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Inhibition by R(+) or S(–) Pramipexole of Caspase Activation and Cell Death Induced by Methylpyridinium Ion or Beta Amyloid Peptide in SH-SY5Y Neuroblastoma

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Cell models of neurodegenerative diseases (NDD) can involve expression of mutant nuclear genes associated with Mendelian forms of the diseases or effects of toxins believed to replicate essential disease features. Death produced by exposing neural cells to methylpyridinium ion (MPP^+) or neurotoxic beta amyloid (BA) peptides is frequently used to study features of the sporadic, most prevalent forms of Parkinson's disease (PD) and Alzheimer's disease (AD), respectively. We examined in replicating SH-SY5Y human neuroblastoma cells the release of cytochrome C into cytoplasm, activation of caspases 9 and 3, and loss of calcein retention as markers of the "mitochondrial" pathway of cell death. Exposure to 5 mM MPP^+, which induces apoptotic cell death within 18–24 hr, released cytochrome C within 4 hr, activated caspases 9 and 3, and reduced calcein accumulation. BA 25–35 peptide produced more rapid and greater elevations of caspase 3 activity; no effects were observed with the nontoxic BA 35–25 reverse sequence. The dependence on mitochondrial transition pore (MTP) activity of MPP^+-induced caspase activations was demonstrated by preincubation with bongkreckic acid, which blocked elevations of caspases 9 and 3. Stereoisomers of pramipexole (PPX), a free radical scavenger and inhibitor of MTP opening, inhibited caspase activation (MPP^+ and BA) and restored calcein accumulation (MPP^+). Our results demonstrate that MPP^+ and BA can induce cell death through MTP-dependent activation of caspase cascades. PPX stereoisomers interfere with activation of these cell death pathways and may be useful clinically as neuroprotectants in PD and AD and related diseases.

Key words: neuroprotection; neurodegenerative diseases; cell death; caspases; pramipexole

The major neurodegenerative diseases (NDD) of adults, Parkinson's disease (PD) and Alzheimer's disease (AD), usually appear sporadically without any obvious Mendelian inheritance patterns but may show maternal biases (Edland et al., 1996; Wooten et al., 1997; Swerdlow et al., 1998a). Effective neuroprotective agents for these debilitating and fatal illnesses not only should be effective in cell culture and animal models of these diseases but must be tolerated chronically in doses high enough to achieve therapeutic levels in nervous tissues. Ideally such agents would also target cellular components involved in control of cell death pathways and interrupt disease pathophysiology.

Although rare or uncommon inherited forms of adult NDD exist, the relevance of pathogenesis in these autosomal genetic variants to that in the much more commonly occurring sporadic forms is a subject of intense debate. Accumulating evidence provides compelling support for mitochondrial dysfunction and resulting increased cellular oxidative stress as a primary etiologic component of sporadic adult NDD. PD and AD brains and non-CNS tissues show reductions in mitochondrial electron transport chain (ETC) activity (Cassarino and Bennett, 1999; Albers and Beal, 2000; Beal, 2000b; Manfredi and Beal, 2000). When selectively amplified in cytoplasmic hybrid ("cybrid") cell models, mitochondrial genes from PD (Swerdlow et al., 1996; Gu et al., 1998) and AD (Swerdlow et al., 1997) subjects recapitulate the ETC deficits and produce increased oxidative stress and a variety of other important mitochondrial and cellular dysfunctions (Swerdlow et al., 1996, 1997; Sheehan et al., 1997a,b; Cassarino et al., 1997, 2000; Gu et al., 1998; Khan et al., 2000;

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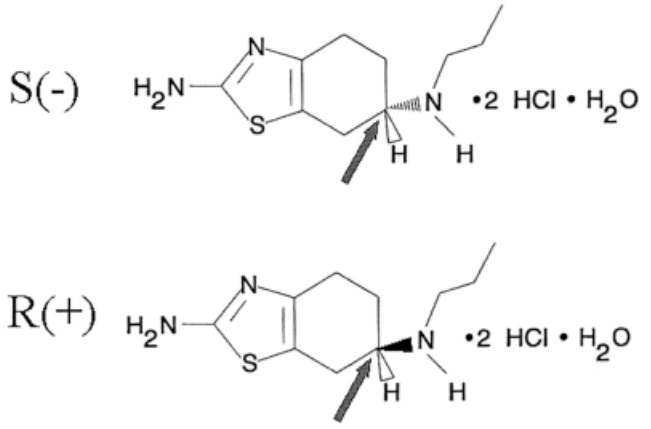

Fig. 1. Structures of S(–) and R(+) PPX. The arrows point to the asymmetric carbons.

