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INHIBITION OF METABOLITE ANION UPTAKE IN MITOCHONDRIA BY TETRAPHENYLBORON

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SUMMARY

1. The effect of the permeant anion, tetraphenylboron, on the uptake of metabolite anions has been examined in rat liver mitochondria.

2. Tetraphenylboron, inhibits the translocation of ADP and ATP, and the uptake of succinate, malonate, and inorganic phosphate. The uptake of malonate is inhibited by picric acid, whereas salicylate and nitrate have no effect on metabolite anion uptake at concentrations examined.

3. Tetraphenylarsonium has little or no stimulatory effect on the uptake of metabolite anions at concentrations up to 10 mM.

4. Mg^{2+} selectively relieves the inhibition of the ATP exchange by tetraphenylboron, but has no similar effect on the uptake of ADP or succinate.

5. Tetraphenylboron decreases the respiratory control ratio by inhibiting ADP exchange, rather than by stimulating State IV respiration. There is only a slight ATPase activity associated with tetraphenylboron in the presence of Mg^{2+}.

6. The primary effect of tetraphenylboron is therefore a generalized inhibition of the binding of all metabolite anions to the membrane.

INTRODUCTION

Studies of the exchange of adenine nucleotides[1] and other substrate anions[2] by mitochondria have shown that the rate of uptake is stimulated by the addition of cations such as K^+, Mg^{2+}, or La^{3+} to mitochondria suspended in low ionic strength media. It has been proposed that cations of protons, by binding to amphoteric groups on the outer surface of the inner membrane, increase the positive charge density, resulting in a decrease of the apparent K_m of the metabolite anion with no change in V (ref. 2).

In an attempt to inhibit metabolite anion uptake, the permeant anion tetraphenylboron, which precipitates K^+ from solution[3], was examined. Utsuma and Packer[4] have found that tetraphenylboron uncouples oxidative phosphorylation in intact mitochondria, presumably by dissolving in the lipid phase of the membrane, and acting as an ion conductor. It will be shown that tetraphenylboron completely

Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; MOPS, morpholinopropane sulfonic acid; EGTA, ethylenegylcol-bis-(β-amino ethyl ether)-*N*, *N'*-tetraacetic acid.

inhibits the binding of all metabolite anions examined, whereas other permeant anions have less (2,4,6-trinitrophenol) or no (salicylate, NO_3^-) effect. A preliminary report has been presented[5].

METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose, 20 mM triethanolamine buffer (pH 7.2), and 1 mM EDTA, and washed twice in 0.3 M sucrose adjusted to pH 7 with Tris. The kinetics of uptake of metabolite anions were studied as previously described[1,6] by adding unlabelled nucleotide to [^{14}C]ADP or [^{14}C]ATP prelabelled mitochondria, or by adding ^{14}C-labelled substrate to unlabelled mitochondria[2], and counting radioactivity in a Nuclear–Chicago scintillation counter.

Protein was measured by a modified biuret method[7].

MATERIALS

Radioactive substrates ([1,4-^{14}C]succinic acid, [1-^{14}C]malonic acid, $^{32}P_i$, [8-^{14}C]ADP, [8-^{14}C]ATP) were purchased from New England Nuclear Corp., Boston, Mass.; antimycin A and oligomycin from Sigma Chemical Co.; rotenone from F. P. Penick and Co., N.Y.; Na^+ salt of tetraphenylboron and tetraphenylarsonium from K. and K. Laboratories, Plainview, N. Y.; 2,4,6-trinitrophenol from Fisher Scientific.

RESULTS

Inhibition of adenine nucleotide exchange

The effect of tetraphenylboron on the adenine nucleotide exchange at pH 6.3 is shown in Fig. 1. In the absence of tetraphenylboron, the exchange of 0.1 mM ATP is 30% that of the ADP exchange, indicative of coupled mitochondria[6]. Tetraphenylboron inhibits completely the exchange of both ADP and ATP, with the apparent K_i being approximately 0.2 and 0.06 mM, respectively. The greater sensitivity of the ATP exchange to tetraphenylboron is analogous to the increased susceptibility to stimulation by cations[1], and is probably due to the extra negative charge on ATP.

