Tamoxifen Inhibits Induction of the Mitochondrial Permeability Transition by Ca\(^{2+}\) and Inorganic Phosphate

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Tamoxifen (TAM) is a synthetic, nonsteroidal antiestrogenic agent that is widely prescribed in the treatment of estrogen-dependent neoplasias, including breast cancer. The mechanism of action has yet to be defined, but likely is independent of estrogen receptor binding. In light of its high lipophilicity and peroxyl radical scavenging activities, we hypothesized that TAM might be an effective inhibitor of the mitochondrial permeability transition (MPT), which is widely implicated in the mechanisms of chemical-induced tissue injury and apoptosis. The MPT was induced in vitro by incubating freshly isolated rat liver mitochondria in 1 mM Pi with increasing concentrations of calcium. Induction of the MPT was characterized by the calcium-dependent depolarization of mitochondrial membrane potential, release of matrix calcium, and large amplitude swelling. Membrane potential and calcium release were measured with ion-selective electrodes; mitochondrial swelling was monitored spectrophotometrically. Preincubation with either cyclosporine A or TAM prevented, in a dose-dependent manner, the calcium-induced MPT. TAM also inhibited the calcium-induced release of matrix glutathione. TAM caused a time-dependent reversal of both the calcium-induced membrane depolarization and calcium release, suggesting that the effect was on the permeability transition pore and not due to inhibition of the mitochondrial calcium uniport. The results suggest that TAM mimics cyclosporine A to inhibit induction of the MPT and that this activity is not related to the antioxidant properties of TAM. © 1998 Academic Press

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Tamoxifen (TAM) is a synthetic nonsteroidal antiestrogenic drug used successfully as a first line chemotherapy in breast cancer, with the potential for much broader utility as a tumor-preventing agent (Jordan, 1990; Nayfield et al., 1991). However, the mechanisms by which TAM inhibits mammary cancer cell proliferation are not yet understood. It is becoming evident, however, that the antiproliferative effects are not restricted to the classical estrogen receptor binding model (Kon, 1989).

Biomembranes are important targets of TAM and its more active metabolite 4-hydroxylamoxifen, both of which are lipophilic molecules with high partitioning in membranes, which explains the high tissue-to-serum ratios of these drugs (Custodio et al., 1991; Lien et al., 1991). Moreover, the high lipophilicity of TAM may explain the membrane-based cytostatic mechanisms. TAM affects the thermotropic behavior of lipid bilayers and is a strong intramembranous scavenger of peroxyl radicals (Custodio et al., 1993, 1994). The decreased energetic efficiency of sarcolemmal reticulum Ca\(^{2+}\)-ATPase is attributed to TAM disrupting the structural characteristics of biomembranes (Custodio et al., 1996). These same membrane-dependent effects may also be responsible for toxic actions of TAM in vivo. Early reports implicated TAM in the incidence of certain tumors, but these effects are often contested and no clear description has been offered. (Mani and Kupfer, 1991; Fendel and Zimminkski, 1992). Studies of the biochemical interactions of TAM are essential to a better understanding of the molecular mechanisms and cytotoxic effects of this important anticancer drug.

At present, data concerning the effects of TAM on mitochondrial membranes have not been reported. Interference with mitochondrial bioenergetics is known to participate in the process of cell injury by assorted agents and by a variety of mechanisms (Imberti et al., 1993; Kass et al., 1992; Nieminen et al., 1995; Wallace et al., 1997). For example, a number of different chemical inducing agents cause mitochondrial Ca\(^{2+}\) overload and induction of the mitochondrial membrane permeability transition (MPT), which is characterized by the opening of a cyclosporine A-sensitive permeability transition pore within the inner mitochondrial membrane (Bernardi et al., 1994; Gunter and Pfeiffer, 1990; Gunter et al., 1994; Zoratti and Szabo, 1995; Wallace et al., 1997). Experimentally, induction of the MPT is characterized by an abrupt swelling and depolarization of membrane potential accompanied by the efflux of mitochondrial calcium, GSH, and NAD(P)H (Bernardi,
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1992; Petronilli et al., 1994a; Savage et al., 1991; Weis et al., 1994), all of which are inhibited by the immunosuppressant cyclosporine A (CyA) (Broekemeier et al., 1989). Induction of the permeability transition is widely implicated in the mechanisms by which many chemical compounds interfere with mitochondria bioenergetics and cell survival both in vitro (Bernardi et al., 1994; Gunter et al., 1994; Hirsch et al., 1997; Imberti et al., 1993; Kass et al., 1992; Nieminen et al., 1995; Palmeira and Wallace, 1997; Richter and Kass, 1991; Solem et al., 1996; Zoratti and Szabo, 1995) and in vivo (Saxena et al., 1995; Solem et al., 1994, 1996).

