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Estradiol Induction of Rhodamine 123 Efflux and the Multidrug Resistance Pump in Rat Pituitary Tumor Cells

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SUMMARY

Rhodamine 123 is a fluorescent dye that localizes in mitochondria, is a substrate for the multidrug resistance pump, and is retained for long periods of time by carcinoma cells. 17β-Estradiol causes GH_4C_1 cells (rat pituitary tumor cells) to lose rhodamine 123 fluorescence faster than untreated cells. We found that estradiol induces accumulation of the mRNA for the multidrug resistance pump 3–5-fold, with maximum induction occurring within 1 day at 10^{-9} M estradiol. Immunoblot analysis demonstrated that estradiol induces a protein of 150 kDa that reacts with an antibody to P-glycoprotein, the multidrug resistance pump. The reduced retention of rhodamine 123 caused by estradiol is prevented by verapamil and cyclosporin, inhibitors of the pump. A clone resistant to the effects of estradiol on rhodamine 123 has greatly reduced levels of mRNA for the pump. The effect of estradiol is more marked on rhodamine 123 retention than it is on that of rhodamine 110 or tetramethylrhodamine methyl ester. We conclude that estradiol enhances rhodamine 123 efflux by inducing the multidrug resistance gene. The specificity for rhodamine 123, compared with other analogs, may be caused by differences in accessibility to the pump.

Rhodamine 123 is a fluorescent dye that accumulates in mitochondria in living cells (1); it is retained much longer in many transformed epithelial cells and carcinoma-derived cell lines than it is in normal epithelial cells (2). As a result, the antitumor properties of this drug are being investigated, including its use in photodynamic therapy (3–6). The causes of rhodamine 123 retention are therefore of interest.

Several properties influence distribution of this drug. Rhodamine 123 is both lipophilic and cationic; it therefore crosses membranes easily and accumulates at negative membrane potentials. This property causes rhodamine 123 to accumulate in mitochondria at equilibrium, because the interior of mitochondria is more negative than cytoplasm. Lipophilic cationic molecules, however, are also substrates for a plasma membrane pump called the multidrug resistance pump, or P-glycoprotein, which prevents such molecules from accumulating in cells (7). This pump appears to cause rhodamine 123 efflux from cells (8, 9). Other factors must also influence the distribution of this drug, at least in carcinoma cells, because rhodamine 123 remains in these cells long after the extracellular drug is removed, when the drug would normally be expected to diffuse out (2); such retention may reflect binding to intracellular components or active accumulation by mitochondria. Finally, rhodamine 123 may be metabolized to rhodamine 110 by cleavage of a methyl ester group (10); this product would have different distribution properties because it has a free carboxyl group with a negative charge.

We previously found that estradiol decreased retention of rhodamine 123 fluorescence in a pituitary tumor cell line (11). One explanation for the decrease is that estradiol induces the multidrug resistance pump; we suggested that other factors may be involved as well because there was some specificity for rhodamine 123. Daunomycin is a substrate for the pump, but we found that estradiol decreased retention of daunomycin much less than that of rhodamine 123. In addition, we found stable variants in which estradiol did not reduce rhodamine 123 retention but still decreased that of daunomycin (11). In this paper, we determined that estradiol induced the multidrug resistance pump. We also investigated the ability of estradiol to influence retention of other rhodamine analogs, to further analyze the specificity of the effect.

Materials and Methods

Rhodamine 123 and rhodamine 110 were laser grade from Eastman Kodak (Rochester, NY). Tetramethylrhodamine methyl ester and tetramethylrhodamine ethyl ester were from Molecular Probes (Eugene, OR). Verapamil was from Sigma (St. Louis, MO). Cyclosporin A was given to us by Dr. Robert Handschumacher (Yale University). The probe for rat *mdr* RNA was a gift from Dr. Jeffrey Silverman (NCI, NIH) (12).

Cell culture. Stocks of GH_4C_1 cells were maintained as described (11). Experiments were performed in 1:1 Dulbecco's minimal essential medium/Ham's nutrient mixture F10 (GIBCO, Grand Island, NY) plus

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ABBREVIATION: HPLC, high performance liquid chromatography.

