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RAPID STIMULATION OF RHODAMINE 123 EFFLUX FROM MULTIDRUG-RESISTANT KB CELLS BY PROGESTERONE

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Abstract—Rhodamine 123 is a mitochondrial dye that is retained for prolonged periods by carcinoma cells. While investigating causes of retention of this dye, we found that 10 μM progesterone caused a rapid stimulation of efflux of rhodamine 123 within 15 min from KB V20C cells, which overexpress the multidrug resistance pump. Progesterone did not stimulate efflux from KB cells that do not overexpress the pump, and verapamil blocked rhodamine 123 efflux in the presence or absence of progesterone, indicating that rhodamine 123 is removed from KB V20C cells by the multidrug resistance pump. Progesterone, however, is unlikely to stimulate rhodamine 123 efflux by simply increasing pump activity for two reasons: (1) progesterone inhibited the efflux of daunomycin from KB V20C cells, so it did not stimulate efflux of all drugs, and (2) progesterone inhibited efflux of rhodamine 123 from L1210/VMDRC cells and had little effect on Adr® MCF7 cells; both overexpress the multidrug resistance pump. In the experiments with KB V20C cells, progesterone was the most active steroid tested. At 10 μM, progesterone caused a 70-fold stimulation, desoxycorticosterone, testosterone, promegestone and estradiol about 20-fold, and others had little or no effect. Progesterone may act by a non-genomic mechanism to decrease intracellular binding of rhodamine 123, making the dye accessible to the multidrug resistance pump.

Rhodamine 123 is a fluorescent dye that is an exceptional stain for mitochondria in living cells [1]. Chen and coworkers [2] made the interesting observation that many transformed epithelial cell lines and carcinoma-derived cell lines retain the dye much longer than do normal epithelial cells. The original observation described the ability of cells to retain rhodamine 123 for 24 hr, but the retention can be much more prolonged; some carcinoma-derived lines retained the dye for weeks after a 10-min exposure (unpublished results). Membrane potential is an important factor in rhodamine 123 distribution at equilibrium. Since rhodamine 123 is lipophilic and is also positively charged, it crosses membranes easily and accumulates at equilibrium where the potential is relatively negative. If membrane potential were the only factor affecting accumulation, the dye should diffuse back out of the cells when external dye is removed. Additional properties, such as binding to intracellular components, must be involved to cause the dye to remain in cells for prolonged times.

An important factor that decreases rhodamine 123 retention in cells is the multidrug resistance pump. It is well known that resistance to cancer chemotherapy may be caused by a pump that removes drugs from cells; effective substrates for the pump are lipophilic and cationic [3, 4]. Compounds with these characteristics, such as verapamil, directly interact with the pump to inhibit transport of other substrates [5]. Rhodamine 123 is a good substrate for the pump, as would be predicted by its structure [6, 7]; retention of this dye has been suggested to be an assay for the presence of the multidrug resistance pump in resistant cancer cells [7–10]. A positive charge is not an absolute requirement for inhibitors; progesterone and other neutral steroids inhibit drug efflux through the multidrug resistance pump and progesterone interacts directly with the pump [11–13]. In the course of analyzing the factors that contribute to prolonged rhodamine 123 retention in carcinoma cells, we found that progesterone did not always inhibit efflux of the dye, as would be expected from its action on the pump. Instead, it stimulated a rapid efflux of rhodamine 123 from KB cells selected for multidrug resistance.

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MATERIALS AND METHODS

Materials. Rhodamine 123 was from Kodak (Rochester, NY). Daunomycin and verapamil were from Sigma (St. Louis, MO). Steroids were from Steraloids Inc. (Wilton, NH). Delipidized calf serum was from Cocalico Biologicals, Inc. (Reamstown, PA).

Cell lines. KB V20C cells, a subclone of the vincristine-resistant cell line KB V20, were developed in the laboratory of Y. C. Cheng. KB V20 cells were developed from the parental KB cells by step-wise selection for resistance with increasing amounts of vincristine. The KB cells, human epidermoid

carcinoma cells with HeLa markers, were from the American Type Culture Collection. The KB V20C cells were shown to overexpress the multidrug resistance-associated protein, P-glycoprotein, by photoaffinity labeling and western blotting [14]; the parental KB cells do not have detectable amounts of that P-glycoprotein. KB V20C cells were maintained in RPMI medium containing 5% fetal bovine serum in the presence of 20 nM vincristine until 3 days before the experiment when the cells were transferred to medium without the alkaloid. L1210/VMDRC cells, L1210 cells transfected with an expression vector for human *mdr*, were a gift from Drs. Jin-ming Yang and William Hait [15]; the cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 60 ng/mL colchicine until 1 day before the experiment when they were transferred to medium without colchicine. Adriamycin-resistant MCF-7 cells (Adr® MCF-7), originally selected by Fairchild *et al.* [16], were obtained from Dr. William Hait, and experiments were performed in Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum.

