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INHIBITION OF AROMATIC L-AMINOACID DECARBOXYLASE IN CLONAL PHEOCHROMOCYTOMA PC12h CELLS BY N-METHYL-4-PHENYLPYRIDINIUM ION (MPP^+)

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SUMMARY. N-Methyl-4-phenylpyridinium ion (MPP^+), a reaction product of a neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was found to inhibit aromatic L-aminoacid decarboxylase activity in rat clonal pheochromocytoma PC12h cells. The enzyme activity was enhanced to several folds by addition of a cofactor, pyridoxal phosphate, and MPP^+ inhibited the enhancement of the activity by exogenously added pyridoxal phosphate. The inhibition was competitive to pyridoxal phosphate, and the K_i value of MPP^+ was 26.7 $\pm$ 0.4 μM, while the K_m value of pyridoxal phosphate was 0.645 $\pm$ 0.053 μM. The inhibition was partly irreversible. The enzyme sample was incubated with MPP^+ and then dialyzed against phosphate buffer. After dialysis, the inhibited enzyme activity was only partly recovered by addition of pyridoxal phosphate, even though MPP^+ was completely removed. Activity of other enzymes, tyrosine hydroxylase and monoamine oxidase could be recovered by dialysis. On the other hand, MPP^+ did not affect the binding of the enzyme with the substrate, L-DOPA or 5-hydroxytryptophan.

N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin to elicit symptoms very similar to those in Parkinsonism (1). The molecular mechanism of the neurotoxicity of this compound has been intensively studied. MPTP is oxidized by type B monoamine oxidase [monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4, MAO] in the brain to N-methyl-4-phenyl-2,3-dihydropyridine, which is non-enzymatically converted to N-methyl-4-phenylpyridinium ion (MPP^+) (2, 3). MPP^+ is a true neurotoxin, which is taken up into dopaminergic neurons in nigro-striatal system by a transport system specific for dopamine

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Abbreviations used: MPTP, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MAO, monoamine oxidase; MPP^+, N-methyl-4-phenylpyridinium ion; DA, dopamine; TH, tyrosine hydroxylase; AADC, aromatic L-amino acid decarboxylase; 5-HTP, 5-hydroxytryptophan; PLP, pyridoxal phosphate; SOS, sodium 1-octanesulfonate; HPLC, high-performance liquid chromatography.

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(DA) (4, 5). MPP^+ is known to cause degeneration of DA neurons, but the mechanism of the cell death has not been well elucidated. On the other hand, MPP^+ was found to inhibit tyrosine hydroxylase [tyrosine tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2, TH] in slices of rat striatum tissue (6), indicating that MPP^+ inhibits DA synthesis. In addition, MPP^+ inhibits MAO, a major enzyme of catabolism of catecholamines in the brain (7), suggesting that MPP^+ perturbs both the synthesis and metabolism of catecholamines in the brain. In addition, by systemic administration of MPTP to mice, reduction of the enzyme activity of TH and aromatic L-aminoacid decarboxylase (aromatic L-aminoacid carboxyl-lyase, EC 4.1.1.28, AADC) was observed in the striatum (8). The effect of MPP^+ on the catecholamine metabolism was studied using rat clonal pheochromocytoma PC12h cells as a model of DA neurons, because PC12h cells synthesize mainly DA (9) and contain only type A MAO (10, 11).

This paper describes enzymatic study on the inhibition of AADC by MPP^+. For this study, a new and simple assay procedure of AADC toward L-DOPA as substrate was devised, using a Coulometric electrochemical detector with minimum sample preparation was devised. The mechanism of inhibition of AADC by MPP^+ was discussed in relation to the pathogenesis of this neurotoxin.

