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Changes in intra- or extracellular pH do not mediate P-glycoprotein-dependent multidrug resistance

(doxorubicin/vinblastine/rhodamine 123)

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ABSTRACT **P-glycoprotein (Pgp)-mediated multidrug resistance (MDR) is thought to result from active extrusion of lipid-soluble, titratable chemotherapeutic agents. Given the lack of demonstration of coupling between ATP hydrolysis and drug transport, the resistance to chemically unrelated compounds, and findings of elevated intracellular pH (pH_i), it has been proposed that reduced intracellular accumulation of drugs in MDR is due to changes in the pH difference across the plasma membrane. Elevation of pH_i or decrease in local extracellular pH (pH_o) could reduce the intracellular accumulation of the protonated chemotherapeutic drugs and account for Pgp-mediated MDR. Alternatively, changes in pH_i or pH_o could increase drug efflux by other mechanisms, such as coupled transport involving H^+ or OH^-, or allosteric effects on Pgp or other proteins. Both mechanisms could operate independently of the charge of the substrate. The possibility of a role of pH_i in drug efflux is important to test because of the clinical significance of the phenomenon of MDR of tumors. We tested this hypothesis and found that MDR can occur in cells with low, normal, or high pH_i. Further, resistant cells exhibited reduced steady-state drug accumulation and increased efflux without changes in local pH_o. Finally, acute changes in pH_i had no appreciable effect on Pgp-mediated drug efflux. We conclude that Pgp-mediated MDR is not a consequence of changes in pH_i or pH_o.**

The available data strongly support the hypothesis that multidrug resistance (MDR) concomitant with overexpression of P-glycoprotein (Pgp) is due to active extrusion of chemotherapeutic agents by Pgp (1–4). However, given the lack of demonstration of coupling between ATP hydrolysis and drug transport, the resistance to chemically unrelated, lipophilic compounds (5, 6), and findings of elevated intracellular pH (pH_i) (7–10), it has been proposed that the lower intracellular drug levels in MDR cells result from changes in the pH difference across the plasma membrane (11, 12). In the case of a substrate that can be protonated, in the absence of active transport the uncharged form of the drug equilibrates across the plasma membrane by simple diffusion, while the intracellular and extracellular concentrations of the charged form depend on pH_i and extracellular pH (pH_o). When pH_i is high, there is less intracellular "trapping" of the protonated form. In addition, a reduction in pH_o protonates the drug, reducing the extracellular concentration of the neutral form, and therefore limits its influx. Hence, changes in pH_i and/or pH_o (in particular an elevation of pH_i and a fall of pH_o) could explain the lower steady-state intracellular concentration of drugs in multidrug-resistant cells. Alternatively, changes in pH_i and/or pH_o could alter drug efflux by other mechanisms, such as coupled transport (drug/H^+ exchange or drug–OH^- cotransport) or allosteric modulation of Pgp or other proteins. These two mechanisms do not require protonatable substrates and could account for the Pgp-dependent efflux of cationic, anionic, and neutral substrates, all of which have been suggested to be transported by Pgp (13, 14). In most but not all studies, an elevation of pH_i has been found in multidrug-resistant cells compared with wild-type cells (7–10, 15, 16). The alkalinization could be due to "direct" transport of H^+ equivalents by Pgp, to stimulation of transporters that extrude H^+ equivalents, or to pumping of a protonated endogenous substrate by Pgp (7).

To understand the role of pH changes in MDR, we decided to study the relationships between pH_i and pH_o, on the one hand, and drug efflux, on the other, in cell lines with different levels of MDR. Specifically, we aimed to ascertain whether intracellular alkalinization is a necessary and/or sufficient condition for MDR, whether an acidified microenvironment surrounding multidrug-resistant cells is necessary for accelerated drug efflux and decreased steady-state drug accumulation, and whether experimentally induced pH_i changes alter Pgp-mediated drug efflux.

