2-PHENYLCARBAMOYLISATOGEN, A NOVEL UNCOUPLER OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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Summary

2-Phenylcarbamoylisatogen has been shown to stimulate succinate oxidation by rat liver mitochondria, but to inhibit the phosphorylation of ADP. The hydrolysis of ATP was also stimulated and an additive action with 2,4-dinitrophenol was demonstrated. The possible mechanism of the uncoupling action of 2-phenylcarbamoylisatogen is discussed.

Introduction

One approach to the study of the mechanisms involved in oxidative phosphorylation is the use of inhibitors, especially those which have well characterised chemical and physical properties. We have been studying the properties of isatogens and isatogen derivatives with the aim of gaining insight into the reactions involved in oxidative phosphorylation. Previous communications have shown that 2-phenylisatogen inhibits the ADP-stimulated respiration [1], the transhydrogenase enzyme [2] and interacts with the NADH dehydrogenase system [3]. Reduction of 2-phenylisatogen gives 2-phenylindolone which inhibits mitochondrial phosphate transport [4] and the transhydrogenase enzyme [2, 5].

In this paper we report the modification of activity produced by using 2-phenylcarbamoylisatogen instead of 2-phenylisatogen. These results have been presented elsewhere in preliminary form [6].

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Materials and Methods

Oxygen Consumption

Oxygen consumption was measured polarographically using an oxygen electrode (Rank Bros., Bottisham, Cambs.).

ATPase

ATP hydrolysis was measured by the method of Beechey [7], phosphate released being determined by the method of Fiske and Subbarow [8].

Mitochondrial Swelling

Swelling of mitochondria in isoosmolar ammonium chloride was determined by measuring the optical density changes at 520 nm according to the method of Cunnarro and Weiner [9].

P:O Ratios

Rat liver mitochondria (5 mg protein) were suspended in 675 μmol sucrose, 10 μmol tris-HCl, pH 7.4, 5 μmol phosphate buffer, pH 7.4, 10 μmol succinate, 1 mg yeast hexokinase and 0.6 μmol glucose to a final volume of 3.5 ml at a temperature of 30°C. Oxygen consumption was recorded as above and inhibitors, if any, were added at this stage. A 0.5 ml sample of the incubation medium was added to 0.2 ml of trichloroacetic acid (10% w/v) and 0.5 μmol ADP added to the oxygen electrode chamber. Four minutes later a further 0.5 ml sample was similarly treated.

The samples were assayed for phosphate by the method of Fiske and Subbarow [8] after removal of precipitated protein by centrifugation. Oxygen consumption was measured from the oxygen electrode trace.

Protein

Protein was determined by the biuret method [10].

Specific reaction conditions are given in the legends to the figures and tables.

Materials

2-Phenylcarbamoylisatogen was synthesised by the method of Robertson [11]. 2-Phenylisatogen was prepared by the method of Bond and Hooper [12]. 2-Phenylindolone was synthesised by the method of Kalb and Bayer [13]. All three compounds were dissolved in dimethylformamide and added in volumes not exceeding 5 μl, at which volumes the solvent had no effect. Oligomycin (Sigma Chemical Co., London,) and tetrachlorotrifluoromethylbenzimidazole (Prof. Beechey, Shell Res. Ltd., Sittingbourne, Kent) were dissolved in ethanol and added in volumes not exceeding 10 μl. Other reagents were obtained in the purest grades available from British Drug Houses, Poole, Dorset or Sigma Chemical Co., London.

Results

Succinate Oxidation

Rat liver mitochondria oxidised succinate at a rate of 15 ng atom oxygen/min/mg mitochondrial protein, and this was stimulated to a rate of

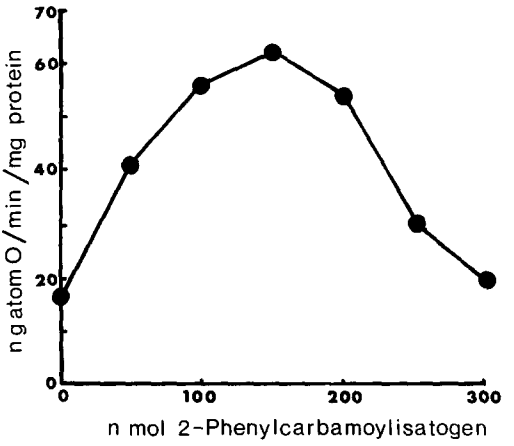

Figure 1: The effect of 2-Phenylcarbamoylisatogen on succinate oxidation in rat liver mitochondria.

