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Evidence that the blockade of mitochondrial respiration by the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) involves binding at the same site as the respiratory inhibitor, rotenone

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SUMMARY. It has been postulated that 1-methyl-4-phenylpyridinium (MPP^+) blocks mitochondrial respiration by combining at the same site as rotenone, a potent inhibitor of NADH oxidation in mitochondria, known to act at the junction of NADH dehydrogenase and coenzyme Q (CoQ). The present experiments show that MPP^+ and two of its analogs indeed act in a concentration dependent manner to prevent the binding of [^{14}C]-rotenone to submitochondrial particles (ETP) and significantly decrease the inhibition of electron transport caused by rotenone. It therefore appears that MPP^+ binds at the same site as rotenone or an adjacent site, supporting the hypothesis that its neurotoxic action is due to the inhibition of mitochondrial respiration.

Expression of the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is initiated by its 2-step oxidation in the central nervous system to the dihydropyridinium ($MPDP^+$) and pyridinium (MPP^+) species, catalyzed by monoamine oxidase (1-5). The synaptic dopamine reuptake system then pumps MPP^+ into the nigrostriatal neurons (6), where it leads to rapid destruction of nigrostriatal cells, consequent loss of dopamine production, and Parkinsonian symptoms.

The mechanism of cell destruction by MPP^+, while not fully understood, appears to result from the inhibition of mitochondrial respiration (7,8). MPP^+, in common with many lipophilic cations (9), is concentrated in the mitochondria in response to the electrical gradient (10), resulting in a high matrix

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concentration, which inhibits NADH dehydrogenase (11-14). The inhibition of respiration results in loss of oxidative phosphorylation, so that ATP levels fall rapidly and cell death ensues. Evidence for this mechanism of nigrostriatal destruction has come from lactate accumulation in striatal slices in response to MPP^+ and on stereotaxic administration of MPP^+ to the nigrostratium of rodents (15), from the protection of dopaminergic neurons in cell culture against MPP^+ by barbiturates (16), and from mitochondrial abnormalities found in the neurons of MPTP-treated monkeys (17) and in humans with idiopathic Parkinson's disease (18,19).

The hypothesis that MPP^+ and its analogs inhibit NADH oxidation by combining at the same site as the classical respiratory inhibitors, namely barbiturates, rotenone, and piericidin A, rests on two types of indirect evidence.First, both the classical inhibitors and MPP^+ and its analogs inhibit only the membrane-bound form of the enzyme (e.g. Complex I), but not the fully active, soluble enzyme. This may suggest that some peptide or lipid component in the membrane, left behind on extraction of the enzyme (20), may be required for binding all these inhibitors. Second, electron spin resonance studies and assays with site-specific dyes show that neither class of inhibitors affects the reduction of the FMN moiety or of the 4 iron-sulfur clusters of the enzyme (21), suggesting that their non-covalent binding occurs between the highest potential iron-sulfur cluster and CoQ. Direct evidence that MPP^+ and other cationic pyridine derivatives bind at the same site as rotenone, barbiturates, and piericidins (or in the immediate vicinity), is provided by the radioligand binding and enzyme inhibition experiments reported below.

MATERIALS AND METHODS

ETP (a mitochondrial inner membrane preparation) was isolated (22) and its NADH oxidase activity assayed spectrophotometrically (23). The sources of MPP^+, its analogs (14) and [^{14}C]-rotenone (24) were as in our previous work. The latter was purified on a silica gel HPLC column to radiochemical purity and had a specific activity of 6.6 mCi/mmol. ETP (3.1 mg protein/ml) was suspended in 7.5 ml of 0.25 M sucrose - 0.05 M K-phosphate, pH 7.6, containing 2% (w/v) bovine serum albumin (BSA) ± 10 mM MPP^+, and incubated for 10 min at 30°C. Aliquots were removed for initial NADH oxidase assay. To the samples, with and without MPP^+, [^{14}C]-rotenone was then added to 0.625 μM concentration and incubated for 10 min at 30°C. Aliquots removed at this time for assay were the "0 wash" in the Figures. The tubes were then centrifuged for 30 min at

146,000 x *g* and the pellets were resuspended by homogenization in sucrose-phosphate BSA, with or without 10 mM MPP^+, to the original volume, incubated for 30 min at 0°C, and then centrifuged as before. Activity measurements were carried out on 5 μl aliquots diluted to 1 ml, taken immediately prior to centrifugation, while the rotenone remaining bound to the particles at that point was determined by removing 0.5 ml aliquots immediately after resuspending the centrifuged pellet.