MATERIALS AND METHODS

Cell Lines. We used Chinese hamster lung fibroblasts (17–19), and human breast cancer cells (20). The Chinese hamster cell lines were V79 (wild type) and LZ-8 (multidrug-resistant). The human breast cancer cell lines were MCF-7 (drug-sensitive), MCF-7/Adr [selected with doxorubicin (Adriamycin)], and BC19/3 [MCF-7 cells transfected with human "*mdr1*" (*PGY1*) cDNA isolated from MCF-7/Adr cells]. Chinese hamster fibroblasts were grown in Ham's F10 medium supplemented with 10% fetal bovine serum [in the case of LZ-8 cells, Adriamycin was added at 8 μg/ml (≈14 μM)]. Both V79 and LZ-8 cells express Pgp in the plasma membrane but at quite different levels, *ca.* 1% and 20% of the total plasma membrane protein, respectively (19). Human breast cancer cells were grown in Eagle's improved minimum essential medium supplemented with 10% fetal bovine serum. In the cases of MCF-7/Adr and BC19/3 cells, 1 or 0.01 μM Adriamycin was added, respectively. Adriamycin, a fluorescent drug, was removed from the culture medium 1 day prior to the experiments.

pH_i. Subconfluent monolayers on glass coverslips were mounted in a chamber placed on the stage of an inverted microscope. All experiments were performed at 37°C. The control solution was 115 mM NaCl/25 mM $NaHCO_3$/5 mM KCl/1 mM $MgCl_2$/2 mM $CaCl_2$/1.5 mM sodium phosphate/8

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Abbreviations: MDR, multidrug resistance; pH_i, intracellular pH; pH_o, extracellular pH; Pgp, P-glycoprotein; R123, rhodamine 123; F_{R123}, R123 fluorescence.

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mM glucose, equilibrated with 95% O_2/5% CO_2, pH 7.42–7.43. pH_i was measured fluorometrically from a population of 100–150 cells by using the pH-sensitive dye 2',7'-biscarboxyethyl-5(6)carboxyfluorescein (BCECF) (21). V79 and LZ-8 cells were loaded at 37°C by a 60-min incubation with 10 μM of the tetrakis(acetoxymethyl) ester of BCECF (BCECF AM). Human breast cancer cells were loaded at room temperature by a 60-min exposure to 5 μM BCECF AM in the presence of pluronic F-127 (Molecular Probes) at 0.07 mg/ml. The loading at room temperature with pluronic F-127 has been shown to reduce intracellular compartmentalization and improve loading of fluorescent probes (22). BCECF fluorescence was measured with a dual-excitation fluorometer (CM1T1; Spex Industries, Edison, NJ) coupled to an inverted microscope (Nikon Diaphot). The dye was alternately excited at 440 and 495 nm, and emitted light was measured at 535 nm. pH_i was calculated from calibrations based on the high-K^+/nigericin technique (23). No differences in BCECF leakage were observed between wild-type and multidrug-resistant cells or between the Chinese hamster lung fibroblasts and the human breast cancer cells (≈20%/hr).

pH_o. pH_o was measured with a liquid–membrane microelectrode (24) positioned either 2–4 μm from the cell surface (pH_s) or in the bulk solution (pH_b). The cells were plated on glass coverslips and superfusion was started with a HCO_3^-/CO_2-buffered control solution containing 10 μM rhodamine 123 (R123). The superfusion rate was 25 ml/min and the volume of solution contained in the chamber was 400 μl. The microelectrode was positioned with a micromanipulator under microscopic observation at ×400. Upon touching the cell surface, small changes in the microelectrode voltage were detected; the microelectrode was then pulled back 2–4 μm, as described (24). Upon measurement of pH_b and pH_s in the presence of R123, the superfusate was switched to one free of R123 and pH_s was continuously measured. At the end of the experiment, pH_b in the R123-free solution was determined.

Steady-State R123 Levels. Cells plated on coverslips were loaded by exposure to 10 μM R123 for 60 min with or without 50 μM verapamil. The coverslips were then washed for 30 sec with an ice-cold R123-free solution containing 100 μM verapamil, which blocks R123 efflux by >95% (see Fig. 1*C*). The coverslips were then placed in a cuvette and exposed to distilled water. Under these conditions, all R123 leaks out of the cells. R123 was excited at 495 nm and fluorescence emission was measured at 535 nm.

Efflux of R123. BC19/3 cells were loaded with the fluorescent Pgp substrate R123 as indicated above. Then, the superfusate was changed to one without R123 and the fall in intracellular R123 fluorescence (F_{R123}) was measured. After 15-sec all fluorescence comes from the cells and the fall in F_{R123} follows a single exponential. These experiments were carried out in the chamber used for the pH_o determinations and with the same superfusion rate (see above).

