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Permeability Transition Pore Closure Promoted by Quinine

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The mitochondrial membrane permeability transition induced by Ca^{2+} is inhibited by quinine in a dose-dependent fashion. Competition experiments strongly suggest that quinine displaces Ca^{2+} bound to the inner membrane. This is supported by experiments showing that quinine inhibits Ca^{2+}-dependent but not Ca^{2+}-independent mitochondrial swelling induced by phenylarsine oxide. As with Ca^{2+} chelators, quinine induces permeability transition pore closure preventing the contraction induced by poly(ethylene glycol) 2000 in mitochondria preswollen by incubation in KSCN medium containing Ca^{2+} and inorganic phosphate. These results suggest that quinine dislodges Ca^{2+} bound to the protein site, which triggers pore opening.

KEY WORDS: Rat liver mitochondria; membrane permeability transition; calcium ion; quinine.

INTRODUCTION

Changes in the cytosolic Ca^{2+} concentration modulate a variety of cellular functions, including secretion, contraction, enzyme activation, synaptic plasticity, cell proliferation, and death (Berridge, 1997). Mitochondria have a low-affinity, high-capacity Ca^{2+} uptake mechanism, which enables the organelle to accumulate Ca^{2+} when it attains concentrations higher than 0.5 μM (Gunter and Pfeiffer, 1990). This phenomenon is of considerable importance in the modulation of mitochondrial physiology (Rizzuto *et al.*, 1993), and possibly in mitochondrial-mediated apoptotic or necrotic cell death (Skulachev, 1996; Marzo *et al.*, 1998; Bradham *et al.*, 1998). A common event in Ca^{2+}-mediated necrosis and apoptosis appears to be a condition known as the mitochondrial permeability transition (MPT), which involves the formation of a cyclosporin A-sensitive, nonselective proteinaceous pore (permeability transition pore, PTP) in the mitochondrial membrane (Marzo *et al.*, 1998; Bradham *et al.*, 1998). The formation of the PTP can be mediated by a concerted action between Ca^{2+} and reactive oxygen species leading to oxidation of membrane protein thiols (Vercesi *et al.*, 1997). According to this model, Ca^{2+} ions are involved in the mechanism of PTP opening by (i) binding to inner membrane cardiolipins and stimulating the production of $O_2^{\bullet-}$ (superoxide union radical) and, hence, H_2O_2, by the respiratory chain (Vercesi *et al.*, 1997; Castilho *et al.*, 1995), (ii) stimulating the Fenton reaction through matrix Fe^{2+} mobilization (Castilho *et al.*, 1995), and (iii) binding to membrane proteins that regulate PTP opening (Gunter and Pfeiffer, 1990; Zoratti and Szabo, 1995; Bernardi *et al.*,1994). The affinity of Ca^{2+} binding to the protein site(s) which trigger(s) pore opening is thought to be increased by thiol oxidation (Halestrap *et al.*, 1997; Chernyak and Bernardi, 1996). In ischemic cells, the cytosolic Ca^{2+} concentration may increase sufficiently to allow the accumulation of this ion in mitochondria once respiration is reestablished by reperfusion (Greene and Paller, 1994; Miyata *et al.*, 1991). This mitochondrial Ca^{2+} accumulation may promote PTP opening and mitochondrial dysfunction that can lead to cell death (Imberti *et al.*, 1992; Nieminem *et al.*, 1995). Compounds which prevent PTP opening, such as cyclosporin A, protect cells against damage induced by ischemia/reperfusion (Imberti *et al.*, 1992; Nieminem *et al.*, 1995). The identification of new MPT inhibitors that may be effective *in vivo* is of great

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relevance for the development of new therapeutic drugs.

In this study, we investigated the effects of quinine, a compound known to block Ca^{2+}-activated K^+ channels (Glavinovich and Trifaro, 1988; Mancilla and Rojas, 1990; and references therein) and Ca^{2+}-activated K^+-dependent swelling of mitochondria (Halestrap *et al.*, 1986) on MPT induced by Ca^{2+}.

