Evidence that the inhibition sites of the neurotoxic amine 1-methyl-4-phenylpyridinium (MPP⁺) and of the respiratory chain inhibitor piericidin A are the same

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1-Methyl-4-phenylpyridinium (MPP⁺), the neurotoxic bioactivation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), interrupts mitochondrial electron transfer at the NADH dehydrogenase–ubiquinone junction, as do the respiratory chain inhibitors rotenone, piericidin A and barbiturates. Proof that these classical respiratory chain inhibitors and MPP⁺ react at the same site in the complex NADH dehydrogenase molecule has been difficult to obtain because none of these compounds bind covalently to the target. The 4'-alkyl derivatives of MPP⁺ inhibit NADH oxidation in submitochondrial particles at much lower concentrations than does MPP⁺ itself, but still dissociate on washing the membrane preparations, with consequent re-activation of the enzyme. The MPP⁺ analogues with short alkyl chains prevent the binding of [¹⁴C]-labelled piericidin A to the membrane and thus must act at the same site, but analogues with alkyl chains longer than heptyl do not prevent binding of [¹⁴C]piericidin.

INTRODUCTION

Sustained interest in many laboratories in the mechanism of action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) stems from the fact that it is neurotoxic to primates and certain other species, producing Parkinsonian symptoms in humans. The toxic form is the oxidation product, 1-methyl-4-phenylpyridinium (MPP⁺), produced by the action of monoamine oxidase B in brain and accumulated in dopaminergic neurons by the dopamine re-uptake system. Cell death probably ensues from ATP depletion, initiated by the inhibition of mitochondrial respiration on NAD⁺-linked substrates [1–3]. Support for this 'mitochondrial' hypothesis has come from the demonstration [4] that the pyridinium oxidation products of all neurotoxic MPTP analogues inhibit mitochondrial respiration as well as NADH oxidation in inverted mitochondrial membranes. Further evidence for the mitochondrial hypothesis has come from the protective effect of barbiturates against the toxicity of MPP⁺ to dopaminergic neurons in culture [5], from mitochondrial abnormalities in MPTP-treated monkeys, and from reports of Complex I deficiency in idiopathic Parkinsonian patients [6,7].

It was nevertheless desirable to obtain more direct evidence that the reaction site of MPP⁺ is as postulated, i.e. on the high-potential end of NADH dehydrogenase, where ubiquinone (Q) reacts. The localization was based on e.p.r. experiments [8] analogous to earlier work [9], showing that rotenone, piericidin A and barbiturates block NADH oxidase between NADH dehydrogenase and Q. It was also known that piericidin A, rotenone and barbiturates bind at the same site in mitochondria in a competitive manner [10]. None of these inhibitors bind covalently to the inhibitor site, although the non-covalent bonds are tight enough in the case of piericidin A and rotenone that they do not dissociate on dilution, in contrast with MPP⁺ [8].

The recent availability of 4'-alkyl-substituted MPP⁺ analogues with IC₅₀ values (concentrations causing 50 % inhibition) for NADH oxidation in the micromolar range [11,12], which nevertheless dissociate readily and can be removed by centrifugation of membrane preparations, resulting in regeneration of NADH oxidase activity, provided the opportunity to examine whether these tightly bound analogues protect NADH dehydrogenase from inhibition by the binding of piericidin A and rotenone. We have briefly reported [13] that MPP⁺ and certain of its analogues protect NADH dehydrogenase in inverted inner membranes from inhibition by rotenone in a concentration-dependent manner and concurrently prevent the binding of rotenone to the enzyme. The limitation of these studies was that rotenone dissociates partially from the site responsible for inhibition on washing of the inhibited particles, which is necessary to remove spuriously bound rotenone, as well as the MPP⁺ analogue. We have extended this experiment to piericidin A, which does not dissociate on washing of the particles by centrifugation [10] and thus permits quantitative comparison of the prevention of the binding of and inhibition due to piericidin A by the MPP⁺ derivatives.

