ABSTRACT

In central neurons, glutamate receptor activation causes massive calcium influx and induces a mitochondrial depolarization, which is partially blocked by cyclosporin A, suggesting a possible activation of the mitochondrial permeability transition pore (PTP) as a mechanism. It has been recently reported that tamoxifen (an antiestrogen chemotherapeutic agent) blocks the PTP in isolated liver mitochondria, similar to cyclosporin A. In this study, we tested whether tamoxifen inhibits the mitochondrial depolarization induced by glutamate receptor activation in intact cultured neurons loaded with the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide. This dye reports disruptions in mitochondrial membrane potential, which can be caused by PTP activation. We found that glutamate (100 μM for 10 min) causes a robust mitochondrial depolarization that is partially inhibited by tamoxifen. The maximum inhibitory concentration of tamoxifen was 0.3 μM, with concentrations higher and lower than 0.3 μM being less effective. However, although tamoxifen (0.3 μM) blocked glutamate-induced mitochondrial depolarization, it did not inhibit glutamate-induced neuronal death, in contrast to the PTP inhibitor cyclosporin A. A relatively high concentration of tamoxifen (100 μM) caused mitochondrial depolarization itself and was neurotoxic. These data suggest that tamoxifen may be an inhibitor of the PTP in intact neurons. However, the lack of specificity of most PTP inhibitors, and the difficulty in measuring PTP in intact cells, preclude definite conclusions about the role of PTP in excitotoxic injury.

Activation of the mitochondrial permeability transition pore (PTP) has been identified as a possible common effector of the cell death of numerous cell types in response to both necrotic and apoptotic stimuli (Lemasters et al., 1997; Kroemer et al., 1998). The PTP includes proteins located in both the inner and outer mitochondrial membranes and, when opened, allows mitochondrial constituents <1.5 kD to cross the inner membrane. In isolated mitochondria this results in swelling, loss of the proton motive force, and the loss of low molecular weight compounds such as glutathione (Savage and Reed, 1994; Zoratti and Szabo, 1995). Increases in matrix Ca\(^{2+}\) and oxidant levels are important inducers of the PTP. Cyclosporin A is among the most potent inhibitors of the PTP (Broekemeier et al., 1989).

The PTP has been suggested to be involved in the neurotoxicity caused by overactivation of neuronal glutamate receptors (Nieminen et al., 1996; Schinder et al., 1996; White and Reynolds, 1996). Glutamate-induced neurotoxicity is involved in the cell loss caused by stroke and trauma, as well as chronic neurodegenerative diseases (Choi, 1988). Activation of the various subtypes of glutamate receptor leads to opening of an integral ion channel and influx of Na\(^+\), and for the N-methyl-D-aspartate (NMDA) subtype and certain α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate subtypes, Ca\(^{2+}\) (Mayer and Westbrook, 1987). Robust Ca\(^{2+}\) accumulation and the subsequent mitochondrial Ca\(^{2+}\)-dependent depolarization (Dugan et al., 1995; Reynolds and Hastings, 1995; Bindokas et al., 1996). The massive Ca\(^{2+}\)-dependent depolarization caused by NMDA receptor activation also induces a Ca\(^{2+}\)-dependent depolarization of the mitochondrial membrane potential (Δψ\(_{m}\)) that is partially blocked by the PTP inhibitor cyclosporin A (Ankarcrona et al., 1996; Schinder et al., 1996; Bindokas et al., 1996).

ABBREVIATIONS: PTP, mitochondrial permeability transition pore; NMDA, N-methyl-D-aspartate; Δψ\(_{m}\), mitochondrial membrane potential; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; HBSS, HEPES-buffered salt solution; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; LDH, lactate dehydrogenase.
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al., 1996; White and Reynolds, 1996) as well as other PTP blockers such as trifluperazine and dibucaine (Hoyt et al., 1997). Cyclosporin A also inhibits toxicity caused by glutamate receptor activation, although this effect may be mediated by calcineurin inhibition rather than PTP activation (Dawson et al., 1993; Ankarcrona et al., 1996; Schinder et al., 1996; White and Reynolds, 1996). Indeed, it has proven difficult to establish the role of the PTP in excitotoxicity because of the lack of potent and selective inhibitors.

