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Release and aggregation of cytochrome *c* and α-synuclein are inhibited by the antiparkinsonian drugs, talipexole and pramipexole

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Abstract

Recently, it has been shown that release of cytochrome *c* from the mitochondria to the cytosol is required for activation of the caspase-3-dependent cascade in apoptosis, and also for α-synuclein aggregation. In the present study, we examined the effects of talipexole and pramipexole on the release of cytochrome *c* and α-synuclein, their aggregations, and activation of caspases. Treatment of human neuroblastoma SH-SY5Y cells with 1-methyl-4-phenylpyridinium (MPP^+, 1 mM) induced the first event, which was the release of cytochrome *c* from the organellar fraction to the cytosolic fraction, then came the DNA fragmentation, and caused the last event, which was the accumulation of α-synuclein protein in the cytosolic fraction. Talipexole and pramipexole at low concentration (0.1–1 mM) significantly inhibited the accumulation of cytochrome *c* or α-synuclein in the cytosolic fraction. These drugs at high concentration (3–10 mM) inhibited in vitro aggregation of cytochrome *c* by hydrogen peroxide or that of α-synuclein by cytochrome *c* and hydrogen peroxide. In addition, in vitro activation of caspase-3 induced by cytochrome *c* and/or dATP was also inhibited by drugs at high concentration (5–10 mM). These results suggest that talipexole and pramipexole may have protective effects against the neurodegeneration, which is induced by intracellular accumulation of cytochrome *c* and α-synuclein. © 2001 Published by Elsevier Science B.V.

Keywords: Talipexole; Pramipexole; Cytochrome *c*; α-Synuclein; Apaf-1; Parkinson's disease

1. Introduction

Parkinson's disease is one of the major neurodegenerative disorders. Recently, evidence for oxidative stress and free-radical injury as pathogenic factors in Parkinson's disease has accumulated (Dunnett and Björklund, 1999; Olanow and Tatton, 1999). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces an irreversible and severe parkinsonian syndrome associated with selective degeneration of the nigrostriatal dopaminergic neurons in humans (Davis et al., 1979; Langston et al., 1983). Its metabolite 1-methyl-4-phenylpyridinium (MPP^+), the formation of which is catalyzed by monoamine oxidase B, causes oxidative stress and dopaminergic neurodegeneration (Dunnett and Björklund, 1999; Olanow and Tatton, 1999; Kitamura et al., 2000). However, recent papers have found many different results that MPP^+-induced oxidative stress is mediated by the inhibition of mitochondrial complex I (Fallon et al., 1997) and the generation of reactive oxygen species is not mitochondrial in origin, but results from the oxidation of intracellular dopamine (Lotharius and O'Malley, 2000), and that MPP^+-induced cell death is apoptotic (Kitamura et al., 1998b) and it is not apoptotic (Lotharius et al., 1999). Thus, the detail mechanism of MPP^+-induced neurodegeneration is still unclear. On the other hand, Apaf-1, cytochrome *c* and caspase-9 were recently identified as the apoptotic protease-activating factors in one of the major caspase-3-dependent cascade (Liu et al., 1996; Li et al., 1997; Zou et al., 1997).

α-Synuclein is localized predominantly in the presynaptic terminals (Iwai et al., 1995); however, its detailed physiological function is still unknown. Aggregated α-synuclein is markedly included in Lewy bodies in brains of patients with Parkinson's disease and dementia with Lewy body (Spillantini et al., 1998). Overexpression of human α-synuclein in mice (Masliah et al., 2000) and fruitflies *Drosophila* (Feany and Bender, 2000) caused degeneration of dopaminergic neurons and motor dysfunction. In addi-

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tion, a fragment of α-synuclein (amino acid residues 61–95) is also known as a non-amyloid β component in amyloid plaques in Alzheimer's disease brains (Uéda et al., 1993). Thus, α-synuclein may be implicated in the etiology of neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases and dementia with Lewy body (Clayton and George, 1999; Hashimoto and Masliah, 1999).

Several drugs for the treatment of Parkinson's disease are known to have neuroprotective effects (Lange et al., 1994; Hagan et al., 1997; Gassen et al., 1998). Previously, we determined neuroprotective effects of talipexole and pramipexole in MPTP-treated mice (Kitamura et al., 1997) and invertebrate flatworms (Kitamura et al., 1998a). In the human neuroblastoma cell line SH-SY5Y, treatment with talipexole or pramipexole inhibited MPP^+-induced apoptotic cell death (Kitamura et al., 1998b). To clarify detailed mechanisms in the neuroprotection by talipexole and pramipexole, we examined the effects of these drugs against pathogenic functions of cytochrome *c*, α-synuclein and caspases.