Considerations of the lipophilic and antioxidant properties of TAM ( Custodio et al., 1991; 1993; 1994) in conjunction with the current understanding of the chemical reactivities of inducing agents, many of which are oxidants, raise the possibility that TAM might be a potent inhibitor of the MPT. The aim of the present study was to investigate the effects of TAM on the mitochondrial permeability transition with special attention to the possible involvement of antioxidant properties in preventing the permeability transition induced by Ca^{2+} and Pi, purportedly via the generation of reactive oxygen species (Kowaltowski et al., 1996a,b).

MATERIALS AND METHODS

Chemicals. Cyclosporine A (CyA) was a gift-in-kind from Sandoz Pharmaceutical Corp. (East Hanover, NJ). Tamoxifen (TAM) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest purity available from commercial vendors.

Isolation of rat liver mitochondria. Mitochondria were isolated from the liver of fasted adult male Sprague–Dawley rats (200–300 g) by differential centrifugation as described elsewhere (Moreno and Madeira, 1991; Palmeira and Wallace, 1997). The homogenate was prepared in 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), and 1 mM EGTA. The EGTA was omitted from the final washing medium, which was adjusted to pH 7.2. The final mitochondrial pellet was washed twice and immediately used for swelling experiments. The purity available from commercial vendors.

Standard incubation procedure. The experiments were conducted at 30°C in a standard reaction medium containing 200 mM sucrose, 10 mM Tris–Mops (pH 7.4), 1 mM KH2PO4, and 10 mM EGTA, supplemented with 2 mM rotenone and 0.5 μM oligomycin/ml. Typically, and unless noted otherwise, TAM was added in ethanolic solutions (up to 4 μl) to the reaction medium after protein addition and incubated for 2 min before starting the reactions. Other additions were as indicated in the figure legends. The results shown represent typical recordings from experiments of at least three different mitochondrial preparations.

Measurement of mitochondrial swelling. Mitochondrial osmotic volume changes were followed by monitoring the decrease in absorbance (light-scattering) at 540 nm with a Beckman DU-7 spectrophotometer as previously described (Palmeira and Wallace, 1997). The reactions were performed with 1 mg mitochondrial protein in 2 ml of the standard incubation medium and started with 5 mM succinate. Ca^{2+} (150 nmol/mg protein) was added 2 min after energization of mitochondria with succinate. Where indicated, CyA in ethanol was added before starting the reactions unless noted otherwise.

Measurements of mitochondrial membrane potential. The mitochondrial membrane potential (ΔΨ) was monitored by evaluating transmembrane distribution of tetraphenylphosphonium (TPP^+) with an ion-selective electrode prepared according to Kamo et al. (1979) using a calomel electrode as reference. Reactions were carried out in an open vessel with magnetic stirring in 2 ml of the standard incubation medium supplemented with 4 μM TPP^+.

The experiments were started by adding 5 mM succinate to mitochondria in suspension at 0.5 mg protein/ml. After a steady-state distribution of TPP^+ had been reached (ca. 2 min of recording), Ca^{2+} was added and ΔΨ recorded for 15 additional min. Membrane potential was estimated from the decrease TPP^+ concentration in the reaction medium as described elsewhere (Moreno and Madeira, 1991). TAM and CyA were incubated 2 min before succinate or after mitochondrial loading with Ca^{2+} as indicated in the figure legends. Calibration runs in the presence of TAM excluded any direct interference with the signal, although both TAM and CyA slightly decreased ΔΨ.

