Basis for the Selective Cytotoxicity of Rhodamine 123

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ABSTRACT

Using rat liver mitochondria we determined that the primary biochemical target for inhibition of mitochondrial bioenergetic function by rhodamine 123 (Rh123) was F0F1-ATPase and that the amount of Rh123 associated with mitochondria is proportional to the mitochondrial membrane potential. Inhibition of coupled respiration by Rh123 in mitochondria isolated from CX-1, a Rh123-sensitive carcinoma cell type, and CV-1, a Rh123-insensitive normal epithelial cell type, was linearly related to the amount of Rh123 added (μg/mg protein) with CX-1 mitochondria exhibiting 2-fold greater inhibition compared to CV-1 mitochondria at any given amount of dye. The inhibition pattern for mitochondria isolated from MIP101, a Rh123-insensitive carcinoma cell type, was nonlinear, exhibiting greater sensitivity to CV-1 mitochondria at very low amounts of Rh123 but becoming less sensitive than either CV-1 or CX-1 at higher amounts. Rh123 inhibited F0F1-ATPase activity to a similar extent and in a concentration-dependent manner in both CV-1 and CX-1 mitochondria, but a different and complex pattern of inhibition was apparent for MIP101 mitochondria. Moreover, mitochondria from the 2 carcinoma cell types, CX-1 and MIP101, had higher membrane potentials (163 ± 7 and 158 ± 8 mV, respectively) than did mitochondria from the normal epithelial cell type, CV-1 (104 ± 9 mV). It was concluded that differences in both mitochondrial membrane potential and sensitivity of F0F1-ATPase contribute to the selective cytotoxicity exhibited by Rh123 for certain cell types in vitro.

INTRODUCTION

The discovery that Rh123 is a mitochondrial specific vital stain was made several years ago (1). Since then, certain tumor cell types, most notably those of epithelial origin, have been shown to exhibit increased uptake and prolonged retention of Rh123 within their mitochondria (2). This phenomenon appears to correlate well with the selective toxicity exhibited by Rh123 for certain cell types in vitro (3, 4).

The inhibitory effect of Rh123 on bioenergetic function has been demonstrated previously in isolated rat liver mitochondria (5, 6). It was concluded that F0F1-ATPase was the primary site of Rh123 toxicity; however, within the limits of the system used, various components of the electron transport chain could not be ruled out as secondary sites of inhibition (5). One objective of the present study was to determine more precisely the specific biochemical targets for Rh123 toxicity.

We also investigated the mechanism of mitochondrial uptake of Rh123. There is much indirect evidence to support the hypothesis that this cationic lipophilic molecule is taken up by mitochondria in response to the negative-inside membrane potential generated upon energization of the organelle (7). By experimentally manipulating the mitochondrial membrane potential and measuring the amount of Rh123 associated with mitochondria, it was possible to assess this relationship directly.

MATERIALS AND METHODS

Materials. Rh123 was obtained from Eastman Organic Chemicals, Rochester, NY. A 1-mg/ml solution in distilled water was stored at −20°C. 86Rb, [14C]sucrose, and 3H2O were purchased from New England Nuclear.

Cell Cultures. CV-1, a normal monkey kidney epithelial cell line, and CX-1 and MIP101, both human colon carcinomas, were kindly provided by Dr. Lan Bo Chen. CV-1 cells were grown in DME medium (GIBCO) supplemented with 5% calf serum (Hazelton). CX-1 and MIP101 cells were grown in equal volumes DME medium and RPMI 1640 medium (GIBCO) and supplemented with 5% calf serum and 5% Nu serum (Collaborative Research). Cells were grown to confluence and harvested from fifteen to twenty 150-mm plates (Nunc).

Isolation of Mitochondria. Liver mitochondria were isolated by differential centrifugation at 4°C from male Sprague-Dawley rats as described previously (9). Briefly, tissue was homogenized in 250 mM sucrose, 1 mM Tris-HCl, 1 mM EDTA, pH 7.4, 20% w/v, and centrifuged at 600 × g for 10 min. The supernatant was then centrifuged for 10 min at 8000 × g. The mitochondrial pellet was resuspended and washed twice in 250 mM sucrose, 1 mM Tris-HCl, 1 mM EDTA, pH 7.4, followed by 1 wash in 250 mM sucrose, 1 mM Tris-HCl, pH 7.4. The final pellet was resuspended in 250 mM sucrose, 1 mM Tris-HCl, pH 7.4 to desired concentration.

