Electroneutral efflux of Ca^{2+} from liver mitochondria

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1. Respiring liver mitochondria were allowed to export Ca^{2+} on the endogenous Ca^{2+}/nH^+ antiporter in the presence of Ruthenium Red (to inhibit uptake on the Ca^{2+} uniporter) until a steady state was reached. 2. Addition of sufficient of the ionophore A23187 (which catalyses $Ca^{2+}/2H^+$ exchange) to bring the Ca^{2+} and H^+ gradients into equilibrium did not alter the steady state. 3. Thermodynamic analysis showed that if a Ca^{2+}/nH^+ exchange with any value of n other than 2 was at equilibrium, addition of A23187 would have caused an easily measurable change in extramitochondrial free $[Ca^{2+}]$. 4. Therefore the endogenous carrier of liver mitochondria catalyses electroneutral $Ca^{2+}/2H^+$ antiport.

Mitochondria take up Ca^{2+} electrophoretically, by Ca^{2+} uniport. They release it on an antiporter by exchange with H^+ (in many tissues, including liver) or Na^+ (in tissues such as heart and brain) (Saris & Åkerman, 1980; Nicholls & Crompton, 1980; Nicholls & Åkerman, 1982). This calcium cycle is thought to be important in regulation of intra- and extramitochondrial $[Ca^{2+}]$ (Denton & McCormack, 1980; Williamson *et al.*, 1981).

The reaction carried out by the antiporter in liver mitochondria is thought to involve H^+ because artificially imposed pH gradients are able to cause Ca^{2+} uptake or release (Åkerman, 1978; Fiskum & Cockrell, 1978). However, it has not yet been clearly established whether the carrier catalyses $Ca^{2+}/1H^+$, $Ca^{2+}/2H^+$ or $Ca^{2+}/3H^+$ antiport. These different exchanges have very different energetics, being opposed by $\Delta\psi$, electroneutral or driven by $\Delta\psi$ respectively. A knowledge of the exchange catalysed is necessary for quantitative understanding of the calcium cycle and its bioenergetics, and for elucidation of the mechanism of the calcium efflux carrier.

Electroneutral $Ca^{2+}/2H^+$ antiport has been proposed by Fiskum & Lehninger (1979) on the basis of kinetic measurements of Ca^{2+} and H^+ movements during Ca^{2+} efflux stimulated by acetoacetate, but the interpretation of their experiments is open to strong criticism (see Nicholls & Crompton, 1980; Nicholls & Åkerman, 1982). A similar

conclusion that the carrier catalyses $Ca^{2+}/2H^+$ antiport was reached by Maglova *et al.* (1982). Zoccarato & Nicholls (1981, 1982) have suggested that $Ca^{2+}/1H^+$ antiport operates. In heart, the number of Na^+ exchanged for each Ca^{2+} is unknown, but indirect evidence indicates that it is 2 or 3 (Crompton *et al.*, 1976, 1977; Crompton & Heid, 1978; Affolter & Carafoli, 1980; Hayat & Crompton, 1982).

In this paper I show that the calcium efflux carrier of liver mitochondria is electroneutral, i.e. catalyses $Ca^{2+}/2H^+$ antiport.

Experimental

Mitochondria

Mitochondria were isolated by conventional methods (Chappell & Hansford, 1972) in 250 mM-sucrose/5 mM-Tris/HCl/1 mM-(potassium) EGTA, pH 7.4, washed once in 250 mM-sucrose/5 mM-Tris/HCl, pH 7.4, and kept on ice until use. Mitochondrial protein was assayed by a biuret method (Gornall *et al.*, 1949).

Free calcium concentration

This was measured with a Radiometer F2112 calcium-sensitive electrode and K801 reference connected to a Radiometer PHM64 pH meter and Bryans 28000 chart recorder. The electrodes were inserted into a plastic vessel of 3 ml capacity, magnetically stirred and open to the air, maintained at 30°C. Except where otherwise stated all experiments were carried out in a medium containing 250 mM-sucrose, 5 mM-(TMA) Hepes, 10 mM-(TMA) succinate, 10 mM-(TMA) acetate, 1 mM-