MATERIALS AND METHODS

Materials: L-DOPA, DA, L-5-hydroxytryptophan (5-HTP), and kynuramine were purchased from Sigma, NSD-1055 (p-bromobenzyloxyamine) and pyridoxal phosphate (PLP) from Nakarai, 5-hydroxytryptophan (5-HT) from Merck, sodium 1-octanesulfonate (SOS) from Aldrich. Clorgyline was kindly donated from May and Baker. MPP^+ was prepared as reported by Markey, et al. (12). PC12h cells were kindly donated by Dr. Kuzuya, Fujita-Gakuen Health University, Toyoake, Aichi, Japan, and cultured as described previously (11). The cells were washed with phosphate buffered-saline and suspended in 10 mM potassium phosphate buffer, pH 7.4, and then sonicated with a Branson sonicator for 30 sec.

Assay for AADC using L-DOPA and 5-HTP as substrates: PC12h cells (10-50 μg protein) were incubated with 1 mM L-DOPA or 5-HTP in 100 μl of 20 mM sodium phosphate buffer, pH 7.2, containing 5 μM PLP, 0.34 mM ascorbic acid and 10 μM clorgyline. After incubated at 37°C for 20 min, the reaction was terminated by addition of 100 μl of 180 mM sodium acetate-70 mM citric acid buffer, pH 4.35, containing 130 μM EDTA and 460 μM SOS, to which methanol was added to be 21%. After centrifuged at 13,000 g for 10 min, the supernatant was filtered through an Ekicrodisc 3-L filter (Gelman Sciences Japan). The amount of DA formed from L-DOPA was measured using high-performance liquid chromatography (HPLC): a Shimadzu HPLC apparatus (LC-4A) connected with a Coulochem electrochemical detector, 5100A was used. The column used was a reverse-phase Cosmosil 5C18

column (4.6 mm i.d. x 250 mm) connected with a Cosmosil 10C18 precolumn. The mobile phase was 90 mM sodium acetate-35 mM sodium acetate buffer, pH 4.35, containing 130 μM disodium EDTA and 230 μM SOS, to which methanol was added to be 10.5% and the flow rate was 1.0 ml/min. Conditions of the electrochemical detector to detect DA were as follows; a 5021 conditioning cell was set at +400 mV and the first electrode of a 5011 analytical cell was set at +100 mV and the second electrode was at -300 mV. The output of the second electrode was recorded. The amount of 5-HT produced from 5-HTP was measured fluorometrically using a Shimadzu LC4A HPLC apparatus connected with a Shimadzu spectrofluorophotometer, RF500, and fluorescence intensity at 340 nm was measured by excitation at 295 nm. The column used was a Shimadzu ODS-H column (4 mm i.d. x 150 mm). The mobile phase was almost the same as that used for the DA assay, except that SOS and methanol concentrations were 115 μM and 15%, respectively, and the flow rate was 0.6 ml/min. Quantitation of DA or 5-HT was carried out by comparison of the peak height with standard.

Assay for MPP^+ and activity of TH, MAO, and β-galactosidase: MPP^+ amount in PC12h cells was measured as reported previously (13). TH activity was measured by our newly-devised method (14), and MAO activity (15) and β-galactosidase activity (16) were measured as described in our previous papers. The protein concentration was measured according to Bradford (17), using bovine γ-globulin as standard.

Kinetical studies: Kinetic data of AADC were obtained according to Lineweaver and Burk, using 8 different concentrations of L-DOPA and 5-HTP (8.76-100 μM) or PLP (0.87-10 μM).

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC patterns of the reaction mixture of PC12h cells with L-DOPA in the absence and presence of 10 μM MPP^+. The amount of DA produced was markedly reduced in the presence of MPP^+. The inhibition by MPP^+ was dependent on MPP^+ concentrations, as shown in Fig. 2. The data show that AADC activity