In Fig. 2, a Lineweaver–Burk plot of the effect of tetraphenylboron on the exchange of ATP at pH 6.3 reveals a mixed type of inhibition. V is decreased from 2.1 to 1.2 nmoles/mg per min by 0.1 mM tetraphenylboron.

If tetraphenylboron were acting primarily as an anion to inhibit the binding of adenine nucleotides, the positively charged tetraphenylarsonium should stimulate the adenine nucleotide exchange. The results are shown in Fig. 3, where a pH of 7.6 was chosen to provide a more negative surface potential or charge, and hence, a greater possible effect of the cation[1]. It is seen that the ADP exchange is inhibited about 50%, while the ATP exchange is stimulated by tetraphenylarsonium. The stimulatory effect of tetraphenylarsonium on the ATP exchange is assumed to be the result of its uncoupling properties[8]. Other experiments with the uptake of [^{14}C]malonate revealed no effect of tetraphenylarsonium at levels up to 10 mM.

To examine whether the effectiveness of tetraphenylboron is reduced by

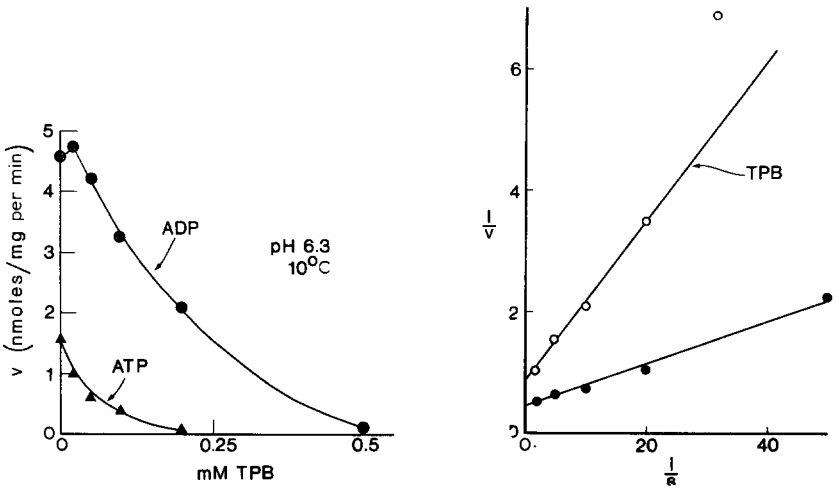

Fig. 1. Effect of tetraphenylboron (TPB) on the rate of ATP and ADP exchange. Mitochondria (1.3 mg), prelabelled with [^{14}C]ADP, were incubated at 10 °C in 1 ml of 0.2 M sucrose, 2 mM Tris–MOPS (pH 6.3) and 0.2 mM Tris–EGTA. The exchange was initiated with 0.1 mM Tris–ATP or Tris–ADP, and stopped at 20 s with 10 μM atractyloside.

Fig. 2. Uncompetitive effect of tetraphenylboron on the rate of ATP exchange. Prelabelled mitochondria (1.0 mg) were incubated at 5 °C in 0.2 M sucrose, 0.2 mM $MgCl_2$, and 2 mM Tris–MOPS (pH 6.3). The exchange was initiated with Tris–ATP, and stopped at 20 s with 10 μM atractyloside.

positive charges, $MgCl_2$ was added to mitochondria suspended in a low ionic strength medium. By increasing the Mg^{2+} concentration to 1 mM, Fig. 4 shows that the inhibition of the ATP exchange by tetraphenylboron is completely overcome, whereas the ADP exchange is initially increased moderately, then inhibited by Mg^{2+}. Since Mg–ATP has a dissociation constant about 10-fold lower than Mg–ADP (ref. 9), it is probably that Mg^{2+} reverses the ATP exchange by complexing selectively with ATP, thus decreasing the negative charge. The inability of Mg^{2+} to prevent the inhibition of the succinate exchange by tetraphenylboron (Fig. 6) substantiates this conclusion.