MATERIALS AND METHODS

Isolation of Rat Liver Mitochondria and Incubation Procedure

Rat liver mitochondria (RLM) were isolated by conventional differential centrifugation from the livers of adult female Wistar rats fasted overnight. The experiments were conducted at 30°C in a standard reaction medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.2, 4 μM rotenone, and 5 mM succinate. Other additions are indicated in the figure legends. The involvement of MPT in Ca^{2+} release, $\Delta\Psi$ decrease, and mitochondrial swelling under these experimental conditions was attested by the sensitivity to cyclosporin A (see also Gunter and Pfeiffer, 1990; Zoratti and Szabo, 1995). The results shown are representative or averages of at least three experiments.

Mitochondrial Transmembrane Electrical Potential

The mitochondrial membrane potential ($\Delta\Psi$) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+ selective electrode constructed in our laboratory, in combination with a calomel reference electrode (Kamo *et al.*, 1977). For calculation of the $\Delta\Psi$ value, the matrix volume of RLM was assumed as 1.4 μl $\times$ mg^{-1} protein. The membrane potential was calculated assuming that the TPP^+ distribution between mitochondria and medium followed the Nernst's equation. Corrections were made for the binding of TPP^+ to mitochondrial membrane as described by Jensen *et al.* (1986). The electrode was calibrated with additions of known concentrations of TPP^+. Signals were amplified and the output was registered with a potentiometric recorder (Kipp & Zonen, BD 121).

Ca^{2+} Concentration

Changes in the Ca^{2+} concentration in the suspending medium were measured using a Ca^{2+}-selective electrode constructed in our laboratory according to Simon *et al.* (1978). The signals from this electrode were amplified and recorded (Kipp & Zonen, BD 121, potentiometric recorder). The electrode response was calibrated in each experiment by adding internal standards to the medium.

Mitochondrial Swelling and Mitochondrial Contraction

Mitochondrial swelling was estimated from the decrease in the absorbance of the mitochondrial suspension at 520 nm measured with an SLM Aminco DW 2000 spectrophotometer. Shrinkage of preswollen mitochondria was performed as described by Halestrap *et al.* (1997). Briefly, RLM (2 mg/ml) were incubated for 20 min at 30°C in 10 mM HEPES, pH 7.2, containing 150 mM KSCN, 1 μM antimycin A, 1 μM FCCP (carbonyl-cyanide-*p*-trifluoromethoxyphenyl hydrazone), 1 μM A_{23187} (Ca^{2+} ionophore), 500 μM Ca^{2+}, and 2 mM inorganic phosphate (P_i), an experimental condition that induces PTP opening and high amplitude swelling (Halestrap *et al.*, 1997). The suspension of swollen mitochondria was then centrifuged and the pellet resuspended to 1 mg/ml in 1.5 ml of 10 mM HEPES, pH 7.2, containing 150 mM KSCN, 0.2 μM antimycin A, 1 μM FCCP, and 1 μM A_{23187}. Shrinkage was induced by addition of 250 μl of 50% poly(ethylene glycol) (PEG) 2000 and measuring the absorbance at 520 nm with a Hitachi U-3000 spectrophotometer.

Materials

HEPES, rotenone, antimycin A, ADP, EGTA, FCCP, succinate, quinine, Ca^{2+} ionophore A_{23187}, and phenylarsine oxide were from Sigma Chemical Company (St. Louis, MO). All other reagents were of the highest grade available.

RESULTS AND DISCUSSION

Effect of Quinine on Mitochondrial Ca^{2+} Transport and Membrane Potential

Figure 1 (line a) shows that when RLM were added to the standard medium containing 55 μM free

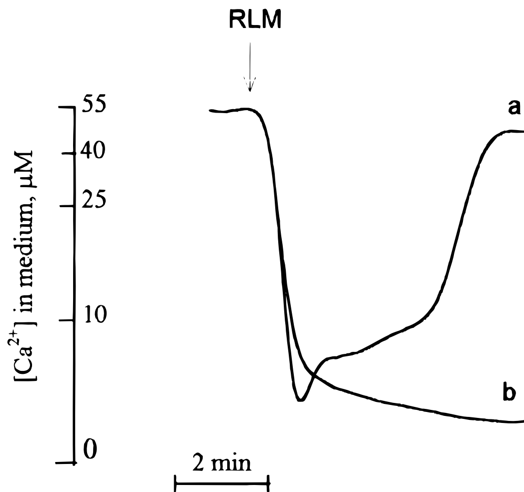

Fig. 1. Effect of quinine on Ca^{2+} accumulation and retention by mitochondria. The reaction was initiated by adding of rat liver mitochondria (RLM, 1.0 mg/ml) to standard medium containing 200 μM P_1 and 55 μM $CaCl_2$. (a) No quinine; (b) 300 μM quinine.