15% gelding serum (Central Biomedia, Irwin, MO). The undiluted serum had <50 pM 17β-estradiol, as determined by radioimmunoassay. The plating density was 1×10^4 cells/35-mm well; medium was usually changed 3 and 6 days after plating, and the cells were assayed on day 7. For the time course experiments, cells were cultured for 1 week in medium with gelding serum and no added estradiol before the experiment was begun.

Flow cytometry. Fresh medium containing 10 μg/ml rhodamine analog was added to the cells, which were incubated at 37° for 30 min. Cells were then rinsed with rhodamine-free medium and incubated for 20 min more, unless indicated, at 37°. Cells were removed from the plate by enzymatic digestion using pancreatin (Sigma). Analysis was performed with a Becton Dickinson FACS IV flow cytometer (San Jose, CA). Rhodamine 123 was excited at 488 nm and the other analogs were excited at 514 nm, using an argon ion laser. The emission was measured as follows: rhodamine 123 above 520 nm, rhodamine 110 between 545 and 575 nm, and tetramethylrhodamine ethyl ester and methyl ester between 560 nm and 590 nm. A minimum of 10^4 cells were analyzed for each sample and fluorescence was plotted on a 5-decade logarithmic scale. The median fluorescence was used as a measure of fluorochrome concentration, and the logarithmically measured fluorescence intensity was recalculated to its linear value (13). These values were usually converted to percentage of controls to compare independent experiments.

Butanol extraction and HPLC analysis. These experiments were performed in phenol red-free medium. After incubation with rhodamine 123 for 30 min, cells were washed three times in phosphate-buffered saline. Three milliliters of butanol were added to the dish, or rhodamine-free medium was added, and the cells were incubated for 20 min more. Medium and cells were then separately extracted with butanol. The sum of the fluorescence recovered in the cells and medium after the 20-min incubation without rhodamine 123 was 97–99% of the fluorescence present at the start of the incubation, indicating that there was not appreciable metabolism to nonfluorescent compounds. Fluorescence was measured with a Hitachi model F1200 fluorescence spectrophotometer; excitation was at 485 nm and emission was measured at 532 nm. HPLC analysis was performed as described (14), on a DuPont Zorbax 3-μm C8 column (3-μm particle size, 60-Å pore size, 8 × 0.62 cm). A linear gradient of 0.05 M phosphate buffer, pH 2.85 (mobile phase A), and acetonitrile (mobile phase B) of 10–80% mobile phase B in 15 min was followed by 40% mobile phase B for 5 min and 10% mobile phase B for 8 min. This analysis demonstrated that the dye was contaminated only by a minor peak that migrated at the position of rhodamine 110. Almost all the fluorescence from all medium and cell extracts migrated as rhodamine 123; the remainder could be accounted for by that initially present as a contaminant. We checked recovery from the column by using the ratio of the height of the peak of fluorescence after HPLC to the amount of fluorescence injected on the column. The ratio was the same for rhodamine 123 standard and cell and medium extracts, so there was not a fluorescent metabolite remaining on the column.

RNA analysis. RNA was extracted by an acid-guandinium thiocyanate-phenol-chloroform technique (15). Approximately 2 μg of RNA, as determined by absorbance, were applied to each lane and electrophoresis and transfer to nitrocellulose were performed. RNA was hybridized to an *Eco*RI/*Hind*III fragment of plasmid p2b13-155 (12), which was labeled with ^{32}P by random priming (Boehringer Mannheim, Indianapolis, IN). Hybridization was at 42° overnight with conditions as described (16). Washes were at 60°. Filters were exposed to X-ray film at −70° with an intensifying screen for 3 days to 1 week. The *mdr* probe was removed from the nitrocellulose with boiling 3 mM NaCl, 0.3 mM Na citrate, 0.001 M EDTA, 0.2% sodium dodecyl sulfate. We used a probe for cyclophilin to normalize amounts of RNA on the filters (17); filters were exposed for 24 hr using this probe.

