Non-respiring Rat Liver Mitochondria Do Not Have a $Ca^{2+}/2H^{+}$ Antiporter

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Liver mitochondria take up Ca^{2+} by the Ca^{2+} uniporter, whereas at steady state efflux is believed to occur mainly by means of a ruthenium red-insensitive $Ca^{2+}/2H^{+}$ antiporter. The latter activity was studied in respiration-inhibited mitochondria in the presence of ruthenium red and was measured as Ca^{2+} uptake following acidification of the matrix by addition of nigericin, which catalyzes K^{+}/H^{+} exchange. Ca^{2+} uptake was stimulated by protonophorous uncoupling agents and inhibited by increasing the concentration of ruthenium red. However, the rates were always smaller than those obtained by addition of valinomycin instead of nigericin. This indicates that under these conditions, Ca^{2+} fluxes are not mediated by a $Ca^{2+}/2H^{+}$ antiporter but by residual uniporter activity.

The distribution of Ca^{2+} between the matrix and extramitochondrial spaces is generally believed to be determined by the rates of influx through the Ca^{2+} uniporter and efflux by various pathways.[1] Of these, the Ca^{2+}/Na^{+} antiporter is of minor importance in liver mitochondria[2] where an electroneutral $Ca^{2+}/2H^{+}$ antiporter may be involved.[3] Evidence in favour of such a putative carrier is efflux of accumulated Ca^{2+} on acidification of the medium[4,5] and influx of Ca^{2+} in response to acidification of the matrix of non-respiring mitochondria whose uniporter activity has been inhibited with ruthenium red.[6,7] The former approach has been criticized on the ground that Ca^{2+} efflux at the uniporter is not readily inhibited by ruthenium red, which makes it difficult to rule out this pathway.[8] Lowering of the pH may lead to release of Ca^{2+} from poorly coupled mitochondria in a heterogeneous population.[8] Furthermore, varying Ca^{2+}/H^{+} stoichiometries at different pH values were found during H^{+}-induced Ca^{2+} release.[8] In the latter approach, the matrix can be acidified by addition of nigericin or dianemycin to mitochondria suspended in a low K^{+} medium, since these ionophores catalyze an electroneutral K^{+}–H^{+} exchange. The activity of the uniporter can then be more effectively inhibited with ruthenium red. This approach has also led to controversial results. Thus, Bernardi and Azzone[9] found negligible ruthenium red-insensitive uptake of Ca^{2+} under these conditions, which had been attributed by Cockrell[7] to inhibition of the putative antiporter activity by Mops*, the buffer used. This question was reexamined and negligible nigericin-induced uptake of Ca^{2+} by rat liver mitochondria found using the medium of Cockrell,[7] where the mitochondrial respiration and uniporter activity were completely inhibited.

Experimental

Materials. Most inhibitors and bovine serum albumin (fraction V) were obtained from Sigma, St. Louis, MO. Ruthenium red was from BDH Ltd., Poole, UK, antimycin A from Boehringer–Mannheim, FRG, safranine from E. Merck, Darmstadt, FRG, arsenazo III from Fluka AG, Buchs, Switz. and Chelex® 100 from Bio-Rad

*Abbreviations: $\Delta\Psi$, mitochondrial inner membrane transmembrane potential, negative inside; EGTA, ethyleneglycol-*O,O'*-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid; FCCP, mesoxalonitrile 4-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

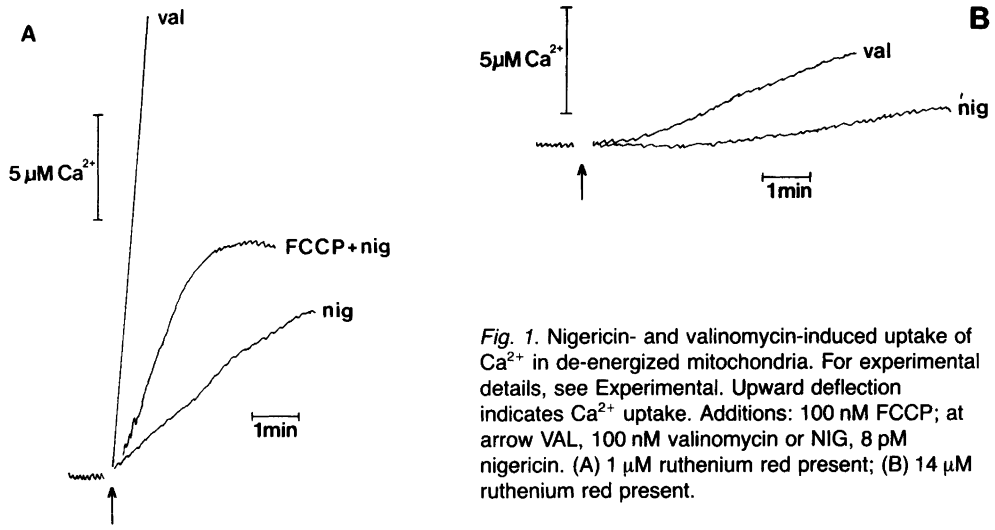

Fig. 1. Nigericin- and valinomycin-induced uptake of Ca^{2+} in de-energized mitochondria. For experimental details, see Experimental. Upward deflection indicates Ca^{2+} uptake. Additions: 100 nM FCCP; at arrow VAL, 100 nM valinomycin or NIG, 8 pM nigericin. (A) 1 μM ruthenium red present; (B) 14 μM ruthenium red present.

