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SPEGAZZININE, A NEW INHIBITOR OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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SUMMARY

The effects of spegazzinine, a dihydroindole alkaloid, on mitochondrial oxidative phosphorylation were studied.

Spegazzinine inhibited coupled respiration and phosphorylation in rat liver mitochondria. The I_{50} was 120 μM. Uncouplers released the inhibition of coupled respiration. Arsenate-stimulated mitochondrial respiration was partially inhibited by spegazzinine. The stimulation of mitochondrial respiration by Ca^{2+} and the proton ejection associated with the ATP-dependent Ca^{2+} uptake were not affected by the alkaloid.

Oxidative phosphorylation and the P_i–ATP exchange reaction of phosphorylating beef heart submitochondrial particles were strongly inhibited by spegazzinine (I_{50}, 50 μM) while the ATP-dependent reactions, reduction of NAD^+ by succinate and the pyridine nucleotides transhydrogenase were less sensitive (I_{50}, 125 μM). Oxygen uptake by submitochondrial particles was not affected.

The 2,4-dinitrophenol-stimulated ATPase activity of rat liver mitochondria was not affected by 300 μM spegazzinine, a concentration of alkaloid that completely inhibited phosphorylation. However, higher concentrations of spegazzinine did partially inhibit it. The ATPase activities of submitochondrial particles, insoluble and soluble ATPases were also partially inhibited by high concentrations of spegazzinine.

The inhibitory properties of spegazzinine on energy transfer reactions are compared with those of oligomycin, aurovertin and dicyclohexylcarbodiimide. It is concluded that spegazzinine effects are very similar to the effects of aurovertin and that its site of action may be the same or near the site of aurovertin.

INTRODUCTION

One of the main experimental approaches that have been used in the attempts to elucidate the pathway of energy transfer from the respiratory chain to the synthesis

Abbreviation: DCCD, *N,N'*-dicyclohexylcarbodiimide.

of ATP is the functional dissection of the process by specific inhibitors. The antibiotics, oligomycin [1] and aurovertin [2], and *NN'*-dicyclohexylcarbodiimide, DCCD [3], are the best characterized and more frequently used among these specific inhibitors of oxidative phosphorylation. In addition to the synthesis of ATP they inhibit coupled respiration, partial reactions of phosphorylation like the P_i–ATP exchange reaction, and the ATP-driven reactions like the NAD^+ reduction by succinate [1–7].

The inhibitory properties and the range of concentrations of oligomycin and DCCD required to inhibit different energy transfer reactions are remarkably similar [4]. Moreover, it has been postulated that they exert their effects at the same site of action [8]. Aurovertin, a scarce antibiotic of unknown structure, shares some of the properties of oligomycin and DCCD [4] but differs in others [2, 4–7]. Its site of action seems to be some component of the mitochondrial ATPase or coupling factor 1 [5, 9, 10].

Spegazzinine is a dihydroindole alkaloid possessing a phenolic and an alcohol group, isolated from the bark of *Aspidosperma chakensis* Spegazzini [11]. Its structure has been elucidated [12]. The present paper reports the effects of this alkaloid on mitochondrial energy transfer reactions. They show that spegazzinine is a specific inhibitor of oxidative phosphorylation that closely resembles aurovertin behavior as an energy transfer inhibitor.

