Inhibition of Complex I by hydrophobic analogues of *N*-methyl-4-phenylpyridinium (MPP^+) and the use of an ion-selective electrode to measure their accumulation by mitochondria and electron-transport particles

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N-Methyl-4-phenylpyridinium (MPP^+), the neurotoxic metabolite of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, kills dopaminergic neurons after its accumulation in mitochondria where it inhibits Complex I of the respiratory chain. MPP^+ inhibits respiration by binding to both a hydrophobic and a hydrophilic site on Complex I and this inhibition is increased by the lipophilic tetraphenylboron anion (TPB^-) which facilitates movement of MPP^+ through membranes and its penetration to the hydrophobic binding site on Complex I. To investigate the inhibition of respiration by MPP^+-like compounds, we have measured simultaneously NADH-linked mitochondrial respiration and the uptake and accumulation of the *N*-benzyl-4-styrylpyridinium and *N*-ethyl-4-styrylpyridinium cations in mitochondria using ion-selective electrodes. The data provide direct evidence that TPB^- increases the inhibition not by increasing matrix concentration but by facilitating access to the inhibitory sites on Complex I. We have also compared the rates of uptake of MPP^+ analogues of varied lipophilicity by the inner membrane and the development of inhibition of NADH oxidation, using an inverted mitochondrial inner membrane preparation and appropriate ion-selective electrodes. These experiments demonstrated that the amount of MPP^+ analogue bound to the inner membrane greatly exceeded the quantity required for complete inhibition of NADH oxidation. Moreover, binding to the membrane occurred much more rapidly than the development of inhibition with all MPP^+ analogues tested. This suggests that the attainment of a correct orientation of these compounds within the membrane and the binding site may be a rate-limiting step in the development of inhibition.

INTRODUCTION

Administration of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to higher primates leads to the selective killing of dopaminergic neurons in the substantia nigra and a syndrome similar to idiopathic Parkinson's disease [for a recent review, see Tipton and Singer (1993)]. MPTP is converted into *N*-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B in the glial cells of the substantia nigra (Chiba et al., 1984; Salach et al., 1984) and this is accumulated by dopaminergic neurons via the dopamine-reuptake system (Javitch et al., 1985). Inside neurons MPP^+ is further accumulated in mitochondria by diffusion of the lipophilic cation through the mitochondrial inner membrane, driven by the membrane potential (Ramsay et al., 1986; Ramsay and Singer, 1986; Hoppel et al., 1987). Ultimately MPP^+ inhibits electron transport via Complex I leading to ATP depletion and cell death (Nicklas et al., 1985; Ramsay et al., 1986). This inhibition is thought to involve binding of MPP^+ to the two rotenone/piericidin-binding sites on Complex I (Krueger et al., 1993; Gluck et al., 1994a). One of these sites is accessible to hydrophilic compounds and MPP^+ binds to this site rapidly. The other hydrophobic site is less accessible to hydrophilic compounds and MPP^+ binds to this site relatively slowly, suggesting that it may be buried within the lipid bilayer. The lipophilic anion tetraphenylboron (TPB^-) potentiates the inhibition of respiration by MPP^+ and its analogues by strong ion-pairing with these cations, thereby accelerating their accumulation in the mitochondrial matrix (Aiuichi et al., 1988; Ramsay et al., 1989ab; Heikkila et al., 1990). TPB^- also stimulates the inhibition of respiration by MPP^+ in electron-transfer particles (ETPs) by facilitating its diffusion to the hydrophobic inhibitory site on Complex I (Ramsay et al., 1989b; Krueger et al., 1993; Gluck et al., 1994a). Both of these actions contribute to its marked effect in enhancing the potency of charged MPP^+ analogues as inhibitors of mitochondrial respiration.

In an effort to define how the inhibitory power of MPP^+-like compounds is determined by their accumulation in mitochondria and by their penetration to the hydrophobic inhibitory site on Complex I, we took advantage of the availability of several *N*-methyl-4-styrylpyridiniums and 4′-alkyl-substituted MPP^+ analogues. Because of their greater hydrophobicity than MPP^+, these compounds are more effective inhibitors of NADH oxidation in both mitochondria and mitochondrial inner-membrane preparations (Ramsay et al., 1989a, 1991; M. J. Kreuger, S. O. Sablin, M. Gluck, W. J. Nicklas, S. O. Bacharin, R. R. Ramsay and T. P. Singer, unpublished work). In the present study we have measured the uptake into mitochondria and inhibition of NADH-linked respiration, with and without TPB^-, for the *N*-benzyl-4-styrylpyridinium and *N*-ethyl-4-styrylpyridinium cations. To determine their rate of uptake and level of accumulation in energized mitochondria, we used an ion-selective electrode system originally developed to measure the accumulation of MPP^+ in mitochondria (Aiuichi et al., 1992; Davey et al., 1992, 1993). These electrodes allow the continuous measurement of uptake of MPP^+ analogues into mitochondria and obviate the need to synthesize radiolabelled compounds and measure their accumu-

Abbreviations used: ETP, electron-transport particle (inverted mitochondrial inner-membrane preparation); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MPP^+, *N*-methyl-4-phenylpyridinium; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TPB^-, tetraphenylboron anion.

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lation by rapid sampling. As these compounds are more hydrophobic than MPP^+, the corresponding electrodes were more sensitive and gave more stable recordings with less noise than MPP^+ electrodes. In a further set of experiments we used these electrodes, in conjunction with an oxygen electrode, to measure simultaneously mitochondrial respiration on NADH-linked substrates and accumulation of these compounds in mitochondria (Davey et al., 1993). Because of the sensitivity of these electrodes and the low concentration of these cations required to inhibit Complex I we were able to measure their uptake into mitochondria and correlate uptake with inhibition of NADH-linked respiration. This is not possible with MPP^+ itself because of the high concentration required to block respiration, the slow onset of inhibition of respiration in the absence of TPB^- and the relative insensitivity of MPP^+ electrodes. We have also compared rate and extent of binding of these *N*-alkyl-4-styryl-pyridiniums and 4′-heptyl-MPP^+ in inverted inner membranes with the development of inhibition. From these experiments we have arrived at some important conclusions about the interaction of MPP^+ analogues with Complex I.