It has been recently reported that tamoxifen, a widely used antiestrogen chemotherapeutic and chemoprevention agent, blocks Ca^{2+}-induced PTP activation in isolated liver mitochondria, with effects similar to those caused by cyclosporin A (Custodio et al., 1998). In addition to its estrogen receptor-blocking effects, tamoxifen is a lipophilic peroxyl radical scavenger (Custodio et al., 1994). However, it does not appear that its antioxidant function is related to its ability to block PTP because the PTP-inducing conditions (Ca^{2+} and phosphate treatment) with which tamoxifen was tested did not alter mitochondrial oxidized glutathione levels (an indication of oxidation) (Custodio et al., 1998).

Tamoxifen rapidly induces apoptosis in neural cell lines (Ellerby et al., 1997; Hashimoto et al., 1997). Whole-cell extracts from cultures treated with 100 μM tamoxifen induced assymetric chromatin formations indicative of apoptosis in naïve isolated nuclei within 1 h. This rapid morphological change was accompanied by caspase cleavage of nuclear substrates (Ellerby et al., 1997). These effects were not blocked by inhibitors of caspases 1 and 4 and could not be reproduced if nuclei were treated with only mitochondrial and cytosolic fractions from tamoxifen-primed cells. This apparent requirement for cellular components other than the mitochondria and cytosol would suggest that tamoxifen does not initiate cell death by directly impairing mitochondrial membrane potential, although this hypothesis has not been directly tested. It also remains to be determined if this compound can provide neuroprotection by altering PTP activation in primary neuronal cultures at concentrations similar to those that inhibit PTP in liver mitochondria (5–25 μM) (Custodio et al., 1998).

There are relatively few drugs available to study PTP activation in intact cells, and we were interested to see whether tamoxifen would be as effective in neurons as it is in isolated mitochondria. We tested whether tamoxifen inhibits the Δψ_m depolarization induced by glutamate receptor activation in cultured neurons. Δψ_m was monitored in neurons loaded with the Δψ_m-sensitive fluorescent dye JC-1 (Molecular Probes), incubated in toxin. Cells were then taken and used to obtain forebrain neurons for cell culture. The Institutional Animal Care and Use Committee of the University of Pittsburgh. Brain tissue was dissociated with trypsin, and then plated on to poly(L-lysine)-coated glass coverslips at a density of 450,000 cells ml^{-1} in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 24 U ml^{-1} penicillin, and 24 μg ml^{-1} streptomycin. Twenty-four hours after plating, the media were removed and replaced with Dulbecco’s modified Eagle’s medium that contained horse serum in place of fetal bovine serum, and the coverslips were inverted to suppress glial proliferation. Neurons were kept in a 37°C, 5% CO_2-humidified incubator for 12 to 18 days until use. All recordings were made with a HEPES-buffered salt solution (HBSS) that contained 137 mM NaCl, 5 mM KCl, 0.9 mM MgSO_4, 1.4 mM CaCl_2, 3 mM NaHCO_3, 0.6 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, and 20 mM HEPES; pH was adjusted to 7.4 with NaOH. All glutamate solutions contained 1 μM glycine. Tamoxifen was dissolved in methanol (<0.02% final methanol concentration) and all control conditions contained 0.02% methanol.

Measurements of Δψ_m. Δψ_m was monitored in neurons loaded with the Δψ_m-sensitive fluorescent dye JC-1 (Molecular Probes, Eugene, OR; White and Reynolds, 1996). Neurons were loaded with the JC-1 (3 μM) for 20 min at 37°C, rinsed with dye-free HBSS for 20 min at room temperature, and then mounted in a recording chamber on the stage of an ACAS 570c laser scanning confocal microscope (Meridian Instruments, Okemos, MI). Fields of neurons were illuminated with the 488-nm line of an argon laser, and emission at 530 and 590 nm was monitored. Solution changes in this protocol were made by rapidly aspirating and replacing the contents of the recording chamber. The fluorescence emission wavelength of JC-1 depends on the aggregation of the JC-1 molecules that in turn depends on the Δψ_m (i.e., the greater the Δψ_m, the greater the aggregation; Reers et al., 1991). By monitoring JC-1 fluorescence at 590 nm (aggregate) and 530 nm (monomer), one can assess relative changes in Δψ_m. Ratio values were obtained by dividing the signal at 590 nm by the signal at 530 nm after background subtraction on a cell-by-cell basis and normalized to a starting value of 1 for comparison between cells. With this approach, a decrease in the normalized ratio represents mitochondrial depolarization, which was confirmed by titration with increasing concentrations of the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 20–750 nM), resulting in graded, concentration-dependent decreases in the JC-1 ratio (K. R. Hoyt and I. J. Reynolds, unpublished observations).

