Ca^{2+}-triggered membrane permeability transition in deenergized mitochondria from rat liver

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Abstract The opening of the cyclosporin A-sensitive permeability transition pore (MTP) in deenergized mitochondria was induced only at millimolar Ca^{2+}. Pretreatment of the mitochondria with 'inducers', such as duroquinone and phenylarsine oxide, allowed observing the pore opening at 0.01–0.1 mM Ca^{2+}. Duroquinone caused a rapid (within 20 s) NAD(P)H oxidation which was followed by a slow (20 min) induction of the pore sensitive to low Ca^{2+}. Phenylarsine oxide capable of cross-linking of vicinal SH-groups caused pore formation without the oxidation of NAD(P)H. The pore opening by both 'inducers' was prevented by *N*-ethylmaleimide. We propose that oxidation or cross-linking of critical dithiol(s) in membrane proteins increase the sensitivity of a putative 'Ca^{2+}-sensor' that regulates the permeability transition pore opening.

Key words: Mitochondria; Permeability transition pore; Ca^{2+}; NAD(P)H oxidation; SH-groups cross-linking

1. Introduction

Accumulation of Ca^{2+} in mitochondria from various animal tissues induces the opening of a large pore in the inner membrane (see [1] for review). This pore (permeability transition pore; MTP) permits an equilibration of solutes with molecular weights of 1,500 or less [2] and is inhibited by cyclosporin A (CsA) [3]. The permeability transition is observed only in the presence of Ca^{2+} and is fully reversed when Ca^{2+} is removed. Various agents which cause oxidation of matrix NAD(P)H or membrane dithiols stimulate the MTP opening [1]. The pore opening is suppressed at the matrix pH values below 7.0 [4] or by Mg^{2+} and ADP [5]. Bernardi et al. [4–7] have found that probability of the MTP opening increases with a decrease in the membrane potential and that various effectors, including ADP, Mg^{2+} and prooxidants, modify the voltage-dependence of the pore.

The present study was intended to bypass the complicated voltage-sensitive MTP regulation. The Ca^{2+}-triggered permeability transition was studied in deenergized mitochondria when the concentration of Ca^{2+} and pH in the matrix were under control. It was shown that the oxidation of NAD(P)H caused by duroquinone and the subsequent formation of disulphides in the membrane resulted in a more than 100-fold increase in the sensitivity of MTP to Ca^{2+}.

2. Materials and methods

Mitochondria from rat liver were isolated as described [8] in the presence of 0.25 mM EGTA. The medium for the last washing and storage contained no EGTA. The measurement medium contained: 200 mM sucrose, 20 mM Tris-MOPS, 20 μM EGTA, 2 μM rotenone, 1 μM A23187, 5 μM CCCP, pH 7.4. The concentration of mitochondrial protein was 0.6 mg/ml. The total concentration of Ca was determined by atomic absorption spectroscopy and the concentration of free Ca^{2+} was calculated using the standard software. NAD(P)H was measured at 340–370 nm with an Aminco DW-2000 spectrophotometer. Light-scattering was measured at 540 nm with a Cary-219 spectrophotometer. The samples were stirred continuously and thermostated at 25°C.

3. Results

3.1. Induction of the permeability transition by duroquinone in deenergized mitochondria

The permeability transition of mitochondria in the sucrose medium results in a high amplitude swelling when the membrane becomes permeable to sucrose. A decrease in the absorbance (at 540 nm) of a mitochondrial suspension is proportional to the fraction of mitochondria with an open permeability transition pore (MTP) [5,6]. In contrast to energized mitochondria, Ca^{2+} fails to cause the pore opening per se up to very high concentrations (1–5 mM) in the presence of an uncoupler. In uncoupled mitochondria at 24 μM Ca^{2+} the addition of 5 μM duroquinone (DQ) induced the pore opening after a lag phase of 2 min (Fig. 1A, trace 1). This effect was not observed in the absence of Ca^{2+} and was completely prevented by cyclosporin A (CsA) (Fig. 1A, traces 5 and 6). Similar transitions but with various lag phases were observed with the well-known 'pore inducers', namely tert-butylhydroperoxide and phenylarsine oxide (not shown).

