Vol. 118, No. 3, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1984 Pages 717-723

RHODAMINE 123 INHIBITS BIOENERGETIC FUNCTION IN ISOLATED RAT LIVER MITOCHONDRIA

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Received December 30, 1983

Summary. Rhodamine 123 accumulates in the mitochondria of living cells and exhibits selective anticarcinoma activity. The biochemical basis of toxicity was investigated by testing the effect of the dye on isolated rat liver mitochondria. Much lower concentrations of rhodamine 123 were required to inhibit ADP-stimulated respiration and ATP synthesis in well-coupled energized mitochondria than were required to inhibit uncoupled respiration and uncoupler-stimulated ATP hydrolysis. The amount of rhodamine 123 associated with the mitochondria was several-fold greater under energized as compared to non-energized conditions, which may explain why coupled functions appeared to be more sensitive than uncoupled functions to inhibition at low concentrations of rhodamine 123. It was concluded that the site of rhodamine 123 inhibition is most likely the F_0F_1ATPase complex and possibly electron transfer reactions as well.

Rhodamine 123 is a cationic lipophilic fluorescent dye which exhibits anticarcinoma activity both *in vitro* (1,2) and *in vivo* (3). The most promising feature of this compound as a potential chemotherapeutic agent lies in its ability at certain doses to selectively kill many epithelial-derived tumorigenic cells while leaving normal epithelial cells viable. However, a biochemical basis for this selective toxicity has yet to be determined.

Rhodamine 123 is accumulated in the mitochondria of living cells (4,5). The uptake and retention of rhodamine 123 appears to be dependent upon the mitochondrial membrane potential, since the addition of ionophores known to dissipate the electrical gradient and respiratory inhibitors which prevent the establishment of the electrochemical gradient diminish mitochondrial specific fluorescence in cells prestained with rhodamine 123 (6). 2-Deoxyglucose, an inhibitor of glycolytic ATP synthesis, potentiates the toxic effect of the dye (2,3). This evidence suggests that the mitochondrion is the primary target for rhodamine 123 cytotoxicity. The

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0006-291X/84 $1.50

purpose of this study was to investigate the possible inhibitory effect of rhodamine 123 on mitochondrial functions.

MATERIALS AND METHODS

Isolation of Mitochondria. Rat liver mitochondria were isolated by differential centrifugation as previously described (7). Protein was determined by the method of Lowry (8).

Respiration. Oxygen consumption was measured polarographically in a 1 ml volume as described previously (9). The order of additions to respiratory assay medium (9) was: mitochondria (.4-.5 mg protein); an oxidizable substrate, either glutamate + malate (5 mM each) or succinate (5 mM + 2 ug/ml rotenone); rhodamine 123 (0-20 ug/ml); ADP (120 nmol). The ADP-stimulated respiratory rate was defined as the rate after ADP minus the rate after rhodamine 123. To determine uncoupled rates, 2,4-dinitrophenol (f.c. 40 uM) was added during State 4.

Measurement of Rhodamine 123 Associated with Mitochondria. Mitochondria (.4-.5 mg protein) were incubated at room temperature for 2 minutes in 1 ml respiratory medium (see above) in the presence or absence of 2,4-dinitrophenol (f.c. 40 uM), oxidizable substrate, ADP, and rhodamine 123 (5-20 ug/ml). The mixture was then layered over silicone oil layered over 12% PCA and centrifuged at 11,000 g for 1 min as described previously (7). The amount of rhodamine 123 in the supernatant was determined by absorbance at 502 nm by comparison with standards. The amount of rhodamine 123 associated with the mitochondrial pellet was determined by subtracting the amount measured in the supernatant from the amount of dye originally added.

ATP Synthesis. ATP synthesis was inferred from oligomycin-sensitive P_i disappearance in the presence of 1 mM ADP and oxidizable substrate in respiratory medium (as above except 1 mM K_2HPO_4 - KH_2PO_4) at 30°C. P_i was assayed colorimetrically (10) in PCA extracts. Net ATP synthesis was calculated from the difference in P_i present in each sample relative to a paired oligomycin (2 ug/ml) blank.