2. Materials and methods

2.1. Materials

Talipexole and pramipexole were obtained from Boehringer Ingelheim (Ingelheim, Germany); MPP^+ from Research Biochemicals International (Natick, USA); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) from Dojindo (Kumamoto, Japan); purified cytochrome *c* (horse heart) from Sigma (St. Louis, USA); *N*,*N*′-dicyclohexylcarbodiimide (DCCD) from Nakarai (Kyoto, Japan); and dATP from Amersham Phar-

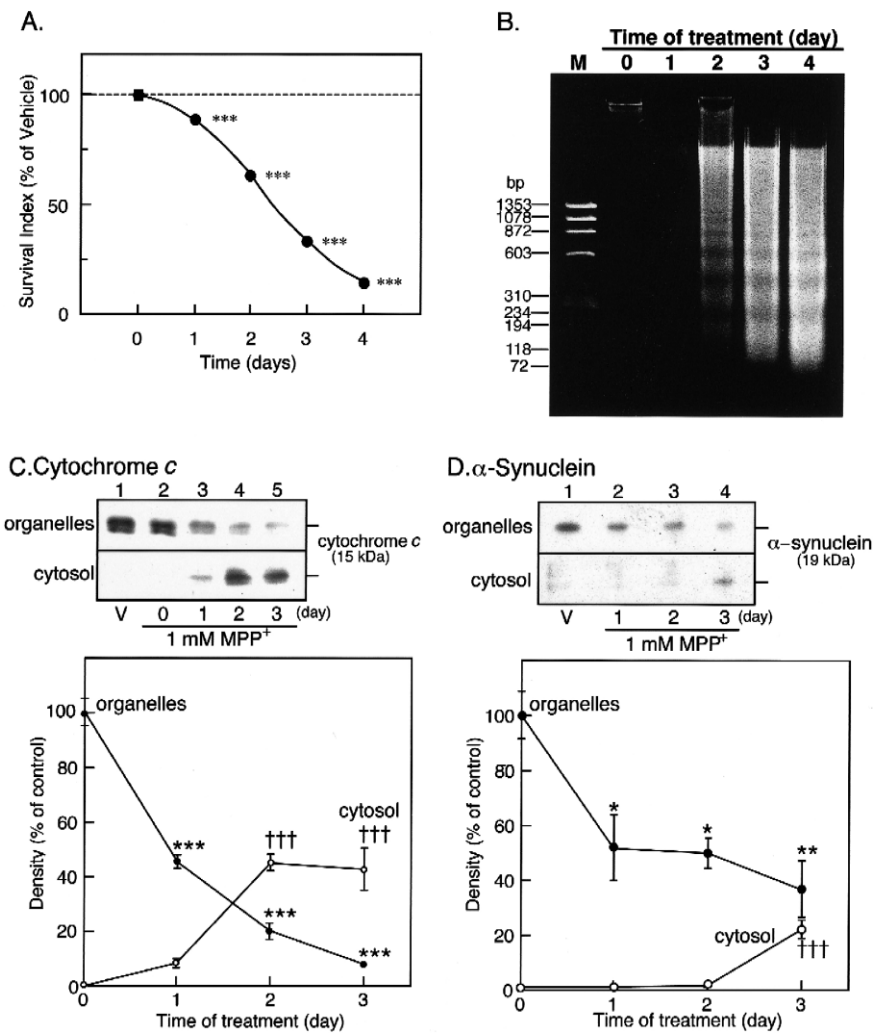

Fig. 1. MPP^+-induced cell death and release of cytochrome *c* and α-synuclein from the organelles to the cytosol. SH-SY5Y cells were incubated with vehicle (V) or 1 mM MPP^+ for 0–4 days. Subsequently, MTT assay (A) and DNA fragmentation (B) were examined. After treatment, cells were scraped, and the cytosolic (○) and organellar (●) fractions were prepared. Each sample (10 μg of protein/lane) was subjected to immunoblot analysis (upper panel) of antibodies against cytochrome *c* (C) and α-synuclein (D), and the protein bands of 15-kDa cytochrome *c* and 19-kDa α-synuclein were assessed (lower panel). The density of the protein band in the organellar fraction at day 0 was taken as 100%. Each value is the mean ± S.E.M. (%) of three determinations. Significance (the post hoc comparisons by Bonferroni/Dunn test): $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the level in the organellar fraction at day 0. †††, $P < 0.001$ vs. the level in the cytosolic fraction at day 0.

macia Biotech (Buckinghamshire, England). Primary antibodies included mouse monoclonal antibodies to cytochrome *c* from PharMingen (San Diego, USA) and α-synuclein from Transduction Laboratories (Lexington, USA); and rabbit polyclonal antibodies to Apaf-1, proenzyme/active fragments of human caspase-9 and proenzyme/active fragments of caspase-3 from PharMingen; and the caspase-3 cleavage site of poly(ADP-ribose) polymerase from Upstate Biotechnology (Lake Placid, USA). Enhanced chemiluminescent detection system kit (ECL kit) from Amersham Pharmacia Biotech, the Bradford protein assay from BioRad Laboratories (Richmond, USA), and the caspase-3 colormetric assay from Clontech Laboratories (Palo Alto, USA) were used.

2.2. Cell culture and assay of cell death

Human neuroblastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and were kept at 37°C in humidified 5% CO_2/95% air. The cells were treated with 1 mM MPP^+ for 0–4 days. Subsequently, the MTT assay and detection of DNA fragmentation were performed as the indexes of cell survival and apoptosis, respectively (Kitamura et al., 1998b).