(TMA) NTA, $5 \mu M$-rotenone, $20 \mu M$-methyl-amine/HCl, 200 nM-methyltriphenylphosphonium bromide, brought to pH 7.1 with TMA hydroxide. Total calcium measured by atomic absorption spectrophotometry was $22 \mu M$. The signal from the electrode was calibrated by adding known amounts of standard 10 mM-$CaCl_2$ to the medium and calculating free $[Ca^{2+}]$ using the algorithm of Fabiato & Fabiato (1979) on a BBC microcomputer, using apparent stability constants at pH 7.1 of $5873 M^{-1}$ for Ca–NTA (Dippenaar & Brand, 1982), $15.68 M^{-1}$ for Ca–succinate and $3.38 M^{-1}$ for Ca–acetate (Martell & Smith, 1977).

$\Delta \psi$, ΔpH and matrix volume

These were measured with radioisotopes. Radiochemicals present were either $0.05 \mu Ci$ of $[^3H]$-methyltriphenylphosphonium/ml for $\Delta \psi$, or $1 \mu Ci$ of ^3H_2O/ml and $0.14 \mu Ci$ of D-$[1-^{14}C]$mannitol/ml for matrix volume, or $1 \mu Ci$ of $[6,6'(n)-^3H]$-sucrose/ml and $0.14 \mu Ci$ of $[^{14}C]$methylamine/ml for ΔpH. Mitochondria (2 mg of protein/ml) were added, then, at the times shown in Fig. 3, 1 ml samples were taken in triplicate and centrifuged. Supernatant and pellet were assayed for radioactivity on an LKB Rackbeta scintillation counter with quench corrections. ΔpH, matrix volume and $\Delta \psi$ (using a binding correction factor of 0.33 for methyltriphenylphosphonium) were calculated as described in Brown & Brand (1985).

Correction for methylamine binding

This was assessed by imposing known pH gradients (acid inside) and measuring $[^{14}C]$-methylamine uptake. Mitochondria (5 mg of protein/ml) were suspended in medium containing 5 mM-(TMA) Hepes, $5 \mu M$-rotenone, $20 \mu M$-methyl-amine/HCl, 1 mM-(TMA) EGTA, $1 \mu M$-antimycin and varying proportions of sucrose and KCl to a total osmolarity of 260 mosM, pH 7.0, 25°C. The medium contained either $1 \mu Ci$ of $[^3H]$sucrose/ml and $0.1 \mu Ci$ of $^{86}RbCl$/ml, or $1 \mu Ci$ of $[^3H]$suc-rose/ml and $0.14 \mu Ci$ of $[^{14}C]$methylamine/ml, or $1 \mu Ci$ of ^3H_2O/ml and $0.14 \mu Ci$ of $[^{14}C]$manni-tol/ml. Nigericin ($0.8 \mu g$/mg of protein) was then added to equilibrate K^+ and H^+ gradients, and after 2 min the suspension was centrifuged and assayed as before. Addition of more nigericin did not affect the results, showing that equilibrium had been attained. Fig. 1 shows that methylamine binding was minimal. Methylamine accumulation was very similar to ^{86}Rb accumulation over a wider range of ΔpH than employed in this paper. From the slope and intercept of the line in Fig. 1 a correction was applied by using the formula:

Corrected methylamine accumulation ratio =
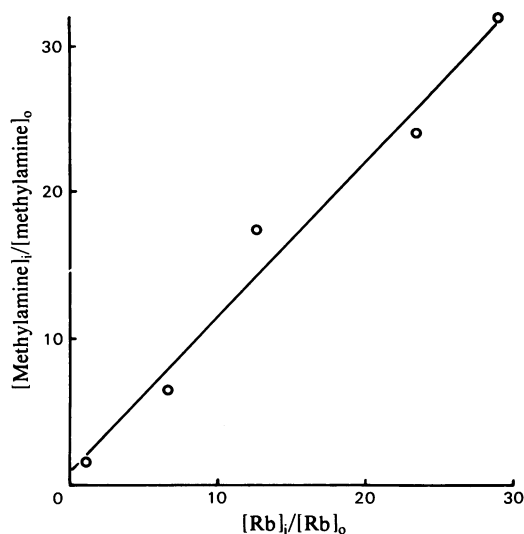0.94 (observed accumulation ratio) − 0.9

Fig. 1. *Calibration of* $[^{14}C]$*methylamine accumulation* Mitochondria were suspended in a sucrose-based medium containing 0, 0.5, 3, 20 or 125 mM added KCl, pH gradients (acid inside) were established by adding nigericin and measured by using ^{86}Rb. For details see under 'Experimental'. The line drawn was calculated by linear regression and had slope of 1/0.94 and intercept on the Rb axis of −0.895.

