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Enhancement of the uptake of 1-methyl-4-phenylpyridinium ion (MPP^+) in mitochondria by tetraphenylboron

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The uptake of 1-methyl-4-phenylpyridinium (MPP^+) by intact mitochondria was measured by an electrode sensitive to MPP^+. The electrode was constructed with a polyvinyl chloride membrane that contained tetraphenylboron (TPB) as an ion-exchanger. MPP^+ was taken up by mitochondria in an energy-dependent process. TPB rapidly enhanced MPP^+ uptake by mitochondria, and then induced release of MPP^+ from mitochondria in medium containing glutamate and malate. No release of MPP^+ from mitochondria after addition of TPB could be observed in medium containing succinate, the oxidation of which is not inhibited by MPP^+. The release of MPP^+ was caused by respiratory inhibition by MPP^+ taken up in mitochondria. Since the release of MPP^+ did not increased O_2 uptake in mitochondria, the major part of MPP^+ released from the matrix, where no respiratory enzyme inhibited by MPP^+ exists. We concluded the following effect of TPB on MPP^+ uptake from the results: (1) The increase of MPP^+ concentration in matrix by addition of TPB increased the amount of bound to the inner membranes of mitochondria. (2) The increase of the amount of MPP^+ in the inner membranes enhanced the respiratory inhibition. (3) The respiratory inhibition induced to release MPP^+ from the matrix. The relation between MPP^+ distribution in the membrane of mitochondria and the respiratory inhibtion by MPP^+ are discussed.

Introduction

The neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) induces a symptom that closely resembles Parkinson's disease in humans and primates [1,2]. MPP^+ inhibits the electron transport system of mitochondria [3–6], and this inhibition is thought to induce neurotoxicity in dopamine neurons. We found that tetraphenylboron (TPB) facilitated MPP^+-induced respiratory inhibition of mitochondria [7] and synaptosomes [8,9], and its effect on mitochondrial respiration has been confirmed in other laboratories [10–12]. TPB also enhances the toxicity of MPTP, the mother substance of MPP^+ [12]. We interpreted the enhancement of MPP^+ toxicity by TPB to be due to increased accumulation of MPP^+ within the mitochondria. However, Ramsay et al. [10] reported, that increase of MPP^+ uptake in mitochondria was not correlated with rapid inhibition of respiration, since rapid MPP^+ uptake did not occur on addition of TPB. The respiratory inhibition was thought, therefore, to depend on the ratio of concentrations of mitochondrial protein and MPP^+. The concentration of mitochondrial protein in the uptake experiment by Ramsay et al. [10] was higher than that in the respiration experiment. We attempted to measure MPP^+ uptake in mitochondria within the concentration range, in which respiration was measured, using an ion selective electrode for MPP^+. In this report, we found that the velocity of uptake of MPP^+ depends on the concentration of mitochondrial

Abbreviations: MPP^+, 1-methyl-4-phenylpyridinium; TPB, tetraphenylboron.

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protein, and the rapid inhibition of respiration by MPP^+ can be explained by the rapid increase of MPP^+ uptake in mitochondria induced by TPB.

Materials and Methods

Mitochondria. Mitochondria were prepared from the liver of 6–10-week-old mice [8]. Protein content was determined by the method of Lowry et al. [13] with bovine serum albumin as a standard.

MPP^+-selective electrode. The membrane used for the MPP^+ selective electrode was made of polyvinyl chloride (PVC), tetraphenylboron and dioctylphthalate by, essentially, the same method used to make membranes for lipophilic cations [14–16]. The membrane was glued on PVC or polystyrene tubing (diameter 0.7–1 cm) with tetrahydrofuran, and the internal solution was 10 mM MPP^+.

Measurement of MPP^+ accumulation by the electrode. The electrode was assembled into a 2.5 ml glass chamber that contained a bridge to a KCl reference electrode. The temperature of the chamber was kept at 25°C by circulating water. Mitochondrial suspension was added to incubation medium (2.0 ml) containing 50 mM sucrose, 100 mM KCl, 10 mM Tris-phosphate (pH 7.2), 2 mM $MgSO_4$ and various concentrations of MPP^+. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously. The signal was stored in a microcomputer (NEC PC-8001mk2, Japan) through an A/D converter, which was made of an ADC-0809 CCN integrated circuit (National Semiconductor, USA). The concentration of MPP^+ and MPP^+ uptake were calculated with the aid of the computer. The concentration of mitochondrial protein was 1–4 mg protein/ml.

