ION FLUX COUPLED TO THE MITOCHONDRIAL OXIDATION OF TETRAMETHYL-*p*- PHENYLENEDIAMINE

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SUMMARY

The respiratory chain inhibitor, antimycin, stimulates ion transport in rat liver mitochondria when energy is provided by the oxidation of tetramethyl-p-phenylenediamine.

Studies of the terminal part of the mitochondrial respiratory chain have frequently employed ascorbate together with catalytic quantities of TMPD* as an electron-donor system (1-3). Investigations have been based on the reaction between TMPD and cytochrome *c* of the intact respiratory chain (3) which enables electrons entering the chain to traverse only the segment between cytochrome *c* and oxygen. Since previous studies (4,5) have indicated a single energy-coupling site in this region, as opposed to the three sites spanned by the oxidation of NADH, investigation of the TMPD-ascorbate system should, in principle, be possessed by relative simplicity.

Investigation of energy conservation coupled to TMPD oxidation has generally revealed surprisingly high efficiencies, with ATP:O ratios of about one (1,2) or somewhat more than one (3). In the instance (3) where ratios of greater than one were obtained, the addition of antimycin led to a stimulation of respiration in the absence of phosphate

*Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; NQNO, 2-nonyl-4-hydroxyquinoline-N-oxide.

acceptor and a diminution of the ATP:O ratio to about one-half of its previous value. The high ATP:O ratios, as well as sensitivity to antimycin, have been taken to suggest contribution to ATP formation by oxidation of endogenous substrates (6,7), although, on this basis, the antimycin-induced stimulation of respiration is somewhat difficult to understand. That explanation is also not confirmed in recent studies by Chamalaum, Tager and Slater (8) who find ATP:O ratios approaching one together with exact stoicheometry between ascorbate oxidation and oxygen uptake.

We have undertaken to examine ion translocation coupled to TMPD oxidation with special reference to the influence of the respiratory inhibitor, antimycin. Our results suggest that the apparent uncoupling by antimycin noted above (3) may be related to a stimulation of ion transport, measured as uptake of ^{45}Ca or ^{32}P by rat liver mitochondria.

METHODS

Rat liver mitochondria were isolated according to the method of Myers and Slater (9). Ion uptake was measured essentially as described by Pressman *et al.* (10) with mitochondria being incubated with shaking in a medium containing ^{45}Ca or ^{32}P and the reaction being terminated by rapid dilution in 10 volumes of 0.25 M sucrose at 0° followed by millipore filtration. The basic reaction medium for Ca^{2+} uptake contained 12.5 mM Tris-HCl, 15 mM Na ascorbate, 60 μM TMPD, 0.25 M sucrose, 2.5 mM phosphate, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, 25 μM rotenone and from 3 to 6 mg of protein in 4.0 ml. The reaction was followed at 20° and at pH 7.5. The reaction medium contained approximately one microcurie of ^{45}Ca per ml.

RESULTS AND DISCUSSION

When calcium uptake is measured in the presence of phosphate, addition of a low concentration of antimycin leads to a small, but consistent, stimulation of translocation and a greater elevation of the Ca:O ratio (Table I). Added phosphate is required for the stimulation to occur. In the absence of phosphate, the rate of calcium transport is little influenced by antimycin while oxygen uptake is stimulated, giving rise to a decline in the Ca:O ratio. In the presence of phosphate, addition of a 40-fold excess of antimycin leads to inhibition of Ca^{2+} uptake and probably reflects the well documented generalized uncoupling by high concentrations of the inhibitor (11). In other experiments, omission of rotenone was without important effect, while the respiratory chain inhibitor NQNO was able to substitute for antimycin in stimulating ^{45}Ca uptake.