As a check on the use of methylamine, some experiments similar to those in Fig. 3 were carried out with ΔpH measured by using $0.8 \mu Ci$ of $[^3H]$acetate/ml. These confirmed that ΔpH was large and reversed, but were less accurate since the acetate was excluded from the matrix space.

Chemicals

Radiochemicals were from Amersham International, except for $[^3H]$methyltriphenyl-phosphonium, which was from New England Nuclear. Ruthenium Red was from Sigma and was purified as described by Luft (1971). Standard 10 mM-$CaCl_2$ was from BDH.

Results

Effect of A23187

The approach I report here to discover the pathway of Ca^{2+} efflux was to allow Ca^{2+} to come to the equilibrium catalysed by the Ca^{2+}/nH^+ antiporter, then to add the ionophore A23187 and investigate whether the equilibrium was shifted. A23187 is thought to catalyse electroneutral $Ca^{2+}/2H^+$ exchange (Reed & Lardy, 1972; McLaughlin & Eisenberg, 1975; Pfeiffer et al., 1976) so if there is no change in the equilibrium when it is added then the endogenous mitochondrial carrier must also catalyse $Ca^{2+}/2H^+$ antiport.

To allow discrimination between different possible values of n in Ca^{2+}/nH^+ antiport (see below), ΔpH had to be made positive, i.e. acid inside. This was achieved by incubating mitochondria in a medium containing no added K^+ and allowing the endogenous K^+/H^+ exchange to equilibrate the K^+ and H^+ gradients. In preliminary experiments I added nigericin to facilitate this exchange, but it made little difference to ΔpH so it was omitted from the experiments reported here. The value of $\Delta\tilde{\mu}H^+$ in this medium was quite low, about $120\,mV$ (Table 1). This was presumably because of the exclusion of succinate from the matrix by the reversed pH gradient and the effect of the acid matrix pH making succinate dehydrogenase more rate limiting. Since both $\Delta\psi$ and ΔpH were measured, the low value of $\Delta\tilde{\mu}H^+$ does not affect the conclusions to be drawn from these experiments.

Fig. 2(a) shows that in this medium the mitochondria accumulated Ca^{2+}, and addition of Ruthenium Red, which inhibits the uniporter, caused efflux. Efflux continued for more than 25 min. At this point addition of A23187 caused further rapid efflux followed by a steady level of $[Ca^{2+}]^f_o$. This steady level represented the equilibrium catalysed by A23187, since addition of more

A23187 did not change $[Ca^{2+}]^f_o$ (results not shown). To reduce the time taken to reach equilibrium, all subsequent experiments were carried out as shown in Fig. 2(b), with Ruthenium Red added before the mitochondria to prevent uptake of Ca^{2+}. This allowed a steady value of $[Ca^{2+}]^f_o$ to be reached within 15 min. This value was the same as that seen after A23187 addition in Fig. 2(a) and did not change when A23187 was added. Clearly the steady state catalysed by the endogenous mitochondrial antiporter (Fig. 2b) is not experimentally different from that catalysed by A23187 (Figs. 2a and 2b), i.e. the endogenous antiporter is not experimentally distinguishable from one catalysing $Ca^{2+}/2H^+$ exchange in this experiment.

The question I then asked was whether $Ca^{2+}/2H^+$ exchange on the antiporter would have been distinguishable from other modes of exchange, particularly $Ca^{2+}/1H^+$ and $Ca^{2+}/3H^+$ antiport. The rest of the results I report here show that it would have been.

