N-Methyl-4-phenylpyridine (MMP^+) together with 6-hydroxydopamine or dopamine stimulates Ca^{2+} release from mitochondria

Balz Frei and Christoph Richter*

Eidgenössische Technische Hochschule, Laboratorium für Biochemie I, Universitätstr. 16, CH-8092 Zürich, Switzerland

Received 21 January 1986

The nigrostriatal neurotoxin *N*-methyl-1,2,3,6-tetrahydropyridine (MPTP) causes Parkinsonism in humans and laboratory animals. MPTP neurotoxicity is dependent on its oxidation to *N*-methyl-4-phenylpyridine (MPP^+). The mechanism by which MPP^+ causes destruction of dopamine-containing nigrostriatal cells is unknown. Here we show that MPP^+ but not MPTP is taken up by energized mitochondria. MPP^+ in the presence of dopamine and particularly of 6-hydroxydopamine stimulates Ca^{2+} release from mitochondria. Ca^{2+} release is accompanied by hydrolysis of intramitochondrial pyridine nucleotides. Our findings suggest that the MPTP-induced model of Parkinson's disease may be due to a disturbed Ca^{2+} homeostasis in dopamine neurons.

Parkinson's disease N-*Methyl-4-phenylpyridine* *Mitochondria* *Dopamine* *6-Hydroxydopamine* *Ca^{2+}*

1. INTRODUCTION

MPTP produces a chemically induced model of Parkinson's disease in primates by selective destruction of dopamine-containing cells in the nigrostriatal tract of the brain [1]. MPTP is metabolized by brain monoamine oxidase type B to *N*-methyl-4-phenyl-2,3-dihydropyridine which is further oxidized to MPP^+ [2–4]. MPP^+ is then selectively taken up into dopamine neurons [5,6]. Once inside the neuron the mechanism by which MPP^+ exerts its neurotoxicity is not known. It has been speculated that the culprit is a cocktail of oxidation products derived from dopamine and MPTP. Since oxidative stress induces release of Ca^{2+} from mitochondria [7–10], and since disturbance of cellular Ca^{2+} homeostasis upon release of mitochondrial Ca^{2+} can cause cell death [11,12], we investigated the ability of mitochondria to retain Ca^{2+} in the presence of MPTP, MPP^+, 6-OHDA and dopamine.

This paper is dedicated to Professor C. Martius on the occasion of his 80th birthday

* To whom correspondence should be addressed

Abbreviations: arsenazo III, 2,2′-(1,8-dihydroxy-3,6-disulphonaphthalene-2,7-bis azo)bis(benzenearsonic acid); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\psi$, mitochondrial transmembrane electrical potential, negative inside; MPP^+, *N*-methyl-4-phenylpyridine; MPTP, *N*-methyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine

2. MATERIALS AND METHODS

2.1. *Materials*

MPP^+ and MPTP were obtained from Research Biochemicals (Wayland); *N*-[*methyl*-^{3}H]MPP^+, *N*-[*methyl*-^{3}H]MPTP and $^{45}CaCl_2$ from New England Nuclear; [*carboxyl*-^{14}C]nicotinic acid from The Radiochemical Centre, Amersham; arsenazo III, dopamine and 6-OHDA from Fluka, Buchs.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50 © 1986 Federation of European Biochemical Societies

2.2. *Isolation of liver mitochondria*

Liver mitochondria of female Wistar rats fasted overnight were isolated as in [13]. Mitochondria were washed twice in 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4 (MSH buffer).

2.3. *Standard incubation procedure*

Mitochondria (2 mg protein/ml) were incubated at 25°C in 3 ml MSH buffer under constant stirring and oxygenation. Reduction of mitochondrial pyridine nucleotides and release of endogenous Ca^{2+} were induced by addition of 5 μM rotenone. Thereafter, when appropriate, Ca^{2+} was added or, to prevent possible reuptake of Ca^{2+}, EGTA was added. Mitochondria were then energized with 2.5 mM K^+-succinate. When appropriate, Ca^{2+} uptake was allowed to proceed for 2 min. The final Ca^{2+} loads given in section 3 are the sum of the endogenous and added Ca^{2+}. Finally, 0.5 mM MPP^+, MPTP, or 6-OHDA were added (time zero).

2.4. *Determination of intramitochondrial radioactivity*

Mitochondria were incubated in the presence of the appropriate radioactive compound ([^{3}H]-MPP^+, [^{3}H]MPTP, $^{45}Ca^{2+}$, or ^{14}C-labeled intramitochondrial pyridine nucleotides). At the times indicated 150-μl aliquots were withdrawn, filtered through Millipore filters (0.45 μm pore size), and rinsed twice with 150 μl cold MSH buffer. The radioactivity remaining on the filters was determined in a liquid scintillation counter.

