Inhibition of the mitochondrial cyclosporin A-sensitive permeability transition pore by the arginine reagent phenylglyoxal

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Abstract The mitochondrial permeability transition pore, a cyclosporin A-sensitive channel, is controlled by the transmembrane electric potential difference across the inner membrane. Here, we show that treatment of rat liver mitochondria with the arginine reagent phenylglyoxal inhibits the permeability transition pore triggered by depolarization with uncoupler after Ca^{2+} accumulation. Phenylglyoxal does not change the extent of mitochondrial Ca^{2+} uptake or the extent of membrane depolarization, indicating that covalent modification of arginine (and possibly lysine) residues directly affects the open probability of the pore. We propose that arginine residues play a role in the physiological control of the permeability transition pore by the mitochondrial transmembrane potential.

Key words: Mitochondrial channel; Permeability transition; Calcium; Cyclosporin A; Voltage-gating; Arginine; Phenylglyoxal (rat liver)

1. Introduction

The inner membrane of mitochondria contains a complex channel, the permeability transition (PT) pore. When fully open, this channel permits passive diffusion of solutes with a molecular weight up to 1500 Da, corresponding to a bore diameter of 2–3 nm [1]. The immunosuppressive peptide cyclosporin A (CsA) inhibits the pore at nanomolar concentrations by binding to matrix cyclophilin, which may lead to its dissociation from the inner mitochondrial membrane and possibly from its putative binding sites on the pore [2,3]. The PT pore is controlled by several ligands as well as by the electric potential difference ($\Delta\psi$) across the inner mitochondrial membrane; at high values of $\Delta\psi$ the closed conformation prevails whereas a decrease in the $\Delta\psi$ can trigger pore opening [4,5]. The pore is activated by Ca^{2+} from the mitochondrial matrix and by an increase in the reduction potential of intramitochondrial pyridine nucleotides and glutathione [6,7].

Chemical modification of specific amino-acid residues has proven of great value in the characterization of the pore in intact mitochondria. Previous work using histidine- and cysteine-specific reagents has allowed the identification of three separate sites that participate in the control of the PT: matrix histidine(s) conferring pH sensitivity [8]; the S-site, a dithiol which undergoes dithiol-disulfide interconversions [6,9] through matrix glutathione [7]; and the P-site, a NEM-reactive site which is in redox equilibrium with pyridine nucleotides [6,7].

In this study, we have tested the potential role of arginine residues in the regulation of the PT pore by characterizing the effect of the arginine-specific reagent, phenylglyoxal (PGO) [10]. Our results show that treatment of intact mitochondria with PGO inhibits the PT pore triggered by depolarization with uncoupler after Ca^{2+} accumulation. PGO does not modify the extent of mitochondrial Ca^{2+} uptake or the extent of uncoupler-induced membrane depolarization, indicating that covalent modification of arginine residues directly affects the open probability of the pore. These findings suggest that arginine residues located on the channel itself or on some of its regulatory component(s) play a crucial role in the regulation of the PT pore by the $\Delta\psi$.

