Original Articles

Inhibitory effects of 1-methyl-4-phenylpyridinium on glutamate uptake into cultured C6 glioma cells[1]

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ABSTRACT

AIM: To investigate the effect of 1-methyl-4-phenylpyridinium (MPP^+) on the glutamate uptake into cultured C6 glioma cells. **METHODS:** The glutamate uptake into C6 glioma cells was measured by radio-ligand binding assay method. The effect of MPP^+ on the morphology of C6 glioma cells was observed under phase contrast microscopy; apoptosis of C6 glioma cells were measured by FITC-labeled Annexin V staining and flow cytometry. Cell viability was measured by MTT method. **RESULTS:** MPP^+ inhibited glutamate uptake into C6 glioma cells. However, MPP^+ failed to induce any morphological changes of C6 glioma cells, and exposure to MPP^+ had no effect on the viability and the apoptotic percentage of C6 glioma cells. Incubation with 12-*O*-tetradecanoylphorbol -13-acetate (TPA), a protein kinase C activator, caused a significant increase in glutamate uptake and completely reversed MPP^+-induced inhibitory effect on glutamate uptake. **CONCLUSION:** The present results indicate that glutamate transporters may have important pathogenetic implications in Parkinson disease. MPP^+-induced inhibition of glutamate uptake was due to the dysfunction of glutamate transporters; TPA enhanced glutamate uptake and completely reversed the inhibitory effect of MPP^+.

INTRODUCTION

Glutamate is a predominant excitatory neurotransmitter in the mammalian central neuron system[1]. Enhanced glutamatergic activity is implicated in the pathology of neurological diseases, such as Parkinson disease (PD)[1]. PD is characterized by a progressive and selective loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) which is the site of a glial activation in PD. Regulation of glutamate level in the synaptic cleft by glutamate transporters located in glia is critical to the maintenance of low level glutamate and the prevention of neurons against excitotoxic injury. A family of glutamate transporters including five members such as EAAT1-5[2,3] mediates this high affinity uptake. The dysfunction of glutamate transporters may contribute to PD as the abnormality leads to excess glutamate in synaptic cleft[4,5]. Therefore, an understanding of the glutamate transporters-mediated regulation of glutamate uptake is of considerable interest.

1-Methyl-4-phenyl-tetrahydropyridine (MPTP) is widely used to prepare animal model for pathological studies of PD[6]. Increasing evidence suggests that the toxic effect of MPTP on dopaminergic neurons of the

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substantia nigra is based on its conversion to MPP^+ by monoamine oxidase type B[7]. But it is still unknown whether the mechanism underlying MPP^+-induced dopamine neuron damage is related to the dysfunction of glutamate transporters. The function of glutamate transporters is regulated by PKC, which is a cell-specific process[8-10]. However, little has been known about the effects of PKC activator on glutamate uptake into C6 glioma cell incubated with MPP^+.

C6 glioma cell, a glia-derived cell line endogenously expressing EAAC-1 (EAAT-3)-like immunoreactivity, was commonly used as an *in vitro* model for the study of glial cell properties, including glutamate transporters-mediated glutamate uptake[3,11]. The present study was undertaken to investigate the effects of MPP^+ and PKC activator-TPA on glutamate uptake into cultured C6 glioma cells so as to clarify whether the dysfunction of glutamate transporters was implicated in MPP^+-induced neurotoxicity and whether glutamate transporters played an important role in the pathology of PD.

MATERIALS AND METHODS

Reagents [^{3}H]*D,L*-glutamate was purchased from Chinese Atomic Nucleus Research Institute (Beijing, China). 1-Methyl-4-phenylpyridinium (MPP^+), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodine (PI) and PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma, USA. Annexin V-FITC apoptosis detection kit was the product of the Genzyme Corporation, USA. RPMI-1640 was obtained from GIBCO, USA. MPP^+ and TPA were dissolved in sterile water and were diluted with culture medium.