The induction of the MTP opening by DQ was inhibited by various agents which suppressed oxidation of NAD(P)H in mitochondria. In the absence of exogenous substrates, 5 μM DQ induced a rapid and complete oxidation of NAD(P)H under our experimental conditions when rotenone, an uncoupler, Ca^{2+}-ionophore and Ca^{2+} were added. The second addition of DQ had no effect (Fig. 1B, trace 1). When glutamate + malate mixture was added before DQ, the steady-state concentration of reduced nucleotides was higher than in control (Fig. 1B, trace 2) and the lag phase of pore induction was longer (Fig. 1A, trace 2). An inhibitor of the respiratory chain, mixothiazole, prevented reoxidation of reduced DQ, allowed the recovery of NAD(P)H after DQ-induced oxidation (Fig. 1B, trace 3) and inhibited MTP opening (Fig. 1A, trace 3). Antimycine produced the same effects whereas in the absence of sub-

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Abbreviations: MTP, mitochondrial permeability transition pore; DQ, duroquinone; PhAsO, phenylarsine oxide; NEM, *N*-ethylmaleimide; EGTA, [ethylenebis(oxoethylene nitrilo)]tetraacetic acid; CCCP, carbonylcyanide-*m*-chloromethoxyphenyl hydrazone; CsA, cyclosporin A.

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strates these inhibitors had little effect on NAD(P)H level and the MTP opening. Dicumarol, an inhibitor of D,T-diaphorase (the enzyme that catalyzes reduction of DQ by NAD(P)H) also suppressed NAD(P)H oxidation (Fig. 1B, trace 4) and the MTP opening (Fig. 1A, trace 4). The effects of dicumarol were observed both in the presence and in the absence of glutamate and malate.

The data presented in Fig. 1 indicate that the DQ-induced permeability transition started within several minutes after the accomplishment of the NAD(P)H oxidation. These experiments, however, allow no analysis of the complete time course of the DQ action. For this purpose, the MTP opening was triggered by Ca^{2+} addition to mitochondria preincubated with DQ for various periods. The lag phase of the Ca^{2+}-triggered transition became shorter with increase of preincubation period with DQ and reached minimal value after 20 min (Fig. 2B, trace 1). Added before DQ, dicumarol much delayed (Fig. 2B, trace 2) whereas glutamate + malate mixture prevented the MTP opening (data not shown). These data demonstrate that the rapid NAD(P)H oxidation induced by DQ is followed by a slow process of the pore 'assembly'. This process was prevented by *N*-ethylmaleimide (NEM) (Fig. 2A, trace 5). NEM did not affect the DQ-induced NAD(P)H oxidation, but probably blocked oxidation of sulfhydryl groups in the membrane proteins, involved in the MTP regulation.

3.2. *The Ca^{2+}-triggered permeability transition in deenergized mitochondria pre-treated with duroquinone*

The Ca^{2+}-induced MTP opening in deenergized beef heart mitochondria has been described earlier by Hunter and Haworth [9]. We reproduced these observations in experiments with rat liver mitochondria and found that the transition could be induced only at a very high concentration of Ca^{2+} (1–5 mM). The kinetics of the process varied significantly from preparation to preparation and depended on the time of mitochondria storage after isolation. It seems probable that the properties of mitochondria depend on the combined action of endogenous inducers and protectors of the pore opening (e.g. NAD(P)H, ADP, Mg^{2+}, etc.).

Micromolar Ca^{2+} induced the MTP opening in the mitochondria preincubated with DQ (Fig. 2A). The dependence of the lag period (the reverse values ($1/\tau$) are plotted as a measure of the rate of induction) on the concentration of Ca^{2+} is presented in Fig. 2C (curve 1). It was strongly affected by ADP and Mg^{2+}, which not only increased the concentration of Ca which induced a half-maximum effect, but also decreased the rate of induction at saturated concentrations of Ca^{2+} (Fig. 2C, curves 2 and 3). These kinetics do not reflect the redistribution of Ca^{2+} between subpopulations of mitochondria (as was proposed for coupled mitochondria [10]) since the membrane potential, Ca^{2+} gradient and restrictions on Ca^{2+} transport were avoided under our experimental conditions.

The experiments were performed in the low-salt sucrose medium to exclude the effects of various ion transport systems in the inner mitochondrial membrane. An intramitochondrial pH, measured in BCECF-loaded mitochondria [4], was equal to 6.7–6.8 in this medium in the presence of protonophore (CCCP) and Ca^{2+}/H-exchanger (A23187). This value was not changed significantly either after a 20-min incubation (with or without 5 μM duroquinone) or on addition of Ca (up to 0.5 mM). These data indicate that the Donnan membrane potential of 40–50 mV (negative inside) exists under our experimental conditions. The effects of duroquinone and phenylarsine oxide in the medium containing 100 mM potassium gluconate and valinomycin, which abolishes the mitochondrial gradient of pH, were similar to these was observed in the presence of CCCP and A23187. Nevertheless, in the presence of potassium gluconate and valinomycin, the Ca^{2+} dependence of the pore opening was slightly shifted to higher Ca^{2+} values.