2. Materials and methods

Rat liver mitochondria were isolated from male Wistar rats (200–300 g) as described previously [11]. To induce chemical modification of arginine residues, mitochondria were pre-incubated with PGO at 20°C for 15 min in a buffer containing 250 mM sucrose, 20 μM ethylene-bis(oxoethylenenitrilo) tetraacetic acid (EGTA)-Tris, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-KOH pH 8.0. The reaction was terminated by adjusting the pH to 6.8 with HEPES and by cooling to 4°C whereupon mitochondria were sedimented by centrifugation at $8000 \times g$ for 10 min. The pellet was resuspended at ≈ 1 mg protein $\times$ ml^{-1} in a medium containing 250 mM sucrose, 0.1 mM EGTA-Tris, 0.1 mM bovine serum albumin, 10 mM Tris-HCl pH 7.4 and the suspension was incubated at 4°C for 15 min. The centrifugation and incubation steps were repeated once and mitochondria were finally re-suspended at ≈ 50 mg protein $\times$ ml^{-1} in the same medium. Control mitochondria were treated exactly as above, except that PGO addition was omitted. Measurements were carried out in a medium containing 250 mM sucrose, 10 mM HEPES-Tris pH 7.4, 3 mM succinate-Tris, 1 mM Pi-Tris, 2 μM rotenone. Mitochondrial swelling was measured at 570 nm with an Aminco DW2a dual-wavelength spectrophotometer operated in the split-beam mode. Ca^{2+} transport was measured with the dye arsenazo III at the wavelength couple 653-684 nm [12] and $\Delta\psi$ was measured with safranine at the wavelength couple 510–530 nm [13] with the Aminco DW2a operated in the dual-wavelength mode. Oxygen consumption was measured polarographically with a Clark electrode. Pi transport was measured in a medium containing 75 mM Pi-KOH pH 7.4, 2 μM rotenone, 1 μM CsA. CsA was a kind gift of Dr. Roland Wenger, Sandoz Pharma (Basel, Switzerland). All other chemicals were of the highest available grade and were purchased from Sigma (St. Louis, MO).

3. Results and discussion

PGO reacts with the guanidino group of arginine under mildly alkaline conditions to form a stable derivative [10].

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Abbreviations: PT, permeability transition; CsA, cyclosporin A; $\Delta\psi$, transmembrane electric potential difference; PGO, phenylglyoxal; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; EGTA, ethylene-bis(oxoethylenenitrilo) tetraacetic acid; RR, ruthenium red; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone

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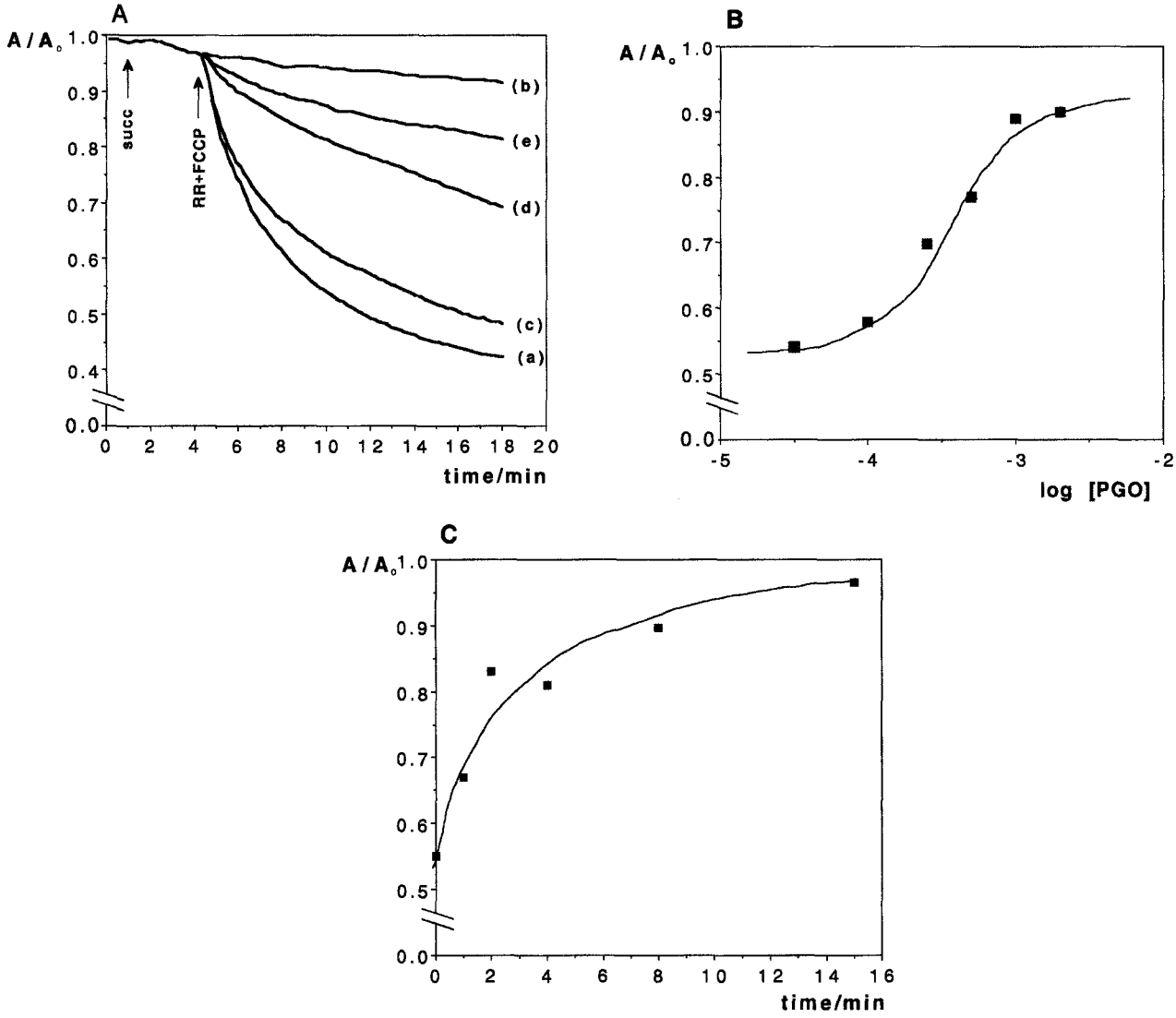