2.3. Preparation of the cytosolic and organellar fractions and immunoblot analysis

After treatment for 0–3 days with 1 mM MPP^+ in the presence or absence of talipexole or pramipexole, the homogenates were separated into the cytosolic and organellar (including mitochondria, membrane and nucleus) fractions by centrifugation at $15{,}000 \times g$ for 30 min at 4°C. The cytosolic and organellar fractions were subjected to the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then the immunoblotting using antibodies against cytochrome *c* (diluted 1:1000), α-synuclein (1:500) and Apaf-1 (1:6000). For semiquantitative analysis, the bands of these proteins on radiographic films were scanned with a CCD color scanner (DuoScan, AGFA, Leverkusen, Germany) and then analyzed. The densitometric analysis was performed using the public domain NIH Image 1.56 program (written by Wayne Rasband at the U.S. National Institute of Health and available from the Internet by anonymous ftp from www.zippy.nimh.nih.gov.).

2.4. In vitro aggregation assay of cytochrome c or α-synuclein

The aggregation of cytochrome *c* or α-synuclein was performed as previously described (Paik et al., 1998; Hashimoto et al., 1999). In brief, purified cytochrome *c* (from horse heart, 100 μM) was incubated at 37°C for 24 h with 100 μM H_2O_2 in the presence or absence of talipexole or pramipexole at 0.1–10 mM. Protein preparations were subjected to the SDS-polyacrylamide gel electrophoresis, and then stained by coomassie brilliant blue.

α-Synuclein protein was partially purified from rat brains as previously described (Nakajo et al., 1990; Tobe et al., 1992). Subsequently, rat α-synuclein was incubated at 37°C for 24 h with 10 μM cytochrome *c*, 100 μM

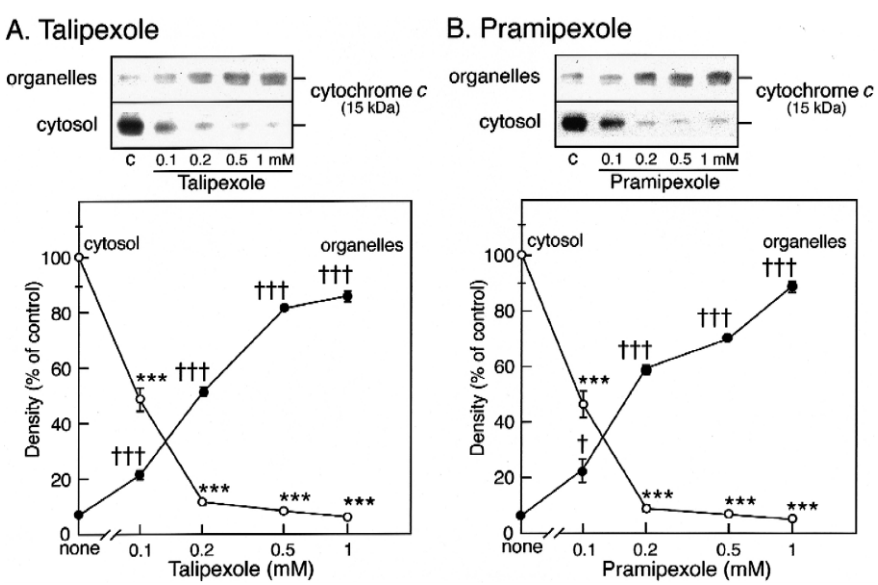

Fig. 2. Inhibitory effects of talipexole and pramipexole on MPP^+-induced release of cytochrome *c*. SH-SY5Y cells were treated for 3 days with 1 mM MPP^+ in the presence of vehicle (C), talipexole (A) or pramipexole (B) at 0.1–1 mM. After preparations of the cytosolic (○) and organellar (●) fractions, each sample was subjected to immunoblot analysis of anti-cytochrome *c* antibody (upper panel). Density of 15-kDa cytochrome *c* is given as the mean ± S.E.M. (%) of three determinations (lower panel), based on MPP^+ treatment without drugs in the cytosolic fraction considered to be 100%. Significance (the post hoc comparisons by Bonferroni/Dunn test): $^{***}P < 0.001$ vs. the level in the cytosolic fraction of treatment with MPP^+ alone. †, $P < 0.05$; †††, $P < 0.001$ vs. the level in the organellar fraction of treatment with MPP^+ alone.