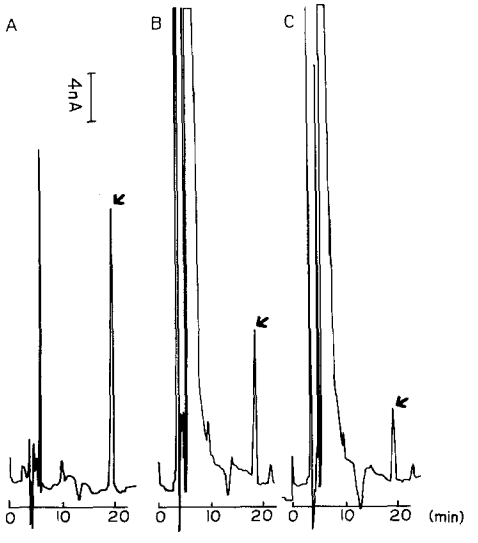

Fig. 1. HPLC patterns of AADC assay. PC12h cells (23.4 μg protein) were incubated in the absence and presence of MPP^+, and AADC activity toward L-DOPA was measured as described in the text. A; 2.5 pmol DA, B; control, and C; sample incubated with 10 μM MPP^+.

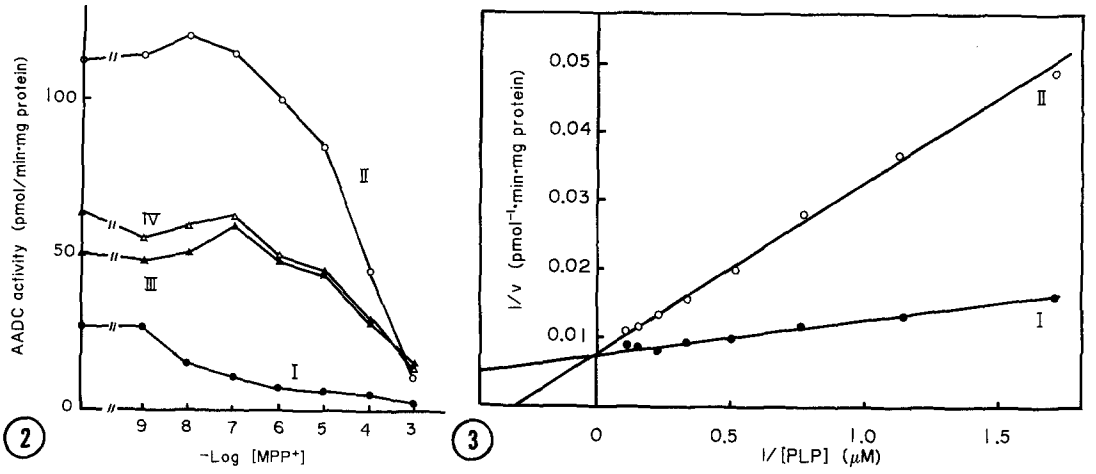

Fig. 2. Effect of MPP^+ on AADC activity. AADC activity was measured in the absence and presence of MPP^+ (1 nM to 1 mM). I and III; AADC activity toward L-DOPA or 5-HTP was measured without addition of PLP, and II and IV; the activity toward L-DOPA or 5-HTP was measured in the presence of 5 μM PLP, respectively. Each point represents mean of triplicate measurements of two experiments.

Fig. 3. Effect of MPP^+ on kinetics of AADC toward PLP concentration. PC12h cells (23.4 μg protein) were incubated with various concentrations of PLP, in the absence and presence of MPP^+, using 1 mM L-DOPA as substrate. The reciprocal of AADC activity observed by addition of PLP was plotted against that of PLP concentration, according to Lineweaver and Burk. I; control and II; AADC activity was measured in the presence of 100 μM MPP^+.

was enhanced by the addition PLP to several folds. Inhibition of AADC by MPP^+ at its low concentrations was prevented by addition of 5 μM PLP into the reaction mixture. The effect of PLP on MPP^+ inhibition was more clearly shown by kinetic study on the AADC activity increased by PLP. The K_m value of AADC toward PLP was 0.65 ± 0.05 μM, and MPP^+ inhibited AADC in competition to PLP, as shown in Fig. 3, Curve II. The K_i value of MPP^+ was 26.3 ± 0.4 μM. The enzymatic properties of AADC in PC12h cells were summarized in Table 1.

As shown in Table 1, MPP^+ did not affect the K_m value of L-DOPA, even at its high concentration. Inhibition of AADC by MPP^+ was also recognized, using 5-HTP as substrate. As shown in Fig. 2, Curve III and IV, the enzyme activity toward 5-HTP was inhibited in a dose-dependent way. The inhibition was competitive to PLP, the K_m value toward 5-HTP was not affected, as summarized in Table 1.