Fig. 1. Inhibition of the PT by PGO. Dependence on concentration and incubation time. Panel A: mitochondria pre-incubated for 15 min with PGO, as described in Section 2, were washed and re-suspended at 0.25 mg protein×ml^{-1} in a buffer containing 250 mM sucrose, 10 mM HEPES-Tris pH 7.4, 1 mM Pi-Tris, 10 μM Ca^{2+}, 2 μM rotenone. The concentration of PGO during pre-incubation was 0 (traces a–b), 0.1 mM (trace c), 0.5 mM (trace d) and 1.0 mM (trace e). In trace b, control mitochondria were re-suspended in the presence of 1 μM CsA. The reaction was started by addition of 3 mM succinate-Tris (succ) followed, were indicated, by 0.1 μM RR and 0.8 μM FCCP (RR+FCCP). Swelling was measured as the decrease in light scattering at 570 nm and the initial absorbance A_0 was set as 1. Panel B: mitochondria were pre-incubated for 15 min with the indicated concentrations of PGO and the experiments were performed as in panel A. Values on the ordinate refer to the absorbance of the suspension 10 min after the addition of RR+FCCP. Panel C: mitochondria were pre-incubated with 1 mM PGO for the time indicated on the abscissa and the experiments were performed as in panel A. Values on the ordinate refer to the absorbance of the suspension 10 min after the addition of RR+FCCP.

To address the potential role of arginine residues in the PT, mitochondria were pre-incubated with PGO for 15 min at pH 8.0, followed by washout of excess reagent. The experiments reported in Fig. 1A show that in respiring, control mitochondria accumulation of Ca^{2+} from the suspension buffer followed by addition of ruthenium red (RR) and the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP) resulted in swelling (trace a), which was completely inhibited by CsA (trace b). This was expected since depolarization of the mitochondrial membrane when the matrix Ca^{2+} concentration is high triggers opening of the PT pore, resulting in extensive diffusion of sucrose from the external medium [4]. In mitochondria pre-treated with PGO, swelling was inhibited (Fig. 1A, traces c–e), the half-maximal effect being observed at 0.3 mM PGO (Fig. 1B). Addition of PGO directly to the cuvette immediately prior to the experiment did not inhibit the pore under these conditions (not shown). Pore inhibition by PGO was dependent on the pre-incubation time, a $t_{1/2}$ of 4 min being obtained at 1 mM PGO (Fig. 1C). These results demonstrate that treatment of mitochondria with PGO at alkaline pH results in a stable protein modification, causing in turn inhibition of the PT pore induced by membrane depolarization.