Ca^{2+}, a rapid decrease in Ca^{2+} concentration was followed by return of the cation to the medium in a triphasic manner. When 0.3 mM quinine was present in the medium (line b), the rate of Ca^{2+} accumulation in the mitochondria was slightly slower and Ca^{2+} was retained inside the organelles throughout the period of observation. Since the mitochondrial Ca^{2+} release associated with PTP opening is preceded by elimination of the mitochondrial membrane potential (Vercesi *et al.*, 1997), experiments were performed to study the dose dependence between the quinine concentration and $\Delta\psi$. In Fig. 2, $CaCl_2$ (55 μM) was added to the medium after the mitochondria had developed a membrane potential of about 195 mV. Following a rapid decrease of about 20 mV, the membrane potential was partially restored, but not retained (line a). Increasing concentrations of quinine (0.1, 0.2, and 0.3 mM, lines b, c and d, respectively) progressively protected against the elimination of $\Delta\Psi$. In the presence of 0.1 mM EGTA (line e), $\Delta\Psi$ was maintained in the absence or presence of quinine.

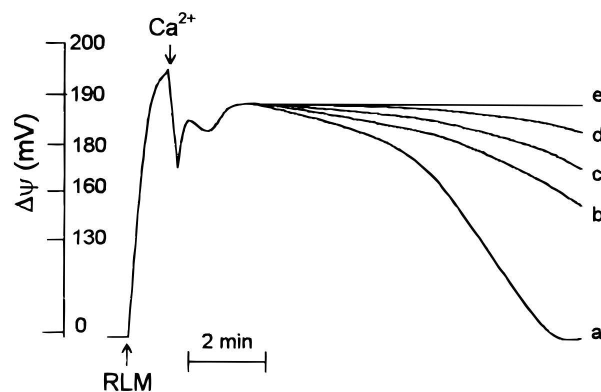

Fig. 2. Effect of quinine on the elimination of the mitochondrial membrane potential ($\Delta\Psi$) caused by Ca^{2+}. RLM (1.0 mg/ml) were added to standard medium containing 200 μM P_1 in the absence (a) or presence of quinine (b) 100 μM, (c) 200 μM, (d) 300 μM or (e) 100 μM EGTA. $CaCl_2$ (55 μM) was added in all experiments, as indicated.

Effect of Ca^{2+} and Quinine Concentration on the Rate of Mitochondrial Swelling Induced by Ca^{2+} and Inorganic Phosphate.

To evaluate a possible competitive relationship between Ca^{2+} and quinine, the rate of swelling in the presence of a constant Ca^{2+} concentration, but different concentrations of quinine, was determined (Fig. 3A, 25–250 μM). In these experiments, a higher concentration (2 mM) of P_i was used to allow opening of the PTP at lower Ca^{2+} concentrations (Vercesi *et al.*, 1997) for the competition experiments. Increasing concentrations of quinine progressively inhibited mitochondrial swelling (Fig. 3A). A similar antagonism between quinine and Ca^{2+} was observed when the Ca^{2+} concentration (10–25 μM) was varied at a fixed concentration (200 μM) of quinine (Fig. 3B). These results resemble those obtained with dibucaine (Vercesi *et al.*, 1988) which, along with other local anesthetics, displaces