C6 glioma cell culture C6 glioma cells were purchased from Shanghai Institute of Biochemistry, Chinese Academy of Sciences (Shanghai, China). Rat C6 glioma cells were cultured in RPMI-1640 supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), benzylpenicillin 200 kU/L and streptomycin 100 mg/L at 37 ºC in atmosphere with 5 % CO_2. For glutamate uptake experiments, the cells were grown in 24-well tissue-culture plates to reach 80 % confluence. Then, the cells were kept in the incubator after drugs and vehicle were added to the culture medium.

Glutamate uptake assay Glutamate uptake was performed as described by Pines, *et al* with modification[12]. Briefly, exposure of cultures to MPP^+ 50, 100, and 150 μmol/L for 12 h, and [^{3}H]glutamate was added to each well (end concentration is 0.25 μmol/L). Exactly 15 min later, the uptake was stopped and the cells were rapidly washed three times with ice-cold NaCl (0.9 %). The cells were harvested, and then solubilized in 0.2 mL $HClO_4$ (1 mol/L) followed by immediate centrifugation (5000×*g* at 4 ºC for 5 min). Radioactive contents and protein contents of the supernatant were determined by liquid scintillation counting and Bradford method, respectively.

Measurement of C6 glioma cell apoptosis by Annexin V staining C6 glioma cells were plated on coverslips. After treatment, the coverslips were washed with PBS three times and were labeled by incubation with Annexin V (5 μL dissolved in 195 μL binding buffer) for 10 min in dark at room temperature. And then, the coverslips were washed with PBS again and were examined using Laser Scanning Confocal Microscope (LSM 510, Heidelberg, German). Quantification of apoptosis was routinely determined by counting the number of cells stained by FITC-labeled Annexin V. Six randomly chosen fields of view were observed after exposure to the conditions indicated.

Measurement of C6 glioma cell apoptosis by flow cytometry C6 glioma cells were treated with MPP^+ 50, 100, and 150 μmol/L at 37 ºC for 12 h, then cells were harvested and washed twice with PBS. After being resuspended in cold 70 % ethanol, the cells were incubated at 4 ºC for 24 h. Then they were stained with 10 mg/L propidium iodide in the presence of 100 mg/L RNase A at 4 ºC for 30 min in the dark. The percentage of apoptotic cells was detected using flow cytometry.

C6 glioma cell morphology C6 glioma cells were exposed to MPP^+ 50, 100, and 150 μmol/L up to 12 h and then examined for morphologic alterations by phase contrast microscopy.

Cell viability assay C6 glioma cells were seeded to 96-well plates and grown to 80 % confluence in RPMI-1640 medium. The cultures were then rinsed with phenol red-free RPMI-1640 medium, and 20 μL of MTT solution (dissolved at 5 g/L in PBS) was added to each well. The reaction was stopped after incubation at 37 ºC for 4 h by discarding the supernatants, and then 100 μL of dimethylsulfoxide (Me_2SO) was added to each well to dissolve the resultant dark blue crystal. The absorbance in each well was determined at 570-nm wavelength with an automatic plate reader (DG3022A).

Statistics Data were expressed as mean±SD and

compared with two-tailed *t*-test. Statistical significance was determined as $P<0.05$.

RESULTS

Effect of MPP^+ on the [^{3}H]glutamate uptake into cultured C6 glioma cells MPP^+ inhibited [^{3}H]glutamate uptake into C6 glioma cells, and the inhibitory rates under different concentrations of MPP^+ (50, 100, and 150 μmol/L) were 21.78 % ($P<0.01$), 37.39 % ($P<0.01$), and 64.57 % ($P<0.01$), respectively (Tab 1).

Effect of MPP^+ on the apoptosis of cultured C6 glioma cells Different concentrations of MPP^+ (50, 100, and 150 μmol/L) could not affect the percentage of apoptotic C6 glioma cells under the same experimental condition using two different apoptosis assay methods (Fig 1, Tab 2, and Fig 2).

Effect of MPP^+ on the morphology of cultured C6 glioma cells Treatment of C6 glioma cells with MPP^+ 50, 100, and 150 μmol/L produced no obvious changes on cell morphology (Fig 3).

