Uncoupling and Specific Inhibition of Phosphoryl Transfer Reactions in Mitochondria by Antibiotic A20668*

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SUMMARY

A20668 A, B, and C are polypeptide antibiotics that inhibit phosphorylation of ADP, MgATPase, and the ATP-driven transhydrogenase of rat liver submitochondrial particles, but not the purified F1 ATPase. In intact mitochondria, A20668 inhibits uncoupler-induced ATPase, State 3 respiration, and phosphorylation; the A and B forms are approximately equipotent with rutamycin, whereas A20668 C is less effective.

Concentrations of A20668 slightly greater than required for complete inhibition of phosphoryl transfer stimulate rapid, uncoupled respiration by mitochondria under State 3 or 4 conditions. A20668 A and B are more effective uncouplers than A20668 C. In the presence of venturicidin or ossamycin, concentrations of A20668, which alone do not uncouple, stimulate oxygen consumption of mitochondria incubated under either State 3 or 4 conditions. A20668 uncoupling is not potentiated by prior inhibition of phosphoryl transfer by venturicidin X, rutamycin, aurovertin, or efrapeptin. A20668 increases mitochondrial permeability to protons in passive swelling experiments where facilitation of proton conductance correlates well with potency to uncouple.

A20668 apparently binds initially at a unique locus to inhibit mitochondrial phosphoryl transfer reactions. When this site is saturated, additional antibiotic may uncouple by increasing proton conductance of mitochondria. Binding of venturicidin or ossamycin appears to interfere with the binding of A20668 to its adjacent inhibitory site, thus effectively increasing the concentration of A20668 available to uncouple.

Antibiotic inhibitors of the electron transport chain (10) and of phosphoryl transfer reactions (2-4, 11-13) have been useful to study the reaction sequence of these pathways (3, 10, 11, 13, 14). Inhibition of phosphoryl transfer in mitochondria produces a concomitant depression of respiration (2, 11, 12) because electron transport and substrate oxidation are tightly coupled to ATP synthesis (15, 16). In a preliminary communication (1), we reported that a new polypeptide antibiotic, A20668 B, inhibited phosphoryl transfer but also uncoupled rat liver mitochondria. This property made A20668 a unique tool with which to probe mitochondrial function. The data presented in this report describe the effects of A20668 on mitochondria and submitochondrial particles from rat liver and are interpreted to suggest that the antibiotic interacts with mitochondria in two distinct ways.

EXPERIMENTAL PROCEDURE

Rat liver mitochondria (17) and submitochondrial particles (18) were prepared by the methods referred to and were suspended in 250 mM mannitol plus 70 mM sucrose. All incubations were at 30°C. ATPase (19), ATP-driven transhydrogenase (20), adenine nucleotide exchange (21), oxidative phosphorylation (16), inorganic phosphate (22), and protein (25) were assayed by the methods indicated. Mitochondrial respiration was measured polarographically with a Gilson oxygraph model K-IC equipped with an IC-pH unit (Gilson Medical Electronics, Middleton, Wis.) Passive swelling of mitochondria in isotonic potassium acetate was measured as a decrease in light scattering at 565 nm (7). A20668 A, B, and C and A20671 (efrapeptin) were generous gifts from Dr. Robert Hamill, Eli Lilly and Co., Indianapolis, Ind. Venturicidin X was kindly provided by Dr. W. Keller-Schierlein, Federal Technical Institute, Zurich. The sources of other antibiotics are found in Refs. 11 and 12. All solutions were brought to pH 7.4 with triethanolamine.

RESULTS

Inhibition of Mitochondrial ATP-utilizing Reactions by A20668—A20668 A, B, and C are closely related polypeptide antibiotics, one of which is probably identical with leukimostatin (24). Leukimostatin has a molecular weight of 1568, is composed primarily of leucine, and contains smaller amounts of four unidentified amino acids (24). Fig. 1 shows that A20668 inhibits the ATPase induced by 2,4-dinitrophenol in rat liver mitochondria. A20668 A and B are more potent than the C compound and diminish this ATPase as effectively as rutamycin, on a molar basis (Fig. 1). A20668 also inhibits mitochondrial ATP hydrolysis produced by several other uncouplers, calcium uptake, and iono-

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1 R. L. Hamill, personal communication.
Inhibition of mitochondrial and submitochondrial particle ATPase by A20668 and rutamycin. Mitochondria (1 to 1.2 mg of protein) were 
incubated for 10 min in 1 ml of medium containing 6 mM ATP-
triothanolamine, pH 7.4, 10 mM Cl-triothanolamine, pH 7.4, 30 mM 
KCl, 75 mM mannitol, and 90 mM sucrose. Dinitrophenol, 1 × 
10⁻⁶ M, induced ATPase of 2.26 μmol of Pi/mg of protein.

### Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Inducer</th>
<th>μmol Pi/mg of protein</th>
<th>Per cent inhibition</th>
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<tr>
<td>Mitochondria</td>
<td>+1 × 10⁻⁴ M 2,4-DNP</td>
<td>2.69 0.38</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>+5 × 10⁻⁷ M m-Cl-CCP</td>
<td>1.67 0.22</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>+1 × 10⁻⁶ M TTFB</td>
<td>0.40 0.0</td>
<td>100</td>
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<tr>
<td></td>
<td>+2 × 10⁻⁶ M CaCl₂</td>
<td>1.22 0.08</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>+6 × 10⁻⁷ M valinomycin</td>
<td>1.92 0.04</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>+2 × 10⁻⁶ M monazomycin</td>
<td>2.07 0.02</td>
<td>99</td>
</tr>
<tr>
<td>Submitochondrial particles</td>
<td>No addition</td>
<td>0.17 0.17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+2 × 10⁻³ M MgCl₂</td>
<td>4.97 1.67</td>
<td>66</td>
</tr>
</tbody>
</table>

Table II

**Effect of A20668 B on oxidative phosphorylation by submitochondrial particles**

Submitochondrial particles (4.2 mg of protein) were added to 3 
ml of medium which contained 2 mM ATP-triothanolamine, pH 
7.4, 3 mM MgCl₂, 13 mM PO₃-triothanolamine, pH 7.4, 15 mM 
KCl, 42 mM mannitol, and 131 mM sucrose. State 3 was 
initiated by addition of 2 μg of hexokinase, 18 μM glucose, and 10 mM 
substrate from the side arm of a Warburg flask and monitored 
for 10 min.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>A20668 B</th>
<th>O₂ Uptake</th>
<th>Pi Uptake</th>
<th>Pi:O</th>
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<tr>
<td>NADH</td>
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<td>2.39</td>
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<td></td>
<td>0.5</td>
<td>1.96</td>
<td>0.55</td>
<td>0.3</td>
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<td></td>
<td>1.2</td>
<td>2.07</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Succinate</td>
<td>0</td>
<td>2.20</td>
<td>2.41</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2.24</td>
<td>0.64</td>
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<td></td>
<td>1.2</td>
<td>2.57</td>
<td>0.25</td>
<td>0.1</td>
</tr>
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</table>

The uncoupler-induced ATPase of intact mitochondria (5 to 10 
μg of A20668 B/mg of protein are required to inhibit completely 
the Mg⁺⁺/ATPase of particles). A20668 does not inhibit adenine 
nucleotide exchange across the mitochondrial membrane. Thus, 
the diminished effectiveness of A20668 to inhibit particle ATPase 
may result from a decreased affinity for the antibiotic at its bind-
ing site in mitochondrial fragments prepared by sonne oscillation. 
A20668 does not inhibit ATP hydrolysis by the F₁ enzyme puri-
from rat liver or beef heart mitochondria. Therefore, the 
antibiotic probably binds at a locus on the inner mitochondrial 
membrane to inhibit phosphoryl transfer reactions.