Immunoblot analysis. We collected 2×10^8 cells each from control and estrogen-treated cultures by rinsing cells with and then scraping them into phosphate-buffered saline, collecting cells by centrifugation, and resuspending them in 0.25 M sucrose, 10 mM Tris·HCl, pH 7.4, 0.2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin. We disrupted cells by nitrogen cavitation, with 15-min equilibration in nitrogen at 250 psi. The lyzed cells were centrifuged at 500 × *g* for 90 sec and the supernatant was layered on top of a discontinuous sucrose gradient of 20, 30, and 50%. The gradient was centrifuged for 16 hr at 100,000 × *g* at 4°. The band at the 30/50% interface was collected and concentrated by centrifugation at 100,000 × *g* for 60 min. The pellet was dissolved in 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, incubated at 55° for 1 hr, subjected to electrophoresis, and transferred to nitrocellulose. Equal amounts of protein (100 μg) were applied to each lane. Monoclonal antibody C219 to the P-glycoprotein, obtained from Dr. William Hait (Yale University), was used as the first antibody, and the second antibody was ^{125}I-rabbit anti-mouse IgG (New England Nuclear, Boston, MA).

Results

We analyzed RNA from control and treated cells; two bands were detected by Northern analysis with the rat multidrug resistance probe, a more pronounced band at about 4.3 kilobases and a much fainter band at about 5.1 kilobases. These mRNAs were barely detectable in control cells and were present in increased amounts in cells treated with estradiol (Fig. 1A). Induction of accumulation of mRNA for the multidrug resistance pump began within 3 hr and reached a maximum within 24 hr; estradiol had little effect on cyclophilin mRNA accumulation at any time (Fig. 1B). mRNA levels for the pump remained elevated for 1 week in the presence of estradiol (data not shown). Estradiol caused maximal stimulation of the mRNA for the pump at 1 nM (Fig. 2). We quantitated the induction by dot blots and normalized the amount of mRNA to cyclophilin mRNA; maximal induction measured by this technique ranged from 3- to 5-fold in different experiments. We also found that estradiol induced a protein of about 150 kDa that reacted with a monoclonal antibody for P-glycoprotein, the protein that acts as the multidrug resistance pump (7) (Fig. 3). Estradiol therefore increases the accumulation of the message for the pump and the protein itself.

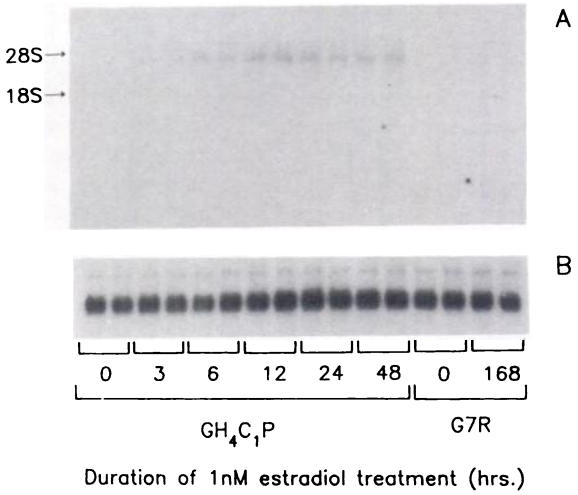

Fig. 1. Time course of estrogen induction. A, mRNA for the multidrug resistance pump; B, mRNA for cyclophin. Cell were passaged for 1 week in the absence of estradiol before the experiment was started. RNA from GH_4C_1 cells was collected at 0, 3, 6, 12, 24, and 48 hr after addition of 10^{-9} M estradiol and from G7R cells at 0 and 168 hr after estradiol. In three independent experiments, the maximal induction occurred within 1 day.

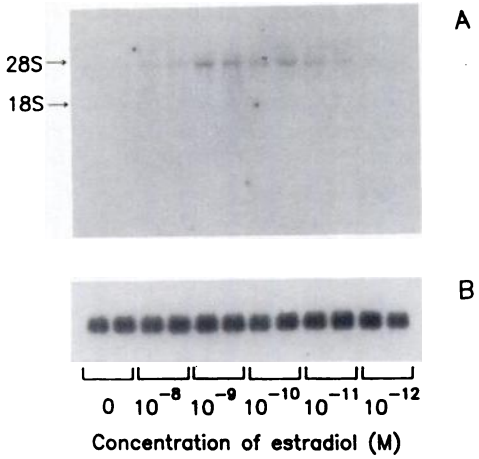

Fig. 2. Dose response of estrogen induction. A, mRNA for the multidrug resistance pump; B, mRNA for cyclophilin. Cells were treated for 1 week with the indicated concentrations. Similar results were seen in two independent experiments.