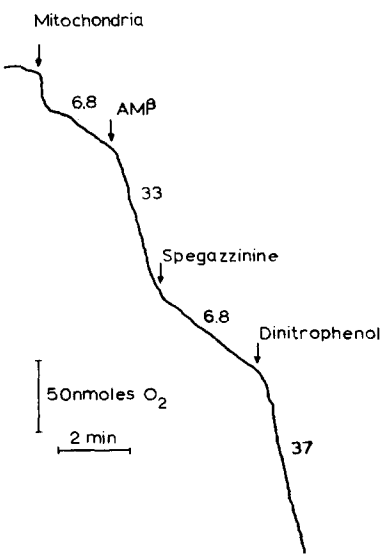

Fig. 1. Effect of spegazzinine on respiration of rat liver mitochondria. Oxygen uptake by rat liver mitochondria (3.25 mg of protein) was measured in a Gilson Oxygraph with a Teflon-covered Clark electrode. The reaction medium (1.65 ml) contained 250 mM sucrose, 30 mM KCl, 6 mM $MgCl_2$, 1 mM EDTA, 25 mM Tris–HCl buffer (pH 7.4), 10 mM potassium phosphate, 5 mM malate and 5 mM glutamate. The temperature was 30 °C. AMP concentration was 1 mM; spegazzinine was 315 μM and 2,4-dinitrophenol was 60 μM. The numerals on the slope indicated nmoles of O_2 $min^{-1} \cdot mg^{-1}$.

RESULTS AND DISCUSSION

Fig. 1 shows that 315 μM spegazzinine totally inhibited the increase, by phosphate acceptor, of the rate of oxygen uptake by rat liver mitochondria with malate and glutamate as substrates. The inhibition was completely released by the addition of an uncoupler, 2-4 dinitrophenol (Fig. 1). Similar effects were observed with succinate as substrate. From titration curves of the inhibitory effect of spegazzinine on phosphorylation coupled to malate-glutamate or succinate oxidation, determined as described in the text a I_{50} value (concentration of the alkaloid that inhibited 50%) of about 120 μM was calculated. 500 μM aspidospermine, a closely related alkaloid, was without effect in the same conditions.

The spegazzinine-inhibited state "3" respiration was also released by other uncouplers namely *p*-trifluoromethoxyphenylhydrazone and 5-chloro-3-butyl-2'-chloro-4'-nitrosalicylanilide but not by arsenate. Fig. 2A shows that, as has been described [13–14], 30 mM arsenate stimulated state "4" respiration of rat liver mitochondria with succinate. The released respiration is inhibited by P_i and stimulated again by ADP or dinitrophenol. Spegazzinine (250 μM) inhibited the respiration stimulated by arsenate (Fig. 2B) and prevented the effect of ADP. The inhibition was released by dinitrophenol. Both oligomycin and aurovertin have been shown to inhibit [13–14] arsenate-stimulated mitochondrial respiration but aurovertin did not inhibit it completely as oligomycin did and high concentrations of the former were not inhibitory at all [14]. Spegazzinine, like aurovertin, did not completely inhibit arsenate-stimulated respiration (Fig. 2C). The inhibition was 81–86% when calculated from the rate of arsenate-stimulated respiration in the absence or in the presence of alkaloid (400–1500 μM) minus the corresponding rate after inhibition by added P_i.