Veech et al., 2000). The combined weight of evidence from tissue studies and cybrid models of sporadic PD and AD requires that relief of oxidative stress by agents capable of scavenging oxygen free radicals and protecting cells from mitochondrially generated cell death be considered as a primary characteristic of compounds developed as neuroprotective agents for these diseases (Cassarino and Bennett, 1999; Beal, 2000a).

Pramipexole (PPX; 2-amino-4,5,6,7-tetrahydro-6-propylaminobenzathiazole) is such a molecule, which exists as two stereoisomers (Fig. 1). The S(–) enantiomer is a potent agonist at D2 family dopamine receptors and is extensively used in the symptomatic management of PD (Bennett and Piercey, 1999; Lieberman et al., 2001). S(–) PPX has been shown by several groups to be neuroprotective in cellular and animal models of increased oxidative stress, including MPTP toxicity to dopamine neurons (Anderson et al., 2001; see references in Bennett et al., 2001). S(–) PPX reduces oxidative stress produced by the parkinsonian neurotoxin and ETC complex I inhibitor methylpyridinium (MPP^+) both in vitro and in vivo (Cassarino et al., 1998; Le et al., 2000; Zou et al., 2000) and can block opening of the mitochondrial transition pore (MTP) induced by MPP^+ and other stimuli (Cassarino et al., 1998). The lipophilic cationic structure of PPX, suggesting the possibility of concentration into mitochondria across $\Delta\Psi_M$, in combination with its low reduction potential (320 mV), may account for these desirable neuroprotective properties.

Dosing with S(–) PPX is limited in humans by its potent dopamine agonist properties (Bennett and Piercey, 1999; Lieberman et al., 2001) and will restrict achievable brain drug levels. Because the R(+) enantiomer of PPX has very little dopamine agonist activity (Schneider and Mierau, 1987) but may retain the desirable molecular/antioxidant properties of S(–) PPX, we have explored its efficacy in cell culture-based models of PD and AD. We find that R(+) and S(–) PPX are effective inhibitors of activation of cell death cascades and loss of viability and deserve further exploration as potential neuroprotective compounds.

MATERIALS AND METHODS

Cell Culture

SH-SY5Y human neuroblastoma cells were obtained from the American Tissue Culture Collection (www.atcc.org) and maintained in culture in a replicating state as previously described (Cassarino et al., 1997, 1998; Fall and Bennett; 1998, 1999). For caspase assays and cytochrome C release studies, they were grown in T75 flasks with Dulbecco's modified Eagle's medium (DMEM)/high-glucose containing 10% fetal bovine serum, antibiotics/antimicotics (100 IU/ml of penicillin, 100 μg/ml of streptomycin sulfate, 0.25 μg/ml of amphotericin B), and 50 μg/ml of uridine and 100 μg/ml of pyruvate in a 5% CO_2 atmosphere at 37°C to approximately full confluence (2×10^7 cell/flask). They were then incubated with 5 mM methylpyridinium iodide (MPP^+; Sigma, St. Louis, MO; www.sigma-aldrich.com) or 100 μM 25–35 or 35–25 beta amyloid (BA) peptides (Bachem, Torrance, CA; www.bachem.com) for varying times, then harvested. For cell death studies, the cells were plated into 96 well black-bottom plates and grown for 24 hr in DMEM media before being exposed to toxin.