Incubation with fluorescent dyes. Fresh medium containing 10 μg/mL rhodamine 123 or daunomycin with or without steroid was added to the cells, and they were incubated at 37° for 30 min. The cells were then rinsed with plain medium once, and incubated again in medium without rhodamine 123 with or without steroid at 37° for 20 min unless otherwise indicated. In the experiments to determine if progesterone stimulated efflux (see Fig. 2), the initial incubation did not contain steroids. Cells were removed from the plates by enzymatic digestion for 4 min at 37° using Pancreatin (Sigma), collected by centrifugation, resuspended in medium, and analyzed by flow cytometry. L1210/VMDRC cells were washed and collected by centrifugation.

Flow cytometry. Flow cytometric analysis was performed with a Becton-Dickinson FACS IV flow cytometer (San Jose, CA). Both fluorochromes were excited at 488 nm with a Spectra-Physics 2025-05 argon laser at 360 mW (Mountain View, CA). Rhodamine 123 and daunomycin fluorescence were measured using a 520 nm long pass filter and a 580 band pass filter, respectively. A minimum of 10^4 cells per sample was analyzed, and fluorescence was plotted on a 5-decade logarithmic scale. The median fluorescence was used as a quantitative measure of fluorochrome concentration, and the logarithmically measured fluorescence intensity was recalculated to its linear value. In most cases, data are presented as stimulation of efflux (median fluorescence of control/median fluorescence of steroid-treated) to facilitate comparison of independent experiments. We confirmed that progesterone decreased the amount of rhodamine 123 and increased the amount of daunomycin in KB V20C cells by collecting cells after cytometric analysis, extracting them with butanol, and measuring the fluorescence emission spectra on an SLM 4800S spectrofluorometer.

RESULTS

KB V20C cells retained less rhodamine 123 than wild type KB cells (Fig. 1); this difference was to be expected because the KB V20C cells express high levels of the multidrug resistance pump, and KB cells do not have detectable amounts of the protein. Treatment with 10 μM progesterone did not change the amount of rhodamine 123 retained in KB cells, but the steroid dramatically reduced the amount of dye retained by KB V20C cells. Progesterone caused a similar reduction in the uncloned resistant cells from which KB V20C cells were derived (data not shown). Pretreating cells with progesterone did not cause a larger reduction, and the effect was not retained when progesterone was removed (data not shown). Progesterone reduced rhodamine 123 retention by stimulating efflux (Fig. 2). KB V20C cells were incubated with rhodamine 123 and washed, and progesterone was added after the rhodamine 123 in the medium was removed. The effect of progesterone was so pronounced that, in the time it took to collect the cells for flow cytometry (approximately 15 min), cells exposed to progesterone but not incubated at 37° (0 time) had already lost substantial amounts of rhodamine 123 compared with the controls (Fig. 2). After 30 min of incubation, the progesterone-treated cells had lost over 98% of rhodamine 123 they contained initially. Untreated cells lost rhodamine 123 much more slowly.

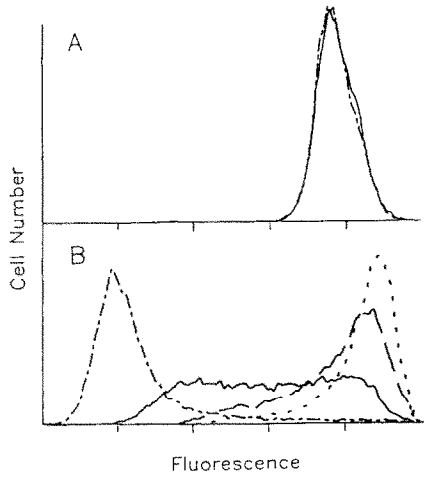

Fig. 1. Rhodamine 123 fluorescence remaining in KB cells (A) and KB V20C cells (B). Key: (——) control cells; (–·–) cells treated with 10 μM progesterone; (– – –) cells treated with 10 μM RU486; and (———) cells treated with 10 μM RU486 and 10 μM progesterone.