2.5. *Spectrophotometric determination of Ca^{2+} uptake and release by mitochondria*

Measurements were made in the presence of 50 μM arsenazo III at 685–675 nm.

3. RESULTS AND DISCUSSION

Energized mitochondria take up MPP^+ but not MPTP (fig.1). With both 100 μM (not shown) and 500 μM MPP^+ (fig.1a), 24%, i.e. 12 or 60 nmol/mg protein, of the offered MPP^+ is taken up. The uptake of MPP^+ is prevented by the uncoupler CCCP; likewise, accumulated MPP^+ is released instantaneously upon addition of CCCP (fig.1a). It is well known that Ca^{2+} is taken up by mitochondria electrophoretically and thereby

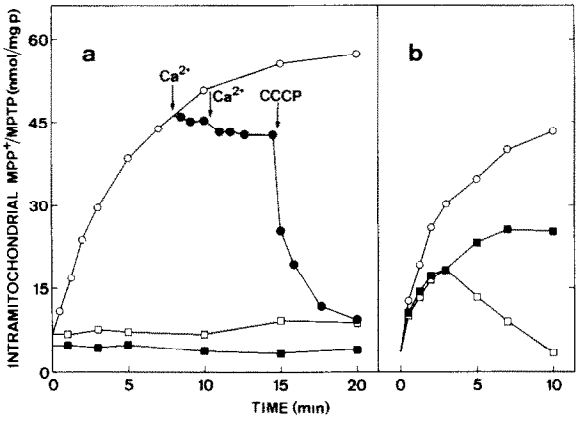

Fig.1. Uptake of MPP^+ and MPTP by mitochondria. Mitochondria were incubated under standard conditions, and (b only) depleted of Ca^{2+} or loaded with 58 nmol Ca^{2+}/mg protein. At zero time, 0.5 mM *N*-[*methyl*-^{3}H]MPP^+ (spec. act. 470 dpm/nmol) or *N*-[*methyl*-^{3}H]MPTP (485 dpm/nmol) was added. Uptake of radioactivity was determined by Millipore filtration. (a) [^{3}H]MPP^+ (○); [^{3}H]MPTP (■); [^{3}H]MPP^+ and, at the arrows, Ca^{2+} (8 nmol/mg protein) or 2.0 μM CCCP (●); 2.0 μM CCCP added before MPP^+ (□). (b) [^{3}H]MPP^+ added to Ca^{2+}-depleted mitochondria (○); [^{3}H]MPP^+ added to Ca^{2+}-loaded mitochondria (■); [^{3}H]MPP^+ together with 0.5 mM 6-OHDA added to Ca^{2+}-loaded mitochondria (□).

lowers the mitochondrial transmembrane potential, negative inside ($\Delta\psi$). When Ca^{2+} is added to energized mitochondria before MPP^+ uptake is completed, further accumulation of MPP^+ is prevented (fig.1a). Fig.1b shows that Ca^{2+}-depleted mitochondria take up more MPP^+ than Ca^{2+}-loaded mitochondria. When MPP^+ is added together with 6-OHDA to Ca^{2+}-loaded mitochondria the initial rate of MPP^+ uptake is the same as in the absence of 6-OHDA. However, MPP^+ uptake is now soon followed by MPP^+ release due to increased Ca^{2+} cycling (see below). The above observations indicate that MPP^+ equilibrates in response to $\Delta\psi$ between the intra- and extramitochondrial compartment.

When MPP^+ or 6-OHDA is added to Ca^{2+}-loaded mitochondria in the absence of EGTA, i.e. when in principle Ca^{2+} reuptake can take place after Ca^{2+} release ('Ca^{2+} cycling'), a pronounced net Ca^{2+} release is observed only after about 15 min (fig.2a). In contrast, when MPP^+ and 6-OHDA are added jointly a rapid and virtual-