RESULTS AND DISCUSSION

Pre-incubation of submitochondrial particles with 10 mM MPP^+ significantly decreases the inhibition of NADH oxidase activity by rotenone and subsequent washing by resuspension in sucrose-phosphate-BSA results in a much greater recovery of activity in the MPP^+-treated sample (Fig. 1A). Aliquots taken from the samples in the experiment of Fig. 1A for radioactivity measurement show the partial prevention of [^{14}C]-rotenone binding by MPP^+ (Fig. 1B). It is known from previous work that 2 mols of rotenone are bound per mol of NADH dehydrogenase at the "specific site", i.e., the site where inhibition of electron transport from NADH dehydrogenase to CoQ occurs, in addition to extensive unspecific binding (24,25). Virtually all the unspecifically bound rotenone is removed by 2 washes with sucrose-phosphate-BSA, whereas the rotenone bound at the specific site dissociates more gradually. In the

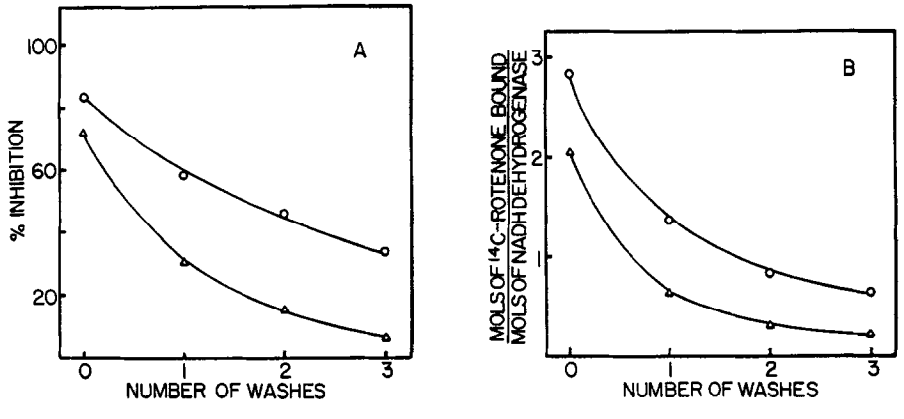

Fig. 1. **A) Inhibition of the NADH oxidase activity of submitochondrial particles (ETP)** by 0.625 μM [^{14}C] rotenone (open circles) and the effect of pre-incubation of the particles with 10 mM MPP^+ on the inhibition by rotenone (triangles). Experimental conditions were as described in the text. The abscissa denotes the number of resuspensions in sucrose-phosphate-BSA buffer and centrifugations to remove unspecifically bound rotenone. In accord with previous work (24) this treatment also causes partial dissociation of rotenone from the specific site and consequent return of activity. **B) Effect of MPP^+ on the binding of [^{14}C]-rotenone in the same experiment.** After 2 washes all bound rotenone is at the specific site responsible for the inhibition.

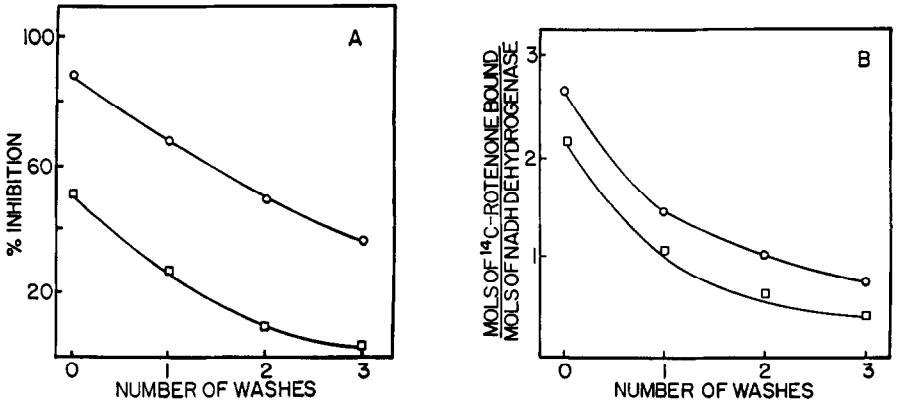

Fig. 2. **Effect of an MPP^+ analog on the inhibition of NADH oxidase activity by rotenone (2A) and on the amount of [^{14}C]-rotenone bound to the particles (2B).** Experimental conditions were the same as in Fig. 1. Symbols: open circles, rotenone alone; squares, pre-incubated and washed with 5 mM 1,1-dimethyl-4-phenyl-tetrahydropyridine; triangles, pre-incubated with 5 mM 1-methyl-4-phenylpyrimidinium.

