	*b*/*a*
Mitochondria	1.21 ± 0.27	1.40 ± 0.24	1.16
Unpurified SMP	1.33 ± 0.04	2.51 ± 0.27	1.89
Fraction 1	1.34 ± 0.11	3.34 ± 0.23	2.49
Fraction 2	1.97 ± 0.28	2.82 ± 0.04	1.43

	Ferricyanide reduction by succinate		
	–antimycin A	+antimycin A	% inhibition
Mitochondria	0.77 ± 0.01	0.05 ± 0.01	93.5
Unpurified SMP	0.17 ± 0.06	0.09 ± 0.02	47.0
Fraction 1	0.21 ± 0.06	0.18 ± 0.05	14.0
Fraction 2	0.13 ± 0.01[a]	0.07[b]	46.0

The assays are described in section 2. Volumes are given in μatoms oxygen min^{-1}. mg $protein^{-1}$ (cytochrome oxidase) and in μmol succinate oxidized. min^{-1}. mg $protein^{-1}$ (ferricyanide reduction by succinate) plus SD. Three different preparations were used, except in [a](2 preparations) and [b]one preparation

lated somewhat by the detergent. The 'purification' achieved with the affinity chromatography column could thus have been only partial. Alternatively, part of the cytochrome *a* could have become somewhat 'scrambled', and thus face both sides of the membrane. Such rearrangement had indeed already been indicated in sonic SMP, [22]. The second part of table 1 suggests indirectly that rearrangement of some cytochrome *a* may have indeed taken place. Clearly, SDH shows that the cytochrome *c* affinity column has separated from SMP a population of vesicles (fraction 1) in which the reduction of ferricyanide is practically antymicyn A insensitive, and which is thus essentially constituted by inverted SMP.

Figure 2 shows that standard SMP (~50% right-side out, see table 1), are able to actively accumulate Ca^{2+}. As in [1], the reaction requires both ATP and succinate, is observed in the presence of phosphate, and is oligomycin-insensitive. (ATP probably stabilizes the Ca-phosphate precipitates inside the vesicles [23].) Since the process is RR-sensitive, it evidently represents the normal uptake of Ca^{2+} in the fraction of SMP oriented as the mitochondria. By contrast,

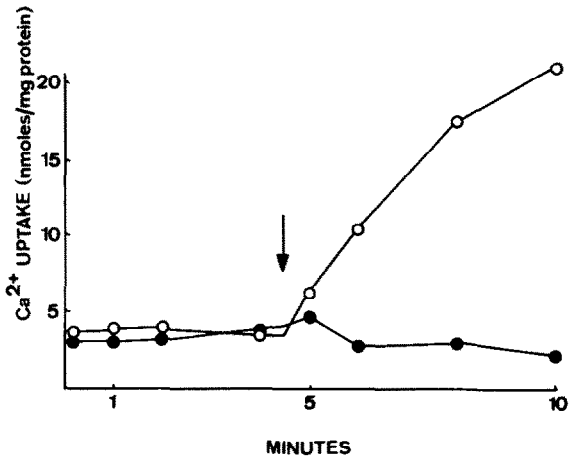

Fig.2. Ca^{2+} uptake in the standard unpurified SMP preparation. The preparation of mitochondria and of SMP, and the conditions for measuring Ca^{2+} uptake are described in section 2. At the arrow, succinate and ATP were added: (○–○) control; (●–●) plus RR.

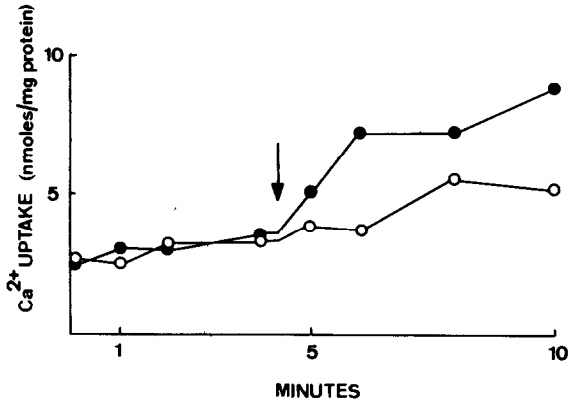

Fig.3. Ca^{2+} uptake in the purified, inside-out SMP preparation. Conditions as in the experiment of fig.2: (o–o) control; (•–•) plus RR.

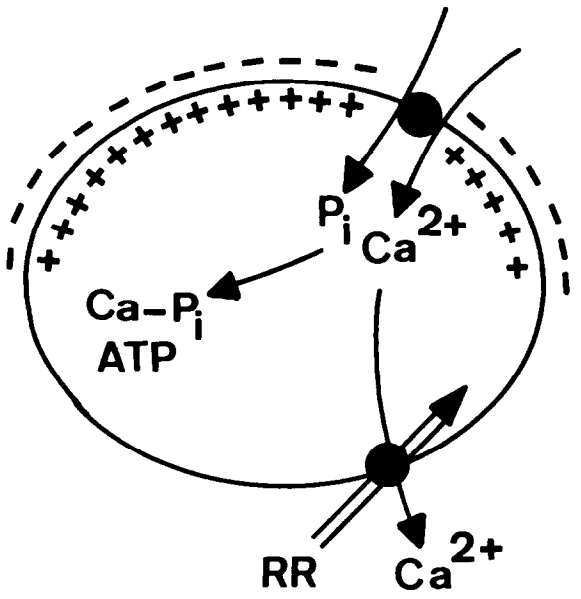

Fig.4. A scheme for the transport of Ca^{2+} in inside-out submitochondrial vesicles.

energization of 'purified' SMP (fig.3) fails to induce uptake of Ca^{2+}, unless the vesicles are treated with RR. A logical interpretation is that in the 'purified', positive-inside SMP, a primary uptake of phosphate occurs, accompanied by the uptake of Ca^{2+}. However, Ca^{2+} is lost to the medium on the uptake uniporter, (now operating in the direction of release) unless the latter is blocked by RR. In the scheme shown in fig.4, energy is visualized to drive the uptake of phosphate, and ATP to stabilize the Ca–phosphate precipitates [23]. Whether the uptake or phosphate occurs on one of the 2 known phosphate carriers, or on a new one (possibly an obligatory Ca–phosphate symporter) is open to question: experiments with inhibitors of a phosphate transport have so far been inconclusive. Another open question is the driving force for the phosphate-linked uptake of Ca^{2+}. Indeed, it is not obvious why the uptake of the phosphate should be accompanied by the uptake of Ca^{2+} in positive-inside energized SMP. Various possibilities are now under study, among them the transport of a negatively-charged Ca–phosphate complex.

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