Reagents. Tetraphenylboron was obtained from Nakarai Chemical Co. (Kyoto). MPP^+ iodide was obtained from Research Biochemical Inc. (Wayland, USA). CCCP and rotenone were from Sigma Chemical Co. All other materials from commercial sources were of the highest purity available.

Results

Properties of the electrode

The response of the electrode was linear with the logarithm of MPP^+ concentration with a slope of 50 mV per decade concentration until the concentration decreased to about $2 \cdot 10^{-6}$ M (Fig. 1). The slope of the electrode was smaller than that of ordinal lipophilic cations [14–16]. The electrode responded to step changes of MPP^+ in solution in less than 5 s. The relative selectivity ratios for interfering ions are defined by Method I of Srinivasan and Rechnitz [17], and the values for NaCl, KCl, sucrose, $MgCl_2$, phosphate buffer (pH 7.4), glutamate, succinate or malate were less than 10^{-5}. This indicates that the measurement of MPP^+ concentration was not affected by the presence of such substances. The slope of the electrode and the response time to various MPP^+ concentrations remained practically unchanged for at least two weeks, when the electrode was stored in 10^{-3} M MPP^+ solution.

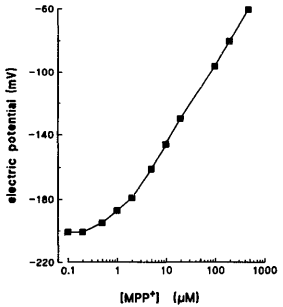

Fig. 1. A typical calibration plot of an MPP^+ electrode.

Energy dependent MPP^+ uptake by mitochondria

In the presence of rotenone, the concentration of MPP^+ in the incubation media was not decreased by addition of mitochondria without glutamate, malate or succinate as energy substrates. Therefore, we could disregard the amount of non specific binding of MPP^+ to mitochondrial membranes under these experimental conditions, which were different from experiments using an isotope of MPP^+ [10,18]. Uptake of MPP^+ can then be calculated by the following equation:

$$\text{Uptake} = (C_0V - C_1(V + v))/C_m(V + v) \quad (1)$$

where C_0 is the initial concentration of MPP^+ (mM); C_1, concentration after addition of mitochondria (mM); C_m, concentration of mitochondria (mg protein/ml); V, initial volume of medium (ml); v, volume of mitochondrial suspension (ml).

MPP^+ was taken up in mitochondria by an energy dependent process, as shown in Fig. 2. The result shown in Fig. 2 is similar to the result obtained using an isotope of MPP^+ [18] and the lipophilic cations, the distribution of which was used to estimate membrane potential of mitochondria [14,15,19]. MPP^+ as a lipophilic cation tends to distribute between medium and intramitochondria (matrix) according the membrane potential difference [14–16], but it can not equi-

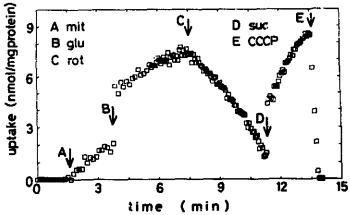

Fig. 2. Energy-dependent accumulation of MPP^+ by mitochondria. Mitochondrial suspensions (final concentration 1.0 mg protein/ml) were added at A in the buffer without energy substrate. Glutamate (10 mM), rotenone (1 μM), succinate (10 mM) and CCCP (1 μM) were added at B, C, D and E, respectively.

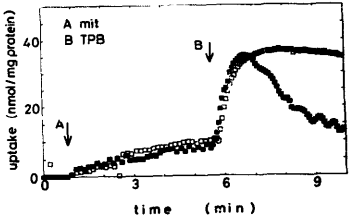

Fig. 4. Effect of substrate on MPP^+ uptake by mitochondria in the presence of TPB. Mitochondrial suspension (final concentration, about 1.0 mg protein/ml) were added to buffer containing glutamate (10 mM) plus malate (10 mM) (■); or succinate (□) at A. TPB added at B. The concentration of MPP^+ was 100 μM.

librate according to the Nernst distribution across the membrane because of its low permeability through mitochondrial membranes. It is reported that dibenzyldimethylammonium was also not distributed between the suspended medium and intramitochondrial space (matrix) in accordance with the Nernst equation without TPB [14,15,19].