MATERIALS AND METHODS

Preparation of mitochondria and ETPs

Fed female Wistar-derived rats (200–300 g) maintained on a standard diet were killed by cervical dislocation after stunning. Liver mitochondria were prepared by homogenization followed by differential centrifugation (Chappell and Hansford, 1972) in ice-cold medium containing 250 mM sucrose, 5 mM Tris and 1 mM EGTA adjusted to pH 7.4 (with HCl) at 25 °C. Mitochondrial protein content was determined by the biuret method with BSA as standard (Gornall et al., 1949). Mitochondria were stored on ice at 40–60 mg of protein/ml and used within 4 h of preparation. ETPs were prepared from bovine heart mitochondria by the method of Crane et al. (1956).

Preparation of electrodes and experimental design

Electrodes selective for *N*-ethyl-4-styrylpyridinium and *N*-benzyl-4-styrylpyridinium were prepared as described previously for MPP^+-selective electrodes (Aiuichi et al., 1992; Davey et al., 1992, 1993). An ion-selective membrane was constructed by dissolving TPB^- in a solution of poly(vinyl chloride) in tetrahydrofuran and dioctyl phthalate. The tetrahydrofuran was evaporated to form a plastic membrane, a section of which was glued to a poly(vinyl chloride) tube using tetrahydrofuran. A platinum wire connected to a coaxial cable was inserted into the electrode case and an Ag/AgCl reference electrode completed the cell. The electrode was made sensitive to the particular cation by soaking overnight in a solution of the iodide salt of the compound. For the mitochondrial experiments the concentration of these solutions was 10 mM and for the ETP experiments the concentrations were 5 mM for *N*-ethyl-4-styrylpyridinium, 2 mM for *N*-benzyl-4-styrylpyridinium and 145 μM for 4′-heptyl-MPP^+. These electrodes are all sensitive to concentrations below 1 μM (the heptyl to 0.25 μM) but perfect linearity of electrode output against log concentration ceased below 1 μM. The three electrodes used for experiments with ETPs gave linear plots of voltage against log concentration with a slope of 58 mV/decade, and the electrodes used for the mitochondrial experiments gave slopes of 56–61 mV/decade. These plots were used to calibrate the electrode (e.g. Figure 2a) and their slopes were unchanged in the presence of TPB^-. For the mitochondrial experiments calibrations were carried out in the presence of mitochondria (e.g. Figure 1), and control experiments demonstrated that rotenone prevented development of a membrane potential (from measurement of the distribution of [^{3}H]methyltriphenylphosphonium) and that in the presence of rotenone the deflection of the electrode trace on addition of mitochondria was negligible. Therefore these electrodes could be calibrated without significant

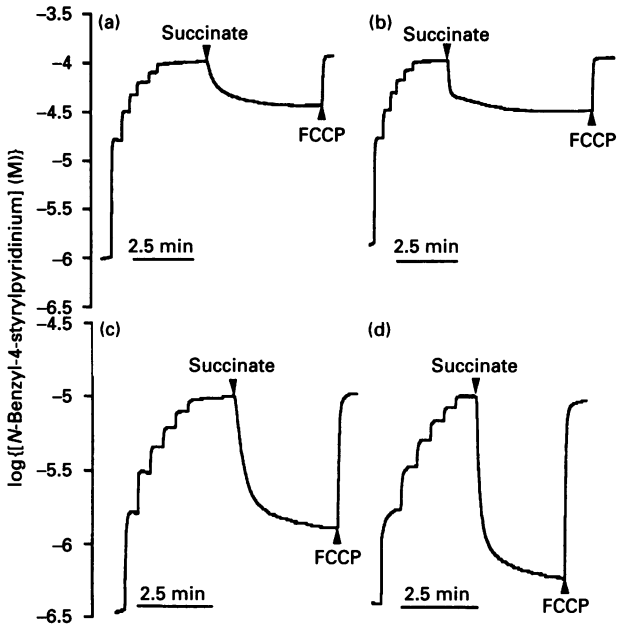

Figure 1 Accumulation of *N*-benzyl-4-styrylpyridinium by mitochondria

Mitochondria were incubated with rotenone as described in the Materials and methods section and the electrode was calibrated by six sequential additions of *N*-benzyl-4-styrylpyridinium up to a final concentration of 10 μM or 100 μM. Potassium succinate was added to 3.33 mM final concentration after 5 min to energize mitochondria and initiate uptake. The initial rate of uptake was calculated by transforming the experimental trace into a plot of the amount of *N*-benzyl-4-styrylpyridinium accumulated in the mitochondria against time, such as that shown in Figure 2(b). FCCP caused efflux of *N*-benzyl-4-styrylpyridinium from the mitochondria. In (**a**) and (**b**) the concentration of *N*-benzyl-4-styrylpyridinium was 100 μM and for (**c**) and (**d**) it was 10 μM. In (**b**) and (**d**) TPB^- was present. Respiration rate was monitored simultaneously but, for the sake of clarity, these data have not been included.

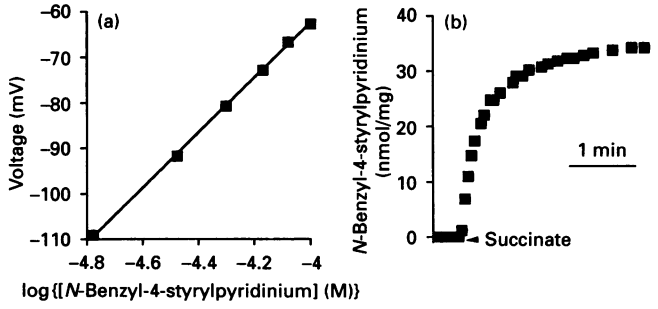

Figure 2 Electrode calibration and measurement of the initial rate of uptake of *N*-benzyl-4-styrylpyridinium by mitochondria

A typical calibration for the *N*-benzyl-4-styrylpyridinium electrode is shown in (**a**). In (**b**) the initial region of uptake of *N*-benzyl-4-styrylpyridinium is expanded and transformed into nmol of *N*-benzyl-4-styrylpyridinium accumulated against time. This was used to calculate the initial rate of uptake of *N*-benzyl-4-styrylpyridinium into mitochondria.