Thermodynamics of Ca^{2+}/nH^+ antiport

For the general case of a Ca^{2+}/nH^+ antiport at equilibrium:

$$\Delta\tilde{\mu}Ca^{2+} = n\Delta\tilde{\mu}H^+$$

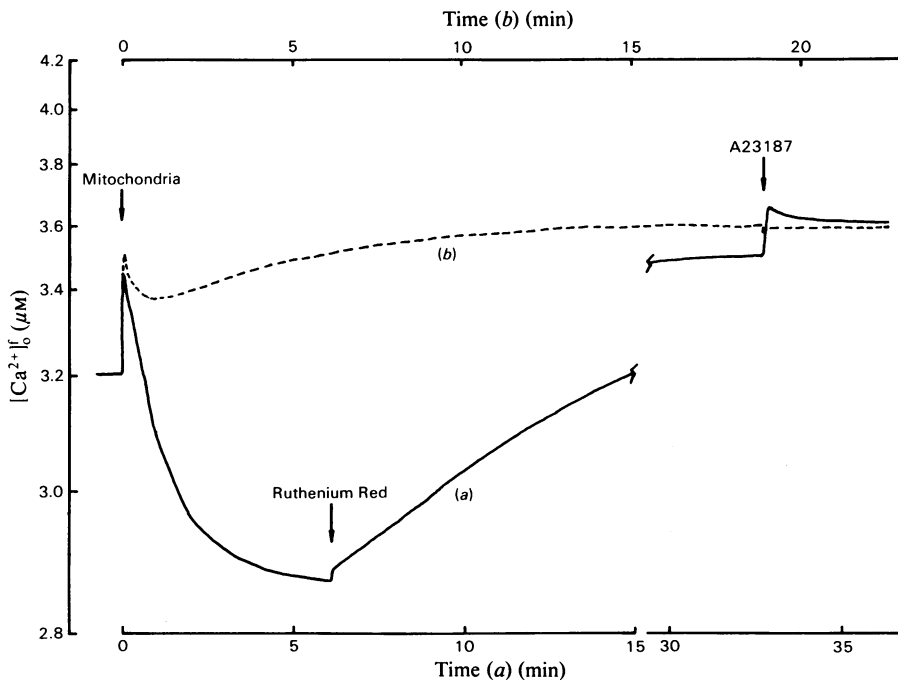Fig. 2. *Approach of the Ca^{2+} antiporter to equilibrium*
Mitochondria (2 mg of protein/ml) were suspended in medium, and $[Ca^{2+}]^f_o$ was measured with a calcium-sensitive electrode as described under 'Experimental'. In (b) Ruthenium Red (0.83 nmol/mg of protein) was added before the mitochondria; in (a) it was added where indicated. Where shown A23187 was added at 0.6 nmol/mg of protein.

therefore:

$$2\Delta\psi - 60\log([Ca^{2+}]^f_i/[Ca^{2+}]^f_o) = n\Delta\psi - 60n\Delta pH$$

where subscripts i and o refer to the intra- and extramitochondrial compartments respectively and superscript f denotes free.

Rearranging:

$$[Ca^{2+}]^f_i/[Ca^{2+}]^f_o = 10\exp[(2-n)\Delta\psi/60 + n\Delta pH] \tag{1}$$

The total amount of Ca^{2+} in the system able to participate in the equilibrium, $Ca^{2+,t}_{io} = Ca^{2+,t}_o + Ca^{2+,t}_i$, i.e.:

$$Ca^{2+,t}_{io} = [Ca^{2+}]^f_o \cdot B_o \cdot V + [Ca^{2+}]^f_i \cdot B_i \cdot v$$

where superscript t denotes total, B_o and B_i are $[Ca^{2+}]^t/[Ca^{2+}]^f$ in compartments o and i, V is the volume of the extramitochondrial compartment and v is the volume of the intramitochondrial compartment.

Rearranging:

$$[Ca^{2+}]^f_o = (Ca^{2+,t}_{io} - [Ca^{2+}]^f_i \cdot B_i \cdot v)/B_o \cdot V \tag{2}$$

Substituting eqn. (1) in eqn. (2):

$$[Ca^{2+}]^f_o = (Ca^{2+,t}_{io} - [Ca^{2+}]^f_o \cdot B_i \cdot v \cdot 10\exp[(2-n)\Delta\psi/60 + n\Delta pH])/B_o \cdot V$$

Rearranging:

$$Ca^{2+,t}_{io} = [Ca^{2+}]^f_o \{B_o \cdot V + B_i \cdot v \cdot 10\exp[(2-n)\Delta\psi/60 + n\Delta pH]\} \tag{3}$$

and

$$[Ca^{2+}]^f_o = Ca^{2+,t}_{io}/(B_o \cdot V + B_i \cdot v \cdot 10\exp[(2-n)\Delta\psi/60 + n\Delta pH]) \tag{4}$$