Uncoupling of Oxidative Phosphorylation by A20668—Phos-
phorylation of ADP by submitochondrial particles is inhibited by 
A20668, whereas substrate oxidation remains unaffected (Table 
II). In this regard, A20668 acts like other inhibitors of phosphoryl 
transfer reactions to uncouple phosphorylating submitochondrial 
particles (2, 12). In intact mitochondria, State 3 succinate oxida-
tion is progressively inhibited by concentrations of A20668 that 
have no effect on State 4 respiration but effectively inhibit un-
coupler-induced ATPase (Fig. 2). During inhibition of phosphate 
esterification by these concentrations of A20668, the P:O ratio 
remains relatively constant (4). At concentrations greater than 
required to inhibit State 3 respiration maximally, A20668 stimu-
lates uncoupled substrate oxidation by mitochondria incubated 
under both State 3 and 4 conditions (Figs. 2 and 3A). A20668 A 
and B are more effective than the C compound both as inhibitors 
of State 3 phosphorylation and as uncouplers (Fig. 2A). A similar 
inhibition, followed by uncoupling of State 3 respiration with in-
creasing concentrations of A20668, is observed when mitochon-
dria are incubated with β-hydroxybutyrate or glutamate plus 
malate (4) as substrates. The rate of uncoupled oxygen uptake 
stimulated by A20668 equals or exceeds the State 3 rate of mito-
chondrial substrate oxidation (Figs. 2 and 3A).

**Effect of Other Phosphoryl Transfer Inhibitors on A20668 Un-
coupling**—Because A20668 appears to have an uncoupling action 
separate from its oligomycin-like effect, attempts were made to 
displace A20668 from its inhibitory site to determine whether 
this would enhance or diminish its uncoupling effect.

When State 3 succinate oxidation is inhibited maximally by 
venturicidin, concentrations of A20668 previously incapable of 
uncoupling, rapidly stimulate respiration (Fig. 3, compare Trace 
B to A). Prior exposure of mitochondria to venturicidin also po-
Fig. 2 (left). Uncoupling of State 3 and 4 respiration by A20668. Mitochondria (1.1 mg of protein) were incubated in 2.3 ml of medium containing 3 mM MgCl₂, 4 mM PO₄-triethanolamine, pH 7.4, 6 mM succinate-triethanolamine, pH 7.4, 1.3 μM rotenone, 6 mM KCl, 5 mM mannitol, and 207 mM sucrose. State 3 was initiated by addition of 0.8 mM ADP-triethanolamine, pH 7.4 (dashed lines, open symbols). State 4, solid lines and symbols.

Fig. 3 (right). Uncoupling by A20668 of mitochondria incubated with various phosphoryl transfer inhibitors. Mitochondria (3 mg of protein) were incubated as described in the legend to Fig. 2. Arrows and numbers indicate additions and cumulative concentrations, respectively, of A20668 A. Phosphorylation inhibitors (1.1 μg/mg of protein) were added at the arrow marked I in Traces B and C.

Fig. 4. Potentiation by venturicidin and ossamycin of uncoupling induced by A20668. Mitochondria (4.3 mg of protein) were incubated in the medium described in the legend to Fig. 2. ○, State 3, 0.8 mM ADP-triethanolamine, pH 7.4; △, ○, △, and ●, State 4. The figures in parentheses indicate the concentration of A20668 A added after venturicidin or ossamycin.

potentiates the release of State 4 respiration by A20668 (Fig. 4A, ref. 4), but not uncoupling due to 2,4-dinitrophenol or carbonyl cyanide m-chlorophenylhydrazone. Venturicidin X (the aglycone of venturicidin), rutamycin, aurovertin, and A23871 (4) have no significant effect on the ability of A20668 to uncouple mitochondria incubated under State 3 or 4 conditions, even when these inhibitors are present at concentrations that completely inhibit ADP phosphorylation (Fig. 3 B and C). Ossamycin potentiates uncoupling by A20668, but much less effectively than venturicidin (Figs. 3C and 4B). Concentrations of venturicidin and ossamycin greater than required to inhibit State 3 phosphorylation completely, continue to potentiate further the release of State 4 respiration by A20668 (Fig. 4).

Enhancement by venturicidin and ossamycin of the uncoupling activity of A20668 cannot be due simply to prior inhibition of phosphoryl transfer, because ADP-stimulated respiration was prevented by all the inhibitors tested. Venturicidin and venturicidin X almost certainly bind to the same inhibitory site, yet the aglycone, a more potent inhibitor (4), does not facilitate uncoupling by A20668. Therefore, occupancy of the venturicidin binding site per se is not sufficient to potentiate uncoupling by A20668. The data suggest a specific role for the dideoxy carbamido sugar moiety of venturicidin in this effect. When venturicidin binds to its site, the sugar moiety could extend into an adjacent area where A20668 binds to a unique inhibitory site. If this prevented binding of A20668, it would increase the amount of A20668 available to uncouple at a separate locus. Ossamycin is probably a sugar-containing antibiotic (4) and could displace A20668 from its site in a similar manner. This explanation implies that A20668 alone first binds at one site to inhibit phosphoryl transfer reactions and, when this site is fully saturated, acts in an independent manner to uncouple mitochondrial respiration.