Fig. 3. Immunoblot analysis of GH_4C_1 cell membranes. One hundred micrograms of protein were applied to each lane. *C*, control cells; *E2*, cells treated for 1 week with 10^{-9} M estradiol.

We examined the effects of two inhibitors of the multidrug resistance pump, verapamil and cyclosporin (18, 19), on rhodamine 123 retention in control and treated cells (Fig. 4). Both drugs increased retention of rhodamine 123 by about 50% in control cells and completely prevented the estradiol-induced reduction in retention. The concentrations of cyclosporin and verapamil required are comparable to those that inhibit the pump in other systems (18, 19). The ability of these agents to prevent the action of estradiol are consistent with induction of the multidrug resistance pump by estradiol causing decreased retention of rhodamine 123.

We investigated the specificity for rhodamine 123 by comparing the retention of this analog with that of rhodamine 110 and tetramethylrhodamine methyl ester. The effect was always greatest on rhodamine 123. In the experiment shown in Fig. 5, the median fluorescence of control cells was 38-fold greater than that of estradiol-treated cells for rhodamine 123 and about 3-fold greater for rhodamine 110 and tetramethylrhodamine methyl ester. Results obtained with tetramethylrhodamine ethyl ester were similar to those for tetramethylrhodamine

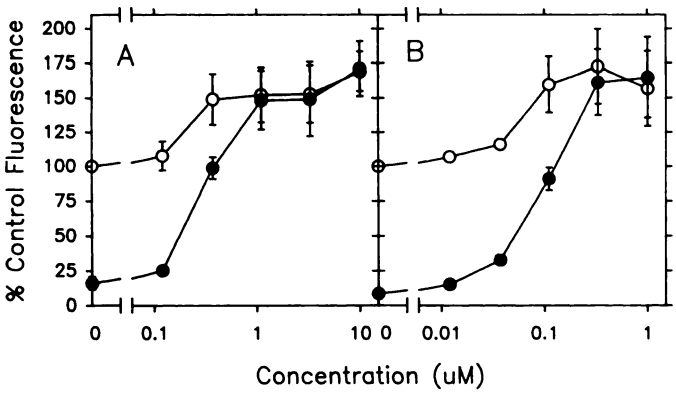

Fig. 4. Effect of inhibitors of the multidrug resistance pump on rhodamine 123 efflux from control and estrogen-treated cells. Cell were incubated with verapamil (A) or cyclosporin (B) and rhodamine 123 for 30 min, and verapamil or cyclosporin was also present in the 20-min incubation period after rhodamine 123 was removed. Median fluorescence in control cells was set at 100%. Each *point* is the average of three independent experiments; *bars*, standard error. Where no bars are shown the standard error fell within the symbol. ○, control cells; ●, estrogen-treated cells.

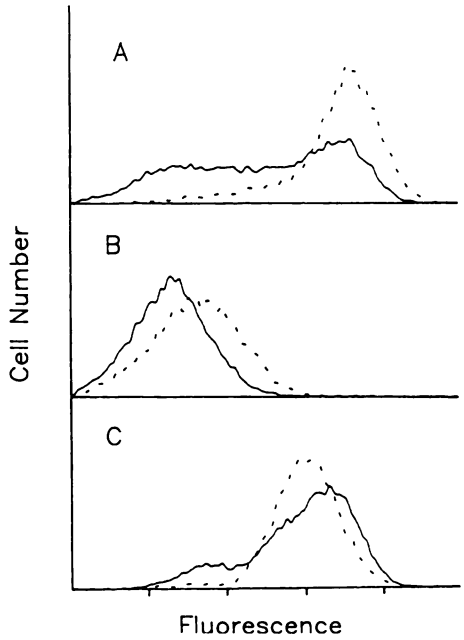

Fig. 5. Retention of rhodamine analogs analyzed by flow cytometry. Fluorescence was analyzed after a 30-min incubation period after the analogs were removed. A, Rhodamine 123; B, rhodamine 110; C, tetramethylrhodamine methyl ester. – – –, Fluorescence of control cells; ——, fluorescence of cells treated with 1 nM estradiol for 1 week before incubation with rhodamine 123.