Predicted $[Ca^{2+}]^f_o$ for different values of n

Eqns. (3) and (4) were then applied to the data obtained from the experiments described in Fig. 3. This allowed calculation of the value of $[Ca^{2+}]^f_o$ which would be predicted by different values of n in Ca^{2+}/nH^+ antiport. Fig. 3(a) shows the steady state $[Ca^{2+}]^f_o$ achieved by mitochondria treated as in Fig. 2(b). Fig. 3(b) shows an identical experiment but with extra $CaCl_2$ added to the medium before the addition of mitochondria. At the points marked in Fig. 3, samples were taken from exactly parallel incubations and assayed for v, $\Delta\psi$ and ΔpH. v was found to be 0.21 μl/mg of protein. This was a low value, but it is not unreasonable since the major intramitochondrial cation is K^+, and much of this would have left the matrix in exchange for H^+ in the long approach to the steady state. Table 1 shows the values of $\Delta\psi$ and ΔpH at each of the points marked in Fig. 3.

B_i was easily determined by applying the principle of conservation of matter following addition of a pulse of $CaCl_2$. It was calculated as $\Delta[Ca^{2+}]^t_i/\Delta[Ca^{2+}]^f_i$ before and after addition of 30 nmol of $CaCl_2$ in the presence of A23187 at known ΔpH and matrix volume, as shown in Fig. 3, where $[Ca^{2+}]^f_i = [Ca^{2+}]^f_o \cdot 10^{2\Delta pH}$, $\Delta[Ca^{2+}]^t_i = (3 \times 10^{-8} - \Delta Ca^t_o)/v$ and $Ca^t_o = V \cdot [Ca^{2+}]^f_o \cdot B$.

The values of B_i obtained in this way were 12.2 and 10.2 for the experiments in Figs. 3(a) and 3(b) respectively. These are similar to those of Nicholls (1978) but much lower than the ones found by Coll et al. (1982) and Denton & McCormack (1980) under rather different conditions. The differences may be due to the very acid matrix pH of less than pH 6 in my experiments.

The values of $[Ca^{2+}]^f_o$, $\Delta\psi$, ΔpH, V, v, B_o and B_i for sample 2 were then substituted in eqn. (3) to calculate Ca^t_{io} in the presence of A23187, where $n = 2$. The values obtained were 15.2 nmol/mg of protein for the experiment in Fig. 3(a) and 19.3 nmol/mg of protein for Fig. 3(b). These agreed with, but were a little less than, the values found by direct measurement using atomic absorption spectrophotometry (16.8 and 25.2 nmol/mg of protein), showing that most, but not all, of the calcium in the mitochondria was able to participate in the equilibrium in these experiments.

Finally the predicted value of $[Ca^{2+}]^f_o$ at equilibrium from sample 1 was calculated from eqn. (4) for different values of n. This assumed that B_i did not change when A23187 was added, which seems reasonable. The values calculated in this way are reported in Table 2.

