Evidence for the presence of a reversible Ca^{2+}-dependent pore activated by oxidative stress in heart mitochondria

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Rat heart mitochondria became permeabilized to sucrose when incubated with 100 nmol of Ca^{2+}/mg of protein in the presence of P_i. Ca^{2+} chelation with EGTA restored impermeability to sucrose, which became entrapped in the matrix space. *t*-Butylhydroperoxide markedly promoted permeabilization in the presence of Ca^{2+} but not in its absence, and Ca^{2+}-plus-*t*-butylhydroperoxide-induced permeabilization was reversed by EGTA. The data suggest that Ca^{2+} and oxidative stress synergistically promote the reversible opening of an inner membrane pore.

INTRODUCTION

The capacity of excessive Ca^{2+}, particularly in the presence of P_i, to induce leakiness of the mitochondrial inner membrane to low-M_r solutes generally is well known (Broekemeier *et al.*, 1985, and references therein). Recent studies with liver mitochondria in this laboratory indicate that the lesion may reflect the presence of a non selective Ca^{2+}-activated pore, which opens when matrix Ca^{2+} exceeds normal limits and closes on Ca^{2+} removal (Al Nasser & Crompton, 1986*a*). Although its natural role is as yet obscure, pore reversibility has proved useful practically in allowing the entrapment of Ca^{2+} indicators and Ca^{2+} buffers in the matrix space (Al Nasser & Crompton, 1986*b*; Hayat & Crompton, 1987).

In addition, pore opening in liver mitochondria uncouples energy transduction (Al Nasser & Crompton, 1986*a*), and it would seem worthwhile to explore the possibility that the permeation state of the pore may be a factor in disease associated with excess cellular Ca^{2+}. Of these, ischaemia/reperfusion-induced injury in heart has received considerable attention in view of its clinical importance. This form of injury is associated with a large uptake of Ca^{2+} into the tissue and mitochondrial Ca^{2+} overload (Ferrari *et al.*, 1982; Poole-Wilson *et al.*, 1984; references therein), and with oxidative stress, in which cellular defensive mechanisms against the adverse effects of O_2-derived radicals become impaired during ischaemia so that the tissue is more susceptible to these radicals on reoxygenation (Guarnieri *et al.*, 1980; reviewed in Halliwell & Gutteridge, 1985). The present study therefore exmaines whether heart mitochondria contain such a pore and, in particular, whether it is influenced by oxidative stress.

METHODS

Mitochondrial preparation

Mitochondria were prepared from hearts of female Sprague–Dawley rats (250–300 g body weight), and their protein content determined, by standard procedures (Crompton *et al.*, 1983).

Measurement of mitochondrial permeabilization and resealing

Mitochondria (10 mg of protein/ml), containing 6–10 nmol of Ca^{2+}/mg of protein (assayed as in Al Nasser & Crompton, 1986*a*) were preincubated for 5 min at 25 °C in standard medium (pH 7.2) containing 120 mM-KCl, 10 mM-Tris/Hepes, 0–10 mM-phosphate (K^+ salt), 10 μg of rotenone/ml and 0.1 μCi of ^3H_2O/ml; 50 μM-TPP^+ was included when indicated (Fig. 1*a*). Succinate (5 mM, K^+ salt) was added followed, 1 min later, by 100 nmol of $CaCl_2$/mg of protein and 0.05 μCi of [U-^{14}C]sucrose/ml; 50 μM-*t*-butylhydroperoxide (Fig. 2) was added simultaneously. Samples (200 μl) of the incubate were withdrawn at intervals for measurement of the sucrose-inaccessible space by standard techniques (e.g. Al Nasser & Crompton, 1986*a*).

Resealing was initiated by addition of 10 mM-EGTA to the incubate. Resealing was assessed by entrapment of [^{14}C]sucrose in the matrix space; this was measured in two ways. In the first method, 0.1 ml samples of the incubate were diluted in 0.9 ml of medium containing KCl, Tris/Hepes, succinate and P_i at the same concentrations as the standard medium. The diluted samples were immediately centrifuged in an Eppendorff bench centrifuge for 2 min and the ^{14}C and ^{3}H contents of the pellets and supernatants were determined. The supernatant values were used to correct the pellet ^{14}C values for ^{14}C in the residual ^3H_2O of the pellet, giving [^{14}C]-sucrose entrapped. In the second method, 200 μl incubate samples (without dilution) were filtered under vacuum on Whatman GF/B glass microfibre filters, which were then washed with 1–15 successive 100 μl volumes of KCl/Tris/Hepes/succinate/P_i medium (above) containing 10 mM-sucrose. The ^{14}C contents of the filters were determined.