Effect of TPB on MPP^+ uptake

The effect of TPB on MPP^+ uptake by mitochondria is shown in Fig. 3. After incubation of MPP^+ for 5 min with mitochondria in state 4 in the absence of TPB, the addition of 10 μM TPB induced rapid uptake of MPP^+ followed by release of MPP^+. TPB enhanced the respiratory inhibition of mitochondria by MPP^+ [7–12]. When the concentration of MPP^+ exceeded 0.05 mM, the maximum uptake of MPP^+ occurred within 1 min after addition of TPB. The respiratory inhibition by MPP^+ was induced within 1 min [7,12], and the time dependence of MPP^+ uptake in mitochondria was similar to that of respiratory inhibition by MPP^+. The release of MPP^+ (above 0.05 mM) after addition of TPB must respond to the respiratory inhibition by MPP^+ taken up in mitochondria. MPP^+ acted as a respiratory inhibitor like rotenone, and induced the release of MPP^+ as shown in Fig. 2. That is, no decrease of MPP^+ uptake could be observed in the medium containing succinate (Fig. 4), the oxidation of which is not inhibited by MPP^+ [3–7].

Effect of change in ratio of the amounts of mitochondrial protein and MPP^+

MPP^+ uptake was increased by the addition of TPB (Fig. 3), as reported by Ramsay et al. [10]. However, Ramsay et al. [10] did not observe the following release after rapid increase of MPP^+, and the amount of uptake reported by them was smaller than that shown in Fig. 3. They used higher concentrations of mitochon-

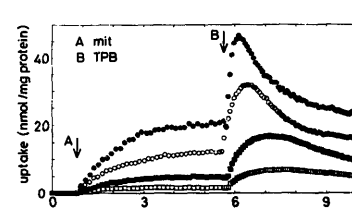

Fig. 3. Effect of TPB on MPP^+ uptake by mitochondria. Mitochondrial suspensions (final concentration, about 1 mg protein/ml) were added to buffer containing glutamate (10 mM) plus malate (10 mM) at A. TPB was added at B. MPP^+ concentrations: ●, 200 μM; ○, 100 μM; ■, 50 μM; □, 20 μM.

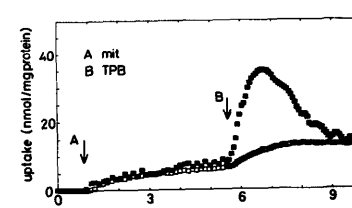

Fig. 5. Effect of concentration of mitochondrial protein on MPP^+ uptake with TPB. Mitochondrial suspensions were added to buffer containing glutamate (10 mM) and malate (10 mM) at A. ■, 1.0 mg protein/ml; □, 4.0 mg protein/ml. TPB was added at B. The concentration of MPP^+ was 100 μM.

drial protein (8 mg protein/ml) than that used in the present study. The concentration of mitochondrial protein was used for experiments on respiration inhibition by MPP^+ is 1–2 mg protein/ml [7,10,11]. When the concentration of mitochondrial protein was increased to 4 mg protein/ml, the MPP^+ uptake without TPB was similar to that observed at low concentration of mitochondria (1.0 mg protein/ml). However, the uptake of MPP^+ after addition of TPB became slow and rapid uptake with the following release of MPP^+ did not occur (Fig. 5), similar to the results of Ramsay et al. [10]. Since an increase in concentration of mitochondria was induced to decrease the ratio of MPP^+/mitochondria, and the MPP^+ concentration was not enough to inhibit mitochondrial respiration, MPP^+ release might be undetectable after addition of TPB (Fig. 5).