After incubated with 100 μM MPP^+, the enzyme sample was dialyzed against 10 mM potassium phosphate buffer, pH 7.4, at 4°C overnight. As summarized in Table 2, the enzyme activity was reduced by addition of MPP^+ and also by dialysis.

Table 1. Effect of MPP^+ on kinetics of AADC in PC12h cells

	Control	In the presence of MPP^+ (100 μM)
Toward PLP		
Km (μM)	0.645 ± 0.053	(4.99 ± 2.73)*
Vmax (pmol/min/mg protein)	131.8 ± 3.9	132.2 ± 5.3
Ki of MPP^+ for PLP (μM)	26.7 ± 0.4	
Toward L-DOPA		
Km (μM)	59.7 ± 15.3	53.7 ± 7.1
Vmax (pmol/min/mg protein)	215.1 ± 42.8	191.4 ± 12.9
Toward 5-HTP		
Km (μM)	54.7 ± 21.0	43.3 ± 23.6
Vmax (pmol/min/mg protein)	52.7 ± 6.3	52.1 ± 5.9

Each value represents the mean and SD of triplicate measurements of three experiments.

* The apparent Km value obtained by measurement of AADC activity in the presence of MPP^+.

The enzyme activity in control sample was also reduced by dialysis, but almost full activity was recovered by addition of 5 μM PLP. On the other hand, the activity of AADC incubated with MPP^+ was only partially recovered by addition of PLP. MPP^+ amount in the sample was also measured, but MPP^+ was not detected, even though the method was sensitive enough to detect 10 fmol MPP^+ (16). These results indicate that the inhibition was irreversible. As summarized in Table 2, activities of other enzymes, TH, MAO and a non-specific enzyme, β-

Table 2. Reversibility of the effect of MPP^+ on DDC, TH, MAO and β-galactosidase activity

Enzyme	Enzyme activity (pmol/min/mg protein) MPP^+	Control
DDC	1.38 ± 0.46	5.61 ± 1.76
+ 5 μM PLP	10.6 ± 2.5	85.4 ± 13.4
TH	20.1 ± 2.3	26.3 ± 4.9
MAO	440 ± 74	324 ± 92
β-Galactosidase	671 ± 9	726 ± 52

PC12h cells (620 mg protein) were incubated with or without 100 μM MPP^+ in 1 ml of 10 mM potassium phosphate buffer, pH 7.4 at 37°C for 20 min, then dialyzed against 10 mM potassium phosphate buffer, pH 7.4, at 4°C overnight. Aliquots of the sample (25 to 100 μg protein) were taken for the enzyme assay.

galactosidase, in samples incubated with MPP^+ were almost the same as those in control by removal of MPP^+ by dialysis.

These data clearly demonstrate that MPP^+ inhibited AADC activity toward L-DOPA and 5-HTP, in competition to a cofactor, PLP. Reduction of TH and AADC activity after peripheral administration of MPTP was reported previously by in vivo experiment (8), and the results reported here elucidate the mechanism of the reduction. Previously Siow reported that MPP^+ inhibited AADC activity toward L-DOPA and increased that toward 5-HTP (18). The discrepancy between his data and ours may be due to the assay conditions used by Siow; the PLP concentration in his assay system, 300 μM, was quite higher than that in our system, 5 μM.

The data present in this paper were obtained using rat pheochromocytoma cells, and we should be careful to apply this result to human Parkinsonism or MPP^+-induced parkinsonism in humans. At least we may emphasize that AADC is also taking part in the perturbation of catecholamine levels in PC12h cells. MPP^+ inhibits the binding of PLP to the apoenzyme of AADC and in general apoenzyme is more labile than holoenzyme. Thus, MPP^+ may accelerate the degradation of AADC in neurons and may cause not only direct inhibition of the enzyme activity, but also reduction of AADC protein itself. Study on the effect of MPP^+ on AADC in cells cultured in the presence of MPP^+ is now under way.

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