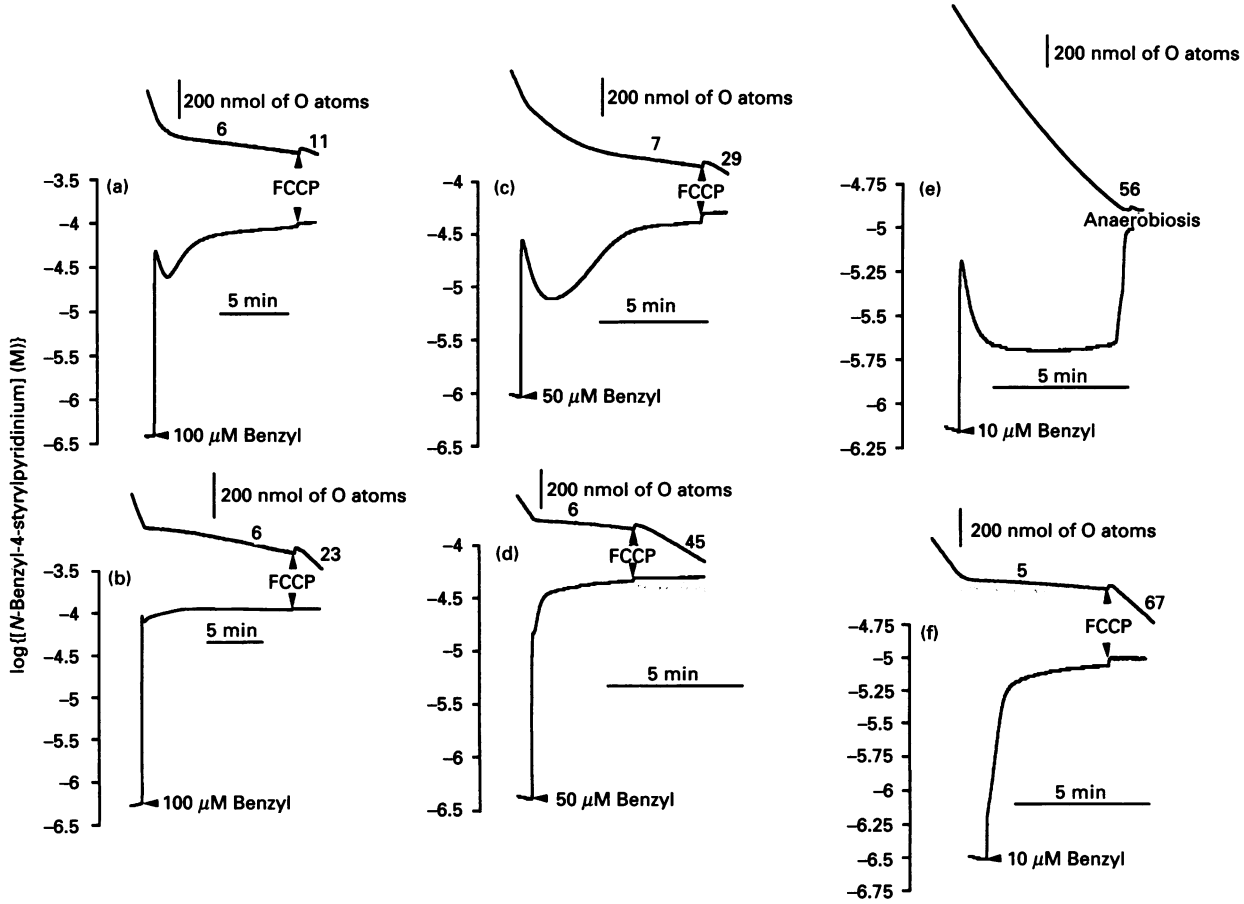

Figure 3 Inhibition of respiration and the simultaneous accumulation of *N*-benzyl-4-styrylpyridinium in mitochondria

Mitochondria were incubated with hexokinase, ADP and malonate. Glutamate and malate were added together 2 min later to initiate respiration and energize the mitochondria. A pulse of *N*-benzyl-4-styrylpyridinium was added 1 min later, and redistribution of the compound and changes in respiration rate were measured simultaneously. TPB^- was present in the experiments shown in (**b**), (**d**) and (**f**) and the concentrations of *N*-benzyl-4-styrylpyridinium added are indicated. FCCP was added at the end of the incubations to cause efflux of all the added compound. The response of the ion-selective electrode was calibrated in adjoining experiments (see Figures 1 and 2). The respiration rate (as a percentage of the initial rate before addition of inhibitor) is shown by the numbers on the experimental traces. Control experiments in the absence of *N*-alkyl-4-styrylpyridiniums showed that the initial rate of respiration was maintained over the timescale shown here, in the presence or absence of TPB^-, and that FCCP did not stimulate respiration rate further.

accumulation or binding of the compound by the mitochondria. Mitochondrial and ETP incubations were carried out in an oxygen-electrode incubation chamber (Rank Brothers, Bottisham, Cambridgeshire, U.K.) stirred by a magnetic flea and thermostically controlled at 25 °C. The ion-selective electrode and a reference Ag/AgCl electrode were passed through a Perspex block equipped with an injection port which fitted on to the oxygen-electrode chamber to give an air-tight seal. This system enabled simultaneous measurement of respiration rate and concentration of the compound in the medium. In studies with mitochondria, which were carried out in New Zealand, the output from the ion-selective and reference electrodes were fed to a voltmeter and from there to a MacLab data-acquisition system and analysed using the MacLab Chart Programme. The output from the oxygen electrode was also sent to the MacLab data-analysis system. In experiments using ETPs, which were conducted in California, the output from the oxygen electrode was fed directly to a strip chart recorder, while the output from the ion-selective electrode was modulated by a Spectrum model 102/A filter amplifier (Newark, DE, U.S.A.) and then sent to a strip chart recorder.