Caspase Assays

After exposure to MPP^+ or BA peptide, cells were collected in phosphate-buffered saline (PBS) and centrifuged at 450*g* for 6 min at 4°C. Cell pellets were resuspended in a hypotonic cell lysis buffer [25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, 1 M dithiothreitol (DTT), and protease inhibitor cocktail; Sigma] at a concentration of 2×10^7 cells/100 μl of lysis buffer. Lysates were subjected to four cycles of freezing and thawing. Cell lysates were then centrifuged at 16,000*g* for 30 min at 4°C. The supernatant fractions were collected, and protein content was measured by Lowry assay (Bio-Rad, Richmond, CA). One hundred micrograms of protein were used to measure caspase activity in 96 well plates, assayed in quadruplicate. The activity was measured using the assay buffer and the protocol provided by the manufacturers (Biomol, caspase 3; Promega, caspases 3 and 9). Caspase activity is based on cleavage of synthetic peptide substrates resulting in liberation of colored [p-nitroaniline (p-NA); Biomol] or fluorescent [aminomethylcoumarin (AMC); Promega] chromogens. Only activated caspases are capable of cleaving these substrates, and chromogen generation is completely inhibited when the caspase inhibitor provided with each assay kit is included in the assay. We observed, under the indicated assay conditions, linear rates of generation of chromogen over 2 hr. We measured chromogen absorbance (p-NA) on an OptiMax plate reader or chromogen fluorescence (AMC) on a SpectraMax Gemini plate reader (Molecular Devices, Palo Alto, CA) at 0 time and after 30 min of incubation at 37°C to estimate relative caspase activities. Chromogen signal at 0 time was subtracted from the readings at 30 min.

Cell Death

Cell death was estimated by measuring loss of calcein retention with the "Live-Dead" assay (Molecular Probes, Eu-

gene, OR; www.molecularprobes.com) in cells grown in 96 well plates and incubated with calcein-AM, according to manufacturer's instructions. Calcein signals were assayed in a SpectraMax Gemini adjustable fluorescent plate reader. Calcein fluorescence from cells preincubated with methanol was substracted from all readings as background. Each assay was performed with eight wells per condition, results from which were averaged. Three to eight independent experiments were performed to evaluate a broad range of concentrations of S(–) and R(+) PPX in this paradigm.

Cytochrome C Western Blot

Cytochrome C was detected by Western blot following polyacrylamide electrophoresis of 100 μg of cell supernatant protein and transfer to nylon membrane. The primary antibody was a mouse monoclonal anticytochrome C, obtained from Pharmingen and used at 1:10,000 dilution. Detection was performed with enhanced chemiluminescence (Pierce, Rockford, IL) and imaged on a Bio-Rad FluorS imaging station.

Drugs

R(+) and S(–) PPX (gifts of Pharmacia Corporation, Kalamazoo, MI) were obtained as their dihydrochloride salts and dissolved directly into culture media. Bongkreckic acid, an antagonist of the adenosine triphosphate (ATP) binding site on the adenine nucleotide translocator, was a kind gift of Prof. J.A. Duine (Delft University, Delft, The Netherlands) and provided as a solution in 1 M NH_4OH. Aristolochic acid (sodium salt), a phospholipase A2 inhibitor, was obtained from Sigma. In the caspase experiments, drugs were added 1 hr before MPP^+ or BA peptide. In the calcein/cell death experiments, drugs were added 4 hr before MPP^+.

Statistical Analysis

Statistically significant differences were sought with unpaired *t*-tests.

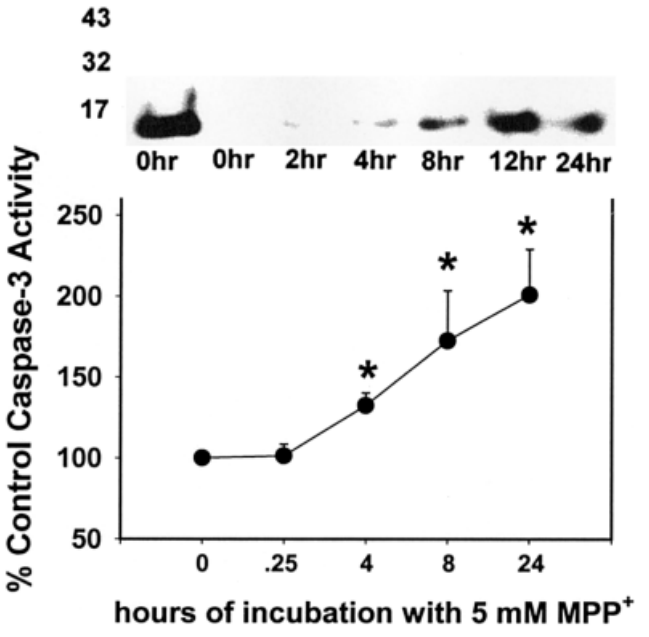

Fig. 2. Time course of MPP^+-induced release of cytochrome C and activation of caspase 3. SH-SY5Y cells were incubated with 5 mM MPP^+ for varying times and harvested. Cells were homogenized in isotonic sucrose and centrifuged, and 100 μg of supernatant protein was electrophoresed using SDS-PAGE, transferred to nylon membranes, and immunostained for cytochrome C with enhanced chemiluminescence detection. The most leftward "0 hr" band corresponds to mitochondrial cytochrome C at time 0, and the other bands represent electrophoresed cytoplasmic protein immunostained for cytochrome C. Positions of MW markers are shown. Other batches of cells were assayed for caspase 3 using a commercial system, according to the manufacturer's instructions (Biomol). Caspase assays are the results of three or four independent experiments. $\star P < 0.05$ compared with activity at 0.25 hr.

