Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 568-573

USE OF RHODAMINE 123 TO INVESTIGATE ALTERATIONS IN MITOCHONDRIAL ACTIVITY IN ISOLATED MOUSE LIVER MITOCHONDRIA

Jose Enrique O'Connor, Jose Luis Vargas, Bruce F. Kimler*
Jose Hernandez-Yago, and Santiago Grisolia

Instituto de Investigaciones Citologicas de la Caja de Ahorros de Valencia
Amadeo de Saboya 4, Valencia, Spain

* Radiation Biology Laboratory, Department of Radiation Oncology
University of Kansas Medical Center, Kansas City, KS

Received January 22, 1988

SUMMARY: The fluorescent dye Rhodamine-123, which selectively stains mitochondria depending on the mitochondrial membrane potential, was used with flow cytometry to evaluate alterations in activity of mitochondria isolated from mouse liver. Under **in vitro** conditions, with succinate and ADP present in the buffer, mitochondrial activity was affected by a variety of metabolic inhibitors that modify membrane potential. These results demonstrate clearly that flow cytometric techniques using Rhodamine-123 can be employed to study activity in isolated mitochondria.

Rhodamine 123 (Rh-123) is a positively charged fluorochrome at physiological pH which selectively stains mitochondria in living cells [1,2]. The uptake of Rh-123 is believed to be correlated with the mitochondrial transmembrane potential [3,4]. Because of this, the dye has been used to study mitochondrial properties in different cell types [5,6]. Flow cytometry has been used to monitor mitochondrial activity in whole cells stained with Rh-123 [6]. In general, flow cytometry techniques have been applied to the study of whole cells, with little effort applied to the study of subcellular organelles such as isolated mitochondria.

Recently, isolated mitochondria have been studied by flow cytometry, **e.g.**, the labelling of isolated potato mitochondria with FITC-labelled Concanavalin A [7] and preliminary work with mouse liver mitochondria stained with Rh-123 [8]. Our interest in using flow cytometry to study the uptake of Rh-123 by isolated mammalian mitochondria was to provide a more direct method with which to study their functions in heterogeneous whole cell preparations. In addi-

Abbreviations: Rh-123, rhodamine-123; FALS, forward angle light scatter; IGFL, integral green fluorescence.

0006-291X/88 $1.50

tion, this provides the opportunity of using isolated mitochondrial fractions to assay the cytostatic effects of Rh-123 itself. In this regard, it has been observed that Rh-123 inhibits the proliferation of some tumor cells [9], and is retained for longer times in the mitochondria of neoplastic cells than in normal cells [10]. However, the mechanisms for both effects remain unknown. Finally, decreases in mitochondrial function may prove useful for the assessment of the cytotoxic properties of cancer chemotherapeutic agents [11,12].

MATERIAL AND METHODS

Mouse liver mitochondria were isolated by the method of Hogeboom **et al.** [13] after homogenation of liver with 2 ml of buffer/gram of liver. The homogenation buffer was 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, and 1 mM EDTA, at pH 7.4. Isolated mitochondria were suspended in incubation medium consisting of 225 mM sucrose, 5 mM Mg_2Cl, 20 mM triethanolamine, and 1 mM EDTA in 10 mM phosphate buffer at pH 7.4. Mitochondria were treated with specific inhibitors or additives (all from Sigma Chemical Co., St. Louis, Mo.), as indicated in Table I for 30 min at 37°C, prior to the addition of 10 mg Rh-123 per ml (Eastman Kodak, Rochester, NY). After 30 min incubation at 37°C, mitochondria were rinsed and were suspended in incubation medium and analyzed on an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL), by means of an argon ion laser tuned at 488 nm and 250 mW. Blocking filters were 515 nm, splitting filter was 590 nm dichroic, and the filter in front of the green photomultiplier tube was a 590 nm short pass filter. Forward angle light scatter (FALS, an estimate of particle size) and integral green fluorescence (IGFL) were measured for 50,000 events. The median channel for each histogram was determined by integral analyses in the MDADS software interfaced to the EPICS V and was used to compare the effects of different treatment conditions.