Measurement of TPP^+ accumulation

Extramitochondrial [TPP^+] was determined with an electrode as described before (Al Nasser & Crompton, 1986*a*). $\Delta\psi$ was calculated assuming a Nernstian distribution of TPP^+ and assuming that TPP^+ partitioning

Abbreviations used: $\Delta\psi$, mitochondrial inner-membrane potential; TPP^+, tetraphenylphosphonium ion.

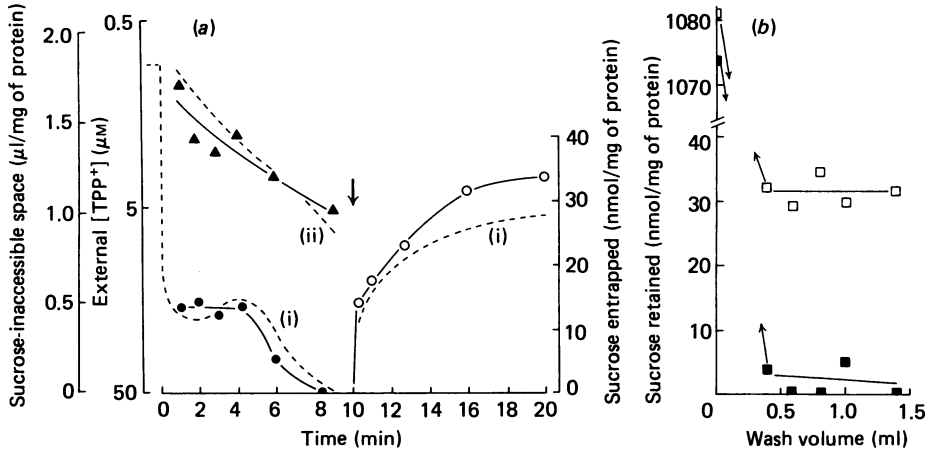


Fig. 1. Heart mitochondrial permeabilization and resealing

(*a*) Mitochondria were preincubated as described under 'Methods'. Ca^{2+} was added at zero time; EGTA was added at 10 min (arrow). Symbols: ●, sucrose-inaccessible space with 10 mM-P_i; ▲, sucrose-inaccessible space with 2 mM-P_i; ○, sucrose entrapment with 10 mM-P_i measured after dilution (see under 'Methods'). The broken traces give the TPP^+ uptake with 10 mM-P_i (i) or 2 mM-P_i (ii). (*b*) Samples of the incubate of (*a*) with 10 mM-P_i were withdrawn at 20 min, filtered and washed with the volumes indicated on the abscissa. Symbols: sucrose retained with (□) and without (■) EGTA addition (at 10 min, Fig. 1*a*).

into the membrane was effectively equivalent to an increase in matrix volume of 8 μl/mg of protein as determined by Rottenberg (1984).

RESULTS AND DISCUSSION

The essential objective of this study was to examine the influence of oxidative stress on Ca^{2+}-induced permeabilization (pore opening, below) and its reversibility in heart mitochondria. A prerequisite was reliable conditions for permeabilization/resealing and assay based on permeability to sucrose. Fig. 1(*a*) reports the changes in the sucrose-inaccessible matrix space of respiring heart mitochondria after addition of Ca^{2+} (at zero time). The amount of Ca^{2+} present (106–110 nmol/mg of protein) very greatly exceeds the amount of intramitochondrial Ca^{2+} thought to be present normally *in vivo* (< 3 nmol/mg: Crompton *et al.*, 1983; McCormack & Denton, 1984), but is similar to that measured in mitochondria isolated from hearts subjected to ischaemia/reperfusion (40–160 nmol/mg: Henry *et al.*, 1977; Peng *et al.*, 1980; Ferrari *et al.*, 1982). The resistance to permeabilization depended on [P_i], since the space disappeared completely within 10 min with 10 mM-P_i, but more slowly with 2 mM-P_i. Other experiments (not shown) established that 10 mM-P_i caused negligible permeabilization in the absence of Ca^{2+} (with 10 mM-EGTA; i.e. < 15% decrease in space in 9 min) in agreement with previous data for liver mitochondria (Al Nasser & Crompton, 1986*a*). TPP^+ uptake correlated reasonable well with the space (Fig. 1*a*), confirming that permeabilized heart mitochondria are uncoupled. The above data are typical of four such experiments conducted.