RESULTS

Activation of Caspases by MPP^+ and BA 25–35

Figure 2 shows the time course of caspase 3 activity during incubation of SH-SY5Y cells with 5 mM MPP^+. Increased activity was detectable by 4 hr and had increased about twofold by 24 hr. At the top of Figure 2 is shown the Western blot result for cytochrome C protein released into cytoplasm. Similarly to the biochemical activity curve, cytoplasmic cytochrome C is detectable in small amounts by 4 hr and increases substantially by 12 hr.

Figure 3 shows that the neurotoxic BA 25–35 fragment caused an even more rapid and substantial increase in caspase 3 activity than did MPP^+. In this paradigm caspase 3 activity reached its maximum of about a fourfold increase by 8 hr. The nontoxic BA 35–25 fragment at equimolar concentration did not cause any increase in caspase 3 activity.

Blockade of Caspase Increases by R(+) and S(–) PPX, Bongkreckic Acid, and Aristolochic Acid

Activation of caspases by MPP^+ and BA 25–35 peptide was blocked by PPX enantiomers and agents active at the MTP. S(–) PPX reduced by about 70% the increases in caspase 3 activity after incubation with BA 25–35 peptide and had no suppressive effect by itself (Fig. 3). Figure 4 shows that both R(+) and S(–) PPX enantiomers suppressed caspase 3 activation during MPP^+ exposure. MPP^+-induced increases in caspase 3 activity were also blocked by bongkreckic acid, a specific antagonist of the ATP binding site on the inner membrane site of the adenine nucleotide translocator. Aristolochic acid, a phospholipase A2 inhibitor, which we have shown blocks MPP^+-induced apoptosis of SH-SY5Y cells (Fall and Bennett, 1998), also prevented increases in caspase 3 activity. MPP^+ exposure also increased activity of caspase 9 with a time course similar to that of activation of caspase 3 (Fig. 5). The increases in caspase 9 activity were also blocked by S(–) and R(+) PPX, bongkreckic acid, and aristolochic acid.

R(+) and S(–) PPX Block MPP^+-Induced Cell Death at Nanomolar Concentrations

Figure 6 shows the effects on cellular calcein retention of incubating SH-SY5Y cells with varying concen-

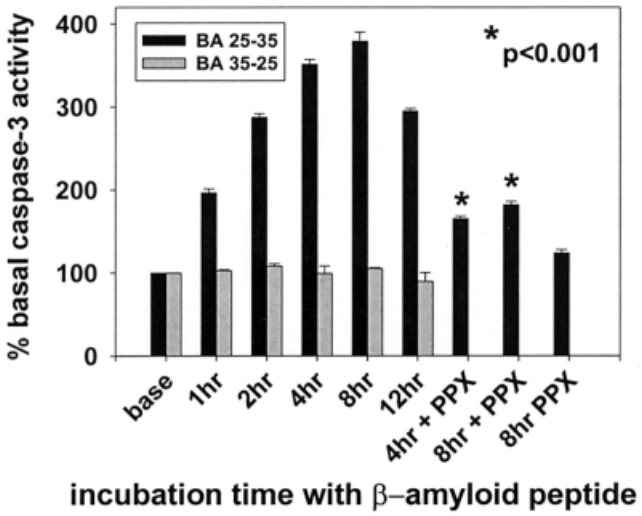

Fig. 3. Time course of caspase 3 activation by BA 25–35, lack of activation by BA 35–25, and inhibition of activation by S(–) PPX. SH-SY5Y cells were incubated with 100 μM BA peptides for the indicated times, then assayed for caspase 3 activity. In some dishes 1 mM S(–) PPX was added before BA peptide. Shown are the mean ± SEM results from three independent experiments. $*P < 0.001$ compared with the same time point without PPX.