Assay conditions

Mitochondria (6 mg of protein) were suspended in 3 ml of incubation medium [120 mM KCl, 5 mM KH_2PO_4, 5 mM glucose, 2 mM $MgCl_2$, 10 mM Hepes, 1 mM EGTA adjusted to pH 7.2 (with KOH) at 25 °C to which nigericin (0.2 μg/ml) was added]. In the presence of high external [KCl] nigericin eliminates the mitochondrial pH gradient and increases and stabilizes the membrane potential. Omission of nigericin decreased the maximum level of accumulation of *N*-benzyl-4-styrylpyridinium, in agreement with earlier findings for MPP^+ (Ramsay and Singer, 1986), and therefore for all mitochondrial experiments shown here nigericin was present and for some experiments rotenone (13.3 μM), ADP (200 μM), hexokinase (20 μg/ml) or potassium malonate (10 mM) was also present from the start of the incubation. *N*-Alkyl-4-styrylpyridinium compounds were added to the mitochondrial incubation chamber from 10 mM stock solutions in water. Other additions of potassium succinate (3.33 mM), TPB^- (10 μM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (333 nM) or glutamate with malate (10 mM of each) were made during incubations. Rates of uptake

and degree of accumulation are expressed as means ± S.E.M. of measurements on a minimum of thee separate mitochondrial preparations, and the experimental traces shown in Figures 1–5 are from typical experiments which were repeated on a minimum of three separate mitochondrial preparations. NADH oxidase activity and IC_{50} values were determined at 25 °C as in earlier work (Gluck et al., 1994a).

Inhibitors

4′-Heptyl-MPP$^+$ was prepared as described previously (Youngster et al., 1990). The *N*-alkyl-4-styrylpyridiniums were kindly given by Mr. Sergei E. Tkachenko, Russian Academy of Sciences, Chernogolovka, Russia.

RESULTS AND DISCUSSION

Uptake and accumulation of *N*-ethyl-4-styrylpyridinium and *N*-benzyl-4-styrylpyridinium by energized mitochondria

Figure 1 describes experiments using an ion-selective electrode to measure the uptake of *N*-benzyl-4-styrylpyridinium into mitochondria. The electrode was calibrated by sequential addition of portions of *N*-benzyl-4-styrylpyridinium up to a final concentration of 10 or 100 μM and the electrode response was plotted against the log of the concentration of the compound (e.g. Figure 2a). Succinate was added to energize the mitochondria and initiate uptake of *N*-benzyl-4-styrylpyridinium. The experimental trace over the region of accumulation was transformed into nmol of uptake against time, in order to measure the initial rate of uptake (Figure 2b). FCCP was added to dissipate the membrane potential and bring about complete efflux of the compound from the mitochondria. The rapid initial accumulation of *N*-benzyl-4-styrylpyridinium on energization slowed after 2–3 min to give a stable distribution between the external medium and the mitochondria. A concentration of 100 μM *N*-benzyl-4-styrylpyridinium led to an initial rate of uptake of 82 ± 22 nmol/min per mg of protein and a final level of accumulation of 34.5 ± 3 nmol/mg of protein (Figure 1a). Assuming a Nernstian distribution and a mitochondrial matrix volume of 0.6 μl/mg of protein (Davey et al., 1992), this level of accumulation indicates an apparent membrane potential of 194 mV, which is higher than the maximum potential under these conditions of about 170 mV (Davey et al., 1992), suggesting some binding of the compound to the mitochondrial inner membrane. TPB$^-$ (Figure 1b) increased the initial rate of accumulation about 5-fold to 425 ± 59 nmol/min per mg of protein, but had little effect on the level of accumulation which was 35.9 ± 3.3 nmol/mg of protein. A concentration of 10 μM *N*-benzyl-4-styrylpyridinium led to an initial rate of uptake of 19.6 ± 3.8 nmol/min per mg of protein and a level of accumulation of 4.5 ± 0.11 nmol/mg of protein (Figure 1c). This level of accumulation indicates an apparent membrane potential of 230 mV, suggesting that about 97% of the *N*-benzyl-4-styrylpyridinium inside the mitochondria is membrane-bound. TPB$^-$ increased this rate of accumulation 4.4-fold to 86.4 ± 13 nmol/min per mg of protein while the level of accumulation was practically unchanged at 4.78 ± 0.18 nmol/mg of protein (Figure 1d). Therefore ion-pairing to TPB$^-$ present in the membrane does not contribute substantially to the level of accumulation of *N*-benzyl-4-styrylpyridinium.

Similar experiments to those described in Figures 1 and 2 were carried out to determine the initial rate of uptake and the level of accumulation of *N*-ethyl-4-styrylpyridinium in mitochondria (results not shown). A concentration of 100 μM *N*-ethyl-4-styrylpyridinium led to an initial rate of uptake of 20.9 ± 3.2 nmol/min per mg of protein and a level of accumulation of 32 ± 3.5 nmol/mg of protein. TPB$^-$ increased the initial rate of uptake about 20-fold to 432 ± 97 nmol/min per mg of protein and the level of accumulation about 1.4-fold to 44.15 ± 0.75 nmol/mg of protein. Ion-pairing to TPB$^-$ within the membrane may contribute to this increased level of uptake. Decreasing the concentration of *N*-ethyl-4-styrylpyridinium to 10 μM decreased the initial rate of uptake to 3.3 ± 2.5 nmol/min per mg of protein and the level of accumulation to 2.35 ± 0.2 nmol/mg of protein. TPB$^-$ increased the initial uptake about 17.5-fold to 57.7 ± 21.8 nmol/min per mg of protein and the degree of uptake about 1.85-fold to 4.02 ± 0.55 nmol/mg of protein. The level of accumulation from 100 μM *N*-ethyl-4-styrylpyridinium corresponds to an apparent membrane potential of 189 mV, which increased to 226 mV in the presence of TPB$^-$. The accumulation from 10 μM *N*-ethyl-4-styrylpyridinium corresponds to a potential of 170 mV, which increased to 210 mV in the presence of TPB$^-$. These data suggest that *N*-ethyl-4-styrylpyridinium binds less readily to the mitochondrial inner membrane than *N*-benzyl-4-styrylpyridinium.