Mitochondria were isolated from cells in culture by a modification of the procedure of Maltese and Aprille (10). Typically, 5 × 106 cells were harvested in DME. Cells were pelleted and washed once with homogenization buffer (250 mM sucrose, 1 mM Tris-HCl, 1 mM EDTA, 1 mg/ml bovine serum albumin, pH 7.4) at low speed in a tabletop clinical centrifuge. The final pellet of cells was resuspended to a volume of 7 ml and homogenized in a Dounce tissue grinder with a tight pestle until at least 95% of the cells were disrupted (approximately 125 up/ down strokes). The homogenate was centrifuged at 800 × g for 10 min at 4°C. The supernatant was removed and saved, and the pellet was
resuspended and centrifuged again at 800 × g for 10 min. The supernatants were then pooled and centrifuged at 9400 × g (10 min, 4°C); the pellet was resuspended and centrifuged again at 9400 × g and finally suspended to a volume of about 5 mg protein/ml. Protein was determined by the method of Lowry et al. (11).

Respiration. Oxygen consumption was measured polarographically with a Clark electrode in a 1 ml water-jacketed chamber maintained at 30°C (12). The basic respiratory assay medium consisted of 225 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM K2HPO4-KH2PO4, 5 mM MgCl2, and 10 mM Tris-HCl, pH 7.4, (1 mg/ml bovine serum albumin was included for mitochondria from cultured cells) to which additions were made in the following order: mitochondria (0.18–0.27 mg protein); rotenone (2 µg/ml); succinate (10 mM); Rh123 (0–20 µg/ml); and ADP (120 nmol). The ADP-stimulated respiratory rate was defined as the rate after ADP minus the rate before ADP.

Rh123 Associated with Mitochondria. In the manner described previously (9), mitochondria (0.2–0.25 mg/ml) were incubated at room temperature for 1 min in 250 mM sucrose, 50 mM Tris-HCl, 5 mM succinate, pH 7.5, and Rh123 (10 µg/ml). One ml of the mixture was then layered over silicone oil which was in turn layered over 12% PCA and centrifuged at 9980 × g for 2 min in an Eppendorf tabletop centrifuge. The amount of Rh123 in the supernatant was determined by absorbance at 502 nm by comparison with standards. The amount of Rh123 associated with the pellet was determined indirectly by subtracting the amount remaining in the supernatant from the amount of dye originally added.

Enzyme Assays. All enzyme activities were assayed in freeze-thawed (−20°C) preparations of isolated mitochondria. F0F1-ATPase activity at 30°C was measured spectrophotometrically at 340 nm by coupling an ATP-regenerating reaction (pyruvate kinase) to the oxidation of NADH via lactate dehydrogenase essentially as described previously (13). The 1-ml reaction medium contained 300 mM sucrose, 25 mM Tris-HCl, pH 7.4; 25 mM KCl; 2 mM MgCl2; 0.4 mM NADH; 1 mM phosphoenolpyruvate; 2 mM ATP; 3 units each pyruvate kinase and lactate dehydrogenase; and 0–100 µg/ml Rh123. Mitochondria were added to a final concentration of about 0.05 mg protein/ml. ATP was added to start the reaction and the oligomycin-sensitive rate was defined as the rate after ATP minus the rate after adding 10 µg oligomycin.

Cytochrome c oxidase activity was determined spectrophotometrically at 550 nm by oxidation of reduced cytochrome c at 37°C (14).

Both NADH-cytochrome c reductase (rotenone-sensitive) and succinate-cytochrome c reductase activities were determined spectrophotometrically, by reduction of cytochrome c at 550 nm at 30°C (15) as follows. For NADH-cytochrome c reductase, the 1-ml reaction mixture contained 25 mM potassium phosphate, pH 7.4; 1 mg cytochrome c (oxidized); 4 mM KCN; 0–40 µg/ml Rh123; and 0.2 mM NADH. The reaction was initiated by addition of mitochondria (freeze-thawed, 16–18 µg protein/ml final concentration) and activity was calculated from the initial rate minus the rate after addition of 0.025 mM rotenone. For succinate-cytochrome c reductase, the 1-ml reaction mixture contained 50 mM potassium phosphate, pH 7.4; 20 mM NaCN; 0–40 µg/ml Rh123; and 20 mM succinate. Freeze-thawed mitochondria were added to a final concentration of 8–9 µg protein/ml and the sample was preincubated for 15 min to activate succinate dehydrogenase. Oxidized cytochrome c (1 mg/ml) was added to start the reaction.