Inhibition of substrate uptake

Specific inhibitors of adenine nucleotide translocation such as atractyloside or bongkrekic acid have not been observed to affect the uptake of other metabolite anions, nor do inhibitors of substrate uptake affect the adenine nucleotide exchange. However, Fig. 5 reveals that the uptake of malonate (Fig. 5A) and phosphate (Fig. 5B) is very sensitive to tetraphenylboron, being 50% inhibited by approx. 0.025 and 0.07 mM tetraphenylboron respectively. With both substrates, there is a slight, but reproducible, stimulation of substrate uptake at low tetraphenylboron concentrations, an effect that can also be seen with all of the other metabolites examined.

Fig. 6 shows that tetraphenylboron readily inhibits the uptake of 0.5 mM succinate, after an initial stimulation at 0.02 mM tetraphenylboron. Addition of

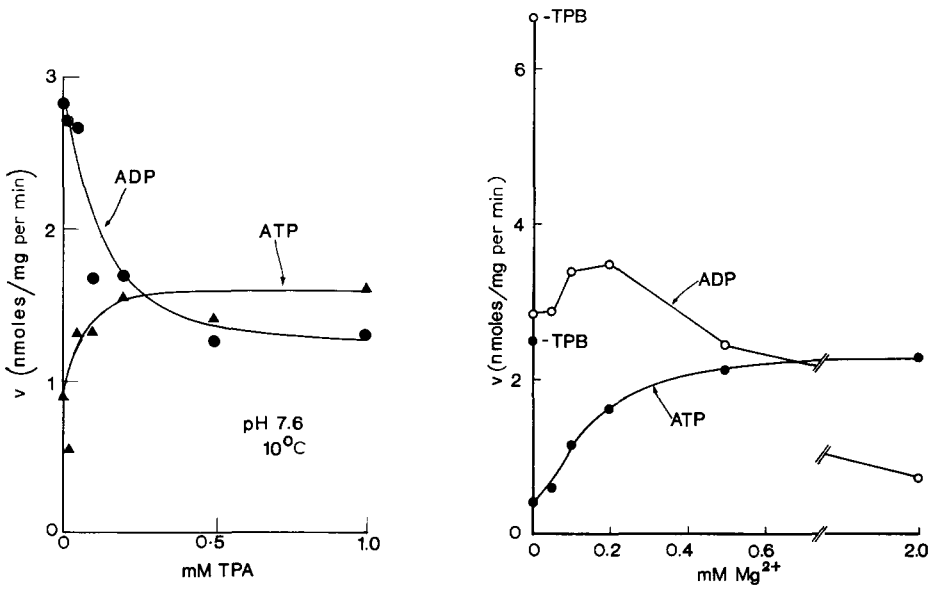

Fig. 3. Effect of tetraphenylarsonium (TPA) on the rate of ADP, ATP exchange. Prelabelled mitochondria (1.04 mg) were incubated at 10 °C in 0.2 M sucrose, 0.2 mM EGTA, and 2 mM Tris–TES (pH 7.6), and back-exchanged with 0.1 mM Tris–ADP or Tris–ATP.

Fig. 4. Stimulation of the tetraphenylboron-inhibited exchange of ATP and ADP by Mg^{2+}. Mitochondria (1.3 mg) were incubated at 10 °C in 0.2 M sucrose, 5 mM Tris–MOPS (pH 6.3) *plus* 0.1 mM tetraphenylboron (ATP exchange) or 0.5 mM tetraphenylboron (ADP exchange) and $MgCl_2$ as indicated. The exchange was initiated with 0.1 mM Tris–ATP or ADP, and stopped with atractyloside at 20 s.