[Ca^{2+}^-], Measurements. [Ca^{2+}^-], was measured from individual neurons loaded with the Ca^{2+}^-sensitive fluorescent dye indo-1 (White and Reynolds, 1995). Neurons were rinsed with HBSS and then loaded with 5 μM indo-1 AM (Molecular Probes) in HBSS containing 5 mg/ml BSA for 50 min at 37°C, and incubated in dye-free HBSS for a further 20 min at 37°C to allow for dye cleavage. Coverslips were then mounted in a recording chamber (1-ml volume) on the stage of a Nikon Diaphot microscope. Cells were illuminated at 350 nm with light from a 75-W mercury arc lamp. Indo-1 emission was simultaneously monitored at 405 and 490 nm with a dual photomultiplier system. Background subtracted ratios were converted to [Ca^{2+}^], with parameters from an in situ calibration.

In Vitro Toxicity Assay. For neuronal viability experiments, coverslips were washed once in HBSS that had been prewarmed to 37°C, inverted, and transferred to new plates. Cells were then washed twice more in HBSS and incubated in toxin. Cells were exposed to glutamate (100 μM) and glycine (1 μM) or HBSS in the presence or absence of tamoxifen (0.3 μM) and returned to the incubator for 10 min. Glutamate exposure was terminated by washing cells twice with HBSS. After rinsing with HBSS, cells were maintained in the presence or absence of tamoxifen (0.3 μM) in minimal essential medium. For high-dose tamoxifen experiments, cells were maintained in 100 μM tamoxifen in minimal essential medium. Neuronal viability was determined by measuring lactate dehydrogenase (LDH) release with an in vitro toxicity assay kit (Sigma Chemical Co., St. Louis, MO). Forty-microliter samples of medium were assayed spectrophotometrically according to the manufacturer’s protocol to obtain a measure of cytoplasmic LDH released from dead and dying neurons.
FCCP, which collapses the Dsutures was identified (bregma). Malonate (3 incision was made and the confluence of the sagittal and coronal State University. Rats were anesthetized with equithesin, then and have been approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. Rats were anesthetized with equithesin, then placed in a Kopf small animal stereotaxic apparatus. A midline incision was made and the confluence of the sagittal and coronal sutures was identified (bregma). Malonate (3 μmol in 2 μl of 0.9 N NaCl) was administered via a 26-gauge Hamilton syringe at a rate of 0.2 μl/min at the following coordinates relative to bregma: 0.7 mm anterior, 2.8 mm lateral, and 5.0 mm ventral. The needle remained in place for an additional 5 min to limit regurgitation up the needle tract. Tamoxifen or vehicle (dimethyl sulfoxide) treatments were administered i.p. 2 h before and 4 h after malonate exposure. Seven days after malonate exposure, all animals were euthanized with chloral hydrate (500 mg/kg) and rapid decapitation. The cranial contents were removed, coated with embedding matrix, frozen under powdered dry ice, and stored at −70°C until sectioning.

Coronal sections (25 μm) were gathered at 250-μm intervals through the rostrocaudal extent of the striatum with a cryostat and were thaw-mounted onto polylysine-treated slides. Tissue sections were then processed for cytochrome oxidase histochemistry.

Cytochrome Oxidase Histochemistry. Sections were incubated in 100 mM sodium phosphate buffer (pH 7.4) with cytochrome c (10 μM) and 3,3’-diaminobenzidine (1 mM) for 2 h at 37°C in the dark. Sections were postfixed in 10% formalin (10 min), dehydrated in graded alcohol, and coverslipped from xylene. Analysis of striatal lesion volume of cytochrome oxidase-stained sections was performed on a microcomputer-based image analysis program (Image Research, St. Catherines, Ontario, Canada) with area standards to provide a calibration from which three-dimensional volume (cubic millimeters) of the lesioned striatum was estimated.