F_0F_1ATPase Activity. Oligomycin-sensitive, uncoupler-stimulated ATP hydrolysis was determined by the appearance of P_i. Mitochondria (.1-.2 mg protein) were added to 225 mM sucrose, 10 mM Tris-Cl, 1 mM $MgCl_2$, 5 mM ATP and 40 uM 2,4-dinitrophenol, pH 7.4, 30°C. Identical incubations (blanks) containing 2 ug/ml oligomycin were run in parallel. P_i was assayed in PCA extracts as described above for ATP synthesis.

Adenine Nucleotide Translocase Activity. This was assayed on ice by forward exchange in 0.23 M sucrose, 0.9 mM Tris, 2.5 mM each glutamate + malate, and 20 uM (^{14}C)ADP (9).

RESULTS

The Effect of Rhodamine 123 on Respiratory Function. Figure 1 shows the effect of rhodamine 123 on respiratory rates in the presence of substrate alone, as well as on ADP-stimulated and uncoupled respiratory rates. ADP-stimulated respiration was much more sensitive to inhibition by rhodamine 123 than was uncoupled respiration (1/2 max. inhibition at 7 ug/ml and >30 ug/ml respectively). There was some evidence for uncoupling by rhodamine 123; in the presence of substrate alone O_2 consumption was stimulated (Fig. 1). Results similar to those shown in Fig. 1 were obtained using glutamate + malate as the substrate instead of succinate (not shown). This suggested that several possible specific sites of inhibition by rhodamine 123

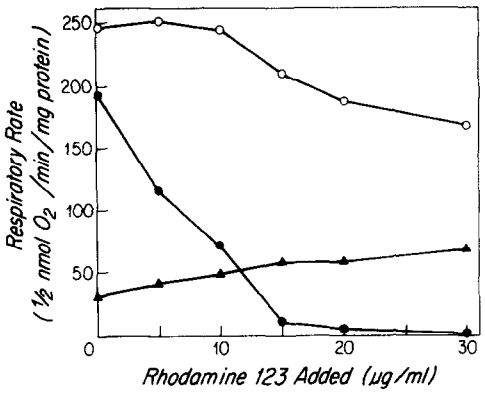

Figure 1. Effect of rhodamine 123 on respiratory rates with succinate as substrate. The order of additions is given under Materials and Methods. Symbols are as follows: respiration in the presence of substrate after the addition of rhodamine 123 (▲); ADP-stimulated respiration (●) and uncoupler-stimulated respiration (o) as defined under Materials and Methods. Points are averages of two separate experiments.

seemed unlikely, including: substrate transport; substrate dehydrogenases; respiratory complexes I and II; and phosphorylation site I of the electron transport chain.

Results like those presented in Fig. 1 (i.e. severe inhibition of ADP-stimulated respiration at concentrations of rhodamine 123 having little effect on uncoupled respiration) are usually interpreted to indicate that the compound being tested affects some reaction unique to the phosphorylation pathway rather than electron transport. However, because uncoupled respiration was inhibited at high concentrations of rhodamine 123, at least some of the inhibition of ADP-stimulated respiration may have been due to an inhibition of electron transport. Furthermore, the addition of uncoupler induces a rapid release of rhodamine 123 from mitochondria in intact cells prestained with the dye (6), suggesting that the different dose-response curves obtained in the presence and absence of uncoupler in Fig. 1 may be attributable to differences in the amount of rhodamine 123 associated with the mitochondria in uncoupled and coupled states. This possibility was investigated next.