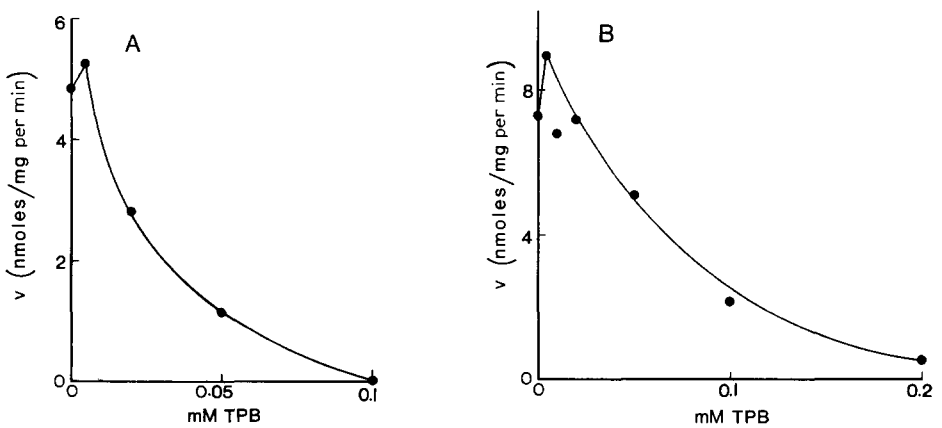

Fig. 5. Inhibition of (A) malonate and (B) phosphate uptake by tetraphenylboron. In A, mitochondria (2.3 mg) were incubated at 5 °C in 0.2 M sucrose, 0.2 mM EGTA, and 2 mM Tris–MOPS (pH 6.3) *plus* 2.5 μg each of rotenone and oligomycin. The reaction was initiated with 0.14 mM [^{14}C]malonate, and stopped at 6 s with 5 mM benzylmalonate. In B, 2.4 mg mitochondria were incubated at 5 °C in 0.2 M sucrose and 2 mM benzylmalonate (pH 6.8) *plus* 2.5 μg each of rotenone and oligomycin. The reaction was initiated with 0.1 mM $^{32}P_i$ (200000 cpm), and stopped at 6 s with 1 mM mersalyl.

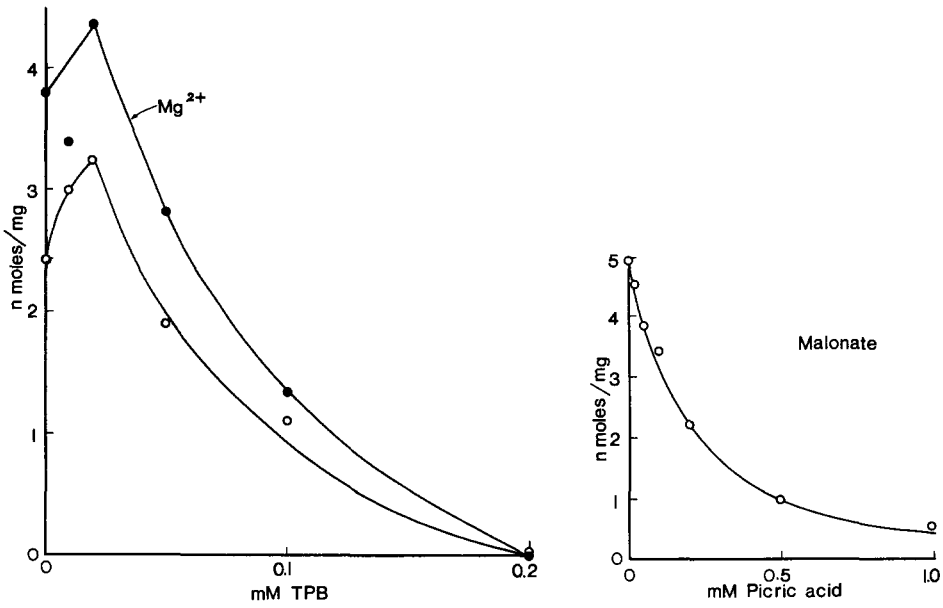

Fig. 6. Effect of Mg^{2+} on the inhibition of succinate uptake by tetraphenylboron. Mitochondria (2.2 mg) were incubated at 10 °C in 0.2 M sucrose, 20 mM NaCl, 5 mM Tris–MOPS (pH 6.5) 2.5 μg each of oligomycin and antimycin A, and 1 mM $MgCl_2$ as indicated. The reaction was initiated with 0.5 mM Tris–[^{14}C]succinate, and terminated at 2 min by centrifugation. Sucrose permeable space was determined under identical conditions, using 0.5 μCi [^{14}C]sucrose, and subtracted from experimental values.