Results

Exposure of neurons to excitotoxic concentrations of glutamate (100 μM) causes a decrease in Δψm that can be monitored with the Δψm-sensitive fluorescent probe JC-1. A decrease in the ratio of JC-1 fluorescence emission at 590 nm relative to the emission at 530 nm indicates Δψm depolarization (Fig. 1A). We have previously shown this depolarization is mediated primarily by the NMDA subtype of glutamate receptor and is Ca2+-dependent (White and Reynolds, 1995, 1997; Hoyt and Reynolds, 1998; Hoyt et al., 1998). The lack of effect of tamoxifen on [Ca2+]i, or on Ca2+ recovery suggests that it does not inhibit glutamate-induced Δψm depolarization because of major alterations in [Ca2+]i handling in response to glutamate.

It has been proposed that PTP activation is involved in the neurotoxicity of glutamate receptor activation, so we were interested to see whether tamoxifen had a neuroprotective action. Tamoxifen (0.3 μM; present during and after glutamate exposure) had no effect on the neuronal death caused by glutamate (100 μM for 10 min) as measured by LDH release from damaged neurons into the media during the 20 h after glutamate exposure (Fig. 4A). Because tamoxifen has been reported to rapidly induce apoptosis in neural cell lines, we tested a higher concentration (100 μM) of tamoxifen alone on neuronal viability and found that a 30-min exposure resulted in significant cell loss expressed 20 h later (Fig. 4B). Continuous exposure of neurons to 100 μM tamoxifen for 3 h also caused an increase in the number of apoptotic nuclei visualized with the fluorescent nuclear dye Hoechst 33342 from 3% in controls to 23% for cells treated with tamoxifen, consistent with previous findings in a neuronal cell line (Ellerby et al., 1997). It appears, therefore, that a low concentration of tamoxifen does not protect cells from excitotoxic injury and that high concentrations of tamoxifen are neurotoxic to primary cultured neurons.

We also tested whether tamoxifen was neuroprotective in an in vivo model of excitotoxic neuronal death. Malonate, an inhibitor of succinic dehydrogenase, causes metabolic inhi-
bition and neuronal damage when injected into the striatum (Fig. 5A). Glutamate receptor antagonists inhibit this neuronal damage, reflecting an excitotoxic component of this neuronal injury (data not shown) (Greene and Greenamyre, 1995; Schulz et al., 1996). Tamoxifen (2 mg/kg i.p. 2 h before and 4 h after striatal malonate injection) did not reduce the volume of the striatal lesion (Fig. 5B). Doses of tamoxifen from 1 to 20 mg/kg were tested and none prevented the striatal damage caused by malonate (Fig. 5C).

Discussion

We found that glutamate (100 μM) causes a robust mitochondrial depolarization that is partially inhibited by tamoxifen. The maximum inhibitory concentration of tamoxifen was 0.3 μM, with concentrations higher and lower than 0.3 μM being less effective. Tamoxifen (0.3 μM) did not inhibit glutamate receptor-activated increases in intracellular Ca²⁺, suggesting that it does not directly inhibit receptor activation, nor does it appear to inhibit [Ca²⁺]i buffering after a glutamate stimulus. Therefore, a decrease in glutamate-in-

Fig. 1. Tamoxifen inhibits glutamate-induced mitochondrial depolarization in neurons loaded with JC-1. A, application of 100 μM glutamate (■) caused a decrease in the normalized 590/530 nm JC-1 emission ratio, reflecting mitochondrial depolarization. Addition of 0.3 μM tamoxifen (□) during the glutamate exposure substantially reduced the extent of the loss of ΔΨm caused by glutamate. Data represent the mean ± S.E. of 54 to 70 neurons per condition. FCCP (750 nM), a protonophore that depolarizes ΔΨm, was added at the end of the experiment for comparison. B, a higher tamoxifen concentration (20 μM) did not inhibit the glutamate-induced decrease in ΔΨm when included during the glutamate exposure. Data represent the mean ± S.E. of 51 to 61 neurons per condition. C, concentration dependence of the inhibition of glutamate-induced mitochondrial depolarization by tamoxifen. Data are expressed as the difference between the normalized JC-1 ratio for tamoxifen-treated versus untreated cells after 5 min of glutamate exposure. Because these data points are not paired, we cannot calculate individual standard error values for these data. As an indication of variability, we report that the range of the standard error for the data points from which the differences were calculated was 0.018 to 0.025 normalized JC-1 fluorescence units. As the tamoxifen concentration was increased, there was a decrease in the inhibitory effect on glutamate-induced depolarization. Data were collected from a total of 41 to 70 neurons.