The Amount of Rhodamine 123 Associated with Mitochondria Under Coupled and Uncoupled Conditions. The association of rhodamine 123 with mitochondria was quantitated by measuring the amount of dye left in the incubation medium after rapid removal of the mitochondria as described under Materials and Methods. Under both

coupled and uncoupled conditions the amount of rhodamine 123 taken up by the mitochondria varied as a function of the concentration of dye added to the medium. For added concentrations of rhodamine 123 between 5 and 20 ug/ml, much more of the dye was associated with mitochondria under energized as compared to non-energized conditions (Table 1). However this particular method was not adequate to study uptake with concentrations > 20 ug/ml.

Rhodamine 123 exhibited a spectral shift upon interaction with mitochondria, the most prominent feature being a change in the absorption maximum from 502 nm to about 512 nm. The peak shift was sensitive to uncouplers, suggesting that it represented the same kind of specific association of rhodamine 123 with mitochondria that is detected in whole cells (6) and in isolated mitochondria (ref. Table 1). This phenomenon was investigated further as an alternative method for comparing the amount of dye associated with the mitochondria under coupled and uncoupled conditions. The absorbance at 580 nm was independent of the amount of dye added so that the net absorbance measured in the difference spectrum between λ_{max} and 580 nm could be used as a quantitative indicator of rhodamine 123 uptake. The results obtained using this method (Figure 2) were in qualitative agreement with data obtained by the first method (Table 1). That is, for concentrations of rhodamine 123 up to 20 ug/ml, there was 5-7-fold more dye associated with the mitochondria under coupled as compared to uncoupled conditions. At concentrations above 30 ug/ml, there was no further increase in the amount of dye taken up under energized

Table 1. The amount of rhodamine 123 associated with mitochondria under energized and non-energized conditions.

Rhodamine 123 Added (ug/ml)	Rhodamine 123 associated with mitochondria (nmol/mg protein)	
	Succinate	Succinate + 40 uM 2,4 dinitrophenol
5	22.3	1.2
10	48.2	2.5
20	63.1	8.0

Values were determined as described under Materials and Methods. The results shown are from a typical experiment using succinate as the substrate. Similar results were obtained using glutamate + malate as the substrate.

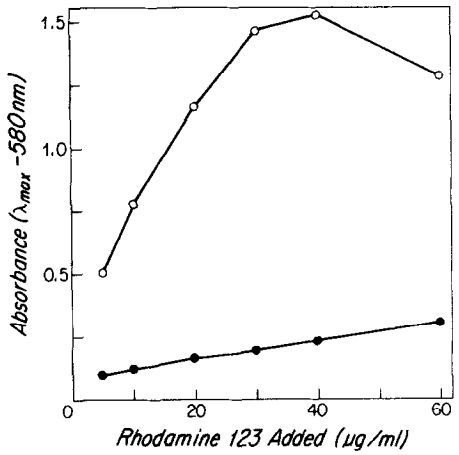

Figure 2. The amount of rhodamine 123 associated with mitochondria under coupled and uncoupled conditions. Absorbance (indicative of uptake) was determined from the difference spectrum obtained for rhodamine 123 in the presence and absence of mitochondria using the wavelength pair λ_{max}-580 nm as described under Results. Points are from one typical experiment with 5 mM succinate in respiratory assay medium with (●) or without (○) 40 uM 2,4-dinitrophenol.

conditions (Fig. 2). This plateau might have been due to saturation of binding sites or to uncoupling by rhodamine 123 at high concentrations, a condition that would inhibit further uptake. The most important point to be derived from Table 1 and Fig. 2 is that at concentrations of rhodamine 123 which appeared to inhibit ADP-stimulated respiration but not uncoupled respiration, the amount of dye associated with the mitochondria was several-fold greater under coupled as compared to uncoupled conditions.