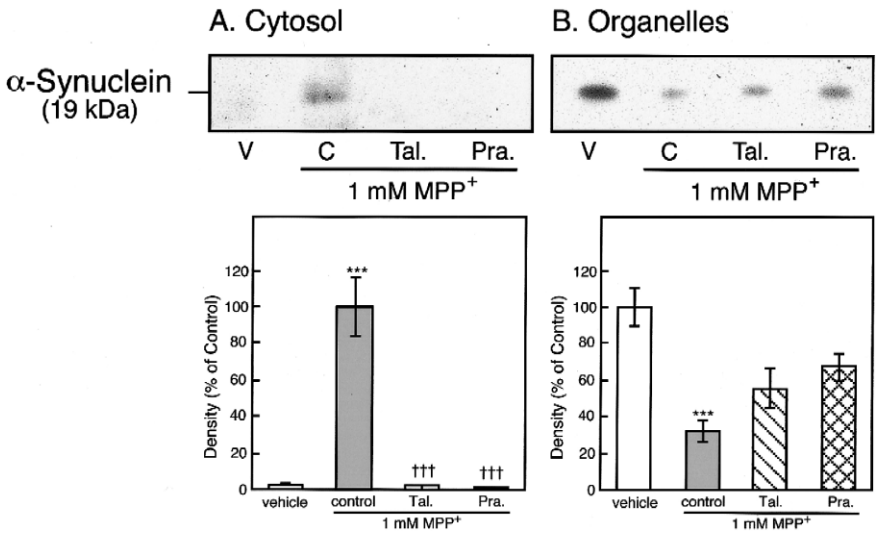

Fig. 3. Inhibitory effects of talipexole and pramipexole on MPP^+-induced release of α-synuclein. Cells were treated for 3 days with vehicle (V) or 1 mM MPP^+, in the presence of vehicle (C, Control), 1 mM talipexole (Tal.) or 1 mM pramipexole (Pra.). After preparations of the cytosolic (A) and organellar (B) fractions, each sample was subjected to immunoblot analysis of anti-α-synuclein antibody (upper panel). Density of α-synuclein protein is given as the mean ± S.E.M. (%) of three determinations (lower panel). Significance: $^{***}P < 0.001$ vs. the level in the vehicle treatment. †††, $P < 0.001$ vs. the level in treatment with MPP^+ alone.

H_2O_2 and 450 μM DCCD, in the presence or absence of talipexole or pramipexole at 1–10 mM. After incubation, samples were subjected to the immunoblotting of anti-α-synuclein antibody.

2.5. Preparation and induction of the cell-free apoptosis system

The cytosolic extract was prepared as previously described (Liu et al., 1996). To initiate caspase activation, an aliquot of the cytosolic extract (50 μg of protein) was incubated with 1 mM dATP and 1 μM cytochrome *c* in the presence or absence of talipexole or pramipexole (1–10 mM), at 30°C for 4 h. Treated extracts were subjected to assay for caspase-3 activity and immunoblot analysis using antibodies against caspase-9 (1:1000), caspase-3 (1:1000) and poly (ADP-ribose) polymerase (1:600).

2.6. Assay for caspase-3 activity

After treatment, the cytosolic extract was incubated with 50 μM *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide at 30°C for 1 h. The amount of released *p*-nitroanilide was measured with a spectrophotometer. The unit of the optical density was converted to nmoles of *p*-nitroanilide using a standard curve generated with free *p*-nitroanilide, according to the manufacturer's protocol.

2.7. Statistical evaluation

Results of densitometric analysis of immunoblotting and caspase-3 activity are given as mean ± standard error of the mean (S.E.M.). The significance of differences was determined by the analysis of variance (ANOVA). Further statistical analysis for post hoc comparisons was performed using the Bonferroni/Dunn test (StatView, Abacus Concepts, Berkeley, USA).

3. Results

3.1. Inhibitory effects of talipexole and pramipexole on MPP^+-induced release of cytochrome *c* or α-synuclein from the organelles to the cytosol in a cell culture system

Treatment of SH-SY5Y cells with 1 mM MPP^+ caused cell death after 1 day and DNA fragmentation was markedly observed after 2 days (Fig. 1A and B). In vehicle-treated cells, 15-kDa of cytochrome *c* and 19-kDa of α-synuclein

Table 1
No change in protein level of Apaf-1

Treatment	(% of Vehicle)	
	Cytosol	Organelles
Vehicle treatment	100 ± 4	n.d.
MPP^+ treatment (1 mM, 3 days)		
+ vehicle	101 ± 5	n.d.
+ talipexole (1 mM)	107 ± 4	n.d.
+ pramipexole (1 mM)	90 ± 4	n.d.

Cells were treated for 3 days with vehicle, or with 1 mM MPP^+ in the presence of 1 mM talipexole or 1 mM pramipexole. After preparations of the cytosolic and organellar fractions, each sample was subjected to immunoblot analysis of anti-Apaf-1 antibody. Density of each protein band is given as the mean ± S.E.M. (%) of three determinations. In the organellar fraction, Apaf-1 was not detected (n.d.) on either vehicle or treatment with MPP^+, talipexole or pramipexole.