Fig. 2. Tamoxifen, at relatively high concentrations, increases the apparent ΔΨm. A, a range of tamoxifen concentrations was tested on the ΔΨm in JC-1-loaded neurons. Concentrations of tamoxifen <1 μM had little direct effect on ΔΨm, whereas higher concentrations (>10 μM) caused an increase in the JC-1 ratio, presumably reflecting an increase in ΔΨm. Data represent the mean ± S.E. of 21 to 41 neurons per condition. B, a prolonged exposure to tamoxifen (100 μM) causes an increase in the ΔΨm followed by a pronounced decrease in ΔΨm. Data represent the mean ± S.E. of 14 neurons from a single culture date and are representative of data collected from a total of three experiments.
duced [Ca\textsuperscript{2+}] levels by tamoxifen is unlikely to explain the inhibitory effect of tamoxifen on mitochondrial Dc\textsubscript{m} depolarization.

Tamoxifen did not completely inhibit glutamate-induced Dc\textsubscript{m} depolarization. This is similar to what we have previously reported for other PTP inhibitors, namely, cyclosporin A, trifluoperazine, and dibucaine (White and Reynolds, 1996; Hoyt et al., 1997; Scanlon and Reynolds, 1998). This may be a matter of time of onset of action of the particular drug, or its duration of action. There are other Ca\textsuperscript{2+}-stimulated effects on mitochondria in addition to activation of the PTP that would result in dissipation of D\textsubscript{psi m}, including mitochondrial Ca\textsuperscript{2+} cycling (Nicholls and Akerman, 1982) and ATP synthesis. Because we are not measuring PTP activation directly and are unable to do so as yet in intact neurons, we cannot differentiate between PTP activation and other direct effects of glutamate receptor activation on Dc\textsubscript{m}. Therefore, definitive conclusions about the role of PTP activation in glutamate-induced mitochondrial depolarization cannot be drawn from the results presented herein. The numerous additional effects of these agents on other cellular signal transduction mechanisms such as calcineurin, calmodulin, and protein kinase C complicate the interpretation of effects of these drugs (Levin and Weiss, 1979; Liu et al., 1991; Rowlands et al., 1995; Gundimeda et al., 1996).

**Fig. 3.** Tamoxifen did not inhibit glutamate-induced increases in [Ca\textsuperscript{2+}]. A, indo-1-loaded neurons were exposed to 15-s pulses of 3 \mu M glutamate/1 \mu M glycine (arrows). When tamoxifen (0.3 \mu M) was included before and during the glutamate stimulus, there was no alteration in the [Ca\textsuperscript{2+}] increase induced by glutamate. Data are representative of Ca\textsuperscript{2+} traces collected from seven neurons. B, tamoxifen does not affect [Ca\textsuperscript{2+}] recovery after a glutamate stimulus. Neurons were exposed to 100 \mu M glutamate/1 \mu M glycine for 5 min and then immediately exposed to Ca\textsuperscript{2+}-free HBSS or tamoxifen (0.3 \mu M) in Ca\textsuperscript{2+}-free HBSS for 2 min immediately after glutamate exposure. Note that there is no apparent effect of tamoxifen on the rate or shape of the [Ca\textsuperscript{2+}] recovery. Data are representative of Ca\textsuperscript{2+} traces collected from five to seven additional neurons.