The rates of uptake and levels of accumulation for these *N*-alkyl-4-styrylpyridinium cations were substantially greater than those found for MPP$^+$. The initial rate of uptake from 100 μM *N*-benzyl-4-styrylpyridinium (82 nmol/min per mg of protein) compares with a rate of uptake from 400 μM MPP$^+$ of 5–6 nmol/min per mg of protein, determined under similar conditions (Davey et al., 1992). The level of accumulation from 100 μM *N*-benzyl-4-styrylpyridinium (34.5 nmol/mg of protein) compares with the level of accumulation from 400 μM MPP$^+$ of 34 nmol/mg of protein, under similar conditions (Davey et al., 1992). Under conditions different from those described here the level of accumulation from 500 μM MPP$^+$ was 10–20 nmol/mg of protein (Ramsay et al., 1986; Ramsay and Singer, 1986) and from 100 μM MPP$^+$ was about 10 nmol/mg of protein (Aiuichi et al., 1992). TPB$^-$ increased the rate of uptake of the *N*-alkyl-4-styrylpyridinium cations, as it does for MPP$^+$ (Ramsay et al., 1989a; Davey et al., 1992). However, the small increase in the level of accumulation for the *N*-alkyl-4-styrylpyridinium compounds caused by TPB$^-$ (from almost no effect for the benzyl compound to a maximal stimulation of 40–80% for the ethyl compound) compares with a 250–400% stimulation of the level of accumulation of MPP$^+$ by TPB$^-$ (Ramsay et al., 1989b; Aiuichi et al., 1992).

The benzyl compound was taken up by energized mitochondria about four to six times more rapidly than the ethyl compound. The levels of accumulation for the benzyl and ethyl compounds were similar at an initial concentration of 100 μM but at 10 μM accumulation of the ethyl compound was about 50% less than the benzyl compound. TPB$^-$ stimulated the initial rates of the uptake for both compounds, the benzyl compound by about 4–5-fold and the ethyl compound by about 17–20-fold. It did not substantially affect the level of accumulation of the benzyl compound, but increased that of the ethyl compound by about 40–80%, depending on the concentration. Table 1 presents the results of experiments on ETPs and mitochondria which indicate that both the ethyl and benzyl compounds were effective inhibitors of NADH-linked respiration in mitochondria (IC_{50} values for the benzyl and ethyl compounds were 25 μM and 50 μM respectively) and that TPB$^-$ increased their effectiveness as inhibitors (IC_{50} values in the presence of 10 μM TPB$^-$ for the benzyl and ethyl compounds were 0.6 μM and 2.5 μM respectively). In ETPs the ethyl compound was similarly somewhat less effective an inhibitor than the benzyl compound, the IC_{50} values for the benzyl and ethyl compounds being 330 and 700 μM respectively in the absence of TPB$^-$ and 15 and 100 μM re-

Table 1 Determination of IC_{50} values for *N*-ethyl-4-styrylpyridinium and *N*-benzyl-4-styrylpyridinium in mitochondria and ETPs

IC_{50} values were determined as described in the Materials and methods section and are means ± S.D. for three separate determinations.

Compound	IC_{50} in ETPs (μM)		IC_{50} in mitochondria (μM)	
	$-TPB^-$	$+10\ \mu M\ TPB^-$	$-TPB^-$	$+10\ \mu M\ TPB^-$
N-Benzyl-4-styrylpyridinium	330±20	15±2	25±3	0.6±0.08
N-Ethyl-4-styrylpyridinium	700±40	100±11	50±5	2.5±0.35

spectively in the presence of 10 μM TPB^-. The greater inhibitory potency of the benzyl compound relative to the ethyl compound is consistent with the finding that the benzyl compound passes through the mitochondrial inner membrane more rapidly than the ethyl compound. Presumably the more hydrophobic benzyl compound permeates more rapidly to the hydrophobic inhibitory site of Complex I than the less hydrophobic ethyl compound. These data support the suggestion that the stimulation by TPB^- of inhibition in mitochondria of MPP^+-like compounds is not due primarily to increasing their accumulation in the mitochondrial matrix (Aiuichi et al., 1992), but is caused by stimulating the movement of these compounds across the membrane and to the inhibitory site on Complex I (Ramsay et al., 1989a). The evidence for this is that TPB^- had little effect on the accumulation of the benzyl compound in the mitochondrial matrix (e.g. Figures 1a and 1b), and only a slight effect on the uptake of the ethyl compound, but substantially increased the inhibition of respiration by both these compounds in mitochondria.

Correlation of inhibition of NADH-linked mitochondrial respiration with accumulation of *N*-alkyl-4-styrylpyridinium cations by mitochondria

To investigate further the role of diffusion of MPP^+-like compounds to the inhibitory site on Complex I in the inhibition of respiration, we correlated their uptake into mitochondria with the inhibition of NADH-linked respiration. To this end we measured simultaneously oxygen uptake on malate and glutamate and the accumulation of these compounds in mitochondria. Figure 3 reports the use of the ion-selective electrode, in conjunction with an oxygen electrode, to measure simultaneously accumulation of *N*-benzyl-4-styrylpyridinium in mitochondria and NADH-linked mitochondrial respiration. The upper traces show oxygen consumption, with the numbers on the experimental traces indicating the respiration rate (as a percentage of the initial respiration rate before addition of inhibitor). The lower traces show the concentration of *N*-benzyl-4-styrylpyridinium. Mitochondria were respiring on the NADH-linked substrates glutamate and malate, in the presence of malonate (to block succinate-linked respiration) and an ADP-regenerating system. After respiration had been established a pulse of *N*-benzyl-4-styrylpyridinium was added and the subsequent decrease in respiration rate and redistribution of *N*-benzyl-4-styrylpyridinium between the medium and the mitochondria measured.