Oxygen consumption was measured polarographically at 30°C. The reaction medium contained 5 μmol sucrose, 10 μmol Tris-HCl buffer, pH 7.4, 10 μmol succinate and 5 mg rat liver mitochondrial protein in a final volume of 3 ml. 2-Phenylcarbamoylisatogen was added as indicated.

62 ng atom oxygen/min/mg mitochondrial protein by 150 nmol 2-phenylcarbamoylisatogen (Figure 1). The stimulation of respiration was dependent on the amount of 2-phenylcarbamoylisatogen added up to 150 nmol, above this amount respiration was inhibited by 2-phenylcarbamoylisatogen (Figure 1). This pattern of activity resembled that observed with classical uncoupling agents [14] and so the effects of 2-phenylcarbamoylisatogen on the phosphorylation of ADP was measured. The P:O ratio for succinate oxidation was reduced from 1.78 to 0.18 by 120 nmol 2-phenylcarbamoylisatogen, with no reduction in oxygen consumption.

Uncouplers reverse the inhibition of respiration by energy transfer inhibitors such as oligomycin [15] and 2-phenylisatogen [1]. Table 1 shows that 2-phenylcarbamoylisatogen completely reversed the inhibition of succinate oxidation by oligomycin and 2-phenylisatogen.

Table 1

Effect of energy transfer inhibitors on ADP and 2-phenylcarbamoylisatogen-stimulated succinate oxidation

ADDITIONS	RATE OF RESPIRATION ng atom O/min/mg protein	
	CONTROL	+ 2-PHENYLCARBAMOYLISATOGEN
None	54	55.5
Oligomycin (2 μg)	14	55.0
2-Phenylisatogen (40 nmol)	14	55.0

Respiration was measured as described in the legend to Figure 1 except that the medium also contained 0.5 μmol-ADP and 10 μmol potassium phosphate. Other agents were added as indicated. 100 nmol 2-phenylcarbamoylisatogen was used as indicated.

ATP Hydrolysis

Increasing the amount of 2-phenylcarbamoylisatogen up to 150 nmol caused a progressive increase in the rate of ATP hydrolysis (Figure 2). Above this amount, ATP hydrolysis was slightly inhibited. A similar curve was obtained using 2,4-dinitrophenol, but the maximum rate of ATP hydrolysis was obtained using 20 nmol 2,4-dinitrophenol (Figure 2). In the presence of 50 nmol 2-phenylcarbamoylisatogen, the maximum rate of ATP hydrolysis was obtained using 8 nmol 2,4-dinitrophenol (Figure 2). Thus the two compounds appear to be additive in their action on ATP hydrolysis.

ATP hydrolysis stimulated by either 2,4-dinitrophenol or 2-phenylcarbamoylisatogen was inhibited by oligomycin and by 2-phenylisatogen.

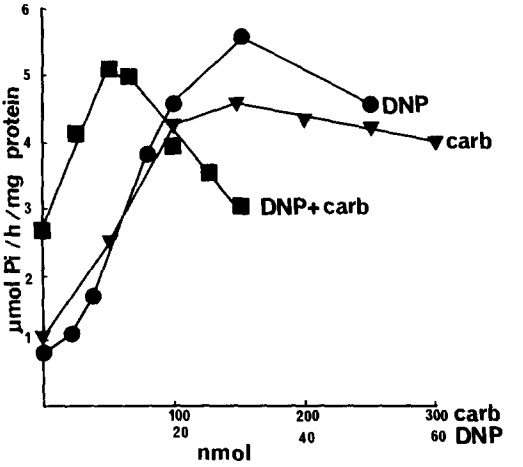

Figure 2: The effect of 2-Phenylcarbamoylisatogen and 2,4-Dinitrophenol on ATP hydrolysis in rat liver mitochondria.

Mitochondria (1 mg protein) were added at zero time to a reaction medium containing 0.125 M sucrose, 60 mM Tris-HCl, pH 8.0, 10 µg antimycin A, 2 µg rotenone, 3 mM ATP and 2,4-dinitrophenol (DNP) or 2-phenylcarbamoylisatogen as indicated. In the combined experiment variable amounts of DNP were added in the presence of 50 nmol 2-phenylcarbamoylisatogen. After 15 min incubation at 30°C the reaction was stopped by the addition of 0.1 ml 30% trichloroacetic acid. After centrifugation the phosphate content of the supernatant was determined by the method of Fiske and Subbarow [8].