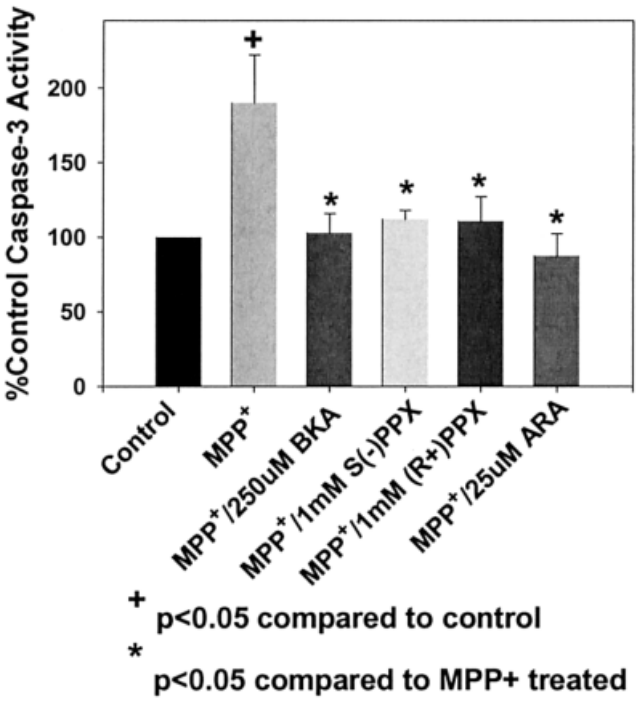

Fig. 4. Inhibition of MPP+-induced caspase 3 activation by PPX, bongkreckic acid, and aristolochic acid. SH-SY5Y cells were incubated with 5 mM MPP^+ for 24 hr in the absence or presence of 1 mM S(–) PPX, 1 mM R(+) PPX, 250 μM bongkreckic acid (BKA), or 25 μM aristolochic acid (ARA). Cells were then assayed for caspase 3 activity. Shown are the mean ± SEM for four or five independent experiments.

trations of R(+) or S(–) PPX prior to exposure to 5 mM MPP^+ for 24 hr. Calcein is a fluorescent dye that is retained inside cells as a function of their ability to maintain a plasma membrane potential. MPP^+ alone reduced calcein uptake by about 60%. Both PPX enantiomers substantially restored calcein uptake at 30 nM levels, and this protective effect was retained through 30 μM PPXs.

DISCUSSION

This study focused on the use of the neurotoxins MPP^+ and BA 25–35 peptide added to replicating SH-SY5Y neuroblastoma cells as cell culture models for studying potential neuroprotective compounds useful for PD and AD, respectively. There are many limitations to this type of model system, which restrict interpetation of our findings.

First, though relatively easy to culture and thus convenient for pharmacological studies, SH-SY5Y cells are neoplastic, dividing cells of neuroectodermal origin, not primary neurons. They are mitotic as a result of a *Ras* mutation, leading to chronic activation of MAPK/ERK signaling. Thus, paradigms inducing cell death in SH-SY5Y may utilize mechanisms not activated in mature primary neurons.

Second, SH-SY5Y cells are relatively insensitive in short-term incubations to MPP^+ compared with primary neurons. We found that 2.5 and 5 mM, but not 1 mM, MPP^+ produced apoptotic morphology and DNA pyknotic fragments within 18–24 hr (Fall and Bennett, 1999). Kitamura et al. (1998) found that 1 mM MPP^+ induced DNA laddering and chromatin condensation in SH-SY5Y within 3 days. Most recently, Gomez et al. (2001) found that longer term (4 days) incubation of SH-SY5Y with 5 μM MPP^+ reduced viability by ~15%, activated caspases, and produced DNA laddering in spite of not causing any detectable increase in cytoplasmic cytochrome C or lowering of mitochondrial membrane potentials. They reported that a 4 day incubation with 100 μM MPP^+ reduced viability ~40% and did increase cytoplasmic cytochrome C and lower mitochondrial membrane potentials. Their findings support the concept that longer incubations with lower concentrations of MPP^+ may more closely approximate the in vivo MPTP model of PD in animals. Their results also showed that longer term exposure to lower MPP^+ levels still can activate the mitochondrial cell death cascade.