The result was unexpected because progesterone acts directly on the pump to inhibit transport of drugs [11–13]. Progesterone, however, has many nonreceptor-mediated effects on membranes that could cause the enhanced efflux of rhodamine 123. Progesterone might allow rhodamine 123 to diffuse more rapidly through membranes to leave the cells, bypassing the pump. We determined if verapamil, an inhibitor of the pump, blocked stimulation of efflux by progesterone. Verapamil completely blocked efflux of rhodamine 123 in KB V20C cells

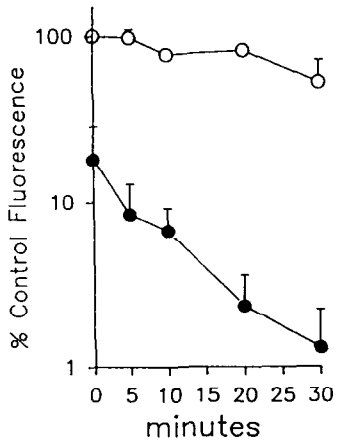

Fig. 2. Effect of progesterone on efflux of rhodamine 123. KB V20C cells were incubated with 10 μg/mL rhodamine 123, for 30 min at 37°, the dye was then removed, and 10 μM progesterone was added to half the cells. The cells were incubated at 37° from 0 to 30 min, collected, and analyzed. The values on the abscissa indicate the time at 37° after removing rhodamine 123. Each point is the mean of independent experiments, and the bars indicate the range; where no bars are shown, the range is smaller than the symbol. Key: (○) control cells; and (●) cells treated with 10 μM progesterone.

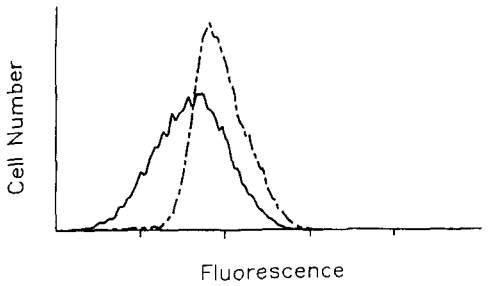

Fig. 4. Effect of progesterone on retention of daunomycin in KB V20C cells. Three independent experiments gave similar results. Key: (—) control cells; and (- - - -) 10 μM progesterone.

in the presence and absence of progesterone (Fig. 3); the ability of progesterone to stimulate efflux was essentially abolished at 10 μM verapamil. These results suggest that rhodamine 123 was leaving the cell via the multidrug resistance pump both in the presence and absence of progesterone.

Progesterone might directly stimulate as well as inhibit the pump. If the stimulation were general, all substrates for the pump should efflux more rapidly. Daunomycin is a substrate for the multidrug resistance pump, but progesterone inhibited daunomycin efflux in KB V20C cells (Fig. 4). Progesterone, therefore, does not stimulate efflux of all drugs from KB V20C cells. Furthermore, progesterone did not stimulate rhodamine 123 efflux from all cell types overexpressing the multidrug resistance pump. In L1210/VMDRC cells, which were transfected with the cDNA for the human multidrug resistance pump, 10 μM progesterone inhibited rhodamine 123 efflux (Fig. 5A). In Adr® MCF7 cells, which were selected to overexpress the human multidrug resistance pump, 10 μM progesterone caused only a slight stimulation of rhodamine 123 efflux (Fig. 5B). Differences in the cell types are most likely to be caused by the amounts of binding sites for rhodamine 123 relative to pump

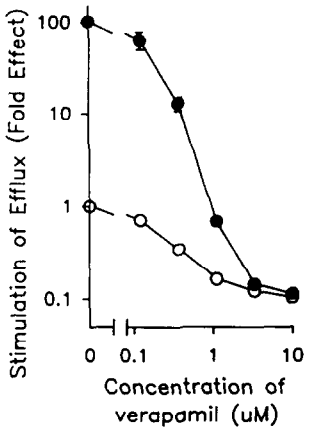

Fig. 3. Effect of verapamil on stimulation of rhodamine 123 efflux in KB V20C cells. Values are ratios of control over treated median fluorescence. Each point is the mean of three independent experiments and the bars indicate the SEM; where no bars are shown, the SEM is smaller than the symbol. Key: control (○); and (●) 10 μM progesterone.