Tab 1. Inhibitory effect of MPP^+ on the [^{3}H]glutamate uptake of cultured C6 glioma cells. *n*=6. Mean±SD. $^cP<0.01$ *vs* control group.

Groups	Glutamate uptake/ nmol·min^{-1}·g^{-1} protein	Inhibition rate/%
Control	30.3±2.8	–
MPP^+ 50 μmol/L	23.7±2.4^c	21.78
MPP^+ 100 μmol/L	19.0±2.8^c	37.39
MPP^+ 150 μmol/L	11±3^c	64.57

Effect of MPP^+ on the viability of cultured C6 glioma cells Similarly, compared with control group, exposure of C6 glioma cells to MPP^+ 50, 100, and 150 μmol/L failed to affect the viability of the cells determined by MTT method (data not shown).

Effect of TPA on the [^{3}H]-glutamate uptake of cultured C6 glioma cells incubated with MPP^+ Preincubation of C6 glioma cells with MPP^+ 50, 100, and

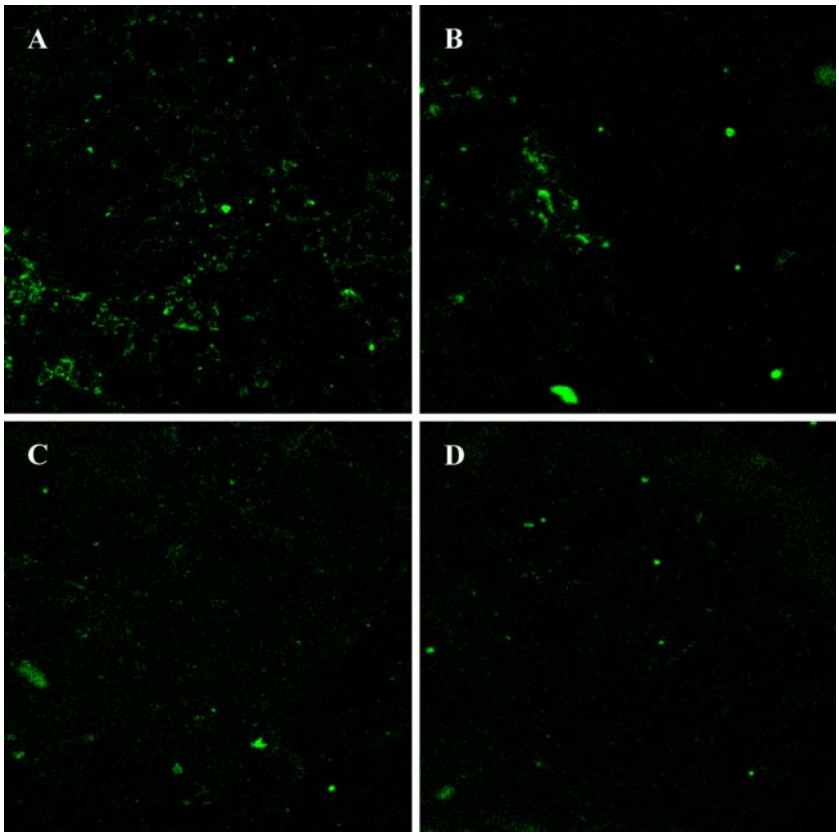

Fig 1. Effect of different concentrations of MPP^+ (50, 100, 150 μmol/L) on the apoptosis of cultured C6 glioma cells. A) Control; B) Cells treated with MPP^+ 50 μmol/L; C) Cells treated with MPP^+ 100 μmol/L; D) Cells treated with MPP^+ 150 μmol/L. ×200.

Tab 2. Effect of MPP^+ on the apoptosis of cultured C6 glioma cells. *n*=6. Mean±SD.

Groups	Apoptotic rate/%
Control	3.1±0.7
MPP^+ 50 μmol/L	3.1±1.2
MPP^+ 100 μmol/L	2.3±1.3
MPP^+ 150 μmol/L	3.0±1.2

150 μmol/L for 1 h was followed by incubation with TPA 0.1 μmol/L for 12 h. TPA alone caused increase in glutamate uptake up to 52.17 % and completely reversed MPP^+ 50, 100, and 150 μmol/L-induced glutamate uptake inhibition (Tab 3).