Mitochondrial Ca^{2+} fluxes. Mitochondrial Ca^{2+} fluxes were measured by monitoring the changes in Ca^{2+} concentration in the reaction medium using a Ca^{2+}-selective electrode according to previously described procedures (Madeira, 1975; Moreno and Madeira, 1991). The reactions were conducted in an open vessel with magnetic stirring in 2 ml of the reaction medium of 0.2 M sucrose, 10 mM Tris–Mops (pH 7.4), and 1 mM KH2PO4 supplemented with 2 mM rotenone and 0.5 μM oligomycin. Mitochondria (0.5 mg protein/ml) were energized with 5 mM succinate after 2 min of Ca^{2+} addition in the absence and presence of TAM or CyA.

Simultaneous measurements of GSH release and ΔΨ. Mitochondria (2.5 mg/ml) were suspended in 2.2 ml of the standard incubation medium at 30°C. ΔΨ was monitored continuously for 15 min after adding Ca^{2+} as described above. The reactions were conducted under a continuous stream of O2 to avoid anaerobiosis. Oxygen tension was monitored polarographically throughout the reaction in order to insure well-oxygenated conditions (Clarke-type, YSI oxygen electrode).

Intra- and extramitochondrial GSH were determined by the HPLC method described by Fariss and Reed (1987) with slight modifications. Sample aliquots (1 ml), taken after 15 min of Ca^{2+} addition (150 nmol/mg protein), were layered over dibutyl phthalate (0.4 ml) which was layered over 10% perchloric acid (PCA) containing 1 mM bathophenanthroline disulfonic acid (0.55 mM). Samples were centrifuged at 5,500 g for 2 min in a Eppendorf table-top centrifuge. The acidified mitochondrial fraction was separated from the extra-mitochondrial supernatant and both fractions analyzed for GSH by HPLC following derivatization. Briefly, 0.5 ml each of the supernatant and acid extract were derivatized by adding 50 μl of 100 mM iodoacetic acid and 0.5 ml of 2 M KOH–2.4 M KHCO3. The samples were allowed to incubate in the dark at room temperature for 1 h. After addition of 0.45 ml of 1.5% 1-flouro-2,4-dinitrobenzene, samples were stored at 4°C overnight and then centrifuged at 1,400 g for 2 min. GSH was quantified using an HPLC system equipped with a Spherisorb S5 amino column (Deeside Ind. Est., UK) and a UV/Vis spectrophotometric detector. The detection wavelength was 365 nm and the mobile phase, delivered at 1.5 ml/min, was a binary gradient of sodium acetate and methanol as described by Fariss and Reed (1987). During each run, an isocratic elution of 1 M sodium acetate in 67% methanol for 15 min was followed by a 10 min linear gradient to 5 M sodium acetate. GSH concentrations were calculated by comparing the integrated peak to commercial standards.

RESULTS

At concentrations above 40 nmol/mg mitochondrial protein (ca. 20 μM), TAM caused a dose-dependent decrease in light scattering by unenergized mitochondria in suspension, which was independent of calcium (Fig. 1). At 100 nmol TAM/mg protein, the mitochondrial light scattering decreased almost instantaneously and was complete within 3 min. The fact that this decrease in light scattering was not inhibited by cyclosporine A (data not shown) indicates that this is not the result of induction of the MPT. Rather, this apparent mitochondrial
swelling likely reflects the direct membrane active, detergent-like properties of TAM (Custodio et al., 1993, 1996).

Lower concentrations of TAM, however, caused a dose-dependent inhibition of mitochondrial swelling induced by the combination of 75 μM calcium plus 1 mM KH₂PO₄ (Figs. 2A and 2B). As reported by Kowaltowski et al. (1996a, 1996b), incubation of succinate-energized mitochondria with calcium in the presence of Pi causes a delayed but large-amplitude decrease in light scattering, which is indicative of mitochondrial swelling. The fact that it is inhibited by CyA is strong evidence implicating induction of the MPT pore, which the authors attribute to the Ca/Pi-induced stimulation of hydrogen peroxide generation. The fact that the dose-dependent inhibition of mitochondrial swelling by TAM occurred regardless of whether TAM was added before or after energization with succinate and loading with calcium is evidence against TAM causing membrane depolarization or interfering with calcium uptake across the uniport via some other mechanism.