Since opening of the PT pore is critically dependent on Ca^{2+} uptake, we studied whether PGO pre-incubation affected the extent of mitochondrial Ca^{2+} accumulation. As expected, control mitochondria rapidly accumulated Ca^{2+} following energization with succinate, as detected from the change in absorbance of arsenazo III (Fig. 2, traces a–b). After completion of Ca^{2+} uptake, the addition of RR and FCCP induced a

rapid Ca^{2+} release (trace a) which was inhibited by CsA (trace b), indicating that the PT pore served as the major pathway for Ca^{2+} release under these conditions. Although mitochondria pre-treated with PGO accumulated Ca^{2+} at a slower rate than untreated mitochondria, the final accumulation was identical (traces c–e). Addition of RR and FCCP to PGO-treated mitochondria after completion of Ca^{2+} uptake, however, resulted in a much slower rate of Ca^{2+} release (traces c–e), consistent with a concentration-dependent inhibition of the PT pore. An identical result was obtained if dinitrophenol was used instead of FCCP (not shown), indicating that PGO was not acting through an effect on the putative FCCP receptor [14]. Furthermore, $\Delta\psi$ measurements in PGO-treated mitochondria in the absence of externally added Ca^{2+} indicated a complete collapse of $\Delta\psi$ by 0.8 μM FCCP (not shown). These results demonstrate that inhibition of the PT by PGO pre-treatment was not the consequence of an impaired capacity of Ca^{2+} uptake, nor was it due to a decreased mitochondrial sensitivity to FCCP.

Since pre-treatment with PGO induced an inhibition of the rate of Ca^{2+} accumulation, we next assessed the basis for this effect. Direct inhibition of the Ca^{2+} uniporter, which mediates Ca^{2+} uptake in energized mitochondria, appeared extremely unlikely because partial inhibition of this channel with ruthenium red dramatically altered Ca^{2+} distribution (not shown), which is not observed here (Fig. 2). We then assessed whether the effect of PGO could be linked to an impaired generation of the major driving force for Ca^{2+} uptake (the $\Delta\psi$) through inhibition of the respiratory chain or of substrate transport.

The experiments of Fig. 3 show the effect of PGO treatment on respiration with succinate as a substrate. While the basal state 4 oxygen consumption was unaffected, a strong inhibition of uncoupled respiration was observed, suggesting that inhibition of Ca^{2+} uptake in PGO-treated mitochondria results from partial inhibition of respiration. Since maximal

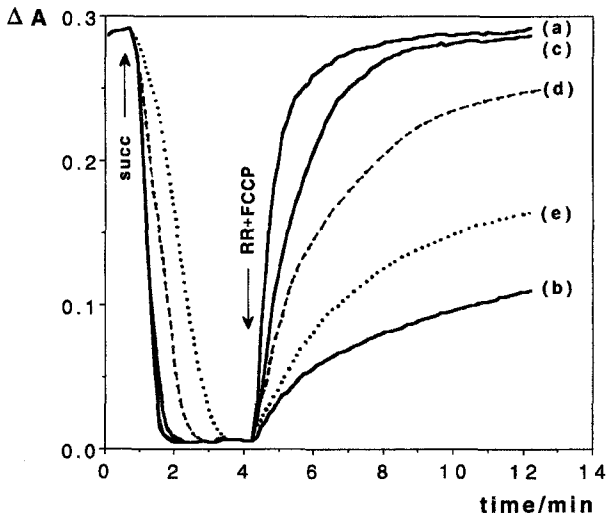

Fig. 2. Inhibition of Ca^{2+} release by PGO. Mitochondria pre-treated for 15 min with PGO at a concentration of 0 (traces a–b), 0.1 mM (trace c), 0.5 mM (trace d) or 1 mM (trace e) were re-suspended in the same incubation medium described in Fig. 1 supplemented with 20 μM arsenazo III. In trace b, control mitochondria were re-suspended in the presence of 1 μM CsA. The reaction was started by addition of 4 mM succinate (succ), followed were indicated by 0.1 μM RR and 0.8 μM FCCP (RR+FCCP). Absorbance was measured at the wavelength couple 653–684 nm.