Addition of 100 μM *N*-benzyl-4-styrylpyridinium rapidly inhibited respiration and this inhibition was only slightly relieved by addition of FCCP (Figure 3a). Reducing the concentration of *N*-benzyl-4-styrylpyridinium to 50 μM (Figure 3c) resulted in a slower development of the inhibition and addition of 10 μM of the compound had substantially less effect on respiration (Figure 3e). Inhibition of respiration by 100 μM *N*-benzyl-4-styrylpyridinium de-energized the mitochondria, and led to almost total efflux of the compound (Figure 3a). Interestingly, the small amount of the compound remaining in the mitochondria was sufficient to maintain inhibition. In contrast, the substantially greater amount of the compound accumulated in mitochondria in Figure 3(e) did not cause inhibition, showing that permeation into the membrane by a small fraction of the compound driven by the high matrix concentration, not uptake into the mitochondria alone, is required for the inhibition of respiration. Addition of FCCP abolished any residual membrane potential and led to efflux of the small amount of the inhibitor remaining in the membrane. It resulted in a slight relief of inhibition at an inhibitor concentration of 100 μM (Figure 3a) whereas at 50 μM inhibitor concentration (Figure 3c) it caused a greater stimulation of respiration. In earlier work we have shown that binding of MPP^+ and its analogues to Complex I is reversible and that the inhibition of Complex I by these compounds is competitive (e.g. Singer et al., 1986; Krueger et al., 1990). Therefore it is most likely that this inhibition of Complex I is not due to a fraction of the MPP^+ analogue being irreversibly bound to Complex I, as had been proposed by Cleeter et al. (1992), but to the fact that the dissociation from the inhibitory site is relatively slow. Therefore within the time frame of the present experiments [and of those of Cleeter et al. (1992)] dissociation of the inhibitor is incomplete because the inhibitor diffuses slowly from its binding site on Complex I. Even in the presence of TPB^- the relief of inhibition by uncoupler was incomplete (Figure 3). This partial relief of inhibition was not due to the establishment of a new equilibrium distribution between the decreased amount of inhibitor in the matrix of the uncoupled mitochondria and the inhibitory site on Complex I. The evidence for this is that addition of a 100 μM concentration of the benzyl compound to uncoupled mitochondria respiring on glutamate and malate caused negligible inhibition of respiration within the period of observation (4 min; results not shown).

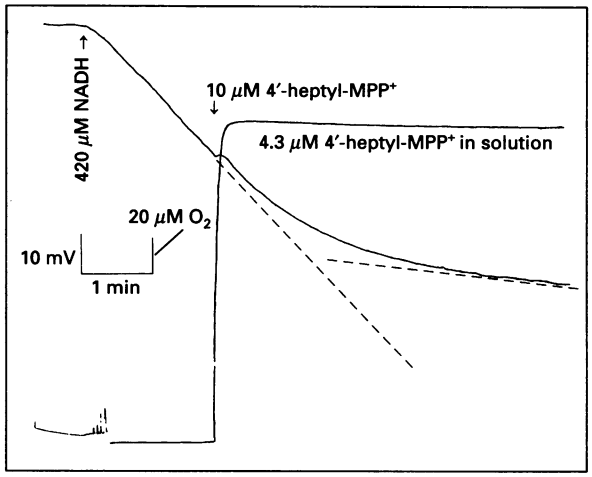

Figure 4 Inhibition of NADH oxidation and the simultaneous measurement of the accumulation of 4′-heptyl-MPP^+ in ETPs

The experiment was conducted as described in the Materials and methods section. The polarographic chamber thermostatically controlled at 25 °C contained 3 ml total volume with 70 μg of ETP protein/ml. The trace that starts in the upper left corner is a measure of oxygen uptake and that which starts in the lower left corner is voltage output by the ion-selective electrode. The total uptake represents 5.7 μM 4′-heptyl-MPP^+. The uninhibited rate of NADH oxidation is 0.58 μmol/min per mg and the inhibited rate is 0.055 μmol/min per mg.

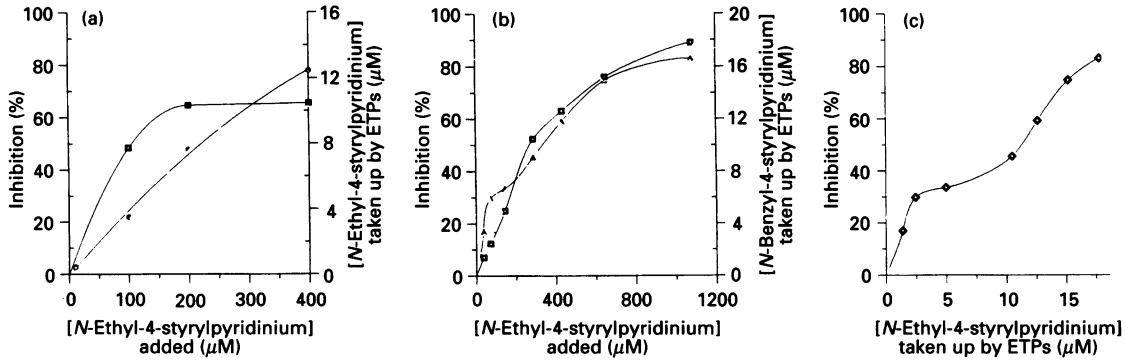

Figure 5 Titration of ETPs with *N*-ethyl-4-styrylpyridinium (a) and *N*-benzyl-4-styrylpyridinium (b)

The experiment was conducted in a polarographic chamber as described in the legend to Figure 4. The protein concentration in (**a**) was 20 μg/ml and that in (**b**) and (**c**) was 50 μg/ml. (**c**) Plot of percentage inhibition versus the amount of benzyl analogue taken up to emphasize the biphasic nature of the effects. □, Percentage inhibition; ●, amount of ethyl analogue taken up by the membrane; △, amount of benzyl analogue bound to the membrane.

Inclusion of 10 μM TPB^- led to the instantaneous and almost complete inhibition of respiration on addition of 10–100 μM *N*-benzyl-4-styrylpyridinium (Figures 3b, 3d and 3f). Inhibition of respiration de-energized the mitochondria and led to rapid efflux of the accumulated compound. The amount of inhibitor retained by the mitochondria was negligible, as was seen in the absence of TPB^-, but this small fraction was again sufficient to maintain inhibition. These experiments confirm that TPB^- does not stimulate inhibition of respiration by increasing the matrix concentration of the inhibitor but by facilitating its access to a hydrophobic inhibitory site on Complex I. This is nicely illustrated by comparing Figure 3(e), in which substantial uptake of the benzyl compound causes partial inhibition, with Figure 3(f) in which TPB^- results in almost complete inhibition with an accumulation of a small amount of the compound in the mitochondria (accurate estimation of the amount of compound bound to the mitochondria is beyond the sensitivity of these electrodes). FCCP dissipated any residual membrane potential and caused efflux of small amounts of the compound from the mitochondria. This resulted in a greater stimulation of respiration than was found in the absence of TPB^-, presumably because TPB^- facilitates the diffusion of the compound from the hydrophobic inhibitory site on Complex I.