Inhibition of Ca/Pi-induced mitochondrial swelling by TAM was a function of elapsed time, the longer the delay in adding TAM the less pronounced was the inhibition of swelling (Fig. 3). In fact, the data of Fig. 3 demonstrate that TAM (and CyA for that matter) is effective at preventing, but not reversing, induction of the MPT by calcium plus phosphate. It can be concluded from these data that TAM arrests the progressive swelling of isolated mitochondria in suspension.

The inhibitory effect of both TAM and CyA on induction of the MPT by Ca/Pi is further demonstrated by the fact that both...
agents prevented the calcium-induced depolarization of mitochondrial membrane potential (Fig. 4). Adding a high concentration of calcium (150 nmol/mg protein) caused a transient depolarization, membrane potential returning to near 200 mV within 1.5 min. This was followed by a dramatic and irreversible depolarization of membrane potential over the course of the next 5–10 min. Adding either CyA or TAM at the beginning of the reaction prior to adding succinate and calcium afforded complete protection against the calcium-induced irreversible depolarization of mitochondrial membrane potential (Fig. 4). Under these conditions, calcium still caused a transient depolarization of membrane potential, the extent of which was the same as controls.

Similar to what was observed for mitochondrial swelling (Fig. 3), the effect of TAM to prevent calcium-induced depolarization of membrane potential was dependent on the time elapsed since adding calcium (Fig. 5). However, rather than arresting the continuance of mitochondrial swelling, TAM actually reversed the calcium-induced membrane depolarization if added before the membranes were completely depolarized (point 4 of Fig. 5). CyA, on the other hand, caused repolarization of membrane potential even if added late in the reaction.

Further evidence that TAM resembles CyA in inhibiting the calcium-induced MPT is provided by the demonstration that both agents prevent the calcium-induced release of mitochondrial calcium (Fig. 6). This protection was evident regardless of whether TAM was added before or after loading of energized mitochondria with calcium. Furthermore, adding TAM during the course of the MPT reversed the calcium-induced calcium release (Fig. 7) in much the same manner as was observed for the calcium-induced membrane depolarization (Fig. 5). Again, there occurred a point in time late in the reaction beyond which TAM did not reverse the calcium-induced calcium release. CyA reversed this process even when added after essentially all of the accumulated calcium had been released from the mitochondria.

Figure 8 illustrates the effect of TAM on calcium-induced depolarization of mitochondrial membranes in reactions monitored concurrently with the measurement of mitochondrial GSH (Fig. 9). Adding high calcium (ca. 150 nmol/mg protein) in the presence of Pi caused the delayed depolarization of membrane potential (Fig. 8, trace 1) accompanied by the release of more than two-thirds of the mitochondrial GSH (Fig.

**FIG. 4.** Inhibition of Ca\(^{2+}\)-induced depolarization of succinate-energized mitochondria by TAM and CyA. Mitochondria (1 mg) in 2 ml of the standard reaction medium supplemented with 4 μM TPP\(^+\) were energized with 5 mM succinate after incubation for 2 min at 30°C. Ca\(^{2+}\) (150 nmol/mg protein) was added after a steady-state distribution of TPP\(^+\) in the absence or presence of either 40 nmol TAM/mg protein or 0.85 μM CyA. Both TAM and CyA were preincubated with mitochondria for 2 min before starting the reactions with succinate. The membrane potential (ΔΨ) was estimated as described in Materials and Methods. Note that both TAM and CyA depress the total developed ΔΨ and the depolarization induced by Ca\(^{2+}\).