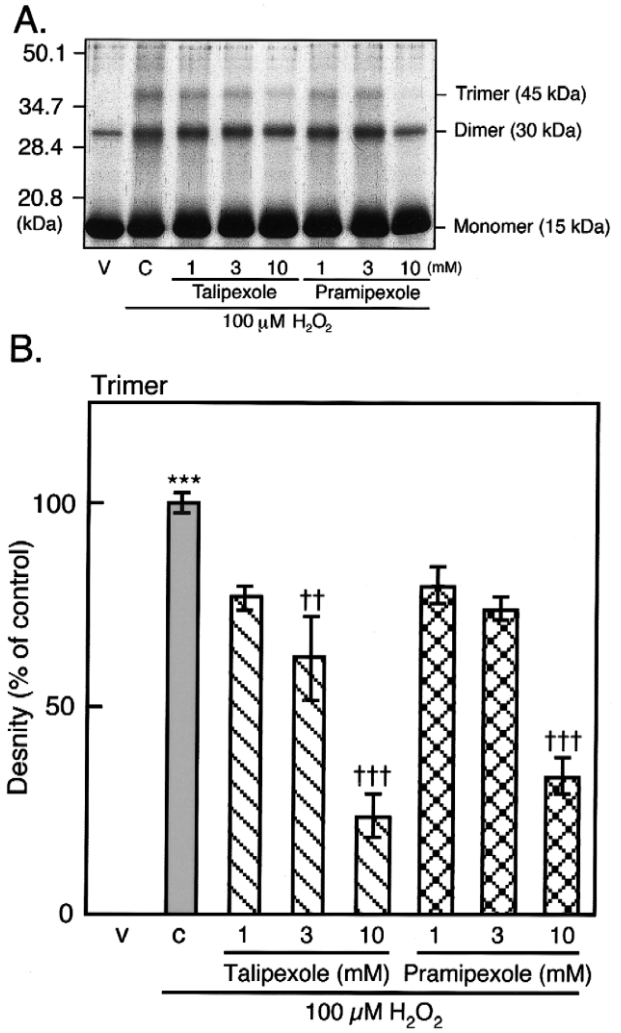

Fig. 4. Inhibition of H_2O_2-induced aggregation of cytochrome *c* by talipexole and pramipexole in vitro. Purified cytochrome *c* (100 μM) was incubated with vehicle (V) or 100 μM H_2O_2 at 37°C for 24 h, in the presence of vehicle (C), talipexole or pramipexole. After incubation, protein preparation was subjected to the polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol and SDS, and then stained by coomassie brilliant blue (A). Density of 45-kDa trimer of cytochrome *c* is given as the mean ± S.E.M. (%) of three determinations (B), based on the density in the treatment with H_2O_2 alone. Significance: $^{***}P < 0.001$ vs. the level in the vehicle treatment (V). ††, $P < 0.01$; †††, $P < 0.001$ vs. the level in treatment with H_2O_2 alone.

were found mostly in the organellar fraction (including mitochondria and membrane); however, these proteins were undetectable in the cytosolic fraction. MPP^+ induced earlier release of cytochrome *c* (after 1 day) from the organellar fraction to the cytosolic fraction (Fig. 1C) than that of α-synuclein (after 3 days) (Fig. 1D).

We previously found that treatment with talipexole or pramipexole inhibited MPP^+-induced apoptotic cell death (Kitamura et al., 1998b). In the present study, treatment with talipexole and pramipexole inhibited MPP^+-induced increase of cytochrome *c* amount in the cytosolic fraction, in a concentration-dependent manner (Fig. 2, IC_{50} values: 0.055 ± 0.010 and 0.048 ± 0.012 mM, respectively). Reversely, these drugs significantly inhibited MPP^+-induced decrease of cytochrome *c* amount in the organellar fraction (Fig. 2, IC_{50} values: 0.215 ± 0.004 and 0.201 ± 0.003 mM, respectively). Thus, these drugs at low concentration markedly inhibited MPP^+-induced release of cytochrome *c* from the mitochondria to the cytosol. Similarly, these drugs at 1 mM inhibited MPP^+-induced increase of α-synuclein amount in the cytosolic fraction and the decrease in the organellar fraction (Fig. 3). On the other hand, although Apaf-1 protein was predominantly included in the cytosolic fraction, either MPP^+ or treatment with talipex-

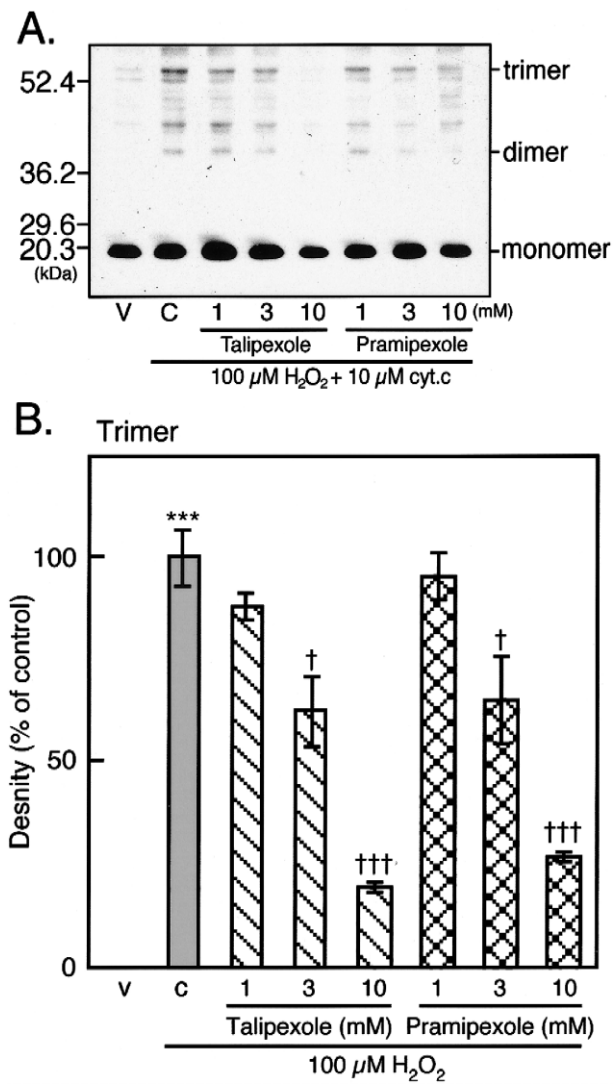

Fig. 5. Inhibitory effects of talipexole and pramipexole on in vitro α-synuclein aggregation induced by H_2O_2 *plus* cytochrome *c*. Partially purified rat α-synuclein was incubated at 37°C for 24 h with vehicle (V), or 100 μM H_2O_2 *plus* 10 μM cytochrome *c*, in the presence of vehicle (C), talipexole or pramipexole. After incubation, protein preparation was subjected to immunoblot analysis of anti-α-synuclein antibody (A). Density of 57-kDa trimer of α-synuclein is given as the mean ± S.E.M. (%) of three determinations (B), based on the density in the treatment with H_2O_2 *plus* cytochrome *c*. Significance: $^{***}P < 0.001$ vs. the level in the vehicle treatment (V). †, $P < 0.05$; †††, $P < 0.001$ vs. the level in treatment with H_2O_2 *plus* cytochrome *c*.

ole or pramipexole did not change this protein level (Table 1).