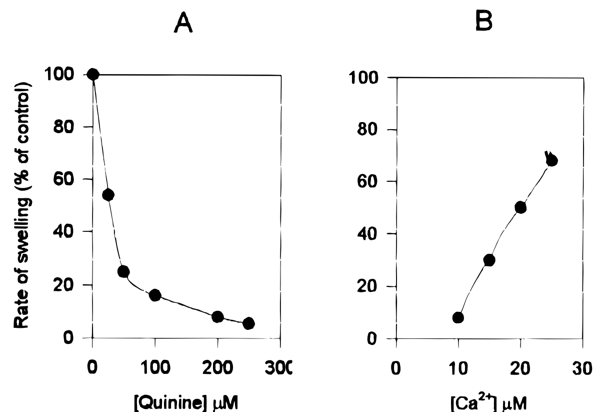

Fig. 3. Influence of Ca^{2+} and quinine on the rate of mitochondrial swelling induced by Ca^{2+} and P_i. RLM (0.5 mg/ml) were incubated in standard medium in the presence of 2 mM P_i. In Panel (A), different concentrations of quinine (0, 25, 50, 100, 200, and 250 μM) and 10 μM $CaCl_2$ were used. In Panel (B), different concentrations of $CaCl_2$ (10, 15, 20, and 25 μM) were tested with a fixed concentration (200 μM) of quinine.

Ca^{2+} bound to the membrane surface (Low *et al.*, 1979). This action is attributed to the ability of these compounds to dissolve in the membrane and to increase the distance between adjacent protein and/or phospholipid components of Ca^{2+} attachment sites (Low *et al.*, 1979). Since quinine and dibucaine have similar functional groups (quinolinic ring, tertiary amines, benzylic nitrogens, esters), similar molecular weights and relatively close pK values (pk_1 = 3.0−5.07; pk_2 = 7.8−9.7), we hypothesized that quinine could displace mitochondrial membrane Ca^{2+} by a mechanism similar to that of dibucaine. Indeed, it was recently shown (Kowaltowski *et al.*, 1998) that dibucaine protects against MPT by displacing Ca^{2+} bound to inner membrane phospholipids, thus decreasing the Ca^{2+}-stimulated generation of reactive oxygen species by mitochondria, which, in turn, prevents membrane protein thiol oxidation.

Effect of Quinine on the Swelling Induced by Phenylarsine Oxide.

The hydrophobic disulfide reagent phenylarsine oxide (PhAsO) induces PTP opening by binding to protein thiol groups and promoting their cross linkage (Lenartowics *et al.*, 1991). At a concentration of 100 μM, this effect is Ca^{2+} independent, while in the presence of Ca^{2+}, this compound induces PTP opening at a concentration as low as 10 μM (Kowaltowski and Castilho, 1997; Kowaltowski *et al.*, 1997). Figure 4A shows that in the absence of Ca^{2+} the mitochondrial swelling induced by 100 μM PhAsO (line a) was sensitive to cyclosporin A (line c), but not to quinine (line b) in contrast with experiments done in the presence of 10 μM Ca^{2+} and 10 μM PhAsO (Fig. 4B) where a significant inhibition was promoted by both quinine and cyclosporin A (lines b and c, respectively). These results support the interpretation that the quinine effect on MPT is mediated by its interaction with Ca^{2+}-binding sites. Line d (A and B) represents control experiments in the absence of PhAsO and presence of 200 μM EGTA.

PTP Closure by Quinine

PEG 2000 has a diameter larger than the PTP and therefore does not penetrate mitochondria, promoting mitochondrial contraction when the PTP is open (Halestrap *et al.*, 1997; Haworth and Hunter, 1979). In this regard, Fig. 5 (line a) shows that the addition of PEG to preswollen mitochondria caused an extensive contraction, indicating that, in agreement with Halestrap *et al.*, (1997), the PTP was open under these experimental conditions. It can be observed that quinine (200 μM) (line b) promoted PTP closure and prevented this contraction in a manner similar to the Ca^{2+} chelator HEDTA [N′-(2-hydroxyethyl)ethylenediamine-*N*,*N*,*N*′-triacetic acid] (Chernyak and Bernardi, 1996). Therefore, in addition to the possible effect of quinine on Ca^{2+} bound to phospholipids, quinine may dislodge Ca^{2+} bound to a protein(s) directly involved in pore opening. This proposition is in line with suggestions (Vercesi *et al.*, 1997; Kowaltowski *et al.*, 1998) that the inner mitochondrial membrane possesses two distinct