RESULTS AND DISCUSSION

Fig. 1A shows the size distribution of particles as estimated by FALS analysis of isolated mitochondria stained with Rh-123. Staining with Rh-123 itself had no effect on the FALS distribution obtained. As can be seen, there is a bell-shaped population of lower size particles and a minor population of particles of increasing size. Associated with this size distribution, there is an IGFL-associated distribution, as shown in Fig. 1B. The use of appropriate "gating windows" in two-parameter flow cytometric analyses allows one to study the variations of one of the parameters as a function of the other. Therefore, we set "gate windows" on the FALS signals in order to collect the IGFL signals correlated with particles displaying FALS signals either in the smaller (channels 9-55) or larger (channels 56-113) sized subpopulations of isolated mitochondria. As can be seen in Fig. 1C and 1D, IGFL signals vary as a function of size, the larger sizes being associated with a more intense IGFL. In view of this, we routinely collected the IGFL associated with the larger sized populations, since this led to better signals. Because of a concern that Rh-123 itself might interfere with mitochondrial membrane poten-

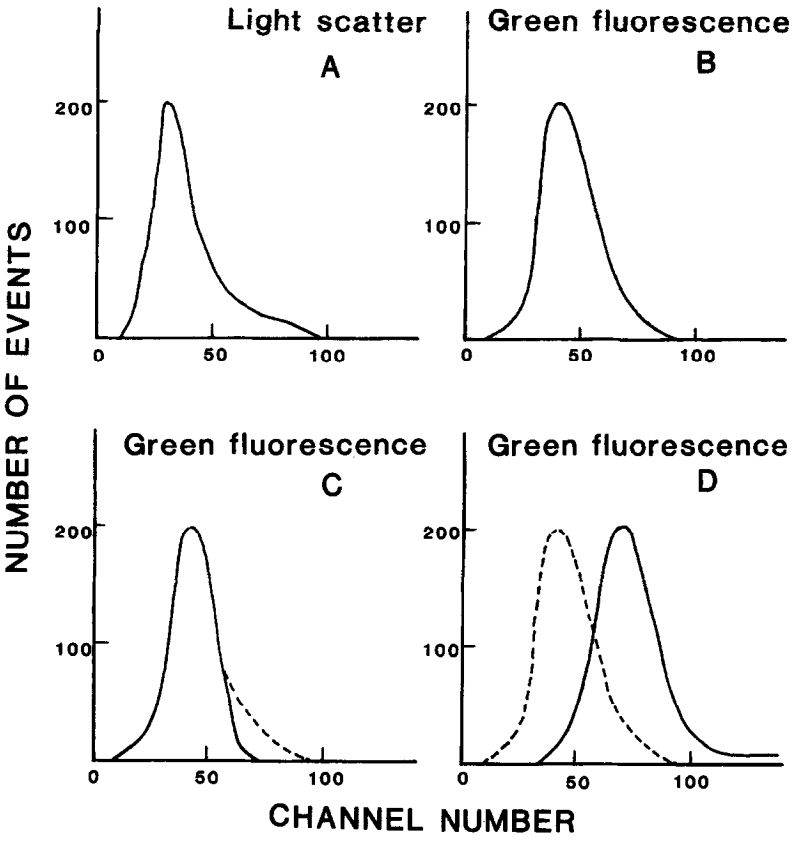

Figure 1. Flow cytometric analysis of size and fluorescence in isolated mitochondria stained with Rh-123. A) size distribution as estimated from FALS. B) IGFL associated with the entire population (FALS channels 9-113). C) IGFL associated with the smaller sized population (sorting window on FALS channels 9-55). D) IGFL associated with the larger sized population (gating window on FALS channels 56-113). The dashed line traces in panels C and D represent the IGFL distribution obtained in panel B. The conditions of the analysis are as indicated in Material and Methods.

tial [9] and thus cause inhibition, samples were periodically assayed over a period of hours following the 30 min incubation with Rh-123. No decrease in IGFL signals was observed under either control or experimental (**vide infra**) conditions. Thereafter, all samples were assayed within 1 hr of preparation.

The above results indicate that isolated mitochondria are easily detected by flow cytometric fluorescence analysis when stained with Rh-123, a selective fluorochrome for measuring mitochondria membrane potential [1,2]. It was our interest to determine if this method could be sensitive enough to detect variations in mitochondria membrane potential, which can be associated with variations in mitochondrial metabolic activity [6], thus providing a suitable system for studying perturbations of mitochondria function in isolated mitochondria. To this purpose, we incubated isolated mitochondria with several well-known agents [14,15] that modify membrane potential by acting as a K^+

Table 1. Incubation conditions in the experiments on Rh-123 uptake by isolated mouse liver mitochondria

Additions	0	1	2	3	4	5	6	7
Valinomycin (0.5 μM)								
Antimycin A (10 μM)	-	-	+	+	+	+	-	-
Oligomycin (10 μM)								
KCl (25 mM)	-	+	-	+	-	+	-	+
NaCl (25 mM)	+	-	+	-	+	-	+	-
Nigericin (10 μM)	-	-	-	-	+	+	+	+

Mitochondrial concentration, 0.2 mg/ml; 3 mM Succinate; 0.3 mM ADP 10 μg/ml Rh-123.