A decrease in sucrose-inaccessible space is not unambiguous since, in principle, the matrix might merely shrink with time but remain sucrose-inaccessible. Accordingly, permeabilization followed by resealing (Al Nasser & Crompton, 1986*a*) was assessed from the entrapment of sucrose in the matrix space after addition of EGTA to mitochondria supposedly permeabilized in the presence of ^3H_2O and [^{14}C]sucrose. Entrapment was measured in two ways; in Fig. 1(*a*), the mitochondria were diluted after EGTA and sedimented. Under these conditions matrix ^3H_2O would be diluted, but any entrapped [^{14}C]-sucrose would not, leading to excess ^{14}C over ^{3}H in the mitochondria with respect to the supernatant. The data (10–20 min) show an initial rapid resealing followed by a slower phase. Since the permeabilization medium contained 28 mM-sucrose, the amount of sucrose finally entrapped (33 nmol/mg) is consistent with sucrose equilibration into a matrix space of about 1.2 μl/mg of protein immediately before resealing (the relative scales of the 'space' and 'entrapped sucrose' axes in Fig. 1*a* are drawn accordingly). In eight such experiments, the resealed space amounted to 1.07±0.07 μl/mg (mean ±S.E.M.). Resealing was accompanied by repolarization (TPP^+ uptake) to a $\Delta\psi$ value of about 117 mV; this compares with about 170 mV before permeabilization.

In the standard dilution technique (Fig. 1*a*), about 15 s elapsed between dilution and mitochondrial sedimentation. Other experiments in which the time delay between dilution and sedimentation was extended to 1 min yielded no significant difference in sucrose entrapment (±6%). The dilution technique therefore provides a means of assessing the state of resealing at the instant of dilution. The validity of the procedure was checked by comparison with an alternative. In Fig. 1(*b*), sucrose entrapment was measured by filtration. The amount of [^{14}C]sucrose retained, presumably intramitochondrial, was similar to that obtained by the first method. Filtration without resealing (no EGTA) produced negligible retained sucrose.

The biphasic course of resealing (Fig. 1*a*) was not always observed; with three out of eight mitochondrial preparations, resealing was complete 15 s after EGTA addition (e.g. Fig. 2*c*, open symbols). The mean initial resealing with the eight preparations amounted to 74±11% (±S.E.M.) of the resealing attained finally 10 min after EGTA. The cause of this variation between preparations is unknown. The essential point however is

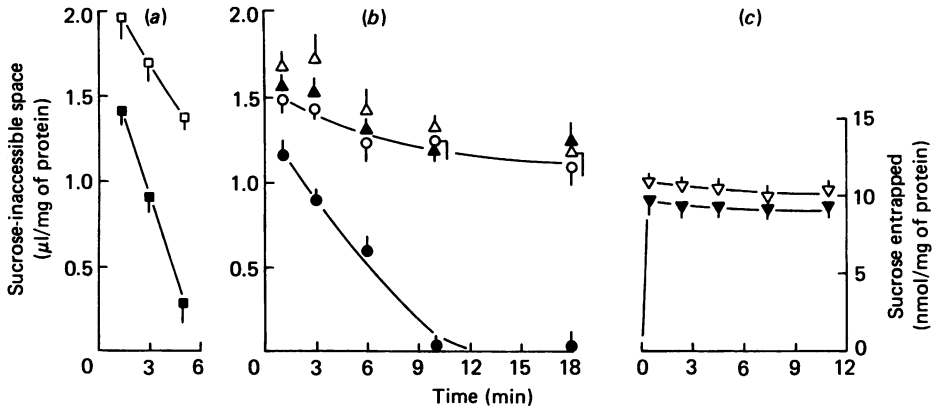


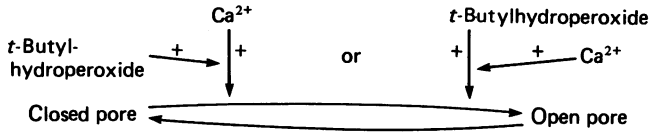
Fig. 2. Effect of 50 μM-*t*-butylhydroperoxide on permeabilization and resealing