EXPERIMENTAL

Electron transport particles (ETP) from bovine heart mitochondria were prepared according to Crane et al. [14], and NADH oxidase activity was measured spectrophotometrically [15]. MPP⁺ and its 4'-substituted analogues, as well as 4'-pentyl-4-phenylpyridine, were synthesized as described elsewhere [11]. [¹⁴C]Piericidin A (specific radioactivity 0.8 Ci/mol) was the sample used in our previous work [10] and was shown to be chemically and radiochemically pure by co-chromatography on t.l.c. with an authentic synthetic sample kindly provided by Dr. N. Takahashi (University of Tokyo, Tokyo, Japan).

Abbreviations used: ETP, electron transport particles (inverted inner membrane preparation); MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP⁺, 1-methyl-4-phenylpyridinium species; TPB⁺, tetraphenylboron anion; IC₅₀, concentration resulting in half-maximal inhibition.

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Table 1. Protection by 4'-alkyl-MPP⁺ analogues of membrane-bound NADH dehydrogenase from inactivation by piericidin A

ETP (2 mg/ml in 0.25 m-sucrose/0.05 m-phosphate, pH 7.4), 1 mM-MPP⁺ analogue, 10 μM-TPB⁻, 0.24 μM-piericidin A and 2 mM-NADH in a volume of 0.2 ml were incubated for 10 min at 30 °C, and then diluted to 1 ml with 0.25 m-sucrose/0.05 m-phosphate/2% BSA containing the MPP⁺ analogue and chilled on ice for 10 min. The samples were then centrifuged and washed once, as described in the Experimental section, and NADH oxidase activity was determined. Parallel samples were incubated without piericidin to determine residual inhibition due to the MPP⁺ analogue. Values are the averages of two assays.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration (mM)</th>
<th>NADH oxidase activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>8.8</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1</td>
<td>8.8</td>
</tr>
<tr>
<td>4'-Methyl-MPP⁺</td>
<td>1</td>
<td>10.0</td>
</tr>
<tr>
<td>4'-Propyl-MPP⁺</td>
<td>1</td>
<td>11.7</td>
</tr>
<tr>
<td>4'-t-Butyl-MPP⁺</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>4'-Pentyl-MPP⁺</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>4'-Heptyl-MPP⁺</td>
<td>0.02</td>
<td>44.1</td>
</tr>
<tr>
<td>4'-Heptyl-MPP⁺</td>
<td>0.003</td>
<td>0 (3*)</td>
</tr>
</tbody>
</table>

*After two additional washes.

RESULTS AND DISCUSSION

We have reported [11,12] that 4'-alkyl-substituted analogues of MPP⁺ are potent inhibitors of mitochondrial respiration on NAD⁺-linked substrates and of NADH oxidation in submitochondrial particles. TPB⁻ greatly increases the inhibitory power of these compounds, as measured by the IC₅₀ values, facilitating their penetration to the hydrophobic binding site on NADH dehydrogenase by ion-pairing [16,17]. Since 4'-alkyl-substituted 4-phenylpyridine analogues are neutral, TPB⁻ does not affect their inhibitory potency. TPB⁻ also does not lower the IC₅₀ values of MPP⁺ analogues with a long alky residue, such as 4'-heptyl- and 4'-decyl-MPP⁺ (M. R. Gluck, S. K. Youngster, M. J. Krueger, R. R. Ramsay & T. P. Singer, unpublished work). The IC₅₀ values (in the presence of 10 μM-TPB⁻) of the MPP⁺ analogues used in the present study were as follows: MPP⁺, 500 μM; 4'-methyl-MPP⁺, 150 μM; 4'-propyl-MPP⁺, 24 μM; 4'-pentyl-MPP⁺, 14 μM; 4'-t-butyl-MPP⁺, 2 μM; 4'-heptyl-MPP⁺, 6 μM; 4'-pentyl-4-phenylpyridine, 2.5 μM.