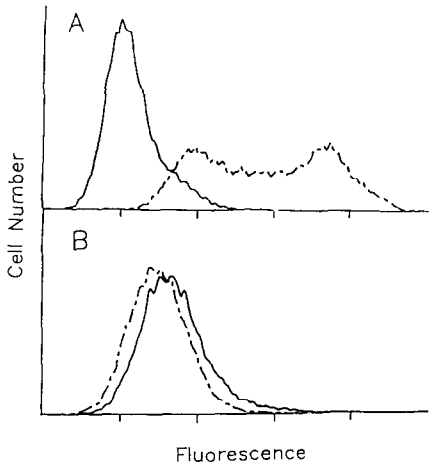

Fig. 5. Effect of progesterone on L1210/VMDRC cells (A) and Adr® MCF7 cells (B). Both cell lines overexpress the multidrug resistance pump. Similar results with L1210/VMDRC cells were detected in two independent experiments, and those with Adr® MCF7 cells in four independent experiments. Key: (—) control; and (- - -) 10 μM progesterone.

Table 1. Stimulation of rhodamine 123 efflux by steroids

Common name	Chemical name	Stimulation of efflux	
		10 μM	1 μM
Progesterone	4-Pregnen-3,20-dione	64.9 ± 7.3	13.7 ± 2.4
Desoxycorticosterone	4-Pregnen-21-ol-3,20-dione	59.6 ± 38.6	4.0 ± 0.5
Testosterone	4-Androsten-17β-ol-3-one	29.2 ± 10.6	1.9 ± 0.3
Allopregnanolone	5α-Pregnan-3β-ol-20-one	28.8 ± 9.8	3.2 ± 1.4
17α-Hydroxyprogesterone	4-Pregnen-17α-ol-3,20-dione	21.3 ± 5.7	3.8 ± 0.8
17β-Estradiol	1,3,5(10)-Estratrien-3,17β-diol	21.1 ± 9.2	1.3 ± 0.1
Promegestone	17α-Methyl-17-propionylestra-4,9-dien-3-one	17.1 ± 10.6	2.6 ± 1.3
5α-Dihydrotestosterone	5α-Androstan-17β-ol-3-one	17.1 ± 6.7	1.3 ± 0.4
Pregnenolone	5-Pregnen-3β-ol-20-one	15.2 ± 6.7	2.3 ± 0.9
Corticosterone	4-Pregnen-11β,21-diol-3,20-dione	9.0 ± 1.5	2.1 ± 0.7
—	5β-Pregnan-3α-ol-20-one	6.8 ± 2.3	1.9 ± 0.5
Cortisone	4-Pregnen-17α,21-diol-3,11,20-trione	5.5 ± 4.1	0.6 ± 0.1
Allotetrahydrodesoxycorticosterone	5α-Pregnan-3α,21-diol-20-one	5.2 ± 1.9	0.9 ± 0.5
11-Dehydrocorticosterone	4-Pregnen-21-ol-3,11,20-trione	4.9 ± 1.3	2.1 ± 0.8
11-Ketoprogesterone	4-Pregnen-3,11,20-trione	4.8 ± 2.1	1.8 ± 0.7
Dehydroisoandrosterone	5-Androsten-3β-ol-17-one	3.3 ± 1.0	1.1 ± 0.03
—	5α-Pregnan-3α-ol-20-one	3.2 ± 0.9	1.0 ± 0.5
Cortisol	4-Pregnen-11β,17α,21-triol-3,20-dione	2.4 ± 0.6	0.8 ± 0.2
Dehydroisoandrosterone sulfate	5-Androsten-3β-sulfate,17-one	1.0 ± 0.2	0.6 ± 0.2
Tamoxifen	2-[4-(1,2-Diphenyl-1-butenyl)phenoxyl]-*N,N*-dimethylethanamine	0.7 ± 0.05	1.9 ± 0.2
Androstenedione	4-Androstene-3,17-dione	0.7 ± 0.1	1.6 ± 0.4
RU486	11β-(4-Dimethylaminophenyl)-17β-hydroxy-17α-(prop-1-ynyl)-estra-4,9-dien-3-one	0.12 ± 0.005	1.2 ± 0.4

Each value is the mean (±SEM) of three independent experiments with the following exceptions: 10 μM progesterone, N = 19; 1 μM progesterone, N = 13; 10 and 1 μM 5α-pregnan-3α-ol-20-one, N = 4; 1 μM RU486, N = 2 (mean ± range). Values are presented as stimulation of efflux (the ratio of median fluorescence of control over treated).

activity. It is clear, however, that the effect of progesterone on rhodamine 123 efflux was not the same in all cell types.