Mitochondrial Membrane Potential. A quantitative determination of the ΔΨ was made from the equilibrium distribution of 45Rb in the presence of valinomycin. Briefly, mitochondria (0.2 mg protein) were added to a 1-ml reaction mixture containing 250 mM sucrose; 50 mM Tris-HCl, pH 7.5; 5 mM succinate; 1 mg/ml valinomycin; and 45Rb (1 µCi/ml), equilibrated to 37°C. In experiments in which ΔΨ was varied, 3–25 mM KCl was added for this purpose (16). The sample was then centrifuged at 9980 × g in an Eppendorf tabletop centrifuge and an aliquot of the supernatant was saved for scintillation counting to determine the amount of 45Rb in the external medium. The pellet was resuspended in 0.10 ml 12% PCA and centrifuged again, and an aliquot of the PCA extract was used to determine the amount of 45Rb in the mitochondrial pellet.

Matrix volume measurements, necessary to calculate 45Rb, were separately determined. Incubation samples containing both 14C-sucrose and 3H2O were layered over oil which was in turn layered over 12% PCA. Aliquots of the PCA extract were sampled for 14C and 3H counts in order to determine sucrose and water space, respectively, of the difference with which corresponds to the mitochondrial matrix volume.

Membrane potential (negative inside) was calculated according to the Nernst equation:

\[ \Delta \Psi = 60 \log \frac{[45\text{Rb}]}{[45\text{Rb}]_{\text{outside}}} \]

RESULTS

Rhodamine 123 Target Site. We investigated the effect of Rh123 on electron transport activity using freeze-thawed preparations of normal rat liver mitochondria. In these disrupted mitochondria Rh123 is not accumulated, and so the local concentration of Rh123 that is relevant to enzyme activities is assumed to be equivalent to that which is added to the medium. Assays were chosen such that only specific, limited segments of the respiratory chain were engaged in electron transport activity at any one time. As shown in Table 1, concentrations of Rh123 up to 40 µg/ml had no effect on either cytochrome c oxidase or succinate-cytochrome c reductase activity, while NADH-cytochrome c reductase activity was inhibited slightly but only at a very high concentration of Rh123. In contrast to these electron transport activities, uncoupler-stimulated F0F1-ATPase activity was shown previously to be very sensitive to Rh123; inhibition was 33% with 20 µg Rh123/ml and 45% with 40 µg Rh123/ml (5).

Amount of Rh123 Associated with Mitochondria in Relation to Mitochondrial Membrane Potential. The amount of Rh123 taken up by mitochondria is severalfold greater under energized compared to nonenergized conditions (5). This has been suggested to occur in response to the negative-inside electrical potential generated upon energization, since various ionophores known to dissipate this potential cause the rapid release of Rh123 from isolated mitochondria (5) and from cells prestained with the dye (7). Using isolated rat liver mitochondria the relationship between mitochondrial membrane potential and the amount of Rh123 associated with mitochondria was investigated directly by adding varying amounts of K+ to the mitochondrial incubations in the presence of valinomycin. Under these conditions the membrane potential is inversely proportional to the K+ ion concentration (16). The results in Fig. 1 show that, above 50 mV, the total amount of Rh123 associated with mitochondria is a function of the mitochondrial membrane potential.

Effect of Rh123 on Coupled Respiratory Rates. Both coupled and uncoupled respiratory rates in the absence of Rh123 were similar in mitochondria isolated from 3 cell lines grown in culture: CV-1, a Rh123-insensitive normal monkey kidney epithelial cell type; MIP101, a Rh123-insensitive undifferentiated human colon carcinoma cell type; and CX-1, a Rh123-sensitive human colorectal carcinoma cell type; and CX-1, a Rh123-sensitive human colorectal carcinoma cell type.
isolated from the 3 different cell types might be due to differences in mitochondrial membrane potential. The results in Table 3 indicate that mitochondria from CX-1, the Rh123-sensitive carcinoma cell type, had a significantly higher membrane potential (163 ± 7 mV) than did mitochondria from CV-1, the Rh123-insensitive normal epithelial cell type (104 ± 9 mV). The membrane potential of mitochondria isolated from MIP101, the Rh123-insensitive carcinoma cell type, was similar to that of CX-1 (158 ± 8 mV).

