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RESERPINE AS AN UNCOUPLING AGENT

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SUMMARY

1. Reserpine, like the uncoupling agent, 2,4-dinitrophenol prevents oxidative phosphorylation but stimulates the rate at which oxygen is reduced.

2. Both reserpine and 2,4-dinitrophenol fail to stimulate oxygen uptake by isolated mitochondria in the presence of arginine.

3. Both 2,4-dinitrophenol and reserpine induce proton permeability in the mitochondrial membrane so that H^+ is absorbed from the suspending medium.

4. When the reaction system contains reserpine, accumulation of Ca^{2+} by mitochondria is inhibited.

5. Reserpine decreases both ADP:O and P:O ratios which strongly suggest that reserpine is an uncoupling agent.

INTRODUCTION

When isolated and tightly coupled mitochondria are suspended in a reaction medium containing a suitable substrate, electrons transverse the respiratory chain to reduce oxygen. The electron transport through the respiratory chain is accompanied by a decrease in free energy: part of which is either converted into conformational energy, ATP, or utilized in the active transport of ions across the inner mitochondrial membrane. The uncoupling agents such as dinitrophenol and dicumarol prevent the process of oxidative phosphorylation. In addition, uncouplers prevent the energy-dependent accumulation of Ca^{2+} by isolated mitochondria.

An investigation of the antihypertensive agent reserpine as an uncoupling agent was undertaken.

METHODS AND MATERIALS

Mitochondria were isolated in 0.25 M sucrose at 0–4 °C from the livers and kidneys of recently captured male vervet monkeys (*Cercopitecus aetiops*) according to the method developed by Schneider et al. [1].

Teflon-coated Clark Oxygen Electrode connected via a voltage divider to a

Sargent R. E. Model SRG Recorder was employed to study the effect of reserpine on oxygen consumption by respiring mitochondria. To determine whether reserpine was capable of preventing energy-dependent Ca^{2+} accumulation by isolated and tightly coupled mitochondria, radioactive $^{45}CaCl_2$ was incubated with the reaction media.

Radioactive $CaCl_2$ was inoculated into a suspension of respiring mitochondria. After 2 min, 0.4 ml was carefully withdrawn from the medium with a microsyringe. The mitochondria were then quickly sedimented by use of Beckman/Spinco Microfuge Model No. 152. 100 μl of the supernatant, containing the untranslocated calcium was placed into 10 ml of scintillating mixture in a glass counting vial and Packard Tricarb Liquid Scintillating Spectrometer Model 3320 used for counting. The same method was employed to study phosphorylation of labelled ADP and phosphate.

ATP was a pure compound from BDH Chemical Ltd. Poole, England, Product No. 42008. ADP was obtained from Boehringer, Mannheim (New York 5318222/Jan. 1969. 15014 NAAG). Radioactive phosphate (^{32}P), calcium ($^{45}Ca^{2+}$), and ADP ([^{14}C]ADP) were purchased from Sigma Chemicals Co. Mo., U.S.A. Sucrose used for the isolation of mitochondria was a very pure product of E. Merck ART 7651. Reserpine was a CIBA product B 16978 R. 900 923. 31C-998 UN which was for both intramuscular and intravenous usage. The scintillation mixture was prepared as follows: 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, 0.6 g; 2,5-diphenyloxazole, 9 g; ethanol, 300 ml; redistilled toluene was used to make the final volume 1000 ml.

Beckman Expandomatic SS connected to the Sargent recorder was used to study the effect of reserpine on H^+ ejection during calcium accumulation.

RESULTS

Stimulation of mitochondrial respiration by reserpine and dinitrophenol

Vervet monkey liver mitochondria suspended in a reaction medium containing added inorganic phosphate responded sufficiently to both ADP and 2,4-dinitrophenol, illustrating that they were tightly coupled and undamaged (Fig. 1, trace A). When these mitochondria were suspended in a reaction medium containing 20 mM arginine (Fig. 1, trace B), adenosine diphosphate, 2,4-dinitrophenol and reserpine were unable to stimulate respiration.

When reserpine was added to the reaction medium containing steadily respiring mitochondria (Fig. 1, trace A), rapid uptake of oxygen was recorded. This was very similar to the observations made in respect to dinitrophenol. Fig. 1, trace B, also shows that where arginine was present in the reaction medium, reserpine, like dinitrophenol, failed to release the inhibition of respiration induced by arginine.

Inhibition of Ca^{2+} accumulation by reserpine

Several recent investigations have demonstrated that approximately 2.0 Ca^{2+}, Sr^{2+} or Mn^{2+} are accumulated by isolated mitochondria as a pair of electrons passes through each energy-conserving site of the respiratory chain [2–7]. In addition, accumulation of divalent cations by mitochondria is an energy-dependent process [4, 7] so that it is inhibited by the uncoupling agents such as dinitrophenol and dicumarol.

