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EFFECTS OF MPTP, MPP⁺ AND PARAQUAT ON MITOCHONDRIAL POTENTIAL AND OXIDATIVE STRESS

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Summary

The effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP⁺) and 1,1-dimethyl-4,4-bipyridinium (paraquat) upon the electrical potential across the plasma and mitochondrial membranes within synaptosomes has been investigated. MPTP selectively depressed plasma membrane potential while MPP⁺ specifically reduced mitochondrial potential. The structurally similar compound paraquat had no effect on either membrane potential. Enhancement of the lipid peroxidative activity with an Fe-ADP complex depressed both potentials. Paraquat effected increased peroxidative activity in brain homogenates that was less pronounced than that due to Fe-ADP. MPTP reduced basal but stimulated Fe-ADP enhanced peroxidation. The mechanisms underlying the toxicity of MPP⁺ are likely to differ from those of paraquat, primarily involving impaired mitochondrial function rather than increased oxidative stress.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been demonstrated to cause selective cytotoxicity to nigrostriatal dopaminergic neurons in man (1). Similar morphological susceptibility was subsequently reported for monkeys (2) and mice (3). However, major behavioral deficits caused by MPTP appear to be largely confined to the primates. The pathogenic mechanism of MPTP occurs by way of its biochemical transformation to 1-methyl-4-phenylpyridinium (MPP⁺), monamine oxidase B is thought to be the enzyme bringing about this oxidation by way of a series of intermediates (4). The specificity of damage appears to be due to selective accumulation of MPP⁺ by a high affinity uptake system within dopaminergic neurons (5).

There are two major hypotheses regarding mechanisms underlying the deleterious effects of MPP⁺. Parallels have been made between MPP⁺ and paraquat, which has a similar pyridinium-based structure. The toxicity of paraquat may involve redox cycling and formation of superoxide free radicals (6). MPP⁺ has been found to cause damage to the rodent lung resembling that brought about by paraquat (7). MPP⁺ and paraquat have been reported to cause similar behavioral changes in the frog (8). MPTP may cause elevation of iron-enhanced lipid peroxidation *in vitro* (9). Finally the toxicity of MPTP in mice is mitigated by a variety of antioxidants (10), and

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ascorbic acid can reduce the biochemical changes in the brain consequent to MPTP treatment (11). However, other reports suggest that production of oxidative stress is not a major outcome following exposure to MPTP or to MPP⁺ (12,13,14), and that MPP⁺ and paraquat, though structurally similar, differ greatly in redox properties (15).

A series of reports point to another locus of action of MPP⁺, namely inhibition of mitochondrial respiration (16). Such inhibition may largely involve NADH-linked mitochondrial oxidation (16) (17), and has been shown to occur in synaptosomal mitochondria (18). The susceptible enzyme within the respiratory chain may be the ubiquinone-linked NADH-cytochrome C reductase (Complex I) (19). Evidence supporting a mitochondrial site of action is that this organelle strongly accumulated MPP⁺ (17).

The studies reported here were designed to distinguish between these two concepts of MPP⁺ action. The ability of MPTP, MPP⁺ and paraquat to produce reactive oxygen species in nervous tissues has been determined under both basal and oxidative conditions. In order to further differentiate the actions of these three compounds upon tissue integrity, their effects on electric potential across the synaptosomal plasma membrane and the mitochondrial membranes were determined.

Materials and Methods

Adult male fisher rats, 4-5 months old weighing 290-330 g were decapitated, the brains quickly excised on ice and the whole brain except the cerebellum and pons-medulla dissected out. For peroxidation studies brain was homogenized to a final concentration of 5% (w/v) in 0.32M sucrose. Synaptosomes were made by the modification of Dodd et al (20) of the differential centrifugation method of Gray and Whittaker (21).