Discussion

TPB potentiated the respiratory inhibition by MPP^+ in mitochondria [7]. We suggested that the effect of TPB on the MPP^+ inhibition of respiration is due to increased accumulation within the mitochondrial matrix [7]. Ramsay et al. proposed another mechanism [10], because the uptake velocity of MPP^+ isotope by mitochondria was not rapid to induce the rapid respiratory inhibition in the presence of TPB. The mitochondrial concentration in the measurement of MPP^+ uptake was 8 mg proteins/ml (Ramsay et al. [10]), and was higher than that of measurement of respiratation (1–2 mg proteins/ml). As shown in Fig. 5, the increase of the concentration of mitochondria decreased in the velocity of MPP^+ uptake. The MPP^+ electrode was able to measure the changes in MPP^+ uptake within short time intervals, and MPP^+ uptake was measured with the MPP^+ electrode in the concentration range 1–2 mg proteins/ml. The time dependence of MPP^+ uptake (Fig. 3) is thought to correspond with that of respiratory inhibition. The results obtained by using a MPP^+ electrode show that TPB potentiation of respiratory inhibition by MPP^+ is due to increased accumulation of MPP^+ within mitochondria, as reported previously [7]. As discussed below in the presence of TPB, we think that the concentration of MPP^+ in the mitochondrial matrix increases according to the Nernst equation. The increase of MPP^+ concentration in the matrix by addition of TPB increases in the amount of bound to inner membranes and enhances the respiratory inhibition.

The inhibitors of respiration suppressed the uptake of MPP^+ by mitochondria [18], and we also showed that in Fig. 2. MPP^+ uptake was enhanced by addition of TPB, and the release of MPP^+ was observed corresponding to the respiratory inhibition in mitochondria (Figs. 3 and 5). The respiratory inhibition in mitochondria by MPP^+ was reversible [3] and the MPP^+ uptake depended on the energized state of mitochondrial membranes [18]. It is possible that MPP^+ taken up inhibits the respiration and decreases in MPP^+ uptake by mitochondria. Adams and Odunze [20] described the similar opinion and the possibility that the cellular toxicity of MPP^+ was reduced by its own respratory inhibition. The release of MPP^+ shown in Figs. 3, 4 and 5 can be explained by this opinion. However, the release of MPP^+ did not decrease the effect of the inhibition of O_2 uptake in mitochondria. Our results (Figs. 3 and 5) show the effect of MPP^+ for respiration is seemingly not reversible in the presence of TPB. MPP^+ inhibited electron transport system in the inner membranes [3–6]. The major part of MPP^+ must release from the matrix, where the electron transport systems do not exist. We discussed the relation between MPP^+ distribution and the respiratory inhibition in mitochondria in the following.

If MPP^+ achieves a Nernst equilibrium across mitochondrial membranes, the concentration of MPP^+ in the matrix (C_{in}) is represented as a function of the concentration of MPP^+ in the medium (C_{out}) by the following equation [14–16,21]:

$$C_{in} = C_{out} \exp(FE/RT) \qquad (2)$$

where E is the electric potential difference between matrix and medium, F, R, and T are the usual thermodynamic constants. Uptake of MPP^+ by mitochondria is the sum of the amount of MPP^+ bound to the (inner and outer) membranes and incorporated in the matrix. Since the volume of membrane is smaller than that of the matrix, uptake of MPP^+ can be approximately represented by the product of MPP^+ concentration and the volume of the matrix. The values of (uptake/C_{out}) must then be proportional to $\exp(FE/RT)$, which is constant in the conditions where E does not change. Fig. 6 shows the relation between (uptake/C_{out}) and time of the results shown in Figs. 3 and 4. As seen from Fig. 6, the values of (uptake/C_{out}) under various conditions have similar time dependence before the increase of the values of (uptake/C_{out}) by addition of TPB. This means that MPP^+ without TPB, did not distribute according to Eqn. 2 and increase of the uptake of MPP^+ within mitochondria resulted in decreased membrane potential of mitochondria corresponding to respiratory inhibition.

Assuming 1 μl matrix volume/mg protein [15,21,22], the concentration of MPP^+ attained in the mitochondria in Fig. 5 is about 9 mM in the absence of TPB, and about 35 mM (maximum level in Fig. 5) in the presence of TPB. The concentration of MPP^+ in the medium is about 90 μM without TPB and about 60 μM with TPB in the experiment of Fig. 5, and the ratio of mitochondrial matrix MPP^+ and outer medium

MPP^+ was calculated to be about 100 (9 mM/90 μM) and 500 (30 mM/60 μM) in the absence and presence of TPB, respectively. The electric potential (E) of mitochondria was estimated to -160 mV from Eqn. 2 in the presence of TPB. This values is reasonable for mitochondria [14,15,21] and this means MPP^+ can distribute a Nernst equilibrium in the presence of TPB.