Uptake of hydrophobic MPP^+ analogues by inverted mitochondrial inner membranes (ETPs)

The use of ETPs has several advantages in studying the interaction of MPP^+ analogues with mitochondria. First, the ETP preparation has been well characterized in terms of Complex I activity and the action of inhibitors that react at the rotenone site. Second, it is free from interfering enzymes, and preparations may be preserved frozen without loss of activity for several months so that samples from the same preparation can be used for several experiments, permitting direct comparison of the results. Third, as the rotenone site is exposed to the medium, the effects of concentration gradients across the membrane are eliminated and the results can be directly related to the permeation of inhibitors to the binding site on Complex I.

The underlying assumption of the experiments described here was that the ion-selective electrode would not respond to the MPP^+ derivatives bound to the membrane, so that it could potentially be used to study the rate and extent of binding of the inhibitors to the membrane. As shown below, the expectation was fully realized although, as expected, the electrode could not distinguish between binding at the specific sites required for inhibition and at other spurious sites, including partition into the lipid phase.

The first question addressed was how the rate of development for the inhibition compares with the rate of binding of the inhibitor in an ETP preparation. For this purpose an electrode sensitive to 4′-heptyl-MPP^+ was prepared. This MPP^+ analogue was chosen because of its low IC_{50} (5 μM in ETPs at 30 °C) and the fact that, as with other hydrophobic MPP^+ analogues, inhibition develops relatively quickly (maximum reached in 5 min without TPB^-) in contrast with more hydrophilic inhibitors which require an hour or longer (Gluck et al., 1994a). Moreover, as shown in Figure 4, the hydrophobicity of 4′-heptyl-MPP^+ resulted in a very sensitive electrode, capable of measuring inhibitor at concentrations below 1 μM.

The data in Figure 4 show that binding of 4′-heptyl-MPP^+ to the membranes was very rapid, being complete within the dead-time of the recorder. In contrast, full development of the inhibition required 3 min. At that point the rate was 55 nmol of O_2/min per mg while the uninhibited rate was 580 nmol of O_2/min per mg. Therefore full development of the inhibition lags considerably behind the initial binding to the membrane. The rate-limiting step may perhaps be the time required to reach the hydrophobic binding site within the membrane or possibly the correct orientation of the inhibitor for interaction with the binding site.

Figure 4 also shows that, of the 300 nmol of inhibitor added, only 57% or 171 nmol were taken up by the membrane. As the amount of ETP protein present in 3 ml was 210 μg, this is several orders of magnitude higher than the amount of NADH dehydrogenase present [0.03 nmol/mg calculated from the estimate of Cremona and Kearney (1964)]. Clearly, the vast majority of the inhibitor is spuriously bound to membrane, as is the case with rotenone and piericidin A (Horgan et al., 1968a,b). Also, as with these hydrophobic inhibitors of the enzyme, the amount of 4′-heptyl-MPP^+ bound/mg of protein varies almost linearly with the amount of inhibitor added, over the range 2–17 μM, well beyond the concentration required for maximal inhibition (results not shown). The amount of 4′-heptyl MPP^+ taken up by ETPs in the experiment described by Figure 4 was not maximal however, as at lower protein concentration (20 μg/ml) up to 260 nmol/mg was taken up. The difference may possibly be due to the fact that at the high protein concentration used in Figure 4 (70 μg/ml) the membrane particles do not remain fully dispersed. This aggregation may interfere with the interactions of inhibitor with

Complex I at the membrane surface. The fact that only a fraction of the inhibitor is bound to the membrane, even in the range in which the amount bound varies linearly with the amount of inhibitor added, suggests the existence of an equilibrium between free and membrane-bound inhibitor, with far less entering the lipid-rich membrane than would be predicted from its lipid partition coefficient, which is 100 for octanol/water at 25 °C (Gluck et al., 1994b).

Figure 5 compares the inhibition of NADH oxidation with the uptake of *N*-ethyl-4-styrylpyridinium and *N*-benzyl-4-styrylpyridinium by ETPs at various inhibitor concentrations. The much less hydrophobic ethyl analogue [its partition coefficient in octanol/water is 0.03 at 25 °C (Gluck et al., 1994b)] is a time-dependent inhibitor, which at low concentrations requires about 1 h to exert its full inhibitory effects (M. J. Kreuger, S. O. Sablin, M. Gluck, W. J. Nicklas, S. O. Bacharin, R. R. Ramsay and T. P. Singer, unpublished work). This is why in polarographic experiments of short duration, such as that in Figure 5(a), the level of inhibition reaches a plateau of about 65%. In contrast, the same titration shows that the binding of the inhibitor continues in an almost linear manner over a wide concentration range.

The somewhat more hydrophobic benzyl analogue [its partition coefficient in octanol/water is 0.056 at 25 °C (Gluck et al., 1994b)] displayed a curious type of behaviour in similar titrations (Figure 5b). The inhibitory effect of this compound on NADH oxidation develops fairly rapidly (less than 5 min) so that at the highest concentration used nearly complete inhibition is reached. However, the curve relating inhibition to inhibitor concentration is complex, and not the hyperbolic curve that most other MPP^+ analogues yield. The binding curve in Figure 5(b) is biphasic, as if the binding affinity of the inhibitor for some parts of the membrane is considerably higher than for others. When percentage inhibition is plotted against the amount of benzyl analogue taken up, this biphasicity is even more evident (Figure 5c). It may be also noted that, whereas in the titration of the ethyl compound (where 20 μg/ml ETP protein was present) at 400 μM inhibitor concentration the binding curve only begins to depart from linearity (Figure 5a), with the benzyl compound (50 μg of protein/ml) at the same ratio of added inhibitor to membrane protein the binding curve has nearly levelled off (Figure 5b). The reason for this difference is not clear but suggests a degree of specificity in binding affinities.