1. Membrane potential

Membrane potential was assayed by estimation of the concentration gradient of a lipophilic cation, tetraphenylphosphonium TPP⁺, that is accumulated by organelles with a potential across their external membrane (22). This method has been applied successfully to synaptosomes (23). Synaptosomes in HEPES buffer (125 mM NaCl, 5 mM KCl, 1.2 mM Na₂HPO₄, 1.2 mM MgCl₂, 5 mM NaHCO₃, 6 mM glucose, 1 mM CaCl₂, 25 mM HEPES, pH 7.4) were incubated at 37°C for 20 minutes in a 1.0 ml volume containing 120 mg synaptosomal protein, 0.2 mCi of ³H-phenyl tetraphenylphosphonium (TPP⁺) (35.5 Ci/mmol) and sufficient TPP⁺ to ensure a final concentration of 2 mM was then added and incubation continued for a further 10 min. Synaptosomes were then collected by filtration through glass-fiber filters (Type A/E, Gelman Sciences Inc), washed three times with 5 ml 0.2 M NaCl and counted. Compounds to be tested were added initially before the 20 min, preincubation as were chemicals designed to allow separation of mitochondrial and plasma membrane contributions to potential.

Plasma membrane

The difference in accumulation of label in the presence of a depolarizing concentration of KCl (0.19M) relative to that in the standard buffer (5mM) was taken as being due to the potential across the plasma membrane. Such determinations were always in the presence of 1 mM carboxyl cyanide m-chlorophenyl hydrazone (CCCP), an uncoupler of oxidative phosphorylation in order to eliminate any mitochondrial contribution to potential. By ascertaining input counts and by taking intrasynaptosomal fluid volume to be 3.6 ml/mg protein (24), it was possible to calculate the ratio of TPP⁺ concentration within synaptosomes to that remaining in the extrasynaptosomal medium, (TPP⁺_i) (TPP⁺_o)

The membrane potential was calculated as $-61 \log \frac{(TPP^+_i)}{(TPP^+_o)}$

Mitochondria

The membrane potential of mitochondria within synaptosomes was measured in the presence of 0.19M KCl in order to eliminate plasma membrane potential. Under such

circumstances, TPP^+ diffuses passively across the plasma membrane without concentration, prior to encountering mitochondria. The difference in accumulation of TPP^+ in the presence and absence of 1 mM CCCP was taken to represent mitochondrial potential. By taking a value of 12% as the proportion of synaptosomal volume occupied by mitochondria (25), it was possible to determine the concentration of TPP^+ within mitochondria. The ability of free mitochondria prepared from the P2 fraction of brain homogenates (20) to accumulate TPP^+ was estimated to be only around 2% of synaptosomal mitochondria. This is probably due to their being damaged by the preparative medium which is not designed for mitochondrial stabilization. Thus, the contribution of any contaminating free mitochondria in the synaptosomal preparation to calculated mitochondrial potential could be discounted.

2. Intrasynaptosomal calcium levels

Free intrasynaptosomal Ca^{2+} , $[\text{Ca}^{2+}]_i$, was measured using the acetoxymethyl ester of fura-2 (fura-2/AM) (27). Samples contained 140-160 mg protein and were allowed to equilibrate for 10 minutes before addition of chemicals. A correction was made for any fura-2 leaking out of the particulate fraction by centrifuging synaptosomes down and determining fluorescence in the supernatant. In order to calculate $[\text{Ca}^{2+}]_i$ before addition of toxic agents, a separate correction was made for each batch of synaptosomes. The correction was around 6% of the fluorescence at 340 nM. For calibration of the synaptosomes fura-2- Ca^{2+} signal (R), R_{min} (the ratio of fluorescence at 340 nm/380 nm in the absence of Ca^{2+}) and R_{max} the ratio when all fura-2 of the sample was saturated with Ca^{2+} were determined for each batch of fura-2 loaded synaptosomes (26). $[\text{Ca}^{2+}]_i$ was calculated using the formula of Grynkiewicz (27).