The respiratory inhibition by MPP^+ in the presence of TPB was stronger than that without TPB even if the amount of MPP^+ uptake was identical (Fig. 3). This agrees with the result of Ramsay et al. [10]. The amount of MPP^+ in the inner membranes, where the enzyme inhibited by MPP^+ exists [3–6], is thought to be proportional to the degree of respiratory inhibition. The amount of MPP^+ in an inner membrane, Q_m, is given by the following equation (Eqn. 3) in the equilibrium condition [23,24]:

$$Q_m = V_m C_i^o (1/L) \int_0^L \exp(-FE(x)/RT)\, dx \qquad (3)$$

where V_m is the effective volume of inner membrane with MPP^+, L is the thickness of the inner membrane, C_i^o is the concentration of MPP^+ at the outer boundary of the inner membrane, and $E(x)$ is the electric potential within the inner membrane. MPP^+ concentrations at the inner and outer boundaries of an inner membrane, represented by C_i^i and C_i^o, respectively, are given by the following equations:

$$C_i^i = b \cdot C_{out} \exp(FE(x)/RT) \qquad (4)$$

$$C_i^o = b \cdot C_{out} \exp(FE_o/RT) \qquad (5)$$

where b is the distribution coefficient of MPP^+, and E_o is the surface potential of outer boundary. As C_{out} increases the values of C_i^o, C_i^i and Q_m, and respiratory inhibition is induced. Since TPB did not alter E_o [10], TPB causes increase in the distribution coefficient of MPP^+ (b) which leads to the formation of a non-charged complex. The increase in b results in increase of Q_m and c_i^o according to Eqns. 3 and 5, as well as

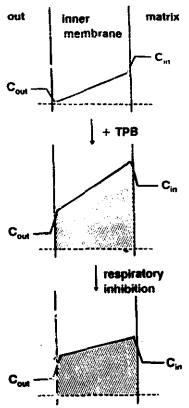

Fig. 7. A hypothetical profile of MPP^+ distribution in inner membrane of mitochondria. The concentration of MPP^+ on the membrane side of either interface is proportional to the concentration of the ion in the adjacent solution as shown in Eqs. 4 and 5. The concentration of MPP^+ is postulated to be linear across the membrane in this model. The hatched area represents the amount of $^{MPP^+}$ in an inner membrane (Q_m) as shown in Eqn. 3. Upper diagram: In the absence of TPB. The distribution coefficient (b) in Eqns. 4 and 5 is less than 1. Middle: In the presence of TPB. The distribution coefficient exceeds 1, and Q_m and C_{in} increase. Lower diagram: The increase in Q_m induces respiration inhibition. The respiratory inhibition leads to a decrease in C_{in} and Q_m.

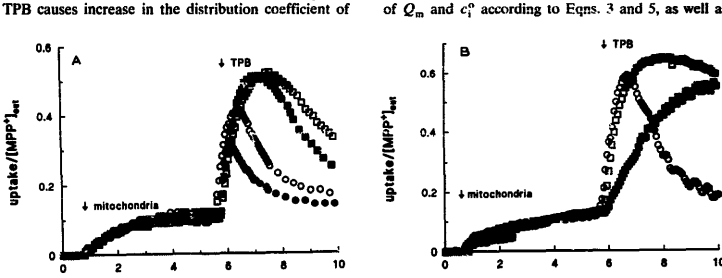

Fig. 6. Relation between (uptake/C_{out}) and time. (A) Results shown in Fig. 3 plotted with the same notations. (B) Results shown in Figs. 4 and 5 plotted with ○, 1.0 mg protein/ml; ■, 4.0 mg protein/ml; □, 1.0 mg protein/ml in the presence of succinate.

increase of uptake in the matrix. Therefore, the distribution of MPP^+ in mitochondrial membranes depends on the presence or absence of TPB as shown in Fig. 7.

The method for measuring MPP^+ uptake with a selective electrode is applicable to other analogs of MPP^+, since preparation of the electrode for lipophilic compounds is not difficult [14–16].

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