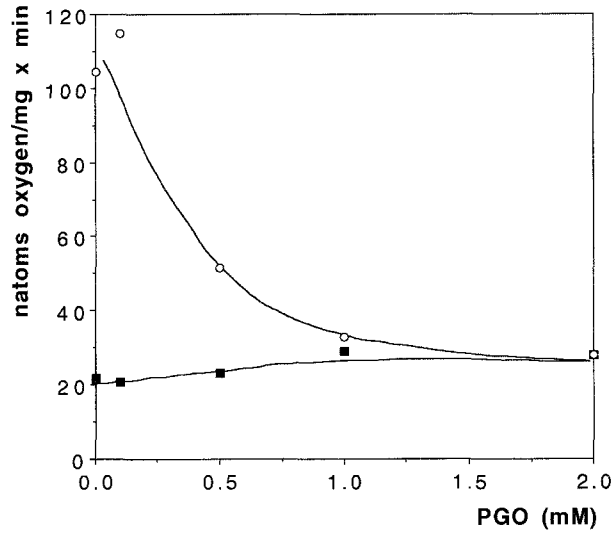

Fig. 3. Effect of PGO on respiration. Mitochondria pre-incubated with the concentrations of PGO indicated on the abscissa were suspended at 0.375 mg protein×ml^{-1} in a buffer containing 250 mM sucrose, 10 mM HEPES-Tris pH 7.4, 5 mM succinate-Tris, 1 mM Pi-Tris, 10 μM EGTA-Tris, 2 μM rotenone. After recording the basal respiration rate (closed symbols), uncoupled respiration was measured after addition of 200 nM FCCP (open symbols).

respiration was not inhibited by PGO treatment in hypotonically ruptured mitochondria (by re-suspension in 1 mM HEPES pH 7.4, results not shown), we conclude that inhibition of respiration by PGO is due to inhibition of substrate transport rather than of the respiratory chain.

It must be stressed that, due to the increased probability of pore opening caused by depolarization [4,5], partial respiratory inhibition by PGO as such should have *promoted* rather than inhibited the PT. To test this prediction, we carried out experiments similar to those reported in Fig. 2. After accumulation of Ca^{2+} by control mitochondria, sufficient antimycin A was added to induce a slow release of Ca^{2+}. Addition of CsA at this point caused a slow but complete reuptake of the released Ca^{2+}, demonstrating that partial inhibition of respiration results in pore opening rather than in its inhibition (results not shown). We conclude that partial inhibition of respiration by PGO cannot explain its inhibitory effect on the pore. Rather, this leads to an *underestimation* of the inhibitory effects of PGO on the PT pore.

Since the PT pore is stimulated by matrix Pi [1], we also investigated whether pore inhibition by PGO could be explained by inhibition of Pi transport. This was not the case since nigericin-induced swelling of deenergized mitochondria in isotonic KPi medium was indistinguishable in PGO-treated and control mitochondria (data not shown).

We think that the most likely mechanism underlying the observed effects of PGO is arginine modification because the reaction was rapid, it occurred at low PGO concentrations and it was favored by mildly alkaline pH (Fig. 1) [10]. We note, however, that PGO can also react to some extent with lysine side chains and N-terminal amino groups. Therefore, we cannot exclude that chemical modification also affects other residues. We note, however, that conserved arginine and lysine residues are believed play a key role in voltage sensing by the plasma membrane K^+, Na^{2+} and Ca^{2+} channels [15].

Taken together, the present observations further point at

the importance of pore modulation by the transmembrane voltage. Although much more work will be needed to clarify the exact mechanism by which PGO causes pore inhibition and the nature and location of the PGO-reactive sites, our study offers a new tool for site-directed modification of pore function at one of its key control points.

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