3.2. Inhibitory effects of talipexole and pramipexole on in vitro aggregation of cytochrome c or α-synuclein

Hashimoto et al. (1999) showed that H_2O_2 induced in vitro aggregation of cytochrome *c* and α-synuclein. Therefore, we examined effects of talipexole and pramipexole on the aggregation of cytochrome *c* and α-synuclein. In the vehicle treatment, dimer of cytochrome *c* was slightly detected. 100 μM H_2O_2 markedly induced dimeric and trimeric formation of cytochrome *c* even in the presence of 2-mercaptoethanol and a potent detergent SDS on the polyacrylamide gel electrophoresis. The in vitro H_2O_2-induced trimeric formation of cytochrome *c* was significantly inhibited by talipexole and pramipexole at high concentration (Fig. 4, IC_{50} values: 4.8 ± 1.0 and 6.3 ± 0.9 mM, respectively), although these drugs at low concentration (less than 1 mM) did not inhibit.

On the other hand, the detection of α-synuclein aggregation was necessary to a zero-length crosslinker DCCD on SDS-polyacrylamide gel electrophoresis. The dimer and trimer of rat α-synuclein were markedly formed by the incubation in the presence of both H_2O_2 (at 100 μM) and cytochrome *c* (at 10 μM) (Fig. 5), although these oligomers were undetectable in the presence of H_2O_2 or cytochrome *c* alone (data not shown). The in vitro trimeric formation of α-synuclein by H_2O_2 *plus* cytochrome *c* was significantly inhibited by talipexole and pramipexole at high concentration (Fig. 5, IC_{50} values: 4.0 ± 0.3 and 5.1 ± 0.4 mM, respectively), similar to inhibitory effects on the aggregation of cytochrome *c* (Fig. 4).

3.3. Inhibitory effects of talipexole and pramipexole on in vitro activation of caspases in a cell-free system

We further examined the effects of talipexole and pramipexole in a cell-free apoptosis system. In the cytosolic extract used in this experiment, Apaf-1 was rich; however, cytochrome *c* was undetectable. Treatment with MPP^+ at 1–3 mM for 4–24 h induced cleavage of neither caspase-3 nor poly(ADP-ribose) polymerase (data not shown). In contrast, 1 mM dATP *plus* 1 μM cytochrome *c* markedly induced cleavages of caspase-9 and -3, and poly(ADP-ribose) polymerase (Fig. 6). In brief, 48-kDa caspase-9, 32-kDa caspase-3 and 116-kDa poly(ADP-ribose) polymerase were cleaved to 37-, 20/17- and 85-kDa fragments, respectively. The in vitro cleavage of caspase-9 and 3, and poly(ADP-ribose) polymerase by dATP *plus* cytochrome *c* was completely inhibited by high concentration of talipexole (Fig. 6A, IC_{50} values: 7.8 ± 0.6, 5.1 ± 0.3 and 7.5 ± 0.7 mM, respectively) or