**FIG. 5.** TAM causes the time-dependent repolarization of mitochondria after exposure to Ca\(^{2+}\) concentrations sufficient to induce pore opening in vitro. The experimental conditions were the same as those described in the legend to Fig. 4, except that, where indicated by arrows, TAM (40 nmol/mg protein) was added at different times after Ca\(^{2+}\) addition. The control trace corresponds to Ca\(^{2+}\) + Pi without either TAM or CyA. Traces 1–4 correspond to increasing delays in time preceding the addition of TAM. Where indicated, 0.85 μM CyA was added after Ca\(^{2+}\)-induced membrane depolarization at the point indicated by the fourth (4) arrow.
The loss of matrix GSH is attributed to its release to the medium since no GSSG was detected in any of the reactions and since the sum total GSH (intramitochondrial plus extramitochondrial) did not change. Adding either TAM or CyA early in the reaction prevented both the calcium-induced membrane depolarization (Fig. 8, traces 2 and 3, respectively) and the release of mitochondrial GSH (Fig. 9). Both agents afforded complete protection against both of these parameters of calcium-induced MPT.

**DISCUSSION**

Induction of the mitochondrial permeability transition is implicated in the mechanism of toxic tissue injury caused by a number of compounds, the majority of which are oxidants (Bernardi et al., 1994; Gunter and Pfeiffer, 1990; Gunter et al., 1994; Imberti et al., 1993; Kass et al., 1992; Nieminen et al., 1995; Palmeira and Wallace, 1997; Richter and Kass, 1991; Solem et al., 1996; Wallace et al., 1997; Zoratti and Szabo, 1995). This is significant in that the MPT pore is under the regulatory influence of the redox status of both mitochondrial pyridine nucleotides and sulfhydryl groups (Chernyak and Bernardi, 1996; Costantini et al., 1996; Fagian et al., 1990; Halestrap et al., 1997; Petronilli et al., 1994a). Accordingly, one would suspect that antioxidants would be effective at preventing induction of the permeability transition, thereby protecting the tissue from such damage.

To date, there are a limited number of inhibitors of the mitochondrial permeability transition that have been identified. The most specific is cyclosporine A, an immunosuppressant...
that competitively prevents cyclophilin from interacting with specific cyclophilin-dependent binding domains of the pore (Broekemeier et al., 1989; Crompton et al., 1988; Nicolli et al., 1996). Other classes of inhibitors of the MPT are far less specific and consist of ligands for the adenine nucleotide transporter (Halestrap and Davidson, 1990; Zoratti and Szabo, 1995), inhibitors of phospholipase A2 (Broekemeier and Pfeiffer, 1995), and antioxidants (Castilho et al., 1996).

Implication of the thiol redox status in the regulation of the MPT pore is owed to the identification of oxidants as important inducers of the transition and by the potent inhibition by sulphydryl reagents (Gunter et al., 1994). It is proposed that a critical factor regulating induction of the MPT is the oxidation state of cysteine residues in the immediate vicinity of the voltage-sensing element of the pore (Petronilli et al., 1994a). Oxidation to form disulfides increases the voltage gating potential and, thus, the probability of pore opening. Conversely, thiol-reducing agents or monofunctional alkylating agents stabilize the pore in the closed state. Oxidants, including hydroperoxides and variously substituted naphthoquinones, cause the accumulation of both glutathione and mixed protein disulfides within the mitochondria (Castilho et al., 1996; Fagian et al., 1990; Olafsdottir and Reed, 1988; Palmeira and Wallace, 1997; Petronilli et al., 1994a), which is prevented by thiol-reducing agents and other antioxidants that are also known to prevent induction of the MPT.