F0F1-ATPase in Mitochondria Isolated from Cultured Cells. We also investigated whether the differential effect of Rh123 on oxidative phosphorylation in mitochondria isolated from these 3 cell types might be due to differences in sensitivity of the target enzyme. The effect of Rh123 on F0F1-ATPase activity was measured directly in freeze-thawed preparations of mitochondria from CX-1, CV-1, and MIP101 cell types. According to Fig. 3, inhibition of enzyme activity in these membrane-disrupted preparations of mitochondria from both CX-1 and CV-1 cell types was a linear function of Rh123 concentration. Disrupted mitochondria from MIP101, on the other hand, showed a complex nonlinear pattern of enzyme inhibition which was quite different from CX-1 and CV-1 mitochondria.

DISCUSSION

The inhibitory effect of Rh123 on bioenergetic function in isolated rat liver mitochondria was demonstrated previously (5, 6). In well-coupled energized mitochondria ATP synthesis was sensitive to very low concentrations of Rh123 (5). The reverse reaction, uncoupler-stimulated F0F1-ATPase activity, was also inhibited by Rh123 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. This apparent difference in sensitivity of F0F1-ATPase to Rh123 inhibition in energized compared to nonenergized mitochondria is most readily explained by the fact that 6–8 times more dye was found to be associated with the mitochondria under energized conditions. Since the effect of Rh123 on adenine nucleotide translocase activity in energized mitochondria was not sufficient to account for inhibition

Table 3 Matrix volume and membrane potential in mitochondria isolated from CV-1, CX-1, and MIP101 cell types

<table>
<thead>
<tr>
<th>Matrix volume (μl/mg protein)</th>
<th>mV</th>
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<tbody>
<tr>
<td>CV-1</td>
<td>0.86</td>
</tr>
<tr>
<td>CV-1</td>
<td>104 ± 9</td>
</tr>
<tr>
<td>CX-1</td>
<td>0.60</td>
</tr>
<tr>
<td>CX-1</td>
<td>163 ± 7</td>
</tr>
<tr>
<td>MIP101</td>
<td>0.69</td>
</tr>
<tr>
<td>MIP101</td>
<td>158 ± 8</td>
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</tbody>
</table>

Fig. 3. Inhibition of F0F1-ATPase activity as a function of Rh123 concentration. F0F1-ATPase activity was measured in freeze-thawed preparations of mitochondria isolated from 3 cell types grown in culture: A, CV-1; B, CX-1; C, MIP101. Values, percentage relative to a control (no Rh123 added) activity. *, mean ± SE of 3 separate preparations; without bars in panel C, n = 1.

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of coupled respiration (5), it was concluded that the primary site of Rh123 toxicity was most probably F0F1-ATPase. However, based on the data available at that time, electron transfer reactions were not rigorously ruled out.

One objective of this study was to determine more precisely the specific biochemical target for Rh123 toxicity. In freeze-thawed (membrane-disrupted) mitochondria, under which condition the "matrix" concentration of Rh123 is assumed to be equivalent to the concentration of dye added, Rh123 had no effect on either succinate-cytochrome c reductase or cytochrome c oxidase activity. This eliminates complexes II, III, and IV as possible target sites of Rh123 toxicity. While NADH-cytochrome c reductase activity did decrease slightly at high concentrations of dye, the magnitude of this inhibition could not account for the effect of Rh123 on oxidative phosphorylation. Respiratory rates using either succinate or glutamate + malate were inhibited by Rh123 to the same extent (5), which also argues against specific inhibition of complex I. Further, F0F1-ATPase activity was shown directly to be much more sensitive to inhibition by Rh123 than was NADH-cytochrome c reductase (Ref. 5; Table 1). Emaus et al. concluded that F0F1-ATPase is the main site of Rh123 toxicity in intact mitochondria (6). Mai and Allison have shown that Rh123 inhibits the activity of F0F1-ATPase reconstituted in phospholipid vesicles (17). A related compound, rhodamine 6G, has been shown to act similarly (18). Thus, while adenine nucleotide translocase and complex I of the electron transport chain may be secondary sites for Rh123 inhibition, especially at the high matrix concentrations of dye that are attained under coupled conditions (see below), the data strongly suggest that oligomycin-sensitive mitochondrial ATPase is indeed the most sensitive biochemical target for Rh123 toxicity.

The fact that Rh123 inhibited energy-linked functions in normal rat liver mitochondria poses many interesting questions about the basis for selective anticancer activity exhibited by the dye in vitro (3, 4) and in vivo (19). Further studies were aimed at understanding the mechanism by which Rh123 exerts a toxic effect on certain tumor mitochondria in the intact cell.