Swelling in Ammonium Chloride

The results presented so far indicate that 2-phenylcarbamoylisatogen is an uncoupler of oxidative phosphorylation. Cunarro and Weiner [9] have reported a correlation between the ability of compounds to uncouple oxidative phosphorylation and their ability to cause mitochondrial swelling in iso-osmolar ammonium chloride. They have suggested that this provides evidence for a proton-carrying mechanism of uncoupling. Table 2 compares the ability of 2-phenylcarbamoylisatogen to induce mitochondrial swelling in an ammonium chloride medium, with that of the uncouplers 2,4-dinitrophenol [16] and tetrachlorotrifluoromethylbenzimidazole [7], and the phosphate transport inhibitors p-hydroxymercuribenzoate [17] and 2-phenylindolone [4].

Table 2

Mitochondrial swelling in ammonium chloride

ADDITIONS	RATE OF SWELLING (OD units/5 min)
None	0
2-Phenylcarbamoylisatogen (150 nmol)	0.05
2-Phenylindolone (90 nmol)	0.05
p-Hydroxymercuribenzoate (100 nmol)	0.05
2,4-Dinitrophenol (60 nmol)	0.155
Tetrachlorotrifluoromethylbenzimidazole (0.2 nmol)	0.15

Rat liver mitochondria (5 mg protein) were suspended in 0.15 M ammonium chloride adjusted to pH 7.4. The final volume was 3.0 ml and inhibitors were added as indicated. Optical density changes at 520 nm were monitored using a Pye-Unicam SP 1800 spectrophotometer with a reaction temperature of 30°C.

2-Phenylcarbamoylisatogen (150 nmol) induced a slow rate of swelling (0.05 OD units/5 min) which was comparable with the rates obtained using 2-phenylindolone and p-hydroxymercuribenzoate. The two uncoupling agents each induced swelling at much faster rates (0.15 OD units/5 min).

Discussion

The stimulation of succinate oxidation with an inhibition of phosphorylation, the reversal of oligomycin inhibition of respiration, and the oligomycin-sensitive stimulation of ATP hydrolysis by 2-phenylcarbamoylisatogen indicate that this compound is an uncoupler of oxidative phosphorylation. It is widely believed [9, 18, 19] that uncouplers act by

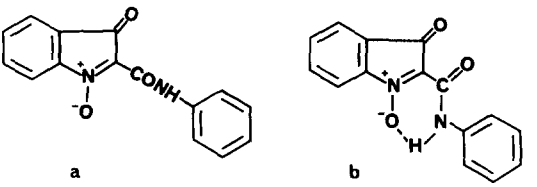

Figure 3: Free (a) and quassi ring form (b) of 2-Phenylcarbamoylisatogen.

transporting protons across the mitochondrial inner membrane. However, this is an unlikely explanation for the mechanism of action of 2-phenylcarbamoylisatogen. The carbamoyl hydrogen would not be expected to be acidic, and so could not be used as a means of transporting protons. Chemical and spectrophotometric evidence indicates that the compounds exist as a quassi ring compound (Figure 3), and in this form the compound would definitely be unable to transport protons across the mitochondrial membrane.

2-Phenylcarbamoylisatogen did produce some mitochondrial swelling in an ammonium chloride medium, however, the rate of swelling did not exceed that obtained using phosphate transport inhibitors. Scot et al., [20], have shown that agents reacting with thiol groups induce chloride permeability in mitochondria and cause the mitochondria to swell in isoosmolar potassium chloride. Thus the slight swelling of mitochondria observed in the presence of 2-phenylcarbamoylisatogen cannot necessarily be equated with proton translocation [9], but may be due to reaction with thiol groups. We have previously suggested that the related compounds 2-phenylisatogen [3] and 2-phenylindolone [4] exert their action, at least in part, by interaction with thiol groups.

The results with 2,4-dinitrophenol on ATP hydrolysis indicate that 2-phenylcarbamoylisatogen acts at the same site as 2,4-dinitrophenol. Cantley and Hammes [21] have reported a direct interaction of 2,4-dinitrophenol with the ATPase and have suggested that this interaction may explain

the uncoupling action of this compound. Thus 2-phenylcarbamoylisatogen may uncouple by binding to the ATPase complex.

It was observed that 2-phenylcarbamoylisatogen reversed the inhibition of respiration by 2-phenylisatogen, a closely related compound. It is possible that slight changes in the structure of the isatogen either alter the site of binding or alter the effect which this binding has on the energy coupling system. Work on the binding of these two isatogens is currently in progress.

To conclude, the introduction of a carbamoyl group into 2-phenylisatogen to give 2-phenylcarbamoylisatogen changes its action in activity in a series of closely related compounds is unusual. We do not believe that the uncoupling can be explained on the basis of proton translocation, but may be due to a direct interaction with the ATPase complex.

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