SH-SY5Y cells are also sensitive to BA peptides. Li et al. (1996) found that serum-starved SH-SY5Y showed extensive DNA nicked end labeling after 3 days of exposure to 100 μM BA 25–35 and exhibited a concentration-dependent increase in DNA laddering. BA peptide-induced activation of caspases has not apparently been described in SH-SY5Y cells, but several reports have shown caspase activations from exposure to BA peptides in various primary neuron lines. These include activation of caspases 2, 3, and 6 by the BA 25–35 analogue in cerebellar granule cells (Allen et al., 2001) and caspase 3 in rat

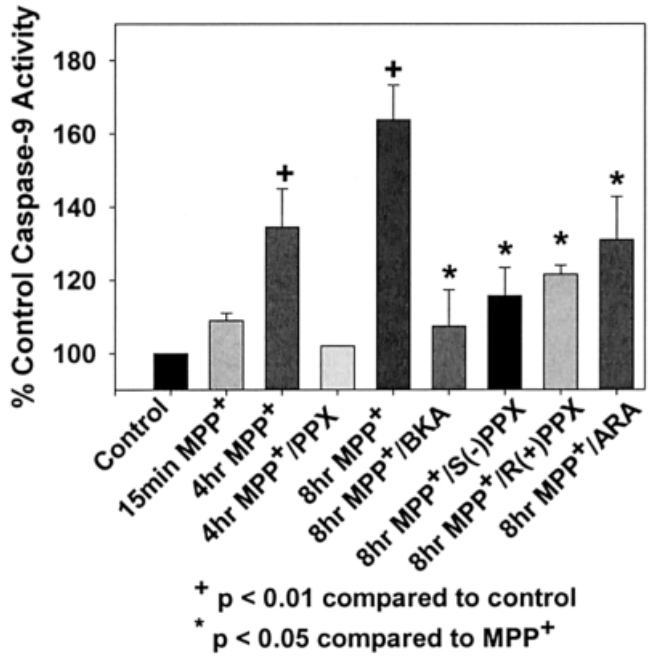

Fig. 5. Inhibition of MPP+-induced caspase 9 activation by PPX, bongkreckic acid (BKA), and aristolochic acid (ARA). SH-SY5Y cells were incubated with 5 mM MPP^+ for 4 or 8 hr in the absence or presence of 1 mM S(–) PPX, 1 mM R(+) PPX, 250 μM BKA, or 25 μM ARA. Cells were then assayed for caspase 9 activity. Shown are the mean ± SEM for three or four independent experiments.