Data presented in Table I serve to establish that vervet monkey liver mitochondria accumulate Ca^{2+} in an energy-linked fashion, since dinitrophenol inhibited the

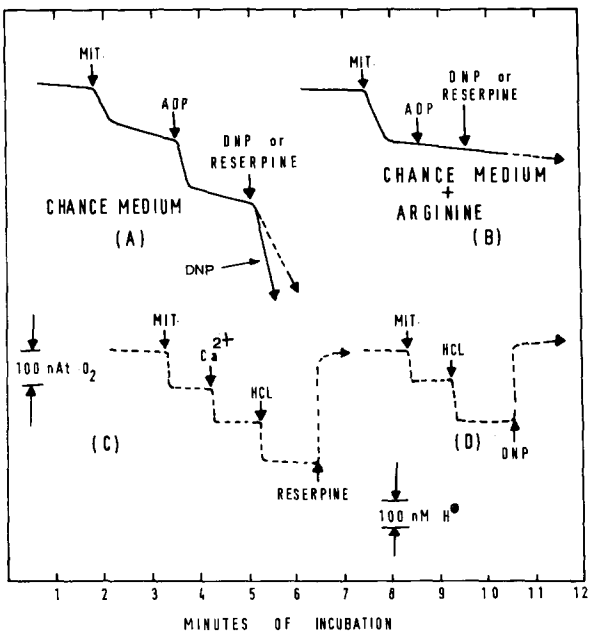

Fig. 1. Stimulation of respiration and inhibition of acid–base gradient by reserpine. The basic reaction medium consisted of 31.25 mM NaCl, 10 mM P_i, 7.5 mM $MgCl_2$, 75 mM KCl, 10 mM succinate and 8.4 mg of mitochondrial protein. Where indicated 250 nM ADP, 200 nM 2,4-dinitrophenol (DNP), 10 μg reserpine, 250 nM $CaCl_2$ and 250 nM HCl were added to the basic reaction medium. The experiments were conducted at 23 °C

TABLE I

INHIBITION OF Ca^{2+} ACCUMULATION BY RESERPINE IN VERVET MONKEY LIVER MITOCHONDRIA

The basic reaction medium contained the following reagents: 31.25 mM NaCl, 10 mM phosphate, 7.5 mM $MgCl_2$, 75 mM KCl, 5 mM succinate and 10 mg mitochondrial protein in a total volume of 2.2 ml. Where indicated 200 nmoles of dinitrophenol; 20 mM arginine and 65.70 μM reserpine were added. 500 nmoles of $^{45}CaCl_2$ was added in all cases. Experiments were conducted at 23 °C.

Additions to the basic reaction medium	Ca^{2+} accumulation (nmoles/mg mitochondrial protein)
None	28.0
2,4-Dinitrophenol	0.8
Reserpine	0.7
Arginine	32·2
Arginine+2,4-dinitrophenol	29.1
Arginine+2,4-dinitrophenol+ reserpine	31.3

transport of these ions. When 65.7 μM reserpine was included in a reaction system containing 10 mg mitochondrial protein the Ca^{2+} uptake was very severely suppressed (Table I), suggesting that reserpine was an uncoupling agent.

TABLE II

EFFECT OF RESERPINE ON OXIDATIVE PHOSPHORYLATION IN VERVET MONKEY LIVER MITOCHONDRIA

The basic reaction medium contained the following reagents: 31.25 mM NaCl, 10 mM phosphate, 7.5 mM $MgCl_2$, 75 mM KCl, 8 mg mitochondrial protein in a total volume of 2 ml. Where indicated 5 mM pyruvate, 5 mM glutamate, 5 mM malate, 65.70 μM reserpine and 200 nM of 2,4-dinitrophenol were added. The experiments were conducted at 23 °C.

Additions to the basic reaction medium	[^{14}C]ADP added (nmoles)	Oxygen consumed (natoms)	ADP taken up (nmoles)	ADP : O ratio
Succinate, 10 mM	250	110	200	1.82
Succinate + antimycin A	250	0	0	0
Succinate + reserpine	250	uncoupled	0	0
Pyruvate	360	120	340	2.83
Glutamate + malate	360	120	350	2.90
Pyruvate + reserpine	360	uncoupled	0	0
Pyruvate + 2,4-dinitrophenol	360	uncoupled	0	0
Glutamate + reserpine	360	uncoupled	0	0
Glutamate − malate + 2,4-dinitrophenol	360	uncoupled	0	0

TABLE III

EFFECTS OF DIFFERENT CONCENTRATIONS OF RESERPINE ON THE P:O RATIO

The reaction medium contained the following reagents: 20 mM NaCl, 7.5 mM $MgCl_2$, 50 mM KCl, 10 mg mitochondrial protein in a total volume of 3.0 ml. Where indicated 10 mM pyruvate and 5 mM succinate were added into the basic reaction medium. $^{32}P_i$ was used at the concentration of 10 mM.