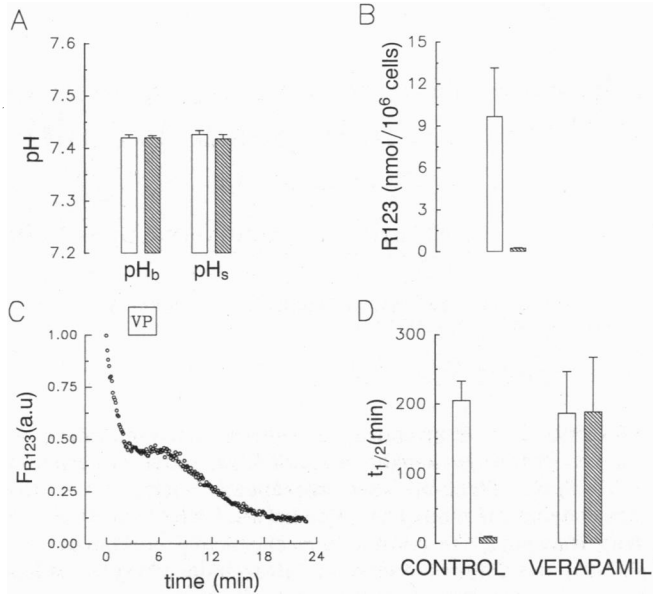

FIG. 1. pH_o and R123 transport in MCF-7 cells (drug-sensitive; open bars) and BC19/3 cells (multidrug-resistant, transfected with the human "*mdr1*" gene; hatched bars). (*A*) Steady-state pH_o. (*B*) Steady-state R123 accumulation in MCF-7 and BC19/3 cells. Under conditions identical to those in *A*, the cells were loaded with R123 by exposure to 10 μM for 60 min. Then, the R123 content was determined by fluorometry. The R123 content in BC19/3 cells was 20-fold that in MCF-7 cells ($P < 0.001$). (*C*) Typical experiment showing the decrease in F_{R123} (in arbitrary units, a.u) in a BC19/3 cell upon removal of extracellular R123. During the period denoted by the box, verapamil (VP, 100 μM) was added to the superfusate. Note the rapid and reversible inhibition of R123 efflux. (*D*) Half-times for the fall in F_{R123} upon R123 removal in MCF-7 and BC19/3 cells, in the absence and in the presence of 50 μM verapamil. Verapamil had no effect on the half-time ($t_{1/2}$) for decay of F_{R123} in MCF-7 cells, but increased $t_{1/2}$ in BC19/3 cells to a value not different from that in MCF-7 cells. This indicates that R123 efflux in BC19/3 cells is Pgp-mediated. These results show that in multidrug-resistant cells, enhanced R123 extrusion and reduced R123 accumulation can occur without an intervening change in pH_o.

RESULTS AND DISCUSSION

Table 1 summarizes steady-state pH_i values and levels of MDR in the cell lines employed in these studies. pH_i was significantly lower in Adriamycin-selected Chinese hamster lung fibroblasts (LZ-8 cells) than in the parent wild-type (V79) cells. In contrast, in human breast cancer cells, the pH_i was elevated in the Adriamycin-selected multidrug-resistant cell line MCF-7/Adr compared with the drug-sensitive parent cell line MCF-7. Finally, transfection of MCF-7 cells with the human "*mdr1*" gene (BC19/3 cells), which results in functional Pgp expression (see Table 1, Fig. 1, and ref. 18), did not alter pH_i. These studies demonstrate that Pgp-mediated

Table 1. Steady-state pH_i and drug sensitivity of Chinese hamster lung fibroblasts (CHLF) and human breast cancer cells (HBCC)

Cell line	Description	pH_i	Adriamycin IC_{50}, μM	Vinblastine IC_{50}, nM
V79	CHLF, wild type	7.27 ± 0.05 (20)	0.064	6.6
LZ-8	CHLF, selected with Adriamycin	7.04 ± 0.03* (24)	190	1100
MCF-7	HBCC, wild type	6.62 ± 0.07 (9)	0.005	0.082
MCF-7/Adr	HBCC, selected with Adriamycin	6.90 ± 0.06* (12)	4.9	520
BC19/3	HBCC, transfected with human *mdr1*	6.64 ± 0.05 (12)	0.1	18

Here and elsewhere, data presented are means ± SE. Number of experiments is in parentheses. The drug concentration that inhibited colony formation by 50% (IC_{50}) was determined by counting cell colonies after a 7-day exposure to Adriamycin or vinblastine. IC_{50} values are averages of two or three experiments.
*Significantly different ($P < 0.05$) from the analogous value in the corresponding wild-type cells.