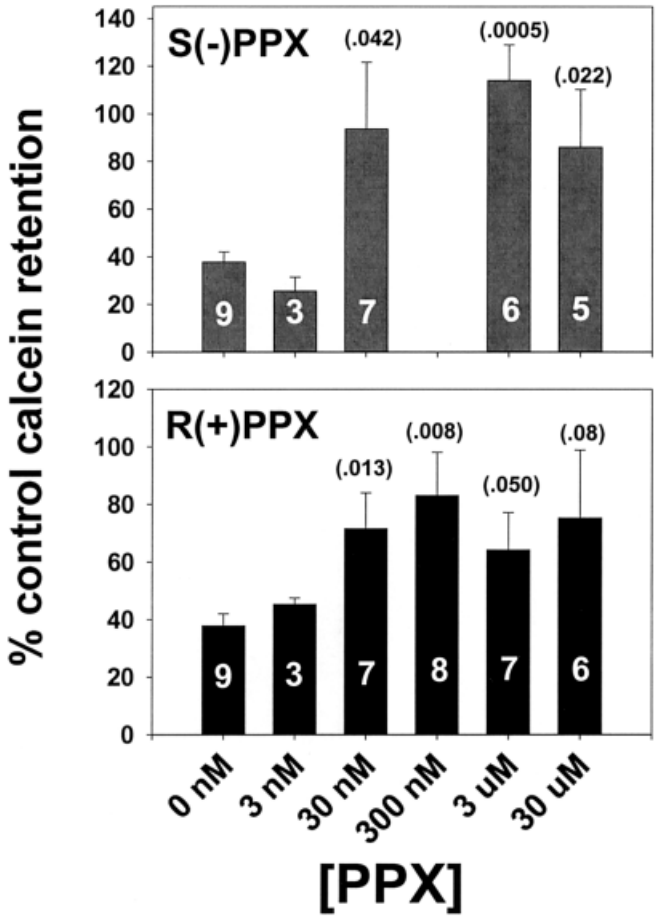

Fig. 6. Inhibition of MPP+-induced cell death by PPX enantiomers. SH-SY5Y cells were incubated with 5 mM MPP^+ for 24 hr in the absence or presence of increasing concentrations of S(–) PPX (**top**) or R(+) PPX (**bottom**). The cells were then incubated with calcein-AM, washed, and read on a fluorescent plate reader. The number of independent experiments is indicated on each bar, and the *P* values by *t*-test for comparison with no PPX (MPP^+ only) are shown above each bar. In the presence of MPP^+, 3 nM, R(+) PPX caused greater calcein retention than S(–) PPX ($P = 0.035$), and 3 μM, S(–) PPX caused greater calcein retention than R(+) PPX ($P = 0.027$).

primary cortical neurons (Harada and Sugimoto, 1999; Marin et al., 2000; Saez-Valero et al., 2000). Thus, it is not surprising that we observed appearance of caspase 3 activity (DEVDase) in SH-SY5Y exposed to 100 μM BA 25–35 but no caspase activation after exposure to the reverse BA 35–25 sequence.

The focus of our experiments was to determine whether PPX enantiomers can prevent activation of caspases and can promote calcein retention as a marker of cell survival in acute toxin exposure, cell culture models of AD and PD. Activation of both the "initiator" caspase 9 and the "executioner" caspase 3 was blocked by both PPX enantiomers in the MPP^+ model for PD, and activation of caspase 3 was blocked by S(–) PPX in the BA 25–35 model for AD. Both PPX enantiomers at nanomolar levels could promote cell survival in the MPP^+ model for PD. Thus, our findings add to the growing body of work describing the neuroprotective actions of PPX (Ferger et al., 2000; Kakimum et al., 2001; see references in Bennett et al., 2001) and suggest the potential clinical utility of this family of compounds in neurodegenerative diseases.

Although this study did not examine the most proximate site of action of PPX, several finding implicate the MTP complex (MTPC) in these cell models. First, bongkreckic acid, a selective adenine nucleotide translocator antagonist and inhibitor of MTP opening, blocked MPP^+-induced activation of both caspases 9 and 3. This would be consistent with MPP^+ bringing about MTP opening either directly or through mechanisms that involve oxidative stress. In isolated liver mitochondria, MPP^+ can bring about a classical MTP opening that is incompletely blocked by free radical scavenging enzymes (Cassarino et al., 1999) and is more completely blocked by S(–) PPX (Cassarino et al., 1998). Neurotoxic BA 25–35 peptide can also stimulate MTP opening in isolated mitochondria (Parks et al., 2001). Both MPP^+ (Chieuh et al., 1992; Smith et al., 1994) and BA 25–35 peptide (Parks et al., 2001) increase oxidative stress in brain microdialysis studies in vivo and in neural cell culture in vitro (Cassarino et al., 1997; Olivieri et al., 2001), and S(–) PPX has been shown to reduce MPP^+-induced oxidative stress in vitro and in vivo (Cassarino et al., 1998).

Although we used relatively high PPX concentrations in the studies of MPP^+ and BA-induced caspase activations, both PPX enantiomers could mitigate MPP+-induced cell death at very low nanomolar levels. This suggests that these compounds may have additional actions, such as increases in antiapoptotic bcl proteins (Takata et al., 2000), that could assist in cell survival. However, if additional mechanisms exist for PPX-induced cell survival, the experiments in this study do not define them.

The interpretation of the results of this study is limited by the relevance of acute, toxin-mediated death paradigms in replicating neurotypic cells to neurodegenerative diseases, which involve postmitotic neurons and evolve over years. Although similar final common cell death pathways may be utilized, the upstream activators of these pathways are not clear in the naturally occurring diseases. Thus agents that might be active in acute toxic paradigms could have minimal efficacy against neurodegeneration in situ.

In spite of these limitations, we believe that our results support the development of R(+) PPX as a neuroprotective compound. Its relative lack of dopamine agonist activity should allow much higher daily doses than those of S(–) PPX that can be tolerated. Preliminary findings involving use of S(–) PPX in early PD support a neuroprotective mechanism (Parkinson Study Group, 2000). Insofar as R(+) PPX appears to share approximately equal potency with S(–) PPX in at least one model of neurodegenerative cell death, it appears to have clinical potential. In addition, R(+) PPX may serve as a structural prototype for other PPX-related lipophilic cations that will be concentrated into mitochondria and also possess antioxidant properties.

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