Effect of Uncouplers and A20668 on Passive Swelling of Mitochondria in Potassium Acetate—Uncoupling agents markedly accelerate valinomycin-induced swelling of nonrespiring mitochondria incubated in isotonic potassium acetate (Fig. 5, Ref. 7). Because uncouplers increase the proton conductance of a variety of membranes (9), they appear to facilitate swelling in the above system by permitting hydrogen ions to leave the mitochondria, thus relieving pH and charge gradients which otherwise limit net penetration of salt and water (7, 9). Fig. 5 shows that A20668 increases mitochondrial permeability to protons in this model system. Furthermore, prior incubation of mitochondria with venturicidin potentiates the rate of swelling produced by less than optimal concentrations of A20668, whereas the other inhibitors of phosphoryl transfer do not show this effect.² A20668 does not catalyze a valinomycin-like potassium uptake or a nigericin-like potassium/hydrogen exchange in this system (Fig. 5) or in respiring mitochondria.

According to the chemiosmotic hypothesis of energy conserva-

² Unpublished observations in this laboratory.
The only anion and is probably limited by the rather slow rate of chloride ion leakage from the mitochondria (27). Oligomycin has been reported to stimulate a chloride-dependent uncoupling and ATPase of mitochondria whose permeability to potassium has been increased by incubation with valinomycin or Triton X-100 (30). Apparently, oligomycin increases mitochondrial permeability to chloride and thus facilitates potassium uptake and energy dissipation (30). A20668 requires neither chloride nor potassium for maximal uncoupling activity and does not increase permeability of mitochondria to ions in a nonselective manner (i.e., a detergent effect).³

A20668 apparently binds to a unique site, other than the $F_{1}$ ATPase, to inhibit phosphoryl transfer reactions in mitochondria. Potentiation by venturicidin and ossamycin of A20668s uncoupling activity is most satisfactorily explained by supposing that these antibiotics interfere with A20668 binding to its inhibitory site and thus increase the concentration of A20668 available to uncouple at another site. A20668 increases the permeability of mitochondrial membranes to protons in a test system where potency to do this correlates well with effectiveness to uncouple mitochondria. The ability of agents to increase proton conductance across black lipid membranes (31–33) or membranes of submitochondrial particles (34) correlates poorly with uncoupling activity. On the other hand, facilitation by uncouplers of proton movements across membranes of liposomes (33) and nonrespiring mitochondria (35) shows an excellent correlation with effectiveness in stimulating uncoupled respiration. Inasmuch as uncouplers also appear to produce organizational changes in multibilayer membranes (36) and bind to mitochondria (37, 38) for both inhibition and uncoupling could explain this observation. However, the data presented in this paper do not rule out the possibility that A20668 affects only one component of the coupling system, in some undefined manner, first to inhibit, then to uncouple mitochondrial respiration.

DISCUSSION

A20668 is both a unique uncoupler and inhibitor of phosphoryl transfer reactions in mitochondria. In contrast to other uncouplers, A20668 does not induce ATPase because it inhibits mitochondrial phosphoryl transfer at concentrations needed to uncouple. A23187 uncouples mitochondria and fails to induce ATPase as a result of its effects on endogenous divergent cations (25). A20668, however, does not have similar effects on mitochondrial divergent cations. In contrast to other inhibitors of phosphoryl transfer reactions (2, 3, 11, 12), A20668 stimulates rapid, uncoupled respiration at concentrations slightly greater than required for complete inhibition of phosphorylation or ATPase.

The trialkyl tins inhibit phosphoryl transfer and uncouple respiration (20–29), but uncoupling is observed only under conditions in which these compounds can produce an anion/hydroxyl exchange (27). The magnitude of uncoupled respiration produced by trialkyl tins is small in a medium that contains chloride as the

REFERENCES

22. Sumner, J. B. (1944) Science 100, 412–414
Uncoupling and specific inhibition of phosphoryl transfer reactions in mitochondria by antibiotic A20668.

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