We investigated the steroid specificity of the stimulation of rhodamine 123 efflux in KB V20C cells, including the antiprogestin RU486. Progesterone was the most active steroid tested (Table 1), although others, including desoxycorticosterone, testosterone and estradiol, caused over 20-fold stimulation at 10 μM. Not all steroids stimulated rhodamine 123 efflux; a range of C_{21} and C_{19} steroids, including cortisol and androstenedione, had little or no effect (Table 1). On the other hand, a diverse group of steroids, 17α-hydroxyprogesterone, 5α-dihydrotestosterone, pregnenolone and promegestone (R5020), while active, were not as active as progesterone. The concentration–response curves of the most active steroids, progesterone and desoxycorticosterone, were biphasic; 10 μM caused a maximal stimulation of rhodamine 123 efflux and higher concentrations were less effective (Fig. 6). These results led us to determine whether androstenedione, which was inactive at 10 μM, might be active at other concentrations. Androstenedione caused no stimulation at concentrations ranging from 100 μM to 13 nM. The antiprogestin RU486 (10 μM) blocked efflux of rhodamine 123; concentrations of 1 μM and lower had no effect (Table 1, Fig. 1B). When 10 μM RU486 was administered with 10 μM progesterone, it almost completely abolished the

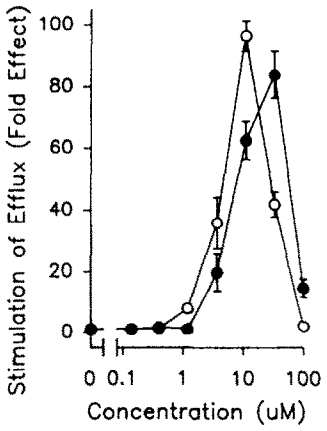

Fig. 6. Concentration–response curves for progesterone and desoxycorticosterone. Each point is the mean of two independent experiments and the bars indicate the range; where no bars are shown, the range is smaller than the symbol. Key: (○) progesterone; and (●) desoxycorticosterone. Stimulation: median fluorescence of control/treated.

stimulation of rhodamine efflux caused by the progestin (Fig. 1B). Although it might appear that this inhibitory effect is a classic action of the antiprogestin at the level of the nuclear progesterone receptor, RU486 also has the characteristics (a lipophilic cation) of a substrate for the multidrug resistance pump and is likely to compete for rhodamine 123 transport. The latter possibility is supported by the high concentration–response and by the inhibitory action without the necessity of the concomitant addition of progestins.

To determine whether endogenous lipids in the serum affect rhodamine efflux, we compared the effects of progesterone in medium containing 5% delipidized bovine serum to medium with 5% fetal bovine serum. The results were identical (not shown).

DISCUSSION

Rhodamine 123 is sequestered in mitochondria in living cells [1]. We found that progesterone stimulated efflux of this dye in KB V20C cells. Rhodamine 123 leaves the cells through the multidrug resistance pump, because efflux was blocked by verapamil (Fig. 3), and because rhodamine 123 remained in the parent cell line that lacks the pump (Fig. 1). Progesterone acts directly on the pump in other cells to inhibit drug transport at concentrations of 10 μM or greater [11–13]; such an action should inhibit rhodamine 123 efflux. Progesterone could stimulate efflux from KB V20C cells through two possible mechanisms: by increasing pump activity or by making more dye available to the pump. In the first model, progesterone has a biphasic effect on the pump to stimulate the pump at low concentrations and inhibit it at higher concentrations. If this model were true, there is specificity involved in the stimulation, in both substrate, because progesterone did not stimulate daunomycin efflux (Fig. 4), and cell type, because all cell lines with the pump did not show the effect (Fig. 5).

In the second model, progesterone shifts the distribution of rhodamine 123 so that there is less in the mitochondria and more in the cytosol accessible to the pump. Progesterone could shift the distribution of the dye either directly by interfering with binding of the dye in the mitochondria or indirectly by causing an action that results in a change in mitochondrial properties so that rhodamine 123 is sequestered there less well. A direct action implies that the dye binding site is not the same in all cells although rhodamine 123 localizes in mitochondria of all cells. An indirect action implies that KB V20C cells have a response to progesterone that L1210 cells do not have. After progesterone shifts the distribution of the dye, cells that express the pump, pump the dye out, but only if the concentration of progesterone is not so high that it blocks the pump. In cells that do not have the ability to shift the dye in response to progesterone, then the only effect we see is inhibition of the pump and retention of the dye, as with L1210 cells (Fig. 5).