methyl ester (data not shown). The population of cells that responded to estradiol with reduced rhodamine 123 retention was clearly heterogeneous (Fig. 5A), and the maximal estradiol effect on rhodamine 123 varied from 10- to 200-fold in this series of experiments. We attempted several times to obtain clones that responded to estradiol as if they were a homogeneous population. We have not found any such clones that are stable. Twenty separate clones that initially looked promising rapidly (within several weeks) began to behave in a heterogeneous fashion again. This instability of sensitive clones is in contrast to clones that are resistant to estradiol, which have remained relatively homogeneous for months (11).

The efflux time course was not the same for the analogs (Fig.

6). Both control and estradiol-treated cells lost 90% or more of rhodamine 110 fluorescence in the first 30 min after removal of the drug (Fig. 6). The remaining fluorescence, about 10-fold more than autofluorescence (data not shown), left the cell at a much slower rate. Both control and treated cells lost tetramethylrhodamine methyl ester more slowly (Fig. 6) and after 60 min still had >100 times more fluorescence than autofluorescence (data not shown). Control cells lost rhodamine 123 fluorescence as slowly as tetramethylrhodamine methyl ester, but estradiol-treated cells lost it rapidly. Induction of rhodamine metabolism could account for the loss, because rhodamine 123 can be converted to rhodamine 110 by deacylation. If estradiol induced esterases to cause this conversion, they would convert rhodamine 123 into a form that is rapidly lost from the cells. We demonstrated by butanol extraction and HPLC analysis that rhodamine 123 was not metabolized (data not shown).

Inhibitors of the multidrug resistance pump did not affect the rhodamine analogs equally (Fig. 7). Verapamil markedly increased the retention of rhodamine 110 in both control and estrogen-treated cells but had little or no effect on tetramethylrhodamine methyl ester. Cyclosporin had similar effects (data not shown).

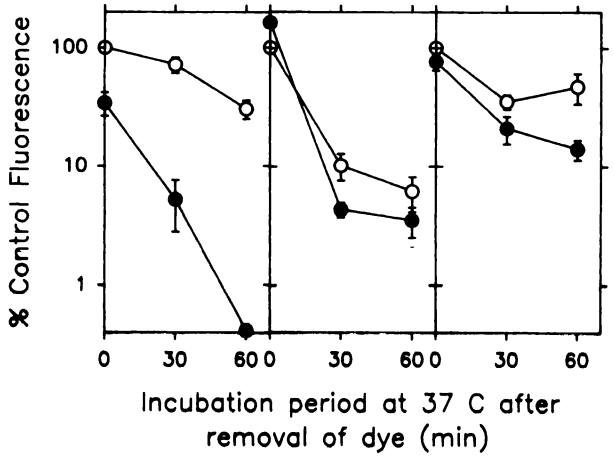

Fig. 6. Time course of retention of rhodamine analogs. *Abscissa*, time of the incubation period after the analogs were removed. *Left*, rhodamine 123; *middle*, rhodamine 110; *right*, tetramethylrhodamine methyl ester. ○, Control cells; ●, estrogen-treated cells. Each *point* is the average of three independent experiments; *bars*, standard error. Where no bars are shown the standard error fell within the symbol.

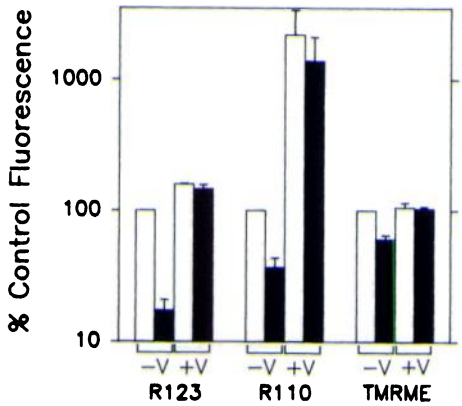

Fig. 7. Effects of verapamil (*V*) on retention of rhodamine 123 analogs. Cells were incubated with the analogs and for 20 min after the analogs were removed. □, Control cells; ■, cells treated with estrogen. Median fluorescence of control cells not treated with verapamil was set at 100%. Each value represents the mean of three independent experiments; *bars*, standard error. *R123*, rhodamine 123; *R110*, rhodamine 110; *TMRME*, tetramethylrhodamine methyl ester.