MDR can occur in cells with "normal," high, or low pH_i. In agreement with a previous report (25), pH_i of MCF-7 cells is lower than that of most mammalian cells.

To test whether increased drug efflux requires acidification of the extracellular microenvironment, we used pH-sensitive microelectrodes to measure pH_o in monolayers of human breast cancer cells (Fig. 1). The steady-state pH_s values were not different in sensitive (MCF-7) and resistant (BC19/3) cells and did not differ statistically from those measured in the bulk solution. Under identical experimental conditions, intracellular accumulation of R123, a Pgp substrate, was significantly less in the multidrug-resistant cells than in the sensitive cells (Fig. 1). Hence, extracellular acidification is not necessary for reduced drug accumulation and increased drug efflux in multidrug-resistant cells.

To test directly whether changes in pH_i modulate Pgp-mediated drug transport, we measured the unidirectional R123 efflux before, during, and after a transient exposure to NH_4Cl (26) (Fig. 2). Upon addition of NH_4Cl, pH_i first increases due to diffusive NH_3 influx, which buffers intracellular H^+, generating NH_4^+. After the peak alkalinization, the pH_i slowly decreases ("plateau acidification"), due to influx of H^+ equivalents in the form of NH_4^+ and/or efflux of base equivalents. Upon removal of NH_4Cl, pH_i falls rapidly to a minimum below control because of the rapid NH_3 efflux and the intracellular trapping of the H^+ generated by dissociation of NH_4^+. Finally, pH_i returns to control levels because of membrane transport mechanisms that extrude H^+ equivalents (27, 28). The changes in pH_i elicited by NH_4Cl had no appreciable effects on the decay of F_{R123}, indicating that R123 efflux persists despite large changes in pH_i. Transient elevations of pH_i by nominal removal of HCO_3^-/CO_2 at constant pH_o, by changing to a Hepes-buffered solution, had no effects on the rate of fall of F_{R123} (Fig. 3). The lack of appreciable changes in F_{R123} upon pH_i alterations indicates that passive diffusion of the unprotonated form of R123 does not contribute significantly to total R123 efflux. Under these experimental conditions, intracellular R123 is mostly contained in mitochondria, and the cytoplasmic concentration is unmeasurably small, as evaluated by confocal microscopy. Virtually all the R123 efflux can be blocked by verapamil (see Fig. 1). R123 influx is possibly by solubility-diffusion (29) and insensitive to verapamil and vinblastine (G.A.A., C. Vanoye, and L.R., unpublished data). BC19/3 cells are more resistant to R123 ($IC_{50} > 150\ \mu M$) than the drug-sensitive MCF-7 cells ($IC_{50} = 2.8\ \mu M$). There were no appreciable changes in efflux

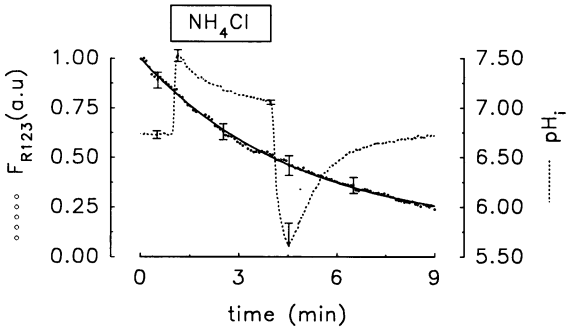

FIG. 2. Effects of pH_i changes elicited by transient exposure to 25 mM NH_4Cl on Pgp-dependent unidirectional efflux of R123. pH_i and the decrease of F_{R123} upon removal of extracellular R123 were measured in BC19/3 cells in separate experiments. F_{R123} data were normalized to the value measured 18 sec after starting superfusion with R123-free solution; the continuous line is the best fit of a single exponential to the data; k values were computed from 1-min segments (60 data points) with the midpoints at 0.5, 2.5, 4.5, and 6.5 min; k values were 0.22 ± 0.03, 0.20 ± 0.03, 0.27 ± 0.10, and 0.22 ± 0.05 min^{-1}, respectively. Each trace is the average of six experiments (bars denote SE).