Tab 3. Effect of TPA on the glutamate uptake of cultured C6 glioma cells incubated with MPP^+. *n*=6. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control group. $^fP<0.01$ *vs* MPP^+ 50 μmol/L group. $^iP<0.01$ *vs* MPP^+ 100 μmol/L group. $^lP<0.01$ *vs* MPP^+ 150 μmol/L group.

Groups	Glutamate uptake/ nmol·min^{-1}·g^{-1} protein
Control	28±8
TPA 0.1 μmol/L	42±5 ^{b}
MPP^+ 50 μmol/L	15.7±2.4^b
MPP^+ 100 μmol/L	12.7±2.3^c
MPP^+ 150 μmol/L	10.4±1.2^c
MPP^+ 50 μmol/L+TPA 0.1 μmol/L	37±5^f
MPP^+ 100 μmol/L+TPA 0.1 μmol/L	35±4^i
MPP^+ 150 μmol/L+TPA 0.1 μmol/L	34±3^l

DISCUSSION

This study demonstrated that dysfunction of glutamate transporter was involved in the neurotoxicity of MPP^+. MPP^+ was known as a mitochondria complex I inhibitor, so MPP^+-induced decrease in glutamate uptake was likely attributable to its direct neurotoxicity as a consequence of the inhibition of mitochondrial function. Inhibition complex I activity will lead to energy failure resulting in changes in cell viability, cell morphology, and cell apoptosis. However, MPP^+, at concentrations 50, 100, and 150 μmol/L that were nontoxic to the cell viability, dramatically inhibited glutamate

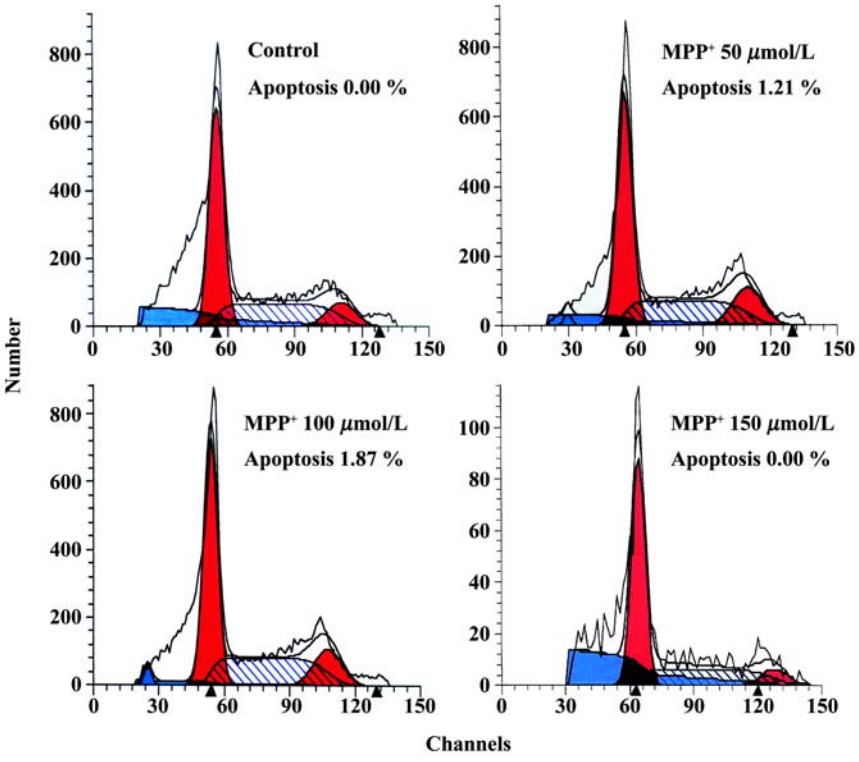

Fig 2. Effect of different concentrations of MPP^+ (50, 100, and 150 μmol/L) on the percentage of apoptotic C6 glioma cells observed by flow cytometry.