Fig. 7. Inhibition of malonate uptake by picrate. Mitochondria (2.0 mg) were incubated at 5 °C in 0.2 M sucrose, 20 mM NaCl, 5 mM Tris–MOPS (pH 6.3) 2.5 μg each of oligomycin and rotenone, *plus* sodium picrate as indicated. The reaction was initiated with 0.14 mM[^{14}C]-malonate, and terminated at 2 min by centrifugation. Sucrose permeable space was determined as in Fig. 6.

1 mM Mg^{2+} stimulates the uptake about 55% in the absence of tetraphenylboron, and is similar to the general stimulation observed by a variety of cations[2]. Mg^{2+} is unable to counter the inhibition of succinate uptake by tetraphenylboron, strengthening the proposition that the activation of ATP translocation by Mg^{2+} in the presence of tetraphenylboron is due to the formation of a Mg–ATP complex.

The inhibitory effects of another permeant anion, 2,4,6-trinitrophenol (sodium picrate), on the uptake of malonate is described in Fig. 7. Although picrate does inhibit malonate uptake, the effectiveness is less than 20% that of tetraphenylboron (see Fig. 5). The effects of salicylate and NO_3^- at levels up to 1 mM were also examined, but there was no significant inhibitory effect on the uptake of any metabolite anion tested.

Although Skulachev *et al.*[8] have pointed out that tetraphenylboron is not taken up by intact mitochondria by virtue of the negative internal potential, Utsuma and Packer[4] report that tetraphenylboron uncouples oxidative phosphorylation supported by succinate, or especially by the tetramethyl-*p*-phenylenediamine (TMPD)–ascorbate system. We have repeated these experiments in mitochondria

respiring with 10 mM succinate at 30 °C. Fig. 8 shows that tetraphenylboron does not stimulate State IV respiration significantly, but rather prevents the ADP supported State III rate, at approximately the same concentration of tetraphenylboron that inhibits the exchange of ADP (see Fig. 1). At 0.4 mM tetraphenylboron, there is a complete inhibition of respiration, which can be partially overcome by raising the concentration of succinate to 30 mM (not shown). Thus the decrease in the respiratory control ratio (+ADP/−ADP) by tetraphenylboron is due entirely to an inhibition in the uptake of ADP.

A more direct measure of the uncoupling properties of tetraphenylboron is seen in Fig. 9, where the effect on ATPase activity is described. If Mg^{2+} is omitted, there is an initial moderate stimulation of ATPase by 0.05 mM tetraphenylboron, followed by a complete inhibition, which may be related to the prevention of ATP exchange by tetraphenylboron. When 5 mM Mg^{2+} is added, in which case ATP translocation is not affected by tetraphenylboron, there is only a 15% increase in ATPase activity at 1 mM tetraphenylboron, compared to a 90% increase by a known uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone.