3. Lipid peroxidation

The formation of thiobarbiturate-reactive (TBA) material was used as an index of peroxidative activity. The precise method used was based on the modification of Uchiyama & Mihara (28) using 1% phosphoric acid as the protein denaturing agent. Color formation at 535 nm was determined in the supernatant with no organic solvent extraction. The molar extinction coefficient of a malonaldehyde (MDA) standard (1.56×10^5) confirmed the calibration of the Cary 210 spectrophotometer. Color formation was found to be proportional to the amount of tissue present only at low tissue concentrations. Therefore, 2% (w/v) homogenates were used for the 2 hour, 37°C incubation. The reaction was shown to be linear over this period.

4. Protein determination

The protein content of homogenates and synaptosomal suspensions were assayed with the method of Bradford (29) using bovine serum albumin as a reference.

5. Chemicals

MPTP-HCl and MPP⁺ were from Research Biochemicals, Inc. (Wayland, MA), TPP^+ was from Aldrich Co. (Milwaukee, WI) and fura-2/AM was obtained from Molecular Probes (Junction City, OR) and $^{45}\text{CaCl}_2$ and [phenyl- ^3H] TPP^+ bromide from New England Nuclear (Amherst, MA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

6. Statistics

Results were analyzed using Fisher's Least Significant Difference Test after one-way analysis of variance. Throughout the results, the symbol (*) means $P < 0.05$ or lower using a two-tailed t distribution.

Results

1. Formation of thiobarbituric acid-reactive material

In view of the key role of transition metals of more than one valency state in enabling the formation of active oxygen species (30), studies were conducted both in the presence and absence of a superoxide-promoting $200 \mu\text{M}$ FeSO_4 - 1 mM Adenosine diphosphate (ADP) chelate (31). Under basal conditions paraquat effected a minor, but significant, increase in peroxidative activity within brain homogenates (FIG. 1A).

FIG. 1

Formation of TBA-reactive products in a 2% (w/v) brain homogenate. Added reagents were present at $5 \times 10^{-3} \text{M}$. Data derived from 6-7 individual determinations $\pm$ SE. *: value differs significantly from control ($p < 0.05$). Parallel incubations were carried out in the absence (FIG. 1A) or presence (FIG. 1B) of $2 \times 10^{-4} \text{M}$ FeSO_4 and 10^{-3}M ADP.

Surprisingly MPTP caused a large reduction of TBA-reactive materials formed during a two-hour incubation. This reduction was not spurious since MPTP did not interfere with color formation between thiobarbituric acid and malonaldehyde. A similar reduction of peroxidative activity by MPTP was found using a synaptosomal suspension (data not shown). In the presence of Fe-ADP, the formation of TBA-reactive material was elevated over 4-fold (FIG. 1B). MPTP stimulated lipid peroxidation under these same conditions, in contrast to its inhibition of basal peroxidation. Both enhancement and inhibition of peroxidative activity have been previously reported for MPTP (10). The effect of paraquat was more pronounced in the presence of Fe-ADP, as described by Sata (31) while MPP^+ had no significant effect on lipid peroxidation in either the presence nor the absence of Fe-ADP. The use of millimolar concentrations of MPP^+ is validated by parallel concentrations reached *in vivo* within neurons accumulating this material (32), and permits our data to be related to those of several other groups using this concentration (14,19,33).

2. Membrane potential

The overall capacity of synaptosomes to accumulate the lipophilic cationic TPP⁺ was initially examined. Both MPTP and MPP⁺, but not paraquat decreased the synaptosomal concentration of TPP⁺ (FIG. 2).

FIG. 2

Accumulation of TPP⁺ by synaptosomes pre-incubated for 20 min at 37°C with various agents present at 10⁻³ M, except iproniazid (INZ) (1.5×10⁻³M). Data derived from 3 individual determinations ±SE *:value differs from control (P<0.05). Mean concentration of accumulated TPP⁺ was 61 nmoles/mg synaptosomal protein.

The effect of MPTP was not abolished in the presence of 10⁻³M iproniazid, an inhibitor of monoamine oxidases A and B (34), suggesting that conversion to MPP⁺, was not essential for this reduction of TPP⁺ uptake.

Subsequent studies involved the pharmacological dissection of this uptake into mitochondrial and plasma membrane components. The resting potential across the synaptosomal outer limiting membrane was 105±2mV (FIG. 3A).