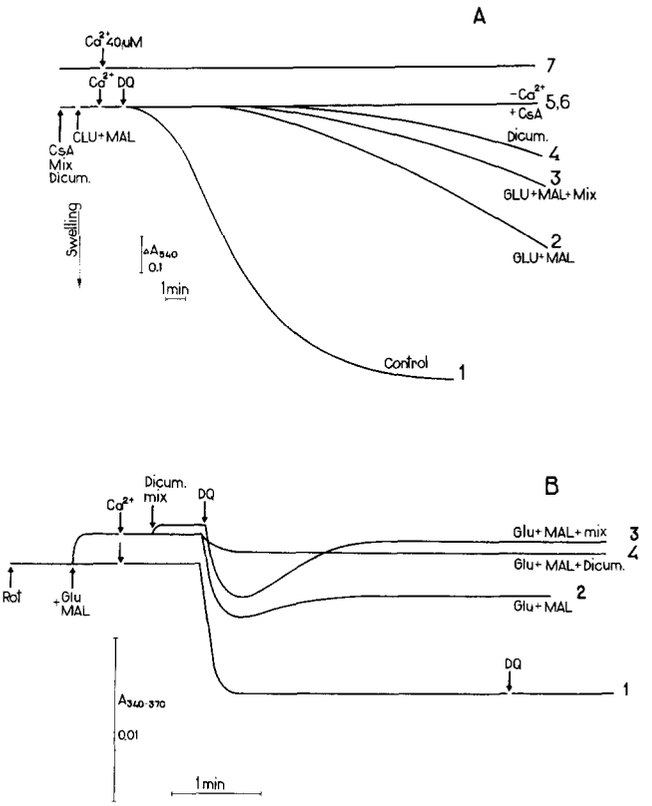
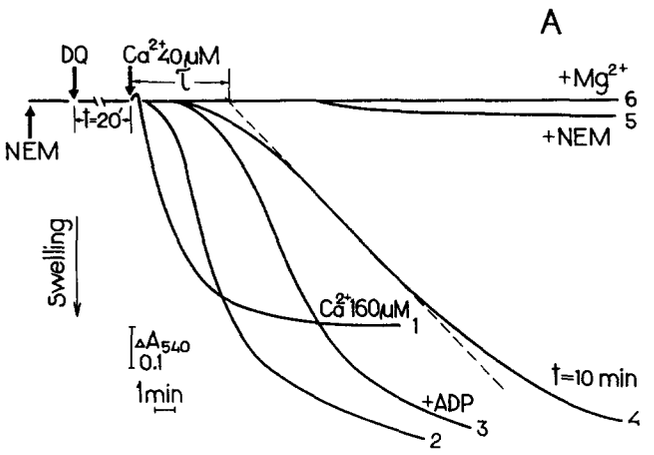


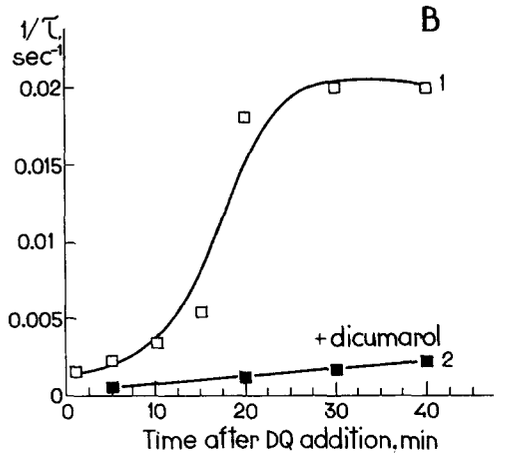
Fig. 1. The effect of duroquinone (DQ) on the swelling of mitochondria (A) and NAD(P)H oxidation (B). The conditions as described in section 2. Experiments in panels A and B were carried out in parallel. Trace numbers are the same for both panels. In all experiments (except for trace 5, panel A) 40 μM of Ca^{2+} were added and the final concentration of free Ca^{2+} was 24 μM. Trace 5 = without added Ca^{2+}, the concentration of free Ca^{2+} was 3×10^{-9} M. Traces 2–4 = 4 mM glutamate and 1 mM malate (Glu + Mal) were added (except for trace 4, panel A). 5 mM duroquinone (DQ; traces 1–6), 5 μM mixothiazole (Mix; trace 3), 1 μM dicumarol (Dicum; trace 4) or 1 μM cyclosporin A (CsA, trace 6) were added when indicated. Trace 7 = without duroquinone.