**Fig. 4.** Effects of tamoxifen on neuronal viability and on excitotoxicity in vitro. A, tamoxifen (0.3 \mu M) does not inhibit glutamate-induced neuronal death. Neurons were exposed to 100 \mu M glutamate for 10 min in the presence or absence of 0.3 \mu M tamoxifen, and neuronal death was assessed 20 h later by LDH release into the media as a measure of neuronal damage. Tamoxifen treatment did not significantly change glutamate neurotoxicity (P > .05). *P < .01, significantly different from untreated control, ANOVA with Bonferroni correction for multiple comparisons. B, a relatively high concentration of tamoxifen (100 \mu M) causes neuronal death. Neurons were exposed to 100 \mu M tamoxifen for 30 min and LDH release was assayed 20 h later. Data represent the mean ± S.E. collected from at least three culture dates. *P < .01, significantly different from untreated control, Student’s t test.
Tamoxifen does not inhibit formation of striatal lesions induced by malonate. A, injection of malonate into the rat striatum induces a lesion that was visualized 7 days postinjection by staining for cytochrome oxidase. B, treatment with 2 mg/kg tamoxifen 2 h before and 4 h after malonate injection did not decrease the size of the malonate-induced lesion. C, a range of tamoxifen doses (1–20 mg/kg) did not protect against striatal malonate lesion formation. Data represent the mean ± S.E. collected from 3 to 13 rats per condition.

The lack of inhibition of glutamate-induced depolarization by tamoxifen at higher concentrations is puzzling. It is possible that at lower concentrations, tamoxifen has a relatively selective effect on glutamate-mediated $\Delta\psi_m$ depolarization, whereas at higher concentrations, its membrane-disruptive effects interact with the glutamate-induced mitochondrial dysfunction, leading to a lack of inhibition at these tamoxifen concentrations. These higher tamoxifen concentrations caused an increase in $\Delta\psi_m$. It is possible that tamoxifen affects one of a number of mitochondrial functions that could result in hyperpolarization. Among these possibilities are inhibition of the mitochondrial Na$^+$/Ca$^{2+}$ exchanger, the F$_{1}$F$_{0}$-ATPase or a direct ionophore effect similar to nigericin (White and Reynolds, 1996; Hoyt et al., 1997), or inhibition of spontaneous depolarizing events (Duchen et al., 1998). These possible mechanisms remain to be tested. High micromolar concentrations of tamoxifen induce rapid apoptotic death in neural cell lines (a finding that we confirmed in our primary cultures) (Ellerby et al., 1997; Hashimoto et al., 1997). The inability of tamoxifen-primed mitochondria to initiate apoptosis in naive cell extracts suggests that nuclear or cell membrane associated caspases mediate the major component of tamoxifen-induced programmed cell death (Ellerby et al., 1997).

Cyclosporin A inhibits glutamate-induced neuronal death in vitro, although the interpretation of the mechanism of this neuroprotective effect is complicated by the multiple effects that cyclosporin A has on cellular function, including inhibition of PTP as well as calcineurin activation (Dawson et al., 1993; Ankarcrona et al., 1996; Schinder et al., 1996; White and Reynolds, 1996). Because tamoxifen inhibited glutamate-induced $\Delta\psi_m$ depolarization in a manner similar to that of cyclosporin A, we were interested to see whether tamoxifen protected neurons from glutamate-induced injury. Tamoxifen did not inhibit glutamate-induced neuronal death, suggesting that PTP activation is not a major contributor to the death caused by glutamate and that other actions of cyclosporin A explain its neuroprotective effect. We also tested whether tamoxifen could lessen the neuronal injury caused by excitotoxic injury to the striatum in an intact animal. Tamoxifen was not an effective inhibitor of striatal injury at the doses tested (1–20 mg/kg). Clinical doses of tamoxifen in humans are 0.4 to 0.8 mg/kg, causing an acute serum concentration of $\sim 0.07 \mu$M and chronic (after 3 months) steady-state concentrations of $\sim 0.2 \mu$M (Physicians’ Desk Reference, 1997). Because tamoxifen is very lipophilic, it is likely that tissue concentrations are higher than the serum concentration. It is possible that a higher and more prolonged tamoxifen exposure than used herein would be neuroprotective. The lack of effect in primary culture argued against further testing this in vivo.

The inhibition of glutamate-induced mitochondrial depolarization by tamoxifen is consistent with its reported action as an inhibitor of PTP activation, although processes other than PTP activation may explain the decrease in $\Delta\psi_m$ caused by glutamate receptor activation. Given the lack of specificity of tamoxifen and other PTP inhibitors and the difficulties in measuring PTP in intact cells, conclusions about the role of PTP in glutamate-induced mitochondrial depolarization and excitotoxic injury are not yet possible and await the development of selective PTP inhibitors, as well as a reliable assay for PTP activation in intact cells.

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