These experiments fulfil the expectation that ion-selective electrodes may be used to study the binding of inhibitors and other compounds to membranes. Although the extensive non-specific binding of the compounds tested precludes direct measurement of the kinetics of binding to, and dissociation from, the active sites specifically involved in blocking electron transport from NADH–ubiquinone oxidoreductase to the Q pool, they suggest that ion-selective electrodes may be developed for sufficiently hydrophobically charged compounds to study their interaction with specific proteins.

Conclusions

The experiments reported here are further evidence that the accumulation of MPP^+-like compounds in the mitochondrial matrix is followed by slow permeation of a small fraction to a hydrophobic site on Complex I. In addition, these data strengthen earlier reports that TPB^- potentiates the inhibition of respiration by MPP^+-like compounds by facilitating diffusion of the compounds to the hydrophobic site on Complex I, and not solely by increasing their concentration in the mitochondrial matrix. The evidence for these conclusions is: (1) inhibition of respiration did not occur even after substantial accumulation of these compounds in the mitochondrial matrix; (2) inhibition was maintained by a small fraction of the inhibitor which remained bound to the mitochondria after depolarization allowed efflux of most of the inhibitor initially accumulated; (3) in ETPs the uptake of 4′-heptyl-MPP^+ into the membrane was rapid but was followed by the relatively slow onset of inhibition of NADH oxidation; (4) in the presence of TPB^-, accumulation of small amounts of these compounds caused complete inhibition of respiration whereas respiration was little affected by accumulation of substantially greater amounts of these compounds in the absence of TPB^-; (5) TPB^- facilitated the inhibition of respiration in mitochondria, even though it had a negligible effect on the level of accumulation of these compounds in mitochondria.

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REFERENCES

Aiuichi, T., Shirane, Y., Kinemuchi, H., Arai, Y., Nakaya, K. and Nakamura, Y. (1988) Neurochem. Int. **12**, 525–531

Aiuichi, T., Syou, M., Matsunaga, M., Kinemuchi, H., Nakaya, K. and Nakamura, Y. (1992) Biochim. Biophys. Acta **1103**, 233–238

Chappell, J. B. and Hansford, R. G. (1972) in Subcellular Components: Preparation and Fractionation (Birnie, G. D., ed.), pp. 77–91, Butterworths, London

Chiba, K., Trevor, A. and Castagnoli, N. (1984) Biochem. Biophys. Res. Commun. **120**, 574–578

Cleeter, M. W., Cooper, J. M. and Schapira, A. H. (1992) J. Neurochem. **58**, 786–789

Crane, F. L., Glenn, J. L. and Green, D. E. (1956) Biochim. Biophys. Acta **22**, 475–487

Cremona, T. and Kearney, E. B. (1964) J. Biol. Chem. **239**, 2328–2334

Davey, G. P., Tipton, K. F. and Murphy, M. P. (1992) Biochem. J. **288**, 439–443

Davey, G. P., Tipton, K. F. and Murphy, M. P. (1993) J. Neural. Trans. **40**, 47–55

Gluck, M. R., Krueger, M. J., Ramsay, R. R., Sablin, S. O., Singer, T. P. and Nicklas, W. J. (1994a) J. Biol. Chem. **269**, 3167–3174

Gluck, M. R., Krueger, M. J., Ramsay, R. R., Sablin, S. O. and Singer, T. P. (1994b) J. Neurochem. **63**, 655–661

Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol Chem. **177**, 751–766

Heikkila, R. E., Hwang, J., Ofori, S., Geller, H. M. and Niklas, W. J. (1990) J. Neurochem. **54**, 743–750

Hoppel, C. L., Greenblatt, D., Kwok, H.-C., Arora, P. K., Singh, M. P. and Sayre, L. M. (1987) Biochem. Biophys. Res. Commun. **148**, 684–693

Horgan, D. J., Ohno, H., Singer, T. P. and Casida, J. E. (1968a) J. Biol. Chem. **243**, 5967–5976

Horgan, D. J., Singer, T. P. and Casida, J. E. (1986b) J. Biol. Chem. **243**, 834–843

Javitch, J. A., D'Amato, R. J., Strittmatter, S. M. and Snyder, S. H. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 2173–2177

Krueger, M. J., Singer, T. P., Casida, J. E. and Ramsay, R. R. (1990) Biochem. Biophys. Res. Commun. **169**, 123–128

Krueger, M. J., Sablin, S. O., Ramsay, R. and Singer, T. P. (1993) J. Neurochem. **61**, 1546–1548

Nicklas, W. J., Vyas, I. and Heikkila, R. E. (1985) Life Sci. **36**, 2503–2508

Ramsay, R. R. and Singer, T. P. (1986) J. Biol. Chem. **261**, 7585–7587

Ramsay, R. R., Salach, J. I. and Singer, T. P. (1986) Biochem. Biophys. Res. Commun. **134**, 743–748

Ramsay, R. R., Youngster, S. K., Nicklas, W. J., McKeown, K. A., Jin, Y.-Z., Heikkila, R. E. and Singer, T. P. (1989a) Proc. Natl. Acad. Sci. U.S.A. **86**, 9168–9172

Ramsay, R. R., Mehlhorn, R. J. and Singer, T. P. (1989b) Biochem. Biophys. Res. Commun. **159**, 983–990

Ramsay, R. R., Krueger, M. J., Youngster, S. K., Gluck, M. R., Casida, J. E. and Singer, T. P. (1991) J. Neurochem. **56**, 1184–1190

Salach, J. I., Singer, T. P., Castagnoli, N. and Trevor, A. (1984) Biochem. Biophys. Res. Commun. **125**, 831–835

Singer, T. P., Salach, J. I., Castagnoli, N., Jr. and Trevor, A. (1986) Biochem. J. **235**, 785–789

Tipton, K. F. and Singer, T. P. (1993) J. Neurochem. **61**, 1191–1206

Youngster, S. K., Gluck, M. R., Heikkila, R. E. and Nicklas, W. J. (1990) Biochem. Biophys. Res. Commun. **169**, 758–764

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