ionophore (valinomycin), as a H^+ ionophore (nigericin), or as inhibitors of phosphorylative oxidation (oligomycin, antimycin A). The conditions of the incubation are indicated in Table I. As seen in Fig. 2, under conditions that are physiological in nature, **i.e.**, when succinate as a respiratory substrate and ADP are present in the incubation medium, some of the treatments markedly affected the uptake of Rh-123 by isolated mitochondria and consequently reduced the IGFL signal. Thus, incubation with valinomycin, antimycin A, and oligomycin reduced the median IGFL channel to 60% of that observed for control mitochondria when incubated in the absence of K^+. The combination of additives in the presence of 25 mM KCl decreased IGFL to 49% of the control (Table 2). KCl by itself had no effect, nor did nigericin when added alone. Nevertheless, the combination of KCl and nigericin induced a reduction of the IGFL to 77% of the control. On the other hand, nigericin reversed partially the decrease of IGFL induced by the addition of valinomycin, antimycin A and oligomycin, both in the absence or presence of KCl.

The results presented here demonstrate clearly that isolated mitochondria can be used to study the mitochondrial membrane potential by using fluorescent staining with Rh-123 and flow cytometry. However, caution is advised in the interpretation of these results, since these commonly used inhibitory agents may affect differently the membrane potential in isolated mitochondria suspended in buffer compared to mitochondria **in situ**. In spite of this, this technique is an interesting extension of the work involving flow cytometric measurements of mitochondrial membrane potential in whole cells, in that it may provide an alternate, quick method for assessing mitochondrial activity under different physiological, pathological, or toxic conditions, **e.g.**, dietary manipulation, alcohol and ammonia toxicity, etc. In addition, the possible heterogeneity of mitochondria subpopulations regarding those pathophysiological conditions may be studied and specific subpopulations separated physically by means of the sorting capacity of the flow cytometer. This method can also be applied to study further the mechanism of Rh-123 uptake by mitochondria as a function of the membrane potential, which is of interest not only on purely