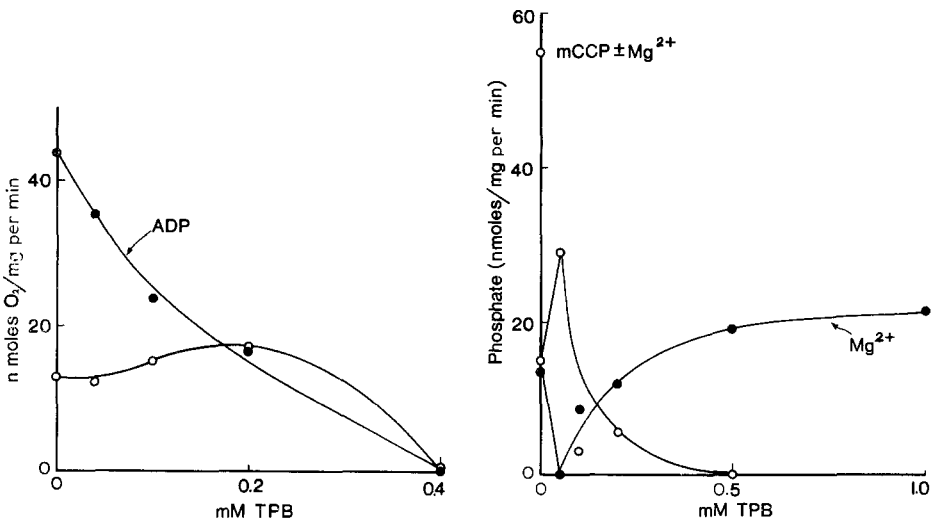

Fig. 8. Inhibition of respiration by tetraphenylboron. Mitochondria (0.9 mg) were incubated at 30 °C in a reaction vessel containing 0.1 M sucrose, 20 mM MOPS (pH 7.2), 2 mM P_i, 1 mM $MgCl_2$, 10^{-6} M rotenone, and 10 mM succinate, followed by tetraphenylboron (○) and 100 nmoles ADP to initiate State III respiration (●). Respiration was determined polarographically with a Yellow Springs oxygen electrode in a total volume of 0.5 ml.

Fig. 9. Effect of tetraphenylboron on ATPase activity. Mitochondria (2.5 mg) were incubated at 25 °C in 1 ml of 50 mM sucrose, 10 mM NaCl, 10 mM Tris–MOPS (pH 7.2) *plus*: 0.1 μM carbonyl cyanide *m*-chlorophenylhydrazone (mCCP), 2.5 μg oligomycin and 5 mM Mg^{2+}. The reaction was initiated with 2 mM ATP, and terminated at 1 min with $HClO_4$. The experimental results were corrected for the oligomycin-insensitive ATPase activity.

DISCUSSION

Tetraphenylboron is known to prevent valinomycin-dependent ATP synthesis[10], and to inhibit the P_i–ATP exchange[4] in mitochondria. It also decreases

the fluorescence of 8-anilino-1-naphthalene sulfonic acid in red blood cells[11] or Ehrlich Lettre ascites cells (Parikh, K. and Meisner, H., unpublished). Based on results presented here, a common mechanism underlying all of these effects may be a generalized, noncompetitive inhibition of the binding of metabolite anions to the membrane. It is proposed that the lipophilic tetraphenyl group binds to the hydrophobic part of the membrane and the high negative charge density of the boron anion creates a negative surface potential that acts to repel the metabolite anions. This would explain the selective stimulation of TMPD supported respiration by picrate, and not glutamate or succinate, as reported by Skulachev *et al.*[8], as well as the increased ATPase activity by tetraphenylboron in the presence of TMPD[4]. The positively charged TMPD is expected to be attracted by the binding of tetraphenylboron or picrate to the membrane, whereas other negatively charged metabolites such as phosphate, ADP, or succinate are repelled. The entry of TMPD into the matrix decreases the membrane potential, or the pH differential, leading to the uncoupled state.

The lack of effect of tetraphenylarsonium should be accounted for, since cations have been shown to stimulate the uptake of metabolite anions under the conditions tested[1,2]. Fortes and Hoffman[11] in their study of the effect of membrane charge on the binding of 8-anilino-1-naphthalene sulfonic acid to red blood cell membranes, used a 20-fold higher concentration of tetraphenylarsonium than tetraphenylboron to record opposite fluorescent effects of similar magnitude. It is suggested that arsenate, which has a low positive charge density by virtue of its relatively large anhydrous radius (1.21 Å compared to 0.80 Å for boron), when bound to the membrane does not provide sufficient positive surface potential to attract the metabolite anions.

Lastly, it should be mentioned that C. Hoppel (personal communication) has recently shown that sulfobromophthalein is a sensitive inhibitor of the adenine nucleotide exchange, and the P_i/OH as well as P_i/malate uptake in mitochondria. Models of mitochondrial carriers must therefore take into consideration the generalized inhibition by these chemically different molecules.

ACKNOWLEDGMENT

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