I then used the predicted values of $[Ca^{2+}]^f_o$ in Table 2 to determine whether the equilibrium catalysed by a Ca^{2+}/nH^+ antiporter would have been measurably disturbed by addition of A23187. Values of n less than 2 predict very low $[Ca^{2+}]^f_o$ because of the contribution of $\Delta\psi$ to uptake. A23187 would have caused huge changes in $[Ca^{2+}]^f_o$. This did not happen, so values of n less than 2 are excluded. With n greater than 2 most of the Ca^{2+} would be extramitochondrial, driven out by $\Delta\psi$, and the differences in predicted $[Ca^{2+}]^f_o$ are not so great. However, they would still have been easily distinguished from the equilibrium catalysed by A23187, and values of n greater than 2 are therefore also excluded. The main reason this experiment can distinguish between $n = 2$ and $n > 2$ is that ΔpH is reversed. This means that $n = 2$ gives substantial accumulation of Ca^{2+} (about 200-fold) at equilibrium, whereas with $n > 2$, $\Delta\psi$ dominates
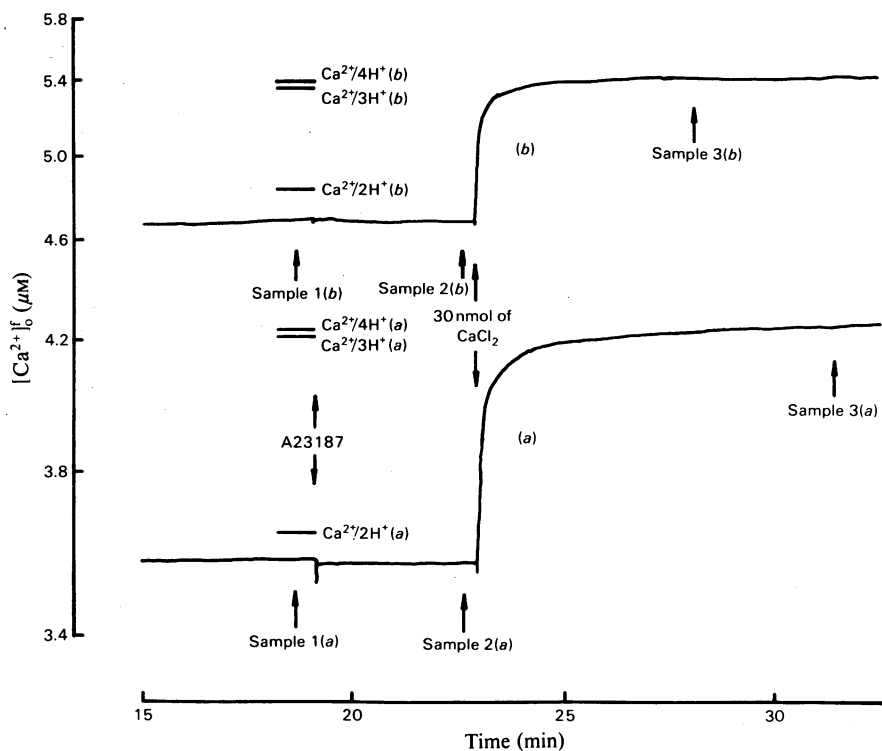
Fig. 3. *Effect of A23187 on $[Ca^{2+}]_o^f$ in the steady state*
Mitochondria were allowed to come to a steady state in the presence of Ruthenium Red as described for Fig. 2(*b*). In (*b*) an additional 8.33 nmol of $CaCl_2$/mg of protein was added to the medium before the mitochondria. Parallel experiments using radioactive markers for measurement of intracellular volume, ΔpH and $\Delta\psi$ were carried out simultaneously, with radioactive samples taken at the times marked on the traces. Where indicated 0.6 nmol of A23187/mg protein was added. The bars marked $Ca^{2+}/3H^+$ (*a*) etc. show the predicted value of $[Ca^{2+}]_o^f$ for that exchange in experiment (*a*) or (*b*).

Table 1. *$\Delta\psi$ and ΔpH for the samples marked in Fig.3*
For details see Fig. 3 and under 'Experimental'.

Sample	$\Delta\psi$ (mV)	ΔpH (pH units)	$[Ca^{2+}]_o^f$ (μM)	$[Ca^{2+}]_o^t$ (μM)	$[Ca^{2+}]_i^f$ (μM)	B_i
1(*a*)	187.4	+1.183	3.58	24.85		
2(*a*)	192.9	+1.210	3.57	24.78	939	
3(*a*)	170.9	+1.198	4.26	29.48	1060	12.18
1(*b*)	187.1	+1.124	4.70	32.46		
2(*b*)	185.9	+1.194	4.70	32.46	1148	
3(*b*)	169.2	+1.150	5.46	37.58	1089	10.16

and most of the Ca^{2+} is excluded from the matrix. The predicted values of $[Ca^{2+}]_o^f$ are shown on Fig. 3 for $Ca^{2+}/2H^+$, $Ca^{2+}/3H^+$ and $Ca^{2+}/4H^+$ antiport to show the size of the changes expected when ionophore is added; changes in $[Ca^{2+}]_o^f$ of this magnitude would not have escaped detection.