We did not detect a shift in the dye by fluorescent microscopy when we added progesterone, either alone or after incubating with verapamil to block activity of the multidrug resistance pump. Detecting such a shift would indicate that the second model was correct. The lack of a visible shift does not rule out the second model, because the shift in distribution may not be sufficient to be detected by fluorescent microscopy, but still be sufficient to make more dye accessible to the pump.

We have attempted to measure binding of rhodamine 123 to mitochondria in the presence and absence of progesterone and to determine if mitochondria isolated from progesterone-treated KB cells retain less dye. We saw no differences, but rhodamine 123 binds to all cell membranes in large amounts in cell-free systems, and the specificity for mitochondria that occurs in living cells is lost. The large amounts of nonspecific binding may prevent detection of more specific effects.

The mechanism that seems most likely to us is that progesterone acts on KB cells to change the accessibility of rhodamine 123 to the pump, although we do not yet know what the action is. The purpose of such an action is obviously not to remove rhodamine 123 from cells because this substrate is not normally present; the action of progesterone may be part of a biological function of the steroid. The concentrations of progesterone that stimulate efflux of the dye are higher than those that occur in adults, but progesterone concentrations are approximately 10 μM in the placenta, and range from 0.5 to over 2 μM in fetal veins [17–20]. Placental and fetal tissues are therefore routinely exposed to progesterone levels that caused large effects in these experiments (Table 1), and hence any tissue in the fetus that can respond to this action of progesterone is exposed to levels sufficient to cause activity.

Determining the tissue specificity of this action may help us to understand the biological significance, but screening for tissue specificity is not straightforward if the action of progesterone is to make more rhodamine 123 accessible to the pump. There are then two components required to detect the action of progesterone; the cells must not only be capable of the initial response to progesterone, but also must express the pump. Determining the initial action of progesterone will allow screening for tissue specificity without relying on the presence of the pump.

There are already several known actions of progesterone, but none of these appear to be the action of progesterone that results in rhodamine 123 efflux based on the steroid specificity of the responses. Progesterone exerts many genomic actions through a nuclear receptor, but the rapid stimulation of rhodamine 123 efflux is unlikely to be mediated through this receptor. Progesterone stimulated rhodamine 123 efflux at lower concentrations than promegestone (Table 1), whereas promegestone is at least an order of magnitude more active than progesterone in binding to the nuclear progesterone receptor and stimulating cell growth [21]. There are several rapid effects of progesterone or its metabolites that are not mediated through the nuclear receptor, but the steroid specificity also differs from the stimulation of rhodamine 123 efflux. Progesterone modulates the binding of *t*-butylbicyclophosphorothionate to the $GABA_A$ receptor [22, 23]. The

progesterone metabolite 5α-pregnan-3α-ol-20-one is the most active steroid to influence the GABA receptor, but this metabolite did not stimulate rhodamine 123 efflux (Table 1). Progesterone causes a rapid rise in intracellular calcium concentrations in human sperm [24, 25], but so does androstenedione, which did not stimulate rhodamine 123 efflux. Progesterone rapidly increases intracellular Ca^{2+} concentrations and decreases cyclic AMP levels in amphibian oocytes [26, 27] and rapidly inhibits K^+ conductance in renal epithelioid MDCK cells [28], but, in both these systems, estradiol has no effect [28, 29], whereas we found that it stimulated efflux (Table 1). Finally, progesterone binds to sigma binding sites, which also bind antipsychotic and psychomimetic drugs [30]. 17α-Hydroxyprogesterone does not bind to these sites, but it did stimulate rhodamine 123 efflux. Therefore, stimulation of rhodamine 123 efflux by progesterone differed in steroid specificity from other previously described rapid progesterone actions.

Efflux of rhodamine 123 has been proposed as an assay to quantitate the presence of the multidrug resistance pump in cancer cells. The results in this paper demonstrate that retention of rhodamine 123 is rapidly changed by factors other than the simple overall activity of the multidrug resistance pump, and so the interpretation of retention is more complex than simply reflecting the amount of the multidrug resistance pump. Determining how progesterone influences rhodamine 123 retention will not only lead to a greater understanding of why this dye is retained so well by carcinoma cells but also may lead to new knowledge of progesterone actions.

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