The Effect of Rhodamine 123 on F_0F_1ATPase and on Adenine Nucleotide Translocase Activities. At 5 ug/ml rhodamine 123 inhibited ATP synthesis by 90% in well-coupled, energized mitochondria (Figure 3). This reaction appeared to be even more sensitive than ADP-stimulated respiration to inhibition by rhodamine 123, possibly because this assay is a more direct measurement of coupled oxidative phosphorylation than is total oxygen consumption. The reverse reaction, uncoupler-stimulated F_0F_1ATPase activity, was also inhibited by rhodamine 123 showing that the dye had a direct effect on this enzyme complex. However much higher concentrations of the dye were needed to inhibit ATP hydrolysis in the presence of an uncoupler than were needed to inhibit ATP synthesis in energized mitochondria (Fig. 3). This apparent difference in sensitivity of the F_0F_1ATPase to

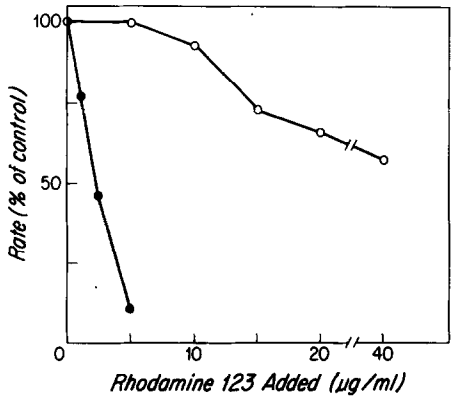

Figure 3. Effect of rhodamine 123 on mitochondrial phosphorylation reactions. Values are plotted relative to a control (no rhodamine 123) for ATP synthesis (•) and ATP hydrolysis (o). Reaction conditions were as described under Materials and Methods.

rhodamine 123 inhibition in energized versus non-energized mitochondria is most readily explained by the fact that more dye associates with the mitochondria under energized conditions.

To determine whether the inhibition of F_0F_1ATPase was secondary to inhibition of the adenine nucleotide translocase, we measured the effect of rhodamine 123 on this transporter. In energized mitochondria adenine nucleotide translocase activity was inhibited by only 43% even at a relatively high concentration (20 ug/ml) of rhodamine 123. In the presence of 40 uM 2,4-dinitrophenol, 20 ug/ml of the dye did not inhibit translocase (not shown).

DISCUSSION

A structural analog of rhodamine 123, rhodamine 6G, has been investigated for its effects on bioenergetic functions (11-13), but there are no detailed studies of this sort for rhodamine 123. In one recent brief report (14), the dye was investigated for possible use in measuring mitochondrial membrane potentials. Inhibition of F_0F_1ATPase and uncoupling activity at high concentrations were described; a spectral shift upon interaction of the dye with mitochondria was also noted. Our results are consistent with those findings (14).

We conclude that rhodamine 123 inhibits oxidative phosphorylation in intact rat liver mitochondria. This probably occurs via inhibition of F_0F_1ATPase, although inhibition of electron transfer reactions may contribute as well. Adenine

nucleotide translocase, substrate transport, substrate dehydrogenases, and respiratory complexes I and II are probably not specifically involved. In interpreting future studies on the biochemical basis of rhodamine 123 toxicity, the finding that more of the dye is taken up under energized as compared to non-energized conditions must be kept in mind. It is not clear at present whether rhodamine 123 binds to the mitochondrial membrane or is sequestered in the matrix. Also, it remains to be determined whether inhibition of F_0F_1ATPase and electron transport is specific or a result of nonspecific membrane perturbation due to the lipophilic nature of the dye.

The fact that rhodamine 123 inhibited energy-linked functions in normal rat liver mitochondria poses many interesting questions about the basis for selective anticarcinoma activity exhibited by the dye in vitro (1,2) and in vivo (3). Further studies, aimed at understanding the mechanism by which rhodamine 123 exerts a toxic effect on certain tumor mitochondria in the intact cell, will be important to the potential use of the dye as a chemotherapeutic agent.

Acknowledgements. This study will be submitted by J.S. Modica-Napolitano in partial fulfillment of the requirement for a Ph.D. in Biology at Tufts University. We thank Caroline A. Brown for technical assistance and Suzanne T. Dobson for preparing the manuscript. The work was supported by NIH grants NS14936, HD16936, and CA22427.

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