Similar Ca^{2+}-dependent induction of the MTP opening was observed with mitochondria preincubated for 5 min with 25 μM phenylarsine oxide (not shown). PhAsO can induce cross-linking of vicinal thiols but fails to cause the NAD(P)H oxidation in an experiment similar to that depicted in Fig. 1. NEM prevents the effect of PhAsO and this is in agreement with the observation reported earlier [11].

4. Discussion

The studies on the permeability transition in deenergized mitochondria allow us to exclude the effects of membrane potential, of pH- and Ca^{2+}-gradients. The effects of 'pore induc-





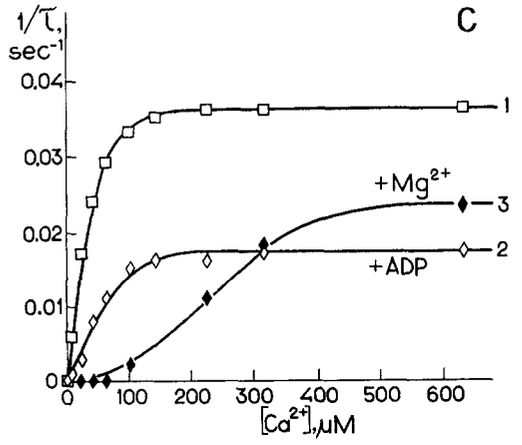


Fig. 2. The swelling of mitochondria induced by Ca^{2+} after preincubation with duroquinone. (A) The swelling was assayed after the incubation of mitochondria with 5 μM duroquinone for 20 min (except for trace 4 where incubation was 10 min) as described in section 2. Trace 1: 160 μM Ca^{2+} was added, the concentration of free Ca^{2+} was 141 μM. Trace 2–6: 40 μM Ca^{2+} was added, the concentration of free Ca^{2+} was 24 μM. 100 μM ADP (trace 3), 25 μM NEM (trace 5), 250 μM $MgSO_4$ (trace 6) were added when indicated. (B) The lag period (τ) of the MTP opening triggered by the addition of 40 μM Ca^{2+} was calculated as it was shown in panel A. The inverse values of lag ($1/\tau$) were plotted against the time of preincubation with 5 μM duroquinone without (curve 1) and with (curve 2) an addition of 1 μM dicumarol. (C) The dependence of the lag period of the MTP opening (the inverse values, $1/\tau$) on the concentration of free Ca^{2+}. Mitochondria were preincubated for 20 min with 5 μM duroquinone. 100 μM ADP (curve 2) or 250 μM $MgSO_4$ (curve 3) were added just before Ca^{2+} addition. Curve 1: without any additions before Ca^{2+}.
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ers' and Ca^{2+} on the MTP opening can be distinguished. In this paper we studied some aspects of the duroquinone-induced pore opening. The pore induction correlated with the extent of NAD(P)H oxidation in the presence of DQ (Fig. 1). Dicumarol, which inhibits the D,T-diaphorase catalyzed reduction of DQ to hydroquinone, strongly suppressed the MTP opening (Fig. 1A, trace 4). These data suggest that DQ reduction to semiquinone catalyzed by the NADPH-cytochrome P_{450} reductase and coupled to the production of superoxide and hydroxyl radicals is not crucial in the mechanism of induction. These reactions are probably more important in the case of menadione [12], benzoquinone [13] and dibromothymoquinone [14] which also induce the MTP opening.

DQ induced slow changes in the pore properties after a rapid NAD(P)H oxidation (Fig. 2B). This effect was completely prevented by NEM (Fig. 2A). NEM also caused a slow ($\tau_{1/2} \approx 20$ min) inhibition of the Ca^{2+}-induced MTP opening if it was added to mitochondria preincubated for 20 min with DQ (not shown). These data indicate that an equilibrium was established between oxidized and reduced states of critical dithiol(s) upon the oxidation of NAD(P)H. NEM modified these thiol groups and stabilized the component of the pore in the 'reduced' state. This modification prevented disulphide formation, when NEM was added before DQ, and shifted the equilibrium to the 'reduced' state, when NEM was added after the accomplishment of the DQ-induced oxidation. Slow NAD(P)H hydrolysis and ADP-ribosylation of membrane proteins, proposed by Richter [15], cannot be excluded but are not obligatory.