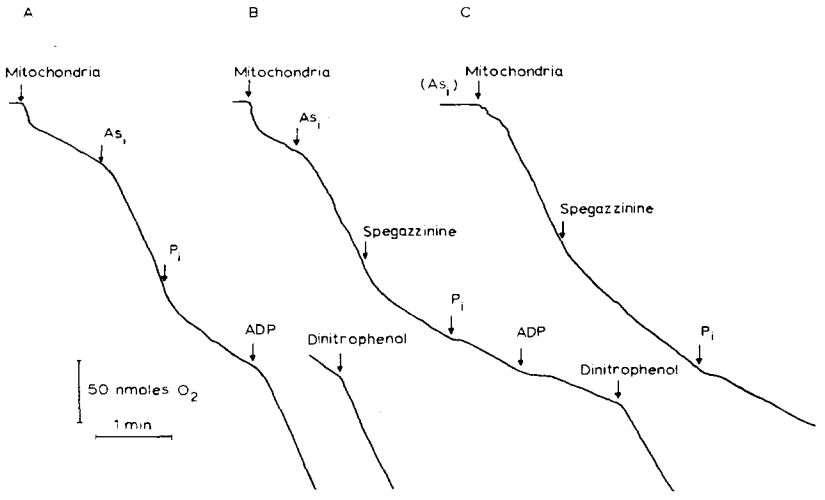

Fig. 2. Effect of spegazzinine on arsenate-stimulated mitochondrial respiration. Oxygen uptake by rat liver mitochondria (2.1 mg) was determined as described in the legend to Fig. 1. except that the reaction medium contained 15 mM KCl, 50 mM Tris–HCl buffer (pH 7.4), 1 mM EDTA, 2.5 mM $MgCl_2$, 60 mM succinate and 50 μM rotenone. As_i, 30 mM arsenate; P_i, 3 mM potassium phosphate; ADP, when added, was 675 μM; 2,4-dinitrophenol was 30 μM and spegazzinine (oxalate) was 250 μM (Expt. B) or 400 μM (Expt. C).

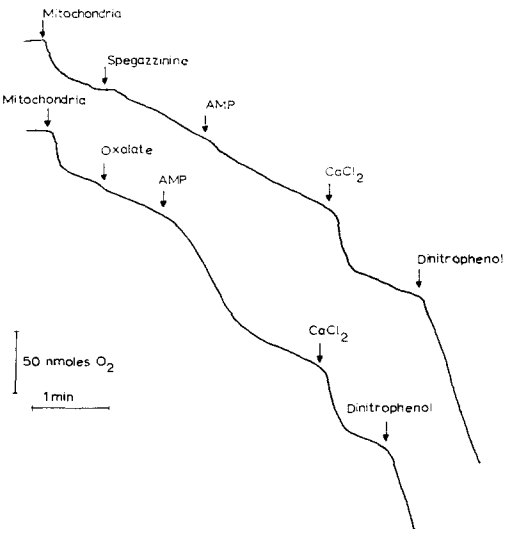

Fig. 3. Effect of spegazzinine on the stimulation of mitochondrial respiration by Ca^{2+}. Oxygen uptake by rat liver mitochondria (3 mg) was determined as described in the legend to Fig. 1 except that the reaction medium contained 80 mM NaCl, 10 mM Tris–HCl buffer (pH 7.4), 20 mM succinate, 2.5 mM P_i and 50 μM rotenone. When added, sodium oxalate concentration was 315 μM; AMP, 125 μM; $CaCl_2$, 315 μM; 2,4-dinitrophenol, 30 μM; and spegazzinine (oxalate), 315 μM.

Uptake of low concentrations of Ca^{2+} by mitochondria is associated with a burst of respiration similar to that observed on addition of phosphate acceptor (ref. 15 and Fig. 3). Fig. 3 shows that spegazzinine like oligomycin [16] prevented the effect of AMP but did not affect the stimulation of respiration by Ca^{2+}. The Ca^{2+} uptake in these experiments was supported by substrate oxidation. It may also be supported by ATP. Oligomycin but not aurovertin [17] inhibited Ca^{2+} uptake and the associated proton ejection by rat liver mitochondria when the Ca^{2+} accumulation was supported by ATP in "limiting loading" conditions. Spegazzinine (333 μM) like aurovertin did not affect the proton ejection associated with ATP-linked Ca^{2+} uptake, when assayed in the same conditions as described for aurovertin [17].

Spegazzinine did not affect the respiration of beef heart submitochondrial particles but strongly diminished oxidative phosphorylation (Fig. 4). The P_i–ATP exchange reaction of Mg–ATP particles was also inhibited by the alkaloid in exactly the same way as was phosphorylation (Fig. 4). Both titrations curves were sigmoidal and the I_{50} values were about 50 μM. The P_i–ATP exchange reaction is considered to be related to the enzyme(s) that catalyze the synthesis of ATP [18]. Oligomycin and aurovertin strongly inhibited this reaction in a similar manner [2, 7]. The ATP-dependent reduction of NAD^+ by succinate and the ATP-dependent pyridine nucleotides transhydrogenase reactions of Mg–ATP particles were also inhibited by spegazzinine but they were less sensitive to the alkaloid and the titration curves were not sigmoidal (Fig. 4). The I_{50} values were about 125 μM. Thus the inhibition of the ATP-driven reactions required twice as much alkaloid as the reactions leading to the synthesis of ATP. The succinate-driven transhydrogenase was not affected by spegazzinine. Similar differential sensitivity of ATP-generating and ATP-consuming reac-