MPP^+ experiments, the wash fluid contained 10 mM MPP^+, to prevent rotenone dissociated from non-specific sites from rebinding at the specific site. It should be noted that the inhibition measured here is due to rotenone itself, since this inhibitor is tightly bound at the specific site and does not dissociate during assay, while the inhibition due to MPP^+ and its analogs is fully reversed on the 200-fold dilution of aliquots taken for activity measurement in the assay mixture (21)

Protection against the inhibition of NADH dehydrogenase by rotenone and prevention of [^{14}C]-rotenone binding have also been demonstrated with a series of MPP^+ analogs of known IC_{50} values (14), such as 1,1-dimethyl-4-phenyltetrahydropyridine, 1-methyl-4-phenylpyrimidinium, as well as some novel, recently synthesized (26) 4'-substituted MPP^+ analogs, which are powerful inhibitors of NADH oxidation in mitochondria and ETP. A typical experiment is illustrated for only one compound in Fig. 2. The protective effect of 1,1-dimethyl-4-phenyl-tetrahydropyridine against inhibition by rotenone and against the binding of [C^{14}] rotenone are comparable, particularly if one considers evidence in the literature (25) that the 2 binding sites of rotenone in Complex I contribute unequally to inhibition when occupied by rotenone or pieridin A. Detailed data on the protective effect of various MPP^+ analogs will be reported elsewhere.

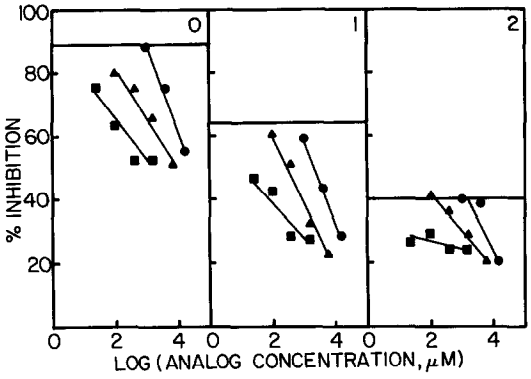

Fig. 3. Concentration dependence of the protection of NADH dehydrogenase by MPP^+ and its analogs from inhibition by rotenone. The panels, left to right, represent residual inhibition after rotenone treatment and resuspension (no wash), after 1 wash, and after 2 washes with sucrose-phosphate-BSA in the presence of the stated concentration of MPP^+ analog. Symbols: solid circles, MPP^+; triangles, 4'-Me-MPP^+; squares, 4'-n-propyl-MPP^+. The horizontal line represents residual rotenone inhibition after each wash with no pyridinium compound present.

The concentration dependence of the protection has been studied with MPP^+ and the 4'-methyl and 4'-n-propyl analogs. Fig. 3 illustrates the strong concentration dependence of the prevention of rotenone inhibition and, hence, suggests that MPP^+ and its analogs compete for the rotenone binding site, much like piericidins and bartiburates. The concentration dependence of the protective effect of the 3 compounds is in satisfactory agreement with their IC_{50} values for inhibition of NADH oxidation of ETP (2.5 to 4 mM for MPP^+, 0.4 to 0.7 mM for 4'-Me-MPP^+, 0.2 mM for 4'-n-propyl MPP^+). A linear correlation between the two values is not necessarily expected, since the IC_{50} is a kinetic parameter, whereas the data in Fig. 2 are thermodynamic parameters.

The results demonstrate that MPP^+ and its analogs protect NADH dehydrogenase from rotenone inhibition and appear to bind very near to or at the same site. This site is well established to be also the binding site of barbiturates, such as amytal, and the very potent NADH oxidase inhibitor piericidin A (24,25). It is not known how the reaction of these inhibitors at this site blocks the reoxidation of NADH dehydrogenase by CoQ. These studies, nevertheless, provide additional evidence for the hypothesis (11) that nigrostriatal cell death is the consequence of inhibition in the Complex I region of the respiratory chain.

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