Induction of the MPT requires, at a minimum, calcium plus phosphate (Petronilli et al., 1993). Kowaltowski et al. (1996a) demonstrated a stimulation of H$_2$O$_2$ generation by hepatic mitochondria incubated with 10 μM calcium plus 1 mM phosphate. The fact that this was associated with induction of the mitochondrial permeability transition, which was inhibited by both catalase and dithiothreitol, implicated the H$_2$O$_2$-dependent oxidation of critical mitochondrial thiols. The authors propose that induction of the MPT by the combination of calcium plus phosphate results from the H$_2$O$_2$-dependent depletion of mitochondrial glutathione and the oxidation of critical mitochondrial protein thiols. However, we measured no change in the oxidation state of mitochondrial glutathione during the course of calcium plus phosphate-induced MPT. The depletion of GSH was due solely to the efflux from the permeabilized mitochondria, which was prevented by inhibiting the MPT with cyclosporine A or TAM. We conclude that unlike oxidants, oxidation of mitochondrial GSH and protein thiols is not a requirement for induction of the MPT by the combination of calcium plus phosphate. This is in accordance with observations by Savage et al. (Savage et al., 1991; Savage and Reed, 1994) using higher concentrations of both calcium (70 μM) and phosphate (3 mM). Accordingly, although TAM is lipophilic and has been shown to scavenge peroxyl radicals (Custodio et al., 1993, 1994), inhibition of the induction of the MPT is due to some factor other than the antioxidant properties of TAM. Instead, TAM may be more global in its effectiveness against inhibiting induction of the MPT by assorted classes of chemical-inducing agents. Since TAM did not alter State 4 respiration (data not shown), the protection against induction of the MPT is not likely the result of a direct effect on the electron transport chain. Although we have no direct evidence, the decrease in probability of pore opening caused by TAM may reflect yet one more membrane-dependent biological activity of this important cytostatic agent. For example, with a pKa ~8.5, TAM exists as the protonated quaternary ammonium cation at physiological pH. Since amphipathic organic cations are known to be potent inhibitors of the MPT (Broekemeier and Pfeiffer, 1995), it is possible that this inhibitory activity of TAM reflects the fact that it increases the membrane surface potential of the mitochondria, thereby affecting the voltage-dependent regulation of the MPT. Many additional experiments are required to test this possibility.

Induction of the MPT is reversible and protection by such agents as EGTA and cyclosporine A is transient (Broekemeier et al., 1989; Crompton and Costi, 1988; Hunter et al., 1976; Petronilli et al., 1994b; Savage et al., 1991). Adding either of these agents soon after induction of the MPT causes a "resealing" of the permeabilized mitochondria. However, Castilho et al. (1996) report a time-limited ability of EGTA to reverse induction of the MPT by oxidants: the more time that elapses after adding the oxidant in vitro, the less complete restoration of mitochondrial volume and membrane potential by EGTA. These authors invoke the progressive oxidation and accumulation of critical membrane protein disulfides as determining the finite capacity to reverse induction of the MPT. We demonstrate a comparable time-restricted capacity of TAM to
reverse induction of the MPT by calcium plus phosphate. However, since we observed no oxidation of mitochondrial glutathione, we must invoke some mechanism other than the accumulation of protein disulfides. In view of the potent membrane-dependent activities of this agent, it is reasonable to suggest that TAM influences the regulation of the transformation of the permeability pore by some as yet undefined allosteric process. The time-dependent irreversibility of the permeability transition pore might reflect a “fixing” of the transformed complex such that it is no longer subject to allosteric influences imposed by membrane-active compounds such as TAM. Regardless, in view of the growing evidence implicating the MPT in assorted chemical-injuries and ischemia-related tissue damage, and as an important apoptogenic event, it has become important to identify strategies to circumvent induction of the MPT in vivo (Bernardi et al., 1994; Gunter and Pfeiffer, 1990; Gunter et al., 1994; Zoratti and Szabo, 1995; Wallace et al., 1997). Cyclosporine A, although extremely useful in vitro, has yielded less than satisfying results in vivo. This newly discovered activity of TAM may provide this long-sought and important opportunity to intervene in the clinical management of such disorders. Therapeutic concentrations of tamoxifen are approximately 120 ng/ml (approximately 0.3 μM). In our incubations, effective concentrations of tamoxifen were between 10 and 50 nmol/mg mitochondrial protein. At 0.5 mg protein/ml, this translates to 5–25 μM. However, one must consider the fact tamoxifen is a very hydrophobic molecule and that in rats and humans the steady-state concentration in liver is 60–70 times higher than in serum (Lien et al., 1991). Thus, it is highly probable that at therapeutic plasma concentrations, the concentration of tamoxifen in the tissues is well within the range found to inhibit induction of the MPT.

REFERENCES