We had previously isolated a clone, G7R, in which estradiol had a greatly reduced effect on rhodamine 123 efflux but still increased daunomycin efflux (11). Estrogen did not reduce retention of tetramethylrhodamine methyl ester in this clone but still reduced retention of rhodamine 110, although to a lesser extent than in GH_4C_1 cells (Table 1). This resistant clone had levels of mRNA for the multidrug resistance pump that were well below those present in GH_4C_1 cells (Fig. 1); little if any mRNA was visible when the autoradiographs were exposed for up to 8 days. This clone behaved as if it had less multidrug resistance pump, because G7R cells lost rhodamine 123 fluorescence in a 2-hr period at a rate >10 times slower than that in GH_4C_1 cells (data not shown). In addition, cyclophilin and verapamil had little if any effect on rhodamine 123 retention in this clone (data not shown). The amount of multidrug resistance pump in this clone therefore appears to be markedly reduced.

Discussion

We have shown that estradiol increases the multidrug resistance pump mRNA and P-glycoprotein in GH_4C_1 cells (rat pituitary tumor cells). In rodents, there are two forms of the pump that appear to be involved in drug efflux (7, 20); the probe we used does not distinguish between these two forms. In mice, multidrug resistance pump mRNA is induced in uterine secretory epithelium during pregnancy, and these changes can be mimicked by giving mice estrogen and progesterone (21). Estradiol induces multidrug resistance pump mRNA accumulation in this rat cell line without requiring added progesterone. The mouse gene sequences have been characterized and no sequences resembling estrogen-responsive elements have yet been reported. Estradiol stimulation of the gene for the pump may be a secondary effect, so that an estrogen-responsive element is not required, or the element may be in a region not yet sequenced. Alternatively, regulation of the rat gene may differ from that of the mouse.

We found that estradiol induction of the multidrug resistance pump mRNA reached a maximum at 12 hr. We had previously reported that estradiol caused >90% of total reduction in rhodamine 123 retention in 2 days and the effect continued to increase (11). We found here that the reduction in rhodamine 123 by estradiol was maximal within 1 day (data not shown), consistent with induction of the mRNA. Maximal induction of the message for the pump occurred at 10^{-9} M estradiol, a concentration above that which causes maximal effects on

TABLE 1

Rhodamine analog retention in wild-type and estrogen-resistant cells

The median fluorescence of rhodamine analogs in untreated cells was set at 100% and that in estrogen-treated cells is expressed as a percentage of control. The data are the mean ± standard error of three independent experiments.

	Rhodamine analog retention	
	GH_4C_1	G7R
	% of control	
Rhodamine 123	10.4 ± 5.0	86.4 ± 10.7
Rhodamine 110	30.6 ± 1.4	58.4 ± 13.6
TMRME[a]	50.6 ± 3.8	94.9 ± 5.8

[a] TMRME, tetramethyl rhodamine methyl ester.

rhodamine 123 retention (10^{-10} M) (11). The amount of the pump may cease being the rate-limiting factor in rhodamine retention once a sufficient amount is induced.

The induction of the mRNA for the multidrug resistance pump, as well as the increase in P-glycoprotein itself, therefore indicates that estradiol reduces rhodamine 123 retention by increasing the multidrug resistance pump.

We examined the ability of estradiol to affect retention of rhodamine analogs because we previously found a much larger effect on rhodamine 123 than on daunomycin (11). We selected rhodamine 110 because it is a possible metabolite of rhodamine 123 and, as a zwitterion, would be less influenced by membrane potential. We also used two methylated analogs, tetramethylrhodamine methyl ester and tetramethylrhodamine ethyl ester. These analogs have been used to measure membrane potential in cytoplasm and in mitochondria of HeLa cells and other cell types (22). The analogs are good indicators of membrane potential in these cells because they rapidly leave the cells when the drugs are removed from the cells; they show less binding to intracellular components than does rhodamine 123. We found these methylated analogs did not behave in the same fashion in GH_4C_1 cells (Fig. 6). Very little tetramethylrhodamine methyl ester is lost from the cell when the drug is removed, indicating that there must be binding to some cellular component that is as great as that of rhodamine 123. There is, therefore, some specificity in the retention of these analogs that will complicate their use as indicators of membrane potential.