Laboratories. FCCP was a kind gift from Dr. P. G. Heytler, E. I. DuPont de Nemours and Co., Wilmington, DE.

Rat liver mitochondria were prepared by a conventional procedure and depleted of endogenous Ca^{2+} by incubation at 20 °C for 10 min at a concentration of 5 mg protein/ml in 210 mM mannitol, 70 mM sucrose, 10 mM Hepes-chloride (pH 7.3) and 1 μg each of oligomycin and rotenone/mg protein. They were then harvested by centrifugation, resuspended to a concentration of 40 mg protein/ml in 250 mM sucrose, 10 mM Hepes-chloride (pH 7.2) (depleted of Ca^{2+} by passing through a Chelex 100 column) and kept in an ice-bath until used. Ruthenium red and arsenazo III were purified as in Refs. 10 and 11.

Incubations were carried out at 3 mg protein/ml at 23 °C in the sucrose medium supplemented with 0.5 μg antimycin/ml, 2 μM rotenone, 2 μg oligomycin/ml and 1 mg bovine serum albumin/ml, unless otherwise indicated. Other details are given in the figure legends. Ca^{2+} uptake was followed by adding 50 μM arsenazo III and measuring the absorbance change at the wavelengths 685 and 665 nm.[11] Establishment of $\Delta\Psi$ was monitored at 554–524 nm by the safranine technique.[12] Protein was estimated by a biuret technique using bovine serum albumin as standard.

Results and discussion

In studies of the putative $Ca^{2+}/2H^+$ carrier by inducing efflux of accumulated Ca^{2+} by an external acid pulse[4,5] rather high Ca^{2+} loads were used,

Table 1. Ruthenium red-sensitivity of valinomycin and nigericin-induced Ca^{2+} uptake.

Ruthenium red Concentration (μM)	Valinomycin-induced uptake		Nigericin-induced uptake	
	Uptake [pmol/(mg × s)]	Inhibition (%)	Uptake [pmol/(mg × s)]	Inhibition (%)
0	20500		18.5	
1	290	98.6	14.5	21
4	54	99.7	6.5	65
10	19	99.9	5.0	73
14	7.8	99.96	2.8	85

>60 nmol/mg protein. The release of Ca^{2+} may be due to the increased permeability induced by a Ca^{2+} overload in a part of the heterogeneous mitochondrial population.[13] Indeed, at loads >40 nmol Ca^{2+}/mg protein, EGTA induces a fast efflux of part of the accumulated Ca^{2+}.[14] Ca^{2+} efflux through the uniporter is not sensitive to inhibition by ruthenium red and cannot be ruled out. Therefore, the other approach to inducing an artificial H^+ gradient, i.e. more acid inside so as to drive Ca^{2+} uptake by the putative carrier[5,6] in the presence of ruthenium red to inhibit the Ca^{2+} uniporter, was used. The Ca^{2+} load was also kept smaller and antimycin was used to completely block endogenous respiration that might otherwise extrude protons and induce the establishment of $\Delta\Psi$. Nigericin was used to exchange matrix K^+ for H^+ from the medium.

Fig. 1A shows the nigericin-induced uptake of Ca^{2+}. The uptake is stimulated by FCCP, which may seem unexpected since FCCP should equilibrate the H^+ gradient formed (see below). Fiskum and Cockrell[6] also used an uncoupler in order to de-energize the mitochondria. Ca^{2+} influx was also induced by addition of valinomycin instead of nigericin. Valinomycin creates an artificial K^+ diffusion potential that can be used to drive Ca^{2+} uptake via the uniporter.[13] Increasing the concentration of ruthenium red decreased the rates of Ca^{2+} uptake to very low levels (Fig. 1B and Table 1). The capacity of the uniporter is orders of magnitude larger than that of electroneutral pathways. Under the present conditions, the rates in the absence of ruthenium red were >1000-fold higher when induced by valinomycin than when nigericin was used. Ruthenium red progressively inhibited both the valinomycin- and the nigericin-induced Ca^{2+} uptake. Although inhibition of the valinomycin-induced uptake approached 100%, the remaining flux still exceeded the corresponding nigericin-induced flux. There is thus no indication of a $Ca^{2+}/2H^+$ antiporter. The antiporter could catalyze an uptake under these conditions of <3 pmol Ca^{2+} mg^{-1} s^{-1}, which corresponds to the rate of ruthenium red-insensitive Ca^{2+} uptake of <0.1 nmol mg^{-1} min^{-1} reported by Bernardi and Azzone.[9] The higher rates reported by Cockrell[7] may have been due to use of less ruthenium red, 4 μM, and incompletely respiration-inhibited mitochondria. Since in other respects a medium identical to that used by Cockrell[7] was used in this study, it is unlikely that the discrepancy is due to inhibition of antiporter activity by Mops as suggested by Cockrell.[7]