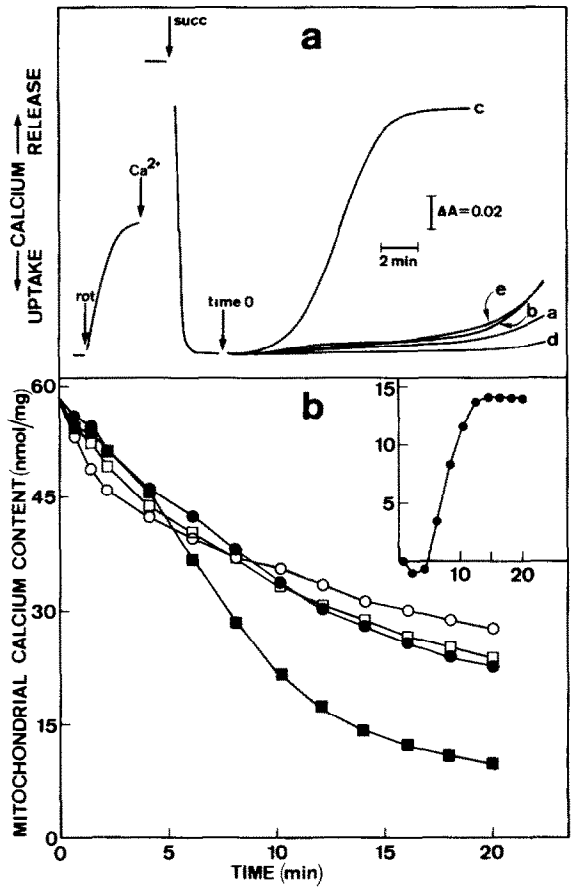

Fig.2. Release of Ca^{2+} from mitochondria in the presence of MPP^+, MPTP and 6-OHDA. Mitochondria were incubated under standard conditions and loaded with 58 nmol Ca^{2+}/mg protein. Ca^{2+} movements were followed either (a) spectrophotometrically or (b) by Millipore filtration and $^{45}Ca^{2+}$ (spec. act. 1100 dpm/nmol). At zero time the following additions (0.5 mM) were made: (a) MPP^+ (curve a); 6-OHDA (curve b); MPP^+ together with 6-OHDA (curve c); MPTP together with 6-OHDA (curve d); MPP^+ and MPTP together with 6-OHDA (curve e). (b) EGTA (□); EGTA and MPP^+ (○); EGTA and 6-OHDA (●); EGTA together with MPP^+ and 6-OHDA (■). (Inset) Difference between curves (□) and (■).

ly complete Ca^{2+} release ensues almost instantaneously (fig.2a). This rapid release is paralleled by increased Ca^{2+} cycling and consequently a fall of $\Delta\psi$ as measured by the safranine technique [14] (not shown). MPTP inhibits the 6-OHDA-stimulated Ca^{2+} release and diminishes the effectiveness of MPP^+ and 6-OHDA in stimulating Ca^{2+} release (fig.2a). Also dopamine, albeit less effectively than 6-OHDA, stimulates Ca^{2+} release from mitochondria in the absence of EGTA the onset of which is much earlier in the presence of MPP^+ (not shown). When Ca^{2+} release is measured under non-cycling conditions due to the presence of EGTA (fig.2b) stimulated release is observed only when MPP^+ and 6-OHDA are offered together to mitochondria. The stimulation is transient under these conditions (cf. inset to fig.2b).

Ca^{2+} release can be the result of a collapsed $\Delta\psi$ due to mitochondrial damage. MPP^+ added to Ca^{2+}-depleted mitochondria energized by K^+-succinate does not alter $\Delta\psi$ (not shown). Ca^{2+} release as a result of damage caused by MPP^+ is therefore ruled out.

The oxidation and subsequent hydrolysis of mitochondrial pyridine nucleotides have been implicated to be important in the Ca^{2+}-release mechanism of mitochondria [8–10]. MPP^+ might be reduced in mitochondria, and 6-OHDA can auto-oxidize [15] and form H_2O_2 which is reduced in mitochondria enzymatically by NAD(P)H [8–10]. We therefore investigated MPP^+- and 6-OHDA-induced pyridine nucleotide hydrolysis