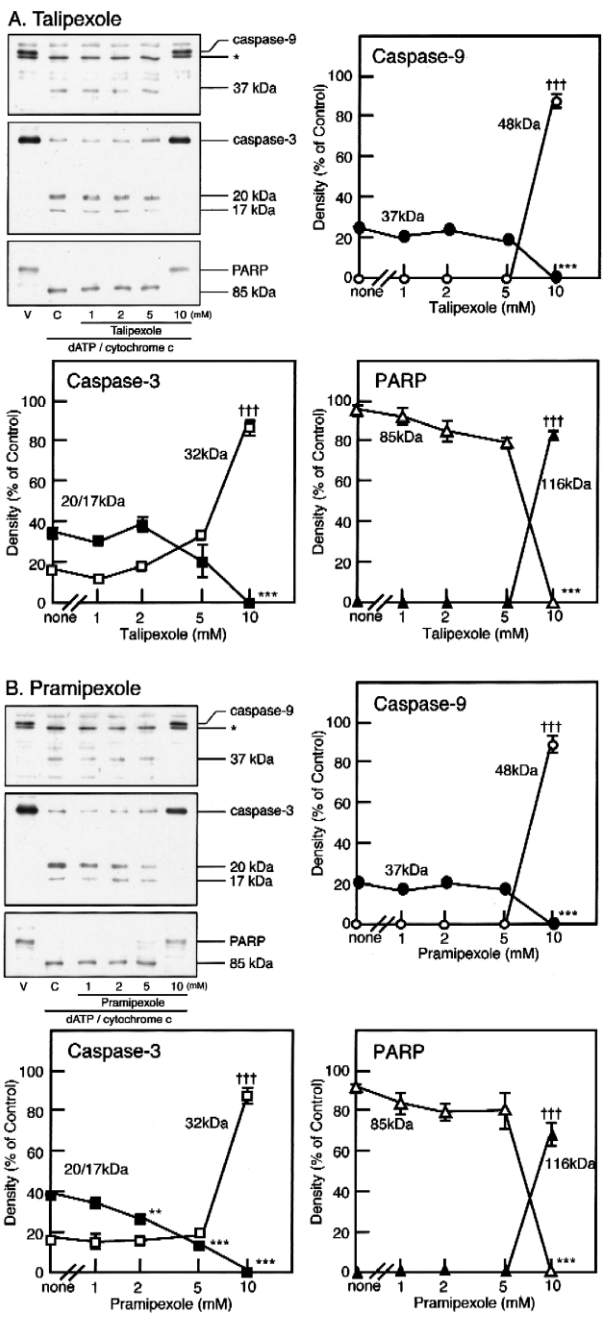

Fig. 6. Inhibition of dATP/cytochrome *c*-induced cleavages of caspase-9 and -3, and poly(ADP-ribose) polymerase by talipexole and pramipexole in a cell-free system. The cytosolic extract from nontreated SH-SY5Y cells was treated for 4 h with vehicle (V) or 1 mM dATP *plus* 1 μM cytochrome *c* (dATP/cytochrome *c*), in the presence of vehicle (C), talipexole (A) or pramipexole (B). Each sample was subjected to immunoblot analysis of antibodies against caspase-9 (upper panel), caspase-3 (middle panel) and poly(ADP-ribose) polymerase (PARP, lower panel). In immunoblots of caspase-9, the asterisk (*) denotes a nonspecific protein band. Density of 48-kDa pro-caspase-9 (○)/37-kDa fragment (●), 32-kDa pro-caspase-3 (□)/20–17-kDa fragments (■) and 116-kDa poly(ADP-ribose) polymerase (△)/85-kDa fragment (▲) are given as the mean ± S.E.M. (%) of three determinations, based on the density of each pro-enzyme in the vehicle treatment considered to be 100%. Significance (the post hoc comparisons by Bonferroni/Dunn test): $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the fragment level in treatment with dATP/cytochrome *c* alone (C). †††, $P < 0.001$ vs. the level of the pro-enzyme in treatment with dATP/cytochrome *c* alone (C).

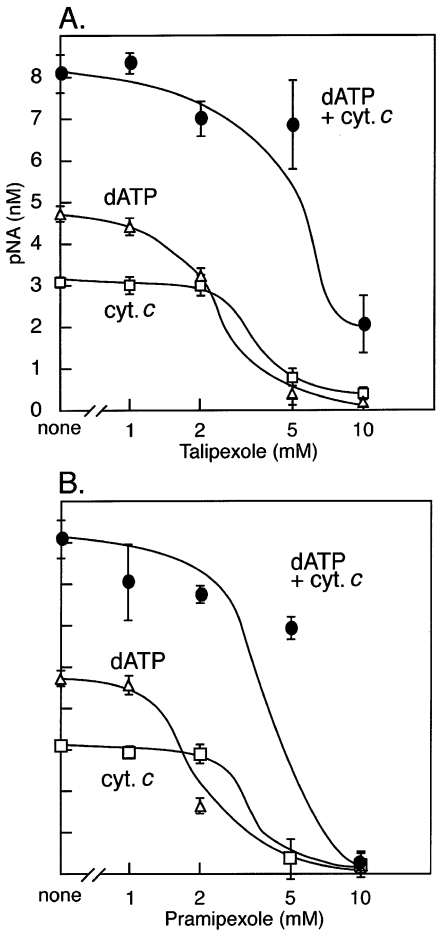

Fig. 7. Inhibitory effects of talipexole and pramipexole on caspase-3 activation induced by dATP and cytochrome *c* in a cell-free system. The cytosolic extract was treated for 4 h with 1 mM dATP (△), 1 μM cytochrome *c* (□) or both (●), in the presence of vehicle, talipexole (A) or pramipexole (B) at 1–10 mM. Subsequently, the substrate of caspase-3 was added to the mixture and then caspase-3 activity was measured. The activity is given as the mean ± S.E.M. of three determinations.

pramipexole (Fig. 6B, IC_{50} values: 8.3 ± 0.7, 5.8 ± 0.5 and 8.9 ± 0.4 mM, respectively).

In addition, activation of caspase-3 by 1 mM dATP alone, 1 μM cytochrome *c* alone and dATP *plus* cytochrome *c* was also inhibited by high concentration of talipexole (Fig. 7A, IC_{50} values: 2.6 ± 0.1 mM, 3.9 ± 0.2 and 8.5 ± 2.6 mM, respectively) or pramipexole (Fig. 7B, IC_{50} values: 2.2 ± 0.2 mM, 3.4 ± 0.2 and 4.1 ± 0.4 mM, respectively).