Addition of nigericin generated a safranine signal that was smaller and formed more slowly than that induced by valinomycin (Fig. 2). The response to nigericin was potentiated by FCCP. Under these conditions with high H^+ activity in

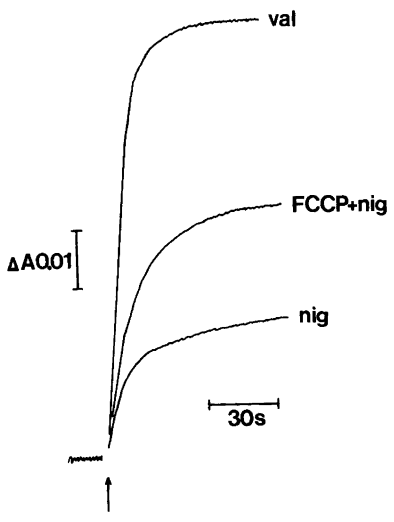

Fig. 2. Changes in $\Delta\Psi$ induced by FCCP and the ionophores. Experimental conditions were as in Fig. 1A but 10 μM safranine was used as the probe, as described in Experimental.

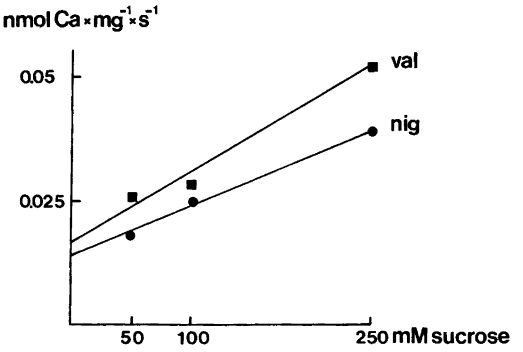

Fig. 3. Ionophore-stimulated Ca^{2+} uptake in hypotonic media. Osmolarity was varied by varying the concentration of sucrose in the medium. 2.5 mg mitochondrial protein/ml; 10 μM ruthenium red; ionophore concentrations as in Fig. 1.

the matrix, FCCP induces an H^+ diffusion potential of the same polarity as the K^+ diffusion potential induced by valinomycin.[12] It seems likely that the safranine signal recorded on addition of nigericin in the absence of FCCP also reflects the establishmeht of such a potential, although the signal could also result from stacking of safranine molecules to additional binding sites on the matrix side of the inner membrane.

The data support the interpretation that the nigericin-induced uptake of Ca^{2+} occurs on the uniporter that is not completely inhibited by ruthenium red and that the differences in influx rates (Fig. 1A) are mainly due to differences in $\Delta\Psi$. Ca^{2+} uptake may also be driven by the difference in Ca^{2+} activity between the medium and the matrix, where competition with K^+ for anionic binding sites is diminished both when valinomycin and nigericin are used. FCCP may stimulate uptake not only by inducing a $\Delta\Psi$ but also by making additional Ca^{2+} binding sites available.

Swelling of mitochondria, either by suspension in hypotonic media under respiratory conditions or in the presence of nitrate under non-energized conditions, has been shown to stimulate Ca^{2+}/H^+ exchange or other Ca^{2+} efflux pathways.[15,16] Sustained oscillatory volume changes of respiring rat liver mitochondria are also observed under hypoosmotic conditions in the presence of Sr^{2+} and permeant anions,[17] and may be due to induction of Sr^{2+}/H^+ exchange when the inner membrane is stretched during swelling induced by uptake of cation salt.[17,18] Therefore, the possibility that $Ca^{2+}/2H^+$ antiporter activity could be stimulated by membrane-stretching was also tested (Fig. 3). Suspending mitochondria in media with lowered sucrose content decreased the rates of both the nigericin- and the valinomycin-induced uptake of Ca^{2+}. Although these rates were close, those induced by valinomycin were always higher than those induced by nigericin. There was thus no evidence for the induction of antiporter activity by membrane-stretching in non-respiring mitochondria.

The present finding that there is no demonstrable direct coupling between Ca^{2+} and H^+ fluxes in non-respiring mitochondria leaves unresolved the question of the mechanism by which efflux of Ca^{2+} and influx of H^+ are coupled in respiring mitochondria. Gunter *et al.*[8] found no increase in the rate of Ca^{2+} efflux with increase in matrix pH and varying Ca^{2+}/H^+ stoichiometries, which is not easily reconciled with the operation of a $Ca^{2+}/2H^+$ antiporter. Coupling might be indirect via symport of Ca^{2+} with anions whose fluxes are coupled to those of H^+. Ca^{2+} and H^+ fluxes may also be associated with a redistribution of charged membrane components such as free fatty acids or acidic phospholipids.

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