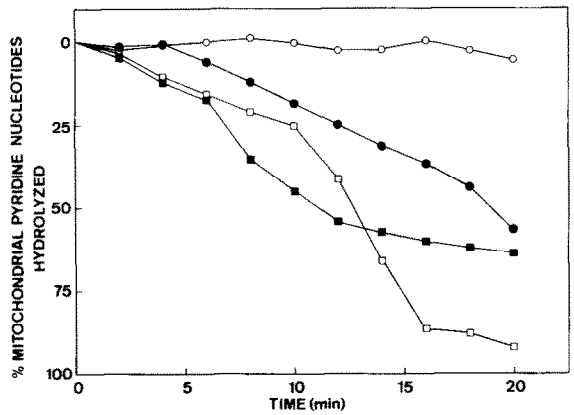

Fig.3. Hydrolysis of mitochondrial pyridine nucleotides induced by MPP^+ and 6-OHDA. Intramitochondrial pyridine nucleotides were labeled in vivo at the nicotinamide moiety [9]. Mitochondria were incubated under standard conditions and loaded with 58 nmol Ca^{2+}/mg protein. At zero time the following additions (0.5 mM) were made: MPP^+ (○); 6-OHDA (●); MPP^+ and 6-OHDA (□); MPP^+ together with 6-OHDA and EGTA (■). Release of intramitochondrial radioactivity, indicating pyridine nucleotide hydrolysis [9], was followed by Millipore filtration.

in mitochondria (fig.3). MPP^+ alone does not cause pyridine nucleotide hydrolysis in Ca^{2+}-loaded mitochondria. About 50% of pyridine nucleotides are hydrolyzed within 20 min in the presence of 6-OHDA whereas in the presence of both MPP^+ and 6-OHDA hydrolysis is almost complete at this time. In the presence of EGTA pyridine nucleotide hydrolysis induced by MPP^+ together with 6-OHDA in Ca^{2+}-loaded mitochondria is less extensive and follows a similar time course to Ca^{2+} release under identical conditions (cf. fig.2b). It is therefore likely that the MPP^+- and 6-OHDA-stimulated Ca^{2+} release takes place by a mechanism linked to pyridine nucleotide oxidation and hydrolysis [8–10].

The present results demonstrate that MPP^+ in combination with 6-OHDA or dopamine, but not alone, greatly decreases the ability of mitochondria to retain Ca^{2+}. Whether this is due to the stimulation of a specific Ca^{2+}-release mechanism or to unspecific damage of mitochondria remains unknown. Disturbance of cellular Ca^{2+} homeostasis upon release of Ca^{2+} from mitochondria can cause cell death [11,12]. Our findings provide experimental support for the hypothesis that a cocktail of oxidation products of both dopamine and MPTP are important for the development of the MPTP-induced model of Parkinson's disease and suggest that destruction of nigrostriatal cells may be due to disturbed Ca^{2+} homeostasis.

ACKNOWLEDGEMENTS

This work was supported by the Schweizerischer Nationalfonds, grant no.3.503.-083. We thank Peter Lohse and Christoph Schickli for help in some experiments.

REFERENCES

[1] Burns, R.S., Chiueh, C.C., Markey, S.P., Ebert, M.H., Jacobowitz, D.M. and Kopin, I.J. (1983) Proc. Natl. Acad. Sci. USA 80, 4546–4550.
[2] Markey, S.P., Johanessen, J.N., Chiueh, C.C., Burns, R.S. and Herkenham, M.A. (1984) Nature 311, 464–467.
[3] Langston, J.W., Irwin, I., Langston, E.B. and Forno, L.S. (1984) Neurosci. Lett. 48, 87–92.
[4] Chiba, K., Trevor, A.J. and Castagnoli, N. jr (1984) Biochem. Biophys. Res. Commun. 120, 574–578.
[5] Javitch, J.A., D'Amato, R.J., Strittmatter, S.M. and Snyder, S.H. (1985) Proc. Natl. Acad. Sci. USA 82, 2173–2177.
[6] Chiba, K., Trevor, A.J. and Castagnoli, N. jr (1985) Biochem. Biophys. Res. Commun. 128, 1228–1232.
[7] Lehninger, A.L., Vercesi, A. and Bababunmi, E.A. (1978) Proc. Natl. Acad. Sci. USA 75, 1690–1694.
[8] Lötscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 76, 4340–4344.
[9] Lötscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) J. Biol. Chem. 255, 9325–9330.
[10] Frei, B., Winterhalter, K.H. and Richter, C. (1985) Eur. J. Biochem. 149, 633–639.
[11] Jewell, S.A., Bellomo, G., Thor, H., Orrenius, S. and Smith, M.T. (1982) Science 217, 1257–1259.
[12] Bellomo, G., Jewell, S.A., Thor, H. and Orrenius, S. (1982) Proc. Natl. Acad. Sci. USA 79, 6842–6846.
[13] Klingenberg, M. and Slenczka, W. (1959) Biochem. Z. 331, 486–517.
[14] Colonna, R., Massari, S. and Azzone, G.F. (1973) Eur. J. Biochem. 34, 577–585.
[15] Sachs, C. and Jonsson, G. (1975) Biochem. Pharmacol. 24, 1–8.