(*a*) After preincubation (see under 'Methods'), mitochondria were permeabilized in the presence of 2 mM-P_i by addition of Ca^{2+} at zero time. Symbols: sucrose-inaccessible spaces with (■) and without (□) *t*-butylhydroperoxide added at zero time. (*b*) Sucrose-inaccessible spaces were measured as in (*a*) but with 0.2 mM-P_i and the following conditions: with Ca^{2+} in the presence (●) and absence (○) of *t*-butylhydroperoxide; without Ca^{2+} in the presence (▲) and absence (△) of *t*-butylhydroperoxide (for clarity, lines connecting these points are omitted). (*c*) Mitochondria were permeabilized in the presence of either 10 mM-P_i (▽; as in Fig. 1*a*) or 0.2 mM-P_i plus *t*-butylhydroperoxide (▼; as in Fig. 2*b*). EGTA was added after 10 min (designated zero time in Fig. 2*c*) and samples were diluted at intervals thereafter (abscissa) and sucrose entrapment was measured. In all cases data are given as means ± S.E.M. (three experiments with the same mitochondrial preparation).

that in all cases permeabilization was reversed on Ca^{2+} removal, which is consistent with the operation of a reversible Ca^{2+}-activated pore.

The influence of oxidative stress was investigated with *t*-butylhydroperoxide. This hydroperoxide is reduced by glutathione peroxidase, leading to oxidation of GSH and, consequently, of NADPH and NADH; it may also produce *t*-butoxy and *t*-butylperoxy radicals leading to peroxidation of unsaturated membrane lipids (review: Sies, 1985). Fig. 2(*a*) shows that 50 μM-*t*-butylhydroperoxide markedly increased the rate of permeabilization with Ca^{2+} and 2 mM-P_i; the rate of permeabilization with 50 μM-*t*-butylhydroperoxide was not changed by increasing the hydroperoxide concentration to 100 μM (results not shown).

In order to examine the potency of the hydroperoxide further, conditions were obtained under which Ca^{2+} itself produced little permeabilization. As shown in Fig. 2(*b*), Ca^{2+} induced negligible permeabilization with 0.2 mM-P_i (open symbols), but the addition of *t*-butylhydroperoxide and Ca^{2+} together caused complete permeabilization within 10 min. However, the hydroperoxide had no significant effect on permeabilization in the absence of Ca^{2+} (Fig. 2*b*). Thus, whereas Ca^{2+} and *t*-butylhydroperoxide alone were essentially ineffective, in combination they induced complete permeabilization. Likewise, in two further experiments, *t*-butylhydroperoxide and Ca^{2+} together caused complete permeabilization within 10 min. However, the hydroperoxide had no significant effect on permeabilization in the absence of Ca^{2+} (Fig. 2*b*). Thus, whereas Ca^{2+} and *t*-butylhydroperoxide alone were essentially ineffective, in combination they induced complete permeabilization. Likewise, in two further experiments, *t*-butylhydroperoxide plus Ca^{2+} markedly stimulated the decrease in sucrose-inaccessible space [e.g., in 6 min, from 8% to 61% (zero-time space, 1.83 μl/mg) and from 12% to 73% (zero-time space, 1.58 μl/mg)]; in contrast, neither agent added alone significantly changed the rate of permeabilization. In terms of the Ca^{2+}-activated pore model (Al Nasser & Crompton, 1986*a*), these data may be rationalized as follows:



Either model predicts that removal of Ca^{2+} would allow pore closure. In agreement with this, EGTA-induced pore closure following permeabilization in either 0.2 mM-P_i plus hydroperoxide or 10 mM-P_i was almost equal (Fig. 2*c*) as judged by the amounts of sucrose entrapped (in this experiment, the medium during permeabilization contained 11 mM-sucrose, and the sucrose entrapment corresponds to a matrix-space resealing of 0.9–1.0 μl/mg). This behaviour was confirmed by three similar experiments in which the matrix space resealing in the absence of hydroperoxide (0.92 ± 0.11 μl/mg at 15 s, 1.11 ± 0.07 μl/mg at 6 min; means ± S.E.M.) was not significantly changed by the hydroperoxide (0.89 ± 0.09, 15 s; 1.05 ± 0.10, 6 min).

In conclusion, there is considerable evidence that oxidative stress and Ca^{2+} overload are critical factors in the progression of ischaemia/reperfusion-induced injury and the finding that these factors promote opening of the (hypothetical) pore synergistically suggests that its permeation state may be relevant to this form of injury. Since pore opening uncouples mitochondria and, presumably, would lead to equilibration of low-M_r solutes across the inner membrane, the consequences for cell recovery might be severe. Resolution of the proteins involved in the synergism might offer suitable targets for pharmacological intervention.

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