A.

FIG. 3

Response of synaptosomal plasma membrane potential (FIG. 3A) or mitochondrial potential (FIG. 3B) incubated with synaptosomes at 37°C for 20 min. prior to the addition of TPP⁺. All compounds were present at a final concentration of 10⁻³M except FeSO₄ (0.2x10⁻⁴). Data derived from 3-7 individual preparations ± SE *:value differs from corresponding control (p<0.05).

This value was reduced in the presence of MPTP. MPP⁺ and paraquat had no significant effect but the peroxidizing environment resulting from Fe-ADP produced a dramatic fall in potential, perhaps due to generalized membrane damage.

Mitochondrial transmembrane potential was 158±3mV (FIG. 3B). This was decreased by MPP⁺ but not by MPTP or paraquat. Thus the depolarizing influence of MPP⁺ was confined to mitochondria while the corresponding effect of MPTP was seen only at the plasma membrane.

3. Ionic calcium within synaptosomes

The responses of synaptosomal levels of free calcium to the agents under study were determined in an attempt to differentiate between plasma membrane and mitochondrial effects. Use of calcium channel blockers, low levels of extracellular calcium and mitochondrial inhibitors has allowed sites of damage within the synaptosome to be distinguished (35,36). The resting level of free calcium within synaptosomes, [Ca²⁺]_i was 319±8 nm (n=34). MPTP, MPP⁺ and paraquat at concentrations between 50 and 2500 mM led to no significant change in this basal level (data not shown).

Discussion

It is believed that the neurotoxicity of MPTP is a result of its biotransformation to the toxic metabolite MPP⁺ (37). MPP⁺ is structurally-related to the compound paraquat, and it has been postulated that the two substances might have similar mechanisms of action. In the present study we have examined the effects of MPTP, MPP⁺ and paraquat on the production of reactive oxygen species in nervous tissue. We have also investigated the effects of these three compounds on both the electrical potential across the synaptosomal plasma and mitochondrial membranes to further distinguish possible modes of action.

Our results demonstrate that neither MPTP or MPP⁺ stimulated lipid peroxidation under basal conditions. Only when non-physiological concentrations of Fe²⁺ were present, did MPTP enhance peroxidation. The effect of paraquat on the formation of TBA-reactive material was minor relative to the effect of Fe-ADP. This is concordant with the finding that paraquat caused little

peroxidation *in vivo* or *in vitro* (38). Since paraquat has also been reported as powerfully catalyzing peroxidation in hepatocytes (14) its mechanism of causing toxic damage remains uncertain.

The depression of plasma membrane potential by MPTP may be related to the uncharged lipophilic nature of this molecule which is in contrast to both MPP⁺ and paraquat. Thus accumulation into lipid-rich areas rather than active transport would largely determine its distribution. The observation that isoniazid, an inhibitor of monoamine oxidase A and B (32), does not inhibit the MPTP induced uptake of TPP⁺ by synaptosomes might indicate that MPTP does not require transformation to MPP⁺ to elicit this depression of plasma membrane potential.

MPP⁺ depresses mitochondrial potential but causes no peroxidative changes under conditions where paraquat enhances peroxidation but does not influence membrane potential. The effects of MPP⁺ then, differ from those of the structurally similar paraquat. The depression of mitochondrial potential by MPP⁺ is in accord with its selective uptake by mitochondria (17,39) and its subsequent ability to disturb enzymes critical for electron transport (16,19). Failure of MPP⁺ to promote peroxidation has been previously described (14,15).

The peroxidative damage caused by Fe-ADP severely and nonspecifically affected both mitochondrial and plasma membrane potentials of synaptosomes whereas MPP⁺ and MPTP effects were confined to the mitochondrion and plasma membrane respectively. Since MPP⁺ did not produce oxidative stress under any of the conditions used here and MPTP is unlikely to be bioconcentrated without being further metabolized, the toxicity of these compounds is probably not primarily related to oxidative damage.

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