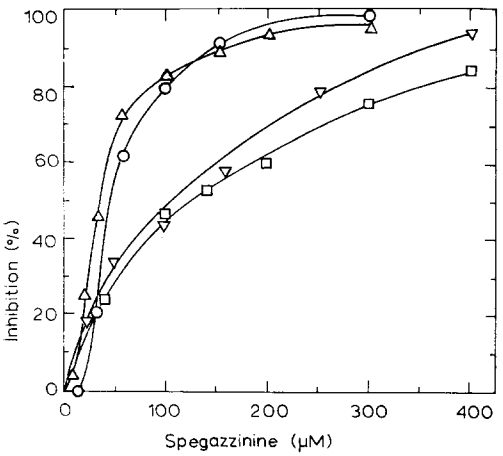

Fig. 4. Effect of spegazzinine on phosphorylation, P_i-ATP exchange reaction and the ATP-driven reactions, NAD^+ reduction by succinate and pyridine nucleotides transhydrogenase by Mg–ATP particles. Experimental conditions were as described in the text. Control values were in nmoles · min^{-1} · mg^{-1}: phosphorylation, 42 (○–○); P_i–ATP exchange reaction, 55 (△–△); ATP-dependent reduction of NAD^+ by succinate, 36 (▽–▽); ATP-dependent pyridine nucleotides transhydrogenase, 62 (□–□).

tions has been described for aurovertin [4, 6] whereas oligomycin and DCCD have analogous effects on both types of reactions [4].

Intact rat liver mitochondria have a latent ATPase that is stimulated by uncouplers and is inhibited by oligomycin and DCCD at nearly the same concentrations required to inhibit phosphorylation. The ATPase activity of submitochondrial particles is also inhibited [4].

Spegazzinine up to 300 μM neither stimulated the latent ATPase of rat liver mitochondria nor inhibited the dinitrophenol-stimulated ATPase (Table I). However, higher concentrations of the alkaloid partially inhibited the latter (Table I, Expt 2). 200 μM spegazzinine, a concentration of the alkaloid that completely inhibited phosphorylation or the P_i–ATP reaction (Fig. 4), diminished the ATPase activity of Mg–ATP particles only 27%. Similarly, 400 μM spegazzinine, a concentration that nearly completely abolished the ATP-driven reactions, NAD^+ reduction by succinate and pyridine nucleotides transhydrogenase (Fig. 4), depressed the ATPase activity by only 54% (Table I, Expt. 2). The activity of the oligomycin-sensitive insoluble ATPase was also partially inhibited by spegazzinine (Table I, Expt. 3). The soluble mitochondrial ATPase has been shown to be insensitive to oligomycin [19] or to DCCD [4] but it is inhibited by aurovertin [4, 9] and by high concentrations of spegazzinine (Table I, Expt. 4).

Low concentrations of oligomycin [7] or DCCD [4] but not of aurovertin were able to stimulate P/O ratios and other energy-dependent reactions in EDTA-particles. Spegazzinine (0.1 to 10 μM) was not able to elicit any enhancement of the P/O ratios of EDTA-preparations that were stimulated by oligomycin.

In conclusion, spegazzinine is a potent and specific inhibitor of mitochondrial energy transfer reactions, that closely resembles the inhibitory effects of aurovertin, and that probably exerts them at or near the aurovertin site of action. That is on the

TABLE I

EFFECT OF SPEGAZZININE ON MITOCHONDRIAL ATPase

Experimental conditions were as described in the text. 1 mg of protein of rat liver mitochondria were used per test tube in Expt 1; 87 μg of Mg–ATP particles in Expt 2; 105 μg of insoluble ATPase in Expt 3 and 20 μg of F_1 in Expt 4.