The predicted value of $[Ca^{2+}]_o^f$ for $Ca^{2+}/2H^+$ antiport differs a little from the observed one. This is because the value of ΔpH was slightly different before and after A23187 addition. This may be caused by the experimental variations in measured ΔpH, or it might represent a residual Ruthenium

Red-insensitive contribution from the Ca^{2+}-uniporter which could pull the steady state $[Ca^{2+}]_o^f$ a little below the equilibrium value for electroneutral exchange. It is possible that $Ca^{2+}/3H^+$ antiport is occurring with the steady state pulled by residual uniport activity to the same $[Ca^{2+}]_o^f$ as the equilibrium for $Ca^{2+}/2H^+$ antiport, but this seems highly improbable.

The difference between the predicted and observed values of $[Ca^{2+}]_o^f$ for values of n other than 2 increases as B_i increases. Thus, if my estimate of B_i is low, then the conclusion that the endogenous

Table 2. *Predicted values of* $[Ca^{2+}]_o$ *for different modes of* Ca^{2+}/nH^+ *antiport at equilibrium for the experiment of Fig. 3*

See the text for details.

Antiport	Predicted $[Ca^{2+}]_o$ (M)	
	Expt. (a)	Expt. (b)
Ca^{2+} uniport	3.320×10^{-9}	5.171×10^{-9}
$Ca^{2+}/1H^+$	2.709×10^{-7}	5.160×10^{-7}
$Ca^{2+}/2H^+$	3.618×10^{-6}	4.873×10^{-6}
$Ca^{2+}/3H^+$	4.216×10^{-6}	5.388×10^{-6}
$Ca^{2+}/4H^+$	4.224×10^{-6}	5.394×10^{-6}
$Ca^{2+}/5H^+$	4.224×10^{-6}	5.394×10^{-6}

antiport is electroneutral would be strengthened. If my estimate of B_i is high the predicted differences are less, but they would still have been seen even when $B_i = 1$.

The possibility that the observed electroneutral antiport represents only the net reaction of, for example, $Ca^{2+}/2C^+$ exchange plus C^+/H^+ exchange is not excluded by my data. Indeed, it is not impossible that there could be some contribution of $Ca^{2+}/2Na^+$ antiport in these experiments (see Goldstone & Crompton, 1982). However, because of the low concentration of Na^+ and K^+ I do not consider these other exchanges to be likely in my experiments.

Discussion

These experiments show unambiguously that the Ca^{2+} efflux carrier of rat liver mitochondria catalyses an electroneutral reaction. The carrier performs the same exchange as the ionophore A23187, i.e. $Ca^{2+}/2H^+$ antiport.

Previous evidence for electroneutrality has been provided by Fiskum & Lehninger (1979), who showed kinetically that about $2H^+$ disappeared from the medium per Ca^{2+} appearing during acetoacetate-induced Ca^{2+} release. As discussed by Nicholls & Crompton (1980) and Nicholls & Åkerman (1982), the interpretation is open to doubt since acetoacetate causes collapse of $\Delta\psi$ (Nicholls & Brand, 1980; Beatrice et al., 1980, 1982; Bardsley & Brand, 1982; Siliprandi et al., 1983). The results of Fiskum & Lehninger (1979) could well have been caused by enhanced H^+ leak and electrically compensating Ca^{2+} efflux on the uniporter, and might have no relevance to the exchange catalysed by the antiporter. Lack of sensitivity to Ruthenium Red cannot exclude the possibility of uniport activity, since Ca^{2+} efflux by uniport can occur under some uncoupled conditions in the presence of this inhibitor (Jurkowitz et al., 1983; Bernardi et al., 1984). This criticism does not

apply to the results of Maglova et al. (1982) who carried out similar kinetic experiments on Ca^{2+} release stimulated by Ruthenium Red and showed that $\Delta\psi$ did not drop during their experiments.

Zoccarato & Nicholls (1981, 1982) have suggested that the enhanced rate of Ca^{2+} efflux seen when uncouplers are added in the presence of Ruthenium Red might be evidence for $Ca^{2+}/1H^+$ antiport. Thus ΔpH would drive efflux but $\Delta\psi$ would oppose it, and uncoupling could stimulate by decreasing $\Delta\psi$ more than ΔpH. The data I present here suggest that this is not the case and support the view of Bernardi et al. (1984) that the effect is due to incomplete inhibition of the uniporter by Ruthenium Red under these conditions.

I conclude that the Ca^{2+} antiporter of liver mitochondria catalyses exchange of Ca^{2+} for $2H^+$.

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