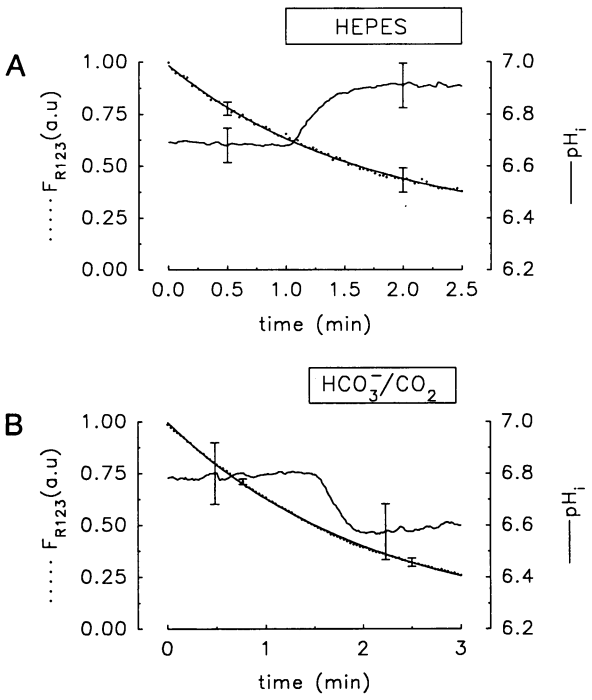

FIG. 3. Effects of pH_i changes elicited by changes in the superfusate buffer on the decay of F_{R123} in BC19/3 cells. (*A*) During the time indicated by the bar, HCO_3^-/CO_2 was replaced by Hepes at constant pH. The cells alkalinized because of the rapid CO_2 loss; apparent R123 efflux did not change. (*B*) Cells were incubated in Hepes medium for 10 min and then Hepes was removed and replaced with HCO_3^-/CO_2 at constant pH. The cells acidified because of rapid CO_2 entry; again, apparent R123 efflux did not change. In each panel, the continuous line is the best fit of a single exponential to the data; k values were computed from 75-sec segments (75 data points); in *A*, k values were 0.60 ± 0.14 and 0.64 ± 0.08 min^{-1} in HCO_3^-/CO_2 and Hepes, respectively; in *B*, k values were 0.48 ± 0.03 and 0.53 ± 0.08 min^{-1} in Hepes and HCO_3^-/CO_2, respectively. There were no significant differences among any of these values. F_{R123} data are from six experiments in each panel; pH data are from six and five experiments in *A* and *B*, respectively.

rate during either the alkalinizing or the acidifying phases of this procedure. The insensitivity of the efflux to pH_i changes spanning almost 2 pH units is surprising in light of the modulatory role of pH_i demonstrated for other transport processes (30–32). As the pK of R123 is >10 (33), and R123 efflux is not near saturation, this result suggests that the Pgp transports the protonated R123. However, we cannot rule out a dual effect of pH_i—e.g., substrate titration and allosteric modulation of Pgp.

Since R123 is contained mainly in mitochondria and has a relatively high pK, the effect of pH_i on its cytoplasmic concentration is probably small. Effects on cytoplasmic concentrations of other drugs could be more significant, in particular if the cytoplasmic concentration is high and the pK lower (e.g., Adriamycin). Under these conditions, changes in pH_i could alter the diffusive flux of drug via the lipid bilayer, and hence modulate MDR, but pH_i changes *per se* do not mediate MDR.

In summary, MDR can be observed in cells with "normal," high, and low pH_i; Pgp-mediated drug efflux does not require external acidification; and acute changes in pH_i do not alter the Pgp-mediated R123 efflux. From these results, we conclude that changes in pH_i, pH_s, or transmembrane ΔpH are not necessary mechanisms for Pgp-mediated MDR. Additional support for our main conclusion comes from observations that MDR modulators can correct MDR, but not cell alkalinization (9), and from our direct demonstration that

acute changes in pH_i do not alter R123 efflux. These results are consistent with the notion that Pgp-mediated MDR is due to active efflux of chemotherapeutic drugs.

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