4. Discussion

The brains of patients with Parkinson's disease have evidence of inhibition of mitochondrial complex I (Mizuno et al., 1989; Schapira et al., 1989) and generation of oxidative stress (Castellani et al., 1996). Similar phenomena were observed in MPTP-treated animals, the parkinsonian models (Sriram et al., 1997; Kitamura et al., 2000). Recent studies have suggested that apoptogenic cytochrome *c*, which is released from the mitochondria into the cytosol, activated the intermediary of Apaf-1 and caspase-9, and then activated caspase-3 (Liu et al., 1996; Li et al., 1997; Zou et al., 1997). Caspase-3 activation may participate in the vulnerability of dopaminergic neurons in Parkinson's disease (Hartmann et al., 2000). Thus, one pathogenic element of Parkinson's disease appears to be dysfunction of the mitochondria. On the other hand, α-synuclein protein, which is normally bound to the membranes (Davidson et al., 1998; McLean et al., 2000), is known as a component of an intracellular inclusion Lewy body in Parkinson's disease and dementia with Lewy body (Spillantini et al., 1998). In several families in the autosomal dominant Parkinson's disease, two mutations in the amino-terminal region of α-synuclein have recently been identified, i.e. substitutions of alanine at amino acid position 30 by proline (A30P) and at position 53 by threonine (A53T) (Clayton and George, 1999; Hashimoto and Masliah, 1999). In contrast, rat, mouse and zebra finch carry the A53T allele as these normal sequences in the α-synuclein proteins, while A30 is the same as the human wild type (Clayton and George, 1999; Hashimoto and Masliah, 1999). It is known that the full length of wild-type and mutant α-synuclein proteins are self-aggregated in which the assembly rates are human A53T α-synuclein = rat α-synuclein = zebra finch α-synuclein > human A30P α-synuclein = human wild-type α-synuclein (Serpell et al., 2000). Such aggregation was facilitated by cytochrome *c* and oxidative stress (Hashimoto and Masliah, 1999). In addition, cytochrome *c* is colocalized with approximately half of the α-synuclein-positive nigral Lewy bodies in Parkinson's disease and dementia with Lewy body (Hashimoto et al., 1999). On the other hand, overexpression of A53T or A30P α-synuclein causes enhancement of vulnerability to oxidative stress (Kanda et al., 2000). From these observations, we examined effects of antiparkinsonian drugs on the release and aggregation of cytochrome *c* or α-synuclein.

In the present study, MPP^+ significantly induced a release of cytochrome *c* from the organellar fraction (probably mitochondria) to the cytosolic fraction, but did not change Apaf-1 level in a culture system. In a cell-free system, MPP^+ induced cleavage of neither caspase-3 nor poly(ADP-ribose) polymerase (data not shown), suggesting that this neurotoxin did not directly activate either Apaf-1, caspase-9 or -3. Thus, MPP^+ neurotoxicity may be mediated by dysfunction of the mitochondria and accumulation of cytosolic cytochrome *c*. On the other hand, talipexole or pramipexole significantly inhibited MPP^+-induced release of cytochrome *c* from the mitochondria to the cytosol in a concentration-dependent manner. Also, these drugs inhibited MPP^+-induced delayed release of α-synuclein from the organellar fraction (probably membranes) to the cytosol. The concentration ranges of these inhibitory effects of talipexole and pramipexole (0.1–1 mM) is similar to

their concentration yielding inhibition of MPP^+-induced apoptotic cell death (Kitamura et al., 1998b).

While cytochrome *c* was easily aggregated by H_2O_2, the aggregation of rat α-synuclein was facilitated by H_2O_2 and cytochrome *c*. The aggregation of cytochrome *c* or α-synuclein was inhibited by talipexole or pramipexole at high concentration (3–10 mM). These results suggest that talipexole and pramipexole may inhibit the formation of intracellular inclusions by reactive oxygen species, cytochrome *c* and α-synuclein. In addition, in vitro activation of caspase-9 and -3 induced by dATP and/or cytochrome *c* was inhibited by talipexole or pramipexole also at high concentration (5–10 mM). Since inhibitory potencies of talipexole and pramipexole were similar between cleavages of caspase-9 and -3, and poly(ADP-ribose) polymerase, these drugs may directly inhibit Apaf-1 activation. However, the inhibitory effects of these drugs on the aggregation of cytochrome *c* or α-synuclein and the activation of Apaf-1 obtained only at higher concentration (3–10 mM) in comparison with those yielding inhibition of release of cytochrome *c* or α-synuclein (0.1–1 mM). These results suggest that talipexole and pramipexole may most strongly affect the inhibition of release of cytochrome *c* and α-synuclein from the organelles to the cytosol. Furthermore, higher concentration of these drugs has inhibitory effects of intracellular aggregation of cytochrome *c* and α-synuclein, and Apaf-1 activation. Thus, talipexole and pramipexole have multifunctionally neuroprotective effects and may be useful for treatment of patients with Parkinson's disease and also other neurodegenerative disorders, such as dementia with Lewy body and Alzheimer's disease.

In conclusion, MPP^+ induced release of cytochrome *c* and α-synuclein from the organelles to the cytosol, and the accumulation of cytochrome *c* in the cytosol may cause Apaf-1 activation, α-synuclein aggregation and its self-aggregation. Talipexole or pramipexole at low concentration inhibited release of cytochrome *c* and α-synuclein from the organelles to the cytosol. In addition, the aggregation of cytochrome *c* by hydrogen peroxide or that of α-synuclein by cytochrome *c plus* hydrogen peroxide was inhibited by these drugs at high concentration. Apaf-1 activation was also inhibited by higher concentration of these drugs. These results suggest that talipexole and pramipexole have protective effects against the neurodegeneration, which is induced by intracellular accumulation of cytochrome *c* and α-synuclein.

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