Table I

INFLUENCE OF ANTIMYCIN ON Ca/O RATIOS OBTAINED WITH TMPD AND ASCORBATE

additions	Ca^{2+} nmoles/min	(O) natoms/min	Ca/O
none	117	68	1.72
+ antimycin 0.1 µg/mg	111	105	1.06
PO_4 1.25 mM	152	90	1.69
PO_4 + antimycin 0.1 µg/mg	167	68	2.46
PO_4 1.25 mM	155	89	1.74
PO_4 + antimycin 4 µg/mg	99	65	1.52

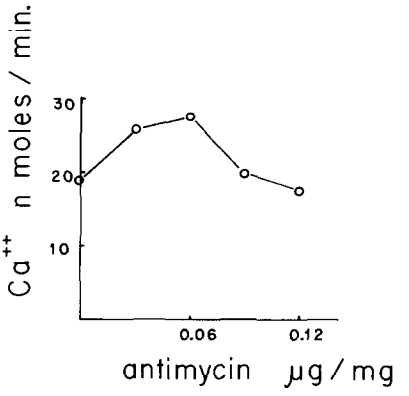

Figure 1. Influence of antimycin concentration upon calcium uptake. Conditions as described in materials and methods.

Figure 1 indicates that maximum stimulation of the rate of Ca^{2+} translocation occurs at about 0.06 µg of antimycin per mg mitochondrial protein, a concentration which corresponds to that required for inhibition of the respiratory chain (12). As this is also the antimycin concentration required for apparent uncoupling of ATP synthesis linked to TMPD oxidation, noted previously (3), inhibition of the respiratory chain, uncoupling of TMPD-linked oxidative phosphorylation, and stimulation of Ca^{2+} transport should probably be regarded as aspects of a single molecular interaction.

Antimycin-induced stimulation of Ca^{2+} transport is only observed when a permeant anion such as phosphate is present, and Table II indicates that, in the presence of Ca^{2+}, phosphate translocation is also stimulated by antimycin. In these experiments, the basic reaction mixture described above was modified so that Tris replaced all cations save an "added cation" listed on the table, which was present as a 5 mM Cl^- salt.

Table II

STIMULATION OF PHOSPHATE UPTAKE BY ANTIMYCIN*

added cation	PO4 uptake nmoles/min/mg -anti.	+anti.	increase %
Ca^{2+}	75.0	90.0	20
Na^{+}	31.9	22.5	-
K^{+}	32.8	72.5	121
Mg^{2+}	39.9	48.4	21
none	20.2	24.4	21

*antimycin: 0.07 µg/mg

It is interesting that phosphate entry is stimulated when Tris is the only cation in the medium. Likewise, antimycin produces the most dramatic influence in the presence of K^{+} and none at all in that of Na^{+}. The ability of an antibiotic, which promotes ion transport, to discriminate between Na^{+} and K^{+} is strongly reminiscent of the action of valimomycin (13).

Thus, in the special case of TMPD oxidation, antimycin uncouples ATP synthesis (3) while promoting ion translocation. Inhibition of endogenous substrate oxidation as explanation of apparent uncoupling noted previously (3) is thus rendered extremely unlikely since it would be expected to produce a decline in ion transport as well as phosphorylation. The action of antimycin in shunting available energy from phosphate esterification toward translocation may be viewed as induced ion transport analogous, in a limited sense, to that associated with the action of valinomycin and gramicidin (13), which promote energy-linked transport of univalent cations.

In this instance, the direct action of antimycin appears to be upon anion transport since phosphate entry is increased, even in the absence of a permeant cation, while Ca^{2+} transport is not stimulated in absence of phosphate. Clearly antimycin differs importantly from valinomycin and gramicidin in being a potent inhibitor of the respiratory chain in the region of cytochrome *b*. Since the inhibitory action of antimycin occurs at the same concentration as its effect on ion transport, one is encouraged to assume that the two effects are related and that the cytochrome *b* region (which is not spanned by TMPD oxidation) may nonetheless play a role in coupled ion transport. Involvement of the cytochrome *b* region in ion transport is consistent with observations by Chance and Schoener (14) of alterations in the low-temperature spectrum of cytochrome *b* on addition of Ca^{2+}, phosphate, or uncoupling agents. It is also supported by observations by Brierley and Settlemire (15) of induction of cation transport by Zn^{2+}, which has also been shown by Skulachev to inhibit the respiratory chain in the cytochrome *b* region (16).

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