Expt	Additions	ATPase activity (nmoles · min^{-1})
1	Rat liver mitochondria	
	None	20
	2-4 dinitrophenol 100 μM	465
	2-4 dinitrophenol 100 μM, spegazzinine 300 μM	446
	2-4 dinitrophenol 100 μM, spegazzinine 600 μM	322
	2-4 dinitrophenol 100 μM, spegazzinine 1200 μM	216
	2-4 dinitrophenol 100 μM, oligomycin 2 μg/ml	60
2	Mg-ATP particles	
	None	18
	Spegazzinine 200 μM	13
	Spegazzinine 400 μM	8
	Oligomycin 2.5 μg/ml	2
3	Insoluble ATPase	
	None	32
	Spegazzinine 400 μM	16
	Oligomycin 3 μg/ml	3
4	Soluble ATPase (F_1)	
	None	89
	Spegazzinine 800 μM	75
	Spegazzinine 1600 μM	63
	Oligomycin 3 μg/ml	89

ATP side of the oligomycin-sensitive site as proposed by Lee and Ernster [7] and Roberton et al. [4] or more specifically on F_1 [10, 20, 21]. More detailed studies may clarify this point and may help to lead to a better understanding of the function of the mitochondrial ATP synthetase.

EXPERIMENTAL

Rat liver mitochondria were prepared as described by Vallejos and Stoppani [22] except that 0.25 M sucrose was used as an isolation medium.

Heavy and light beef heart mitochondria were prepared according to Crane et al. [23] and Hatefi and Lester [24] with slight modifications. Mg–ATP [25] and EDTA-particles [26] were prepared from heavy beef heart mitochondria as described. Insoluble ATPase and soluble ATPase(F_1) were prepared as described [27].

Oxidative phosphorylation of rat liver mitochondria was determined [28] in a Gilson Differential Respirometer. Oxidative phosphorylation of submitochondrial particles was determined in the same apparatus according to Vallejos et al. [27].

The P_i–ATP exchange reaction and ATPase activity of submitochondrial particles and the insoluble and soluble ATPases were measured as described [27].

The ATPase activity of rat liver mitochondria was measured in a reaction medium (1 ml) that contained 100 mM KCl, 50 mM Tris–HCl buffer (pH 7.4) and 5 mM ATP. The temperature was 30 °C. The incubations were stopped after 5 min with trichloroacetic acid (5% final concentration) and the P_i liberated was measured in aliquots of the supernatant solutions according to Sumner [29].

The ATP-dependent reduction of NAD^+ by succinate was measured at 340 nm. in a Beckman DBG spectrophotometer equipped with a recorder. 0.6 mg of Mg–ATP particles were preincubated for 10 min at 30 °C in a reaction medium (1.2 ml) containing 180 mM sucrose, 50 mM Tris–HCl buffer (pH 7.4), 6 mM $MgCl_2$, 1 mM NAD^+, 5 mM succinate and 1.6 mM KCN. The reaction was started by adding 3.6 μmoles of ATP.

The ATP-dependent reduction of $NADP^+$ by NAD^+ was measured at 340 nm in the same spectrophotometer as described [30]. The reaction medium (1 ml) contained 50 mM Tris–HCl buffer (pH 8), 6 mM $MgCl_2$, 250 mM sucrose, 3 μM rotenone, 57 mM ethanol, 0.25 mg alcohol dehydrogenase, 16.7 μM NAD^+, 0.2 mM $NADP^+$, 2 mM ATP and 0.6 mg Mg–ATP particles.

Protein was determined by a modification of the biuret method [31] or by the spectrophotometric method [32].

Spegazzinine (base or oxalate salt) solutions were prepared in dimethyl-sulfoxide. Controls were run for oxalate and the solvent.

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