The location of rhodamine 123 is reported to be in the mitochondria in living cells because the pattern of fluorescent staining directly correlates with mitochondria in the cell as identified by phase microscopy (1, 22); this identification is possible in large spread-out cells. GH_4C_1 cells are small and rounded, with little cytoplasm, and mitochondria are difficult to distinguish. The pattern of staining for both rhodamine 123 and tetramethylrhodamine methyl ester is punctate, however, consistent with localization in the mitochondria.

We found that the effect of estradiol is more marked on the retention of rhodamine 123 than it is on that of other rhodamine analogs (Figs. 5 and 6) or daunomycin (11). A model consistent with our results is the following. Drugs are accessible to the P-glycoprotein pump when they are in the cytoplasm. Tetramethylrhodamine methyl ester, however, is sequestered in the cell by tight binding to components or by active uptake into a noncytoplasmic compartment so that the analog is not available to the pump on the plasma membrane. Consequently, it is retained in GH_4C_1 cells (Fig. 6) and is not affected much by drugs that inhibit the pump or by increases in the level of the pump caused by estradiol (Fig. 7). Rhodamine 110, however, which has a different charge from the other analogs, is not sequestered to the same extent, so it is accessible to the pump. Most of it is rapidly removed from GH_4C_1 cells (Fig. 6), and its retention is substantially increased by drugs that block the pump (Fig. 7). In control cells, rhodamine 123 behaves as tetramethylrhodamine methyl ester; it is retained in cells and only slightly affected by drugs that inhibit the pump, suggesting that it is also sequestered in control cells and not available to the pump. The factors that retain rhodamine 123 and tetramethylrhodamine methyl ester in the cell need not be identical. When estradiol induces the multidrug resistance pump, rhodamine 123 is lost more rapidly from the cell. There are two explanations why estradiol may cause a more pronounced reduction of rhodamine 123 retention than that of the other analogs. The first is that rhodamine 123 may be bound or sequestered less well than the methyl analog but more so than rhodamine 110. In this case, more pump lowers the amount of drug in the cytoplasm, which causes a shift in the rhodamine 123 equilibrium between the sequestered compartment and the cytoplasm and, therefore, keeps supplying more drug for the pump. The second mechanism is that estradiol not only induces the pump but also induces a second change so that rhodamine 123 is sequestered less well. Estradiol therefore causes a change that makes more rhodamine 123 available to the pump as well as increasing the amount of pump.

The G7R clone has much less multidrug resistance pump than the parent GH_4C_1 cells, and this alone explains why control and estrogen-treated G7R cells lost rhodamine 123 much more slowly than did estrogen-treated GH_4C_1 cells. Although the pump levels are greatly reduced a small amount of pump may still be expressed and inducible, although below the level that we could detect. The presence of small amounts of an estrogen-inducible pump would explain why rhodamine 110 fluorescence is still reduced by estradiol, because rhodamine 110 is available to the pump. The reason the loss of response to estradiol in G7R cells was much greater for rhodamine 123 than rhodamine 110 could be explained in two ways. The first is that the amounts of the pump in cells, even if induced, are too low to shift the distribution of the sequestered rhodamine 123; the second is that not only is there less pump but also estradiol is less able to directly decrease sequestration of rhodamine 123. The characterization of other resistant variants may distinguish between these possibilities.

The promotor for the multidrug resistance gene allows expression of a CAT construct in GH_3 cells (23). The ability of estradiol to induce accumulation of multidrug resistance pump mRNA will allow these cells to be used to investigate regulation of expression of this pump. In addition, the ability to isolate clones with different responses will give us tools to distinguish effects on mitochondria and on the pump. Factors that cause retention of rhodamine 123 are of interest because the reasons for the extended binding of this drug in carcinoma cells are not yet known.

Acknowledgments

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