Reserpine concentration added to the basic reaction medium (μM)	P:O ratio	
	Succinate	Pyruvate
None		
4.105	1.80	2.41
8.212	1.42	1.94
12.31	1.06	1.36
16.43	0.78	1.02
20.52	0.58	0.79
24.63	0.40	0.60
28.74	0.27	0.45
32.84	0.21	0.32
36.95	0.16	0.23
41.05	0.10	0.15
49.26	0.05	0.07
57.48	0.04	0.06

Effects of reserpine on oxidative phosphorylation and P:O ratio

Using [^{14}C]ADP and inorganic phosphate ($^{32}P_i$), the effects of reserpine on both oxidative phosphorylation and the P:O ratio, respectively, were determined. Data presented in Table II show clearly that oxidative phosphorylation was inhibited by both dinitrophenol and reserpine. In addition results summarized in Table III give strong support to the suggestion that reserpine is an uncoupling agent.

Effect of reserpine on H^+ ejection into the suspending medium

Recent studies have shown that ejection of H^+ into the suspending medium accompanies energy-linked accumulation of divalent cations such as Ca^{2+} by isolated mitochondria [8–10]. In Fig. 1, trace D, 2,4-dinitrophenol causes absorption of protons (H^+) from the suspending medium by the mitochondria, an indication that Ca^{2+} is released into the medium while protons are taken up by organelles. Fig. 1, trace C, shows that reserpine caused similar absorption of protons from the suspending medium.

Effects of Ca^{2+} and ATP on the arginine-induced inhibition of respiration in the presence of reserpine and dinitrophenol

Results summarized in Fig. 2, trace A, illustrate that when 20 μg of reserpine was added into a reaction system containing 10 mg mitochondrial protein, rapid uptake of oxygen occurred. However, when 20 mM arginine was added during the reserpine-stimulated respiration, complete inhibition of respiration was observed.

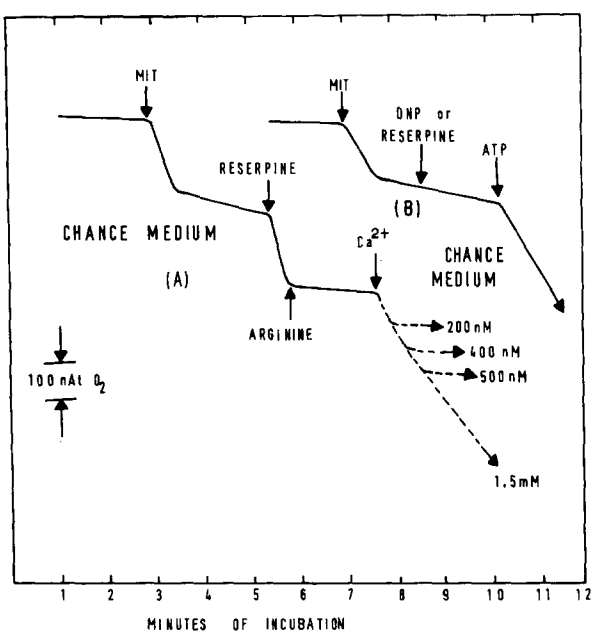

Fig. 2. Arginine-induced inhibition of reserpine stimulation of respiration. The basic reaction medium contained 20 mM NaCl, 5 mM P_i, 7.5 mM $MgCl_2$, 50 mM KCl, 10 mM succinate and 10 mg of mitochondrial protein. Where indicated 10 μg reserpine, 200 nM 2,4-dinitrophenol (DNP), 20 mM arginine, 250 nM $CaCl_2$ and 10 μg ATP were added to the basic reaction medium.

When Ca^{2+} was added following arginine inhibition of respiration in the presence of reserpine or dinitrophenol, there was a stimulation of the respiration (Fig. 2, trace A), whose duration was dependent upon the concentration of Ca^{2+} present in the mitochondria. It is important to note that the respiratory stimulation was followed by a return to the original inhibited state; a reflection that Ca^{2+} had been translocated across the inner mitochondrial membrane into the matrix. This was only observed when the concentration of Ca^{2+} was below 100 nmoles/mg of mitochondrial protein. When this concentration of Ca^{2+} was exceeded, respiratory stimulation was sustained and a return to the inhibited state was not possible.

Fig. 2, trace B, shows that when ATP was added into the reaction medium containing 10 mg of mitochondrial protein in the presence of arginine, inhibition of respiration was released. This release of respiratory inhibition was only observed in the presence of dinitrophenol or reserpine.

Kinetic studies of reserpine stimulation of respiration:

Isolated vervet monkey liver mitochondria were used to study the effects of various concentrations of reserpine on the rate of oxygen uptake. When 5 μg reserpine was added the rate of oxygen uptake was stimulated by over 100% from 36 natoms/min, to 81 natoms/min. With 10 μg reserpine the rate was 104 natoms/min, and 162 natoms/min with 45 μg reserpine. A rectangular hyperbola is obtained when the rate of oxygen uptake (v) is plotted against the concentration of (s) reserpine. At concentrations lower than 10 μg the rate is first order, i.e. it is proportional to the concentration of reserpine. From 80 to 200 μg of reserpine the order of the reaction is zero: the coupling enzymes [11] are saturated with reserpine so that more than 125 μg has no further effect.

CONCLUSION

The virtual identity of the various effects of reserpine and dinitrophenol on mitochondrial function lead to the conclusion that reserpine is an uncoupling agent.

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