From these values one would expect that MPP⁺ analogues bearing a 3-carbon or longer alkyl chain in the 4'-position might protect NADH dehydrogenase from inhibition by piericidin A. This expectation was realized to a large extent, as shown by Table 1 and Figs. 1 and 2. In the experiment of Table 1, a low concentration of piericidin A was used, just sufficient to yield about 90% inhibition in the absence of protective agents, whereas the concentration of MPP⁺ analogues was relatively high (1 mM). Under these conditions, MPP⁺ and 4'-methyl-MPP⁺ afforded little protection, whereas the propyl, pentyl, and t-butyl analogues protected the enzyme extensively. t-Butyl-MPP⁺, in fact, protected the activity completely under these conditions. This is quite remarkable, considering that piericidin A is the most powerful inhibitor of NADH oxidation known, combining with the enzyme stoichiometrically. 4'-Heptyl-MPP⁺ not only failed to protect the enzyme against inhibition but actually lowered the activity below that seen in the piericidin A sample (Table 1). The reason for this is that the heptyl analogue is not removed readily by the single wash with sucrose/phosphate/BSA. At the high concentration used in this experiment (167 times the IC₅₀ value), 4'-heptyl-MPP⁺ by itself caused 94% inhibition, even after washing.

The experiment of Fig. 1 differed in several respects from that in Table 1. First, washing by centrifugation and resuspension in the absence of the 4'-analogue was performed three times, so as to remove most of the highly hydrophobic heptyl and t-butyl analogues and thus minimize residual inhibition by these compounds. Secondly, advantage was taken of the observation that, as in the case of rotenone [13], protection of the enzyme from piericidin A by MPP⁺ analogues is concentration-dependent. Therefore the ratio of MPP⁺ analogue to piericidin A was lowered in order to demonstrate the relative effectiveness of different compounds. Fig. 1 shows that at a concentration of 32 times the IC₅₀ value, 4'-pentyl-MPP⁺ was the most effective, followed by 4'-propyl-MPP⁺ and 4'-methyl-MPP⁺ at concentrations of 63 times and 67 times their IC₅₀ values respectively. The 4'-t-butyl analogue at 30 times its IC₅₀ was much less effective, and 4'-heptyl-MPP⁺ at 5 times the IC₅₀ seemed unable to protect the enzyme. The concentration of this analogue
1-Methyl-4-phenylpyridinium analogues bind at the piericidin site in NADH dehydrogenase

was kept low so as to minimize its own inhibitory effect. Even so, it lowered the residual activity from the 7% of the original control observed with piericidin A alone to virtually zero.

Fig. 1 shows the correlation between the activity protected and the decrease in binding of [14C]piericidin to the specific site. In the absence of MPP+ analogues, 1.7 mol of piericidin A was specifically bound per mol of NADH dehydrogenase. The enzyme concentration was calculated from the NADH ferricyanide activity, in comparison with the turnover number of the highly purified enzyme [18], as in previous work [19]. The data in Fig. 1 show that the 4'-methyl, 4'-propyl and 4'-heptyl analogues prevented the binding of piericidin A and its inhibitory effect to nearly the same extent. In the case of the two highly hydrophobic MPP+ derivatives, limited prevention of binding seemed to exceed protection from inactivation somewhat, but this was due entirely to residual inhibition by these two analogues. When corrected for their inhibitory effect, the two parameters measured were nearly equal. The IC_{50} values for the inhibitory effect of MPP+ analogues on NADH dehydrogenase summarized above do not show a quantitative relationship with their potential protective effect against piericidin A. This is to be expected, of course, from the fact that the former is a kinetic parameter and the latter measures an equilibrium. However, the tightly bound 4'-heptyl analogue (IC_{50} = 6 µM in the presence of TPB+) is virtually ineffective as a protective agent. Even more striking is the fact that under the same experimental conditions with both analogues present at 50 times the IC_{50} 4'-pentyl-MPP+ prevented the inhibition by piericidin A extensively, whereas 4'-pentyl-4-phenylpyridine had no effect (Fig. 2). It seems that, perhaps because of their hydrophobicity, 4'-heptyl-MPP+ and 4'-pentyl-4-phenylpyridine are bound differently in the membrane from the shorter-chain MPP+ analogues. In line with this, the curves relating NADH oxidase activity to analogue concentration are sigmoidal, suggestive of two co-operative sites, for the shorter-chain 4'-MPP+ analogues, but hyperbolic for 4'-heptyl-MPP+ (M. R. Gluck, S. K. Youngster, M. J. Krueger, R. R. Ramsay & T. P. Singer, unpublished work).

Although other interpretations cannot be excluded, the simplest conclusion is that MPP+ and its analogues bind at or near the 'rotenone site' on NADH dehydrogenase, i.e. the region where piericidin A and barbiturates bind and block re-oxidation of the enzyme [9,10].

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REFERENCES


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