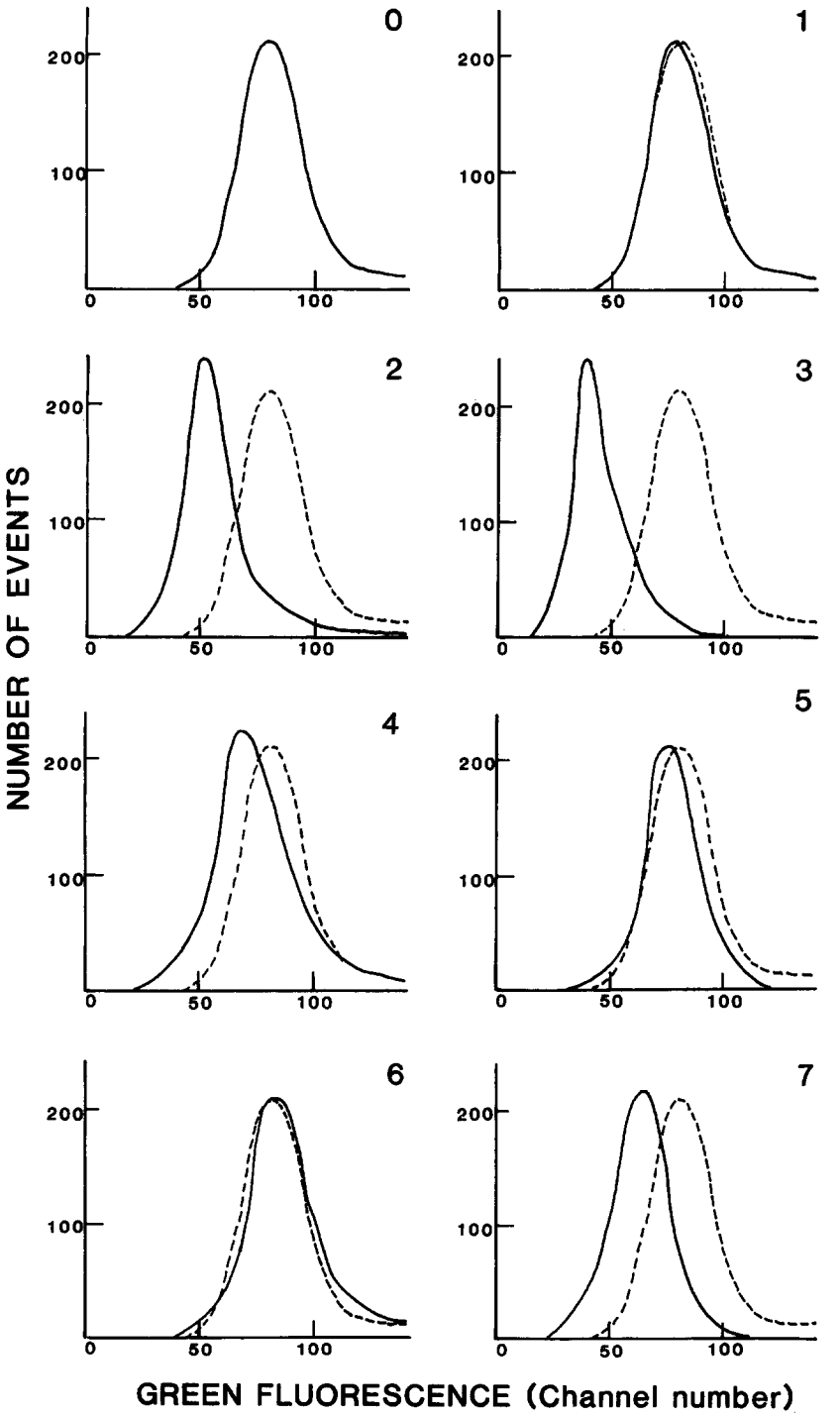

Figure 2. Effect of incubation of mitochondria under various conditions that affect mitochondrial membrane potential. The uptake of Rh-123 by isolated mitochondria was analysed by flow cytometry. The incubation and flow cytometric analyses were carried out as described in Material and Methods. The numbers denote the experimental conditions outlined in Table 1. The dashed line traces represent the IGFL distribution of control mitochondria (condition 0).

Table 2. Results of flow cytometric analyses of treated mitochondria stained with Rh-123

Treatment Number	Median Channel FALS	Median Channel IGFL	Relative IGFL*
0 (Control)	32	35	=1.00
1	29	34	0.97
2	36	21	0.60
3	35	17	0.49
4	31	28	0.80
5	31	30	0.86
6	20	35	1.00
7	32	27	0.77

* Relative IGFL = $IGFL_i/IGFL_0$, calculated from the data displayed in Fig. 2.

theoretical grounds but also could help to explain the anti-tumoral effect of Rh-123 and its increased retention in the mitochondria of neoplastic cells as compared to normal cells [5,9,10].

ACKNOWLEDGEMENTS

This work was supported by U.S.-Spain Joint Committee for Scientific and Technical Cooperation, CCA-8411-110; and The International Center for Cell Biology. The EPICS V flow cytometer at the Instituto de Investigaciones Citologicas is the property of the Tabacalera, S.A.

REFERENCES

1. Johnson, L.V., Walsh, M.L., and Chen, L.B. (1980) Proc. Natl. Acad. Sci. USA 77, 990-994.
2. Chen, L.B., Summerhayes, I.C., Johnson, L.V., Walsh, M.L., Bernal, S.D., and Lampidis, T.J. (1981) Cold Spring Harbor Symp. Quant. Biol. 46, 141-155.
3. Goldstein, S.D., and Korczac, L.B. (1981) J. Cell Biol. 91, 392-398.
4. Johnson, L.V., Walsh, M.L., Bockus, B.J., and Chen, L.B. (1981) J. Cell Biol. 88, 526-535.
5. Chen, L.B., Summerhayes, I.C., Nadakavaukaren, K.K., Lampidis, T.J., Bernal, S.D., and Shepherd, E.L. (1984) in Cancer Cells 1. The Transformed Phenotype (Levine, A.J., Van de Woude, G.F., Topp, W.C., and Watson, J.D., Eds.). pp. 75-86.
6. Ronot, X., Bebel, L., Adolphe, M., and Mounolou, J.-C. (1986) Biology of the Cell 57, 1-8.
7. Petit, P., Diolez, P., Muller, P., and Brown, S.C. (1986) FEBS Letters 196, 65-70.
8. O'Connor, J.E., Vargas, J.L., Kimler, B.F., Hernandez-Yago, J., and Grisolia, S. (1986) Biology of the Cell 58, 14a.
9. Chen, L.B., Weiss, M.J., Davis, S., Bleday, R.S., Wong, J.R., Song, J., Terasaki, M., Shepherd, E.L., Walker, E.S., and Steele, G.D. (1985) in Cancer Cells 3. Growth Factor and Transformation. (Feramisco, J., Ozanne, B., and Stiles, C., Eds.), pp. 433-443.
10. Nadakavakaren, K.K., Nadakavakaren, J.J., and Chen, L.B. (1985) Cancer Res. 45, 6093-6099.
11. Adams, E.G., Barden, K., Badiner, R., Ulrich, R., and Bhuyan, B.K. (1984) Cell Tissue Kinet 17, 670.
12. Wulser, M.J., and Kimler, B.F. (1987) Cell Tissue Kinet 20, 251.
13. Hogeboom, G.M., Scheider, W.C., and Palade, G.E. (1948) J. Biol. Chem. 172, 619-636.
14. Reed, P.R. (1979) Methods in Enzymology 55, 435-454.
15. McLaughlin, S.G.A., and Dilger, J.P. (1980) Physiological Rev 60, 825-863.