Using whole cells, the CX-1 line (human colon carcinoma) was found to be much more sensitive to the cytotoxic effect of Rh123 than the CV-1 and MIP101 lines (normal monkey kidney epithelial and undifferentiated human colon carcinoma, respectively) (3, 8, 9). The present study revealed that respiratory activity in mitochondria isolated from these cell types also exhibited differences in sensitivity to Rh123. Over the range of Rh123 tested, coupled respiration was more sensitive to inhibition in CX-1 mitochondria as compared to CV-1 and MIP101 (Fig. 2).

Fig. 1 firmly establishes that the amount of Rh123 associated with mitochondria is a function of mitochondrial membrane potential. Since the extent of inhibition of respiratory function is dependent upon the amount of Rh123 taken up by mitochondria, the differential effect of Rh123 on oxidative phosphorylation in mitochondria isolated from the 3 cell types might be a function of differences in mitochondrial membrane potential. Indeed, the membrane potentials calculated for CX-1 and CV-1 mitochondria were 163 ± 7 and 104 ± 9 mV, respectively.

It is of interest to compare the matrix concentration of Rh123 at these different membrane potentials since it is this concentration which is relevant to the inhibition F0F1-ATPase in intact energized mitochondria. Rh123 distributes across the inner mitochondrial membrane in accordance with the Nernst equation. Therefore, for any condition, the actual matrix concentration of the dye can be calculated from the in/out ratio of 86Rb distribution (used to determine Δψ) and direct measure of the external concentration of Rh123. Accordingly, when 10 μg/ml Rh123 are added to a sample of isolated rat liver mitochondria, the matrix (free) concentration of the dye is 0.7 μg/μl at a membrane potential of 104 mV versus 3.0 μg/μl at a membrane potential of 163 mV. Thus, a 60-mV difference in membrane potential appears sufficient to account for the more than 2-fold greater sensitivity to inhibition of coupled respiratory activity by Rh123 in CX-1 as compared to CV-1 mitochondria that is shown in Fig. 2.

Although the membrane potential of MIP101 mitochondria is similar to that of CX-1 (158 ± 8 and 163 ± 7 mV, respectively), the biphasic nature of the curve relating inhibition of respiration to Rh123 concentration for MIP101 (Fig. 2) indicates that factors in addition to Δψ are involved. One possibility is a differential sensitivity of the target enzyme, F0F1-ATPase. According to Fig. 3, the effect of Rh123 on F0F1-ATPase activity was a simple linear function of dye concentration, with the percentage of inhibition being similar for both CX-1 and CV-1 mitochondria. The effect on MIP101 mitochondria, however, revealed a completely different and complex pattern of inhibition; low concentrations of Rh123 decreased the activity of the enzyme with no further inhibition except at very high concentrations of the dye. This paralleled the biphasic pattern of Rh123 inhibition of coupled respiration in MIP101 mitochondria, suggesting that differences in sensitivity of MIP101 cells compared to CX-1 cells may be explained at least in part by a lower sensitivity of F0F1-ATPase to Rh123.

In whole cells, the plasma membrane potential preconcentrates Rh123 relative to the extracellular medium, thus affecting the amount of dye available for accumulation by the mitochondria (20). Therefore in situ, total mitochondrial uptake of Rh123 appears to be dependent on both the mitochondrial and plasma membrane potentials. In addition, differences have been noted among various cell types regarding the kinetics of uptake (21) and retention time (2) of Rh123. Ultimately then, the sensitivity of any particular cell type to Rh123 might depend on different cytoplasmic characteristics as well as on properties of the mitochondria.

Our findings have some additional interesting implications. First, regarding both mitochondrial membrane potential and sensitivity of F0F1-ATPase to Rh123, there exist fundamental differences inherent in the mitochondria of different cell types. Interestingly, the mitochondria of the 2 transformed cell types, CX-1 and MIP101, exhibited a significantly higher membrane potential relative to mitochondria of the normal epithelial cell type CV-1. However, the extent to which this holds true for mitochondria of other transformed cell types has yet to be determined. Further, while increased mitochondrial membrane potential is probably necessary for expression of the Rh123-sensitive phenotype, it is not sufficient in that other overriding factors, including variable sensitivity of F0F1-ATPase, appear to be involved.

ACKNOWLEDGMENTS

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REFERENCES