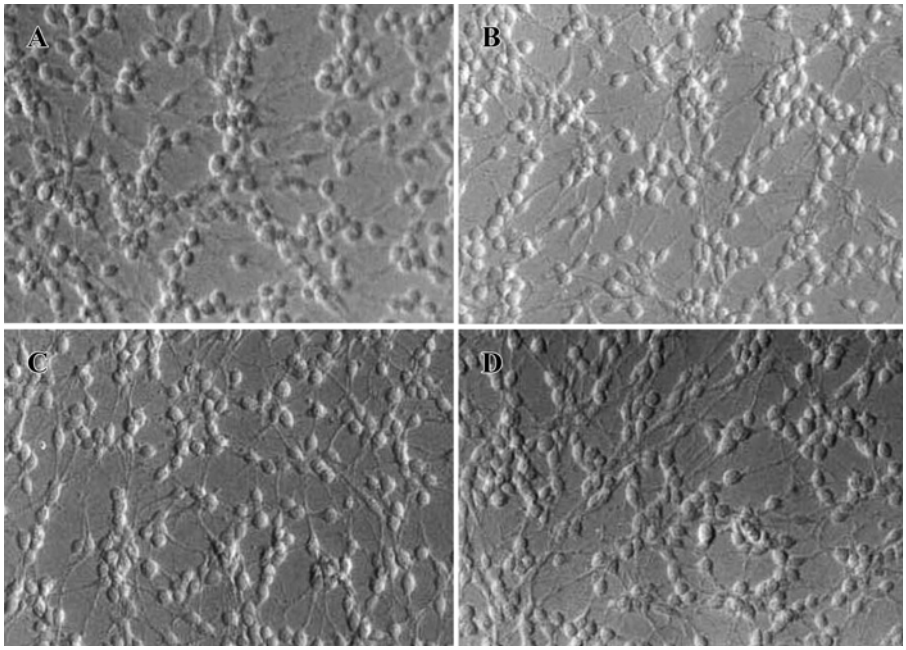

Fig 3. Effect of different concentrations of MPP^+ (50, 100, and 150 μmol/L) on the morphology of cultured C6 glioma cells. A) Control; B) Cells treated with MPP^+ 50 μmol/L; C) Cells treated with MPP^+ 100 μmol/L; D) Cells treated with MPP^+ 150 μmol/L. ×200.

uptake into C6 glioma cells. Furthermore, exposure to the same series of MPP^+ concentrations did not exert any effect on cell apoptosis and cell morphology. These results were consistent with previous reports that treatment of primary cultured astrocytes with MPTP led to a fall in glutamate uptake without any effect on cell viability[7]. As a mitochondria complex I inhibitor, it was anticipated that a fall in ATP level might occur in cells incubated with MPP^+. However, MPTP -treated astrocytes were found to be associated with a small rise in ATP content (22.8 %)[7]. Based on these collective findings, it was concluded that a mechanism other than energy failure must be responsible for the MPP^+-induced decrease in glutamate uptake and the neurotoxic effect of MPP^+ was attributable to the dysfunction of glutamate transporters, which was implicated in the pathology of PD.

PKC-mediated regulation of glutamate transporters was cell-type specific, as direct activation of PKC led to opposite effects on different culture models[10,11,13]. In this study, incubation C6 glioma cells with PKC activator-TPA caused about a 1.5-fold increase in glutamate uptake and completely reversed MPP^+-induced inhibition of glutamate uptake. The possible mechanism underlying the enhancement glutamate uptake of TPA was that TPA activated PKC, and PKC-mediated phosphorylation of glutamate transporters enhanced the activity of these transporters[9,14]. TPA reversed the inhibitory effects of MPP^+ by directly activating PKC. Therefore, MPP^+-induced decrease in glutamate uptake may, at least in part, be attributed to the inactivation of PKC. Continuing exploration of the role of PKC will further our quest into clarifying the mechanism underlying MPP^+-induced glutamate uptake inhibition.

In summary, it was demonstrated that MPP^+ inhibited glutamate uptake via the dysfunction of glutamate transporters, and glutamate transporters may play an important role in the pathology of PD.

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