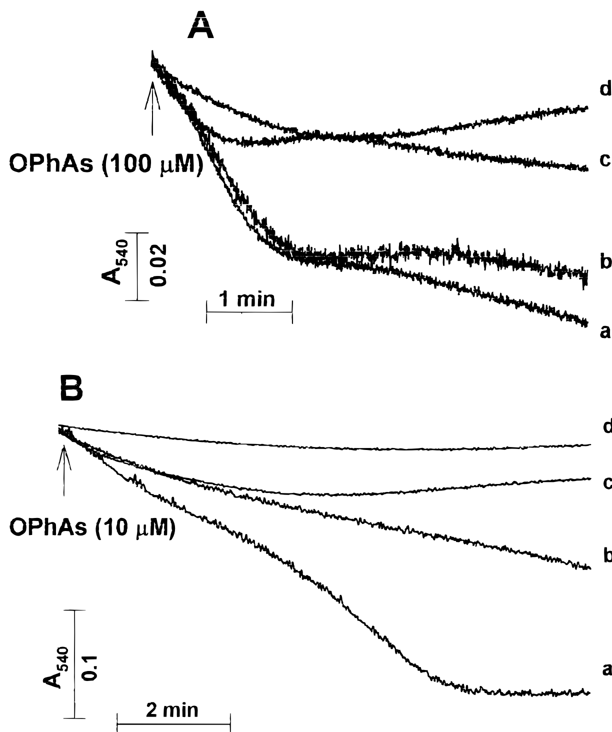

Fig. 4. (A) Mitochondrial swelling induced by 100 μM phenylarsine oxide (PhAsO) in the absence or presence of quinine. RLM (0.5 mg/ml) were incubated in standard medium containing 200 μM EGTA, 2 mM P_i, 100 μM PhAsO, and 5 μM A_{23187} in the absence (a) or presence (b) of 200 μM quinine. Line (c) represents an experiment in the presence of 1 μM cyclosporin A plus 100 μM PhAsO and line (d) represents a control in the absence of PhAsO. (B) Inhibition by quinine of the mitochondrial swelling induced by 10 μM phenylarsine oxide (PhAsO) and Ca^{2+}. RLM (0.5 mg/ml) were incubated in standard medium containing 2 mM P_i, 10 μM PhAsO, and 10 μM Ca^{2+}, in the absence (a) or presence (b) of 200 μM quinine. Line (c) represents an experiment in the presence of 1 μM cyclosporin A plus 10 μM PhAsO and line (d) represents a control in the presence of 200 μM EGTA.

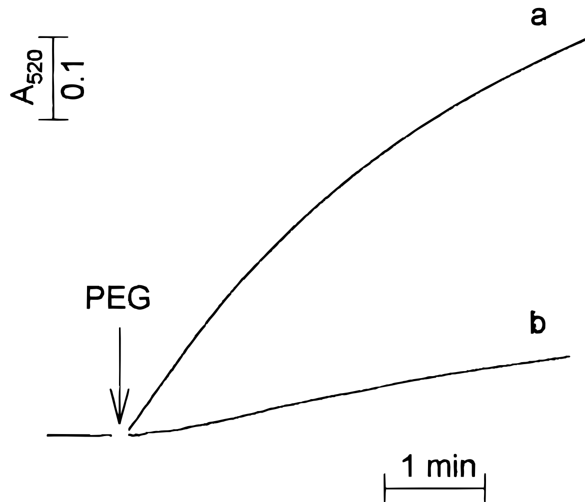

Fig. 5. Effect of quinine on mitochondrial contraction. Preswollen mitochondria were prepared as described in the section on Materials and Methods and incubated in 1.5 ml of KSCN buffer, pH 7.2. Where indicated, 250 μl of 50% PEG 2000 was added (line a). Line b represents an incubation in the presence of 200 μM quinine.

Ca^{2+}-binding sites involved in MPT. The first of these is shown to be sensitive to dibucaine and may be composed of cardiolipin (Kowaltowski *et al.*, 1998). Binding of Ca^{2+} to this site causes lateral phase separation of inner membrane lipids and stimulation in reactive oxygen species production by mitochondria (Vercesi *et al.*, 1997). The second, as shown here, is sensitive to quinine and is probably located on the protein(s) that regulate(s) pore opening. Although the data obtained with isolated mitochondria do not permit extrapolations to conditions *in vivo*, these results are relevant with respect to drug design and the understanding of the molecular mechanisms involved in PTP formation.

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