Phenylarsine oxide not decreasing the NAD(P)H content but directly cross-linking protein thiols induced a similar Ca^{2+}-triggered pore opening under our experimental conditions (not shown). Thiol oxidation has been proposed earlier to be a critical step in the permeability transitions induced by various prooxidants [1,16]. It was attributed to the deenergization of mitochondria by direct membrane modification or through activation of endogenous phospholipase A_2 [16]. The data presented here strongly indicate that thiol oxidation or crosslinking increases the sensitivity of the pore opening mechanism to Ca^{2+}.

In deenergized mitochondria the MTP opening was induced only at millimolar Ca^{2+}. The pretreatment of mitochondria with 'inducers', namely DQ (Fig. 2) or PhAsO, allowed us to observe the pore opening at 0.01–0.1 mM Ca^{2+}. The effect of Ca^{2+} (in the absence of ionophore) was prevented by the inhibitor of Ca^{2+}-uniporter ruthenium red (not shown), therefore the intramitochondrial Ca^{2+}-binding site(s) was involved. The sensitivity of this 'Ca^{2+}-sensor' was increased more than 100 times by oxidation or cross-linking of critical dithiol(s) in membrane proteins and decreased by ADP and Mg^{2+} (Fig. 2C). These properties coincide with the properties of the 'voltage-sensor' that was described by Bernardi et al. [5,7]. It was shown that the probability of the MTP opening in energized Ca^{2+}-loaded

mitochondria was increased with the decrease in the membrane potential. The sensitivity of this system (the gating potential) was enhanced by various prooxidants and thiol cross-linkers [7] and lowered by ADP and Mg^{2+} [5]. The coincidence in the properties of the two 'sensors' can be explained in two ways: (i) the pathways of signals from the 'sensors' intersect and this intersection is a target for the 'tuning' effects described above; (ii) the two sensors are the same molecular device. In the latter case the voltage sensing could be a result of voltage-dependent changes in the affinity of the Ca^{2+}-binding site(s). This hypothesis is in good agreement with the observation of Bernardi et al. [5] that elevation of the Ca^{2+} load increases the gating potential for the MTP opening.

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References

[1] Gunter, T.E. and Pfeiffer, D.R. (1990) Am. J. Physiol. 258, C755–C786.
[2] Hunter, D.R., Harworth, R.A. and Southard, J.H. (1976) J. Biol. Chem. 251, 5069–5077.
[3] Crompton, M., Ellinger, H. and Costi, A. (1988) Biochem. J. 255, 357–360.
[4] Bernardi, P., Vassanelli, S., Veronese, P., Colonna, R., Szabo, I. and Zoratti, M. (1992) J. Biol. Chem. 267, 2934–2939.
[5] Petronilli, V., Cola, C., Massari, S., Colonna, R. and Bernardi, P. (1993) J. Biol. Chem. 268, 21939–21945.
[6] Bernardi, P. (1992) J. Biol. Chem. 267, 8834–8839.
[7] Petronilli, V., Constantini, P., Scorrano, L., Colonna, R., Passamonti, S. and Bernardi, P. (1994) J. Biol. Chem. 269, 16638–16642.
[8] Jonson, D. and Lardi, H. (1967) Methods Enzymol. 10, 90–96.
[9] Hunter, D.R. and Harworth, A. (1979) Arch. Biochem. Biophys. 195, 468–477.
[10] Beatrice, M.C., Stiers, D.L. and Pfeiffer, D.R. (1982) J. Biol. Chem. 257, 7161–7171.
[11] Novgorodov, S.A., Kultayeva, E.V., Yaguzhinsky, L.S. and Lemeshko, V.V. (1987) J. Bioenerg. Biomembr. 19, 191–202.
[12] Rizzuto, R., Pitton, G. and Azzone, F. (1987) Eur. J. Biochem. 162, 239–249.
[13] Moore, G.A., Weis, M., Orrenius, S. and O'Brien, P.J. (1988) Arch. Biochem. Biophys. 267, 539–550.
[14] Harris, E.J. and Baum, H. (1980) Biochem. J. 186, 725–732.
[15] Richter, C. and Frei, B. (1988) Free Rad. Biol. Med. 4, 365–375.
[16] Broekemeier, K.M., Schmid, P.C., Schmid, H.H.O. and Rfeiffer, D.R. (1985) J. Biol. Chem. 260, 105–113.