Novel Cl^--Dependent Intracellular pH Regulation in Murine MDR 1 Transfectants and Potential Implications†

Paul D. Roepe,* Jeffrey H. Weisburg, John Gatley Luz, Mary M. Hoffman, and Li-Yong Wei

Program in Molecular Pharmacology & Therapeutics, Memorial Sloan-Kettering Cancer Center, and Graduate School of Medical Sciences, Cornell University, 1275 York Avenue, New York, New York 10021

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ABSTRACT: Previously [Luz *et al.* (1994) *Biochemistry 33,* 7239–7249], we determined that Cl^-- and $^-HCO_3$-dependent pH_i homeostasis was perturbed in multidrug resistant (MDR) cells created by transfecting LR73 Chinese hamster ovary fibroblasts with wild-type mu (murine) MDR 1 (Gros *et al.*, 1991). Via single-cell photometry experiments performed under various conditions, we are now able to separate Na^+-dependent and Na^+-independent components of $Cl^-/^-HCO_3$ exchange in the MDR transfectants and the parental LR73 cells. Cl^--dependent, Na^+-independent reacidification of pH_i, mediated by the anion exchanger 2 isoform in LR73 cells, is dramatically inhibited by mild overexpression of MDR protein. Analysis of H^+ flux at different pH_i shows that Cl^--dependent reacidification approaches 0.2 mM H^+/s for LR73 cells at $pH_i = 8.0$ but is at least 10-fold slower for MDR 1 transfectants that were never exposed to chemotherapeutics (EX4N7 cells). MDR 1 transfectants selected on the chemotherapeutic vinblastine (1-1 cells), which express approximately 10-fold more MDR protein relative to EX4N7 cells, exhibit similar behavior; however, alterations in Cl^--dependent pH_i regulation are more severe. Hypotonic conditions, which have been shown to increase anomalous Cl^- conductance in some cells overexpressing MDR protein (Valverde *et al.*, 1992), are found to amplify the altered pH_i homeostasis features in the primary transfectants that express lower levels of MDR protein such that they then mimic the behavior of the drug-selected cells that express substantially more MDR protein. Verapamil reverses the anomalous behavior. In addition, removal of CO_2 causes similar pH_i changes in all cell types, whereas removal of $^-HCO_3$ in the presence of Cl^- leads to greater changes in pH_i for the MDR cells. Transfectants harboring mutant MDR protein that is unable to confer the MDR phenotype (K432R/K1074R mu MDR 1) perform Na^+-independent $Cl^-/^-HCO_3$ exchange similar to the parental LR73 cells. These data may help to further refine models for MDR protein function in MDR cells.

Detailed molecular level elucidation of tumor multidrug resistance (MDR[1]), wherein tumor cells exposed to one chemotherapeutic drug acquire resistance to a variety of pharmacologically and chemically distinct compounds (Beck, 1987; Gottesman & Pastan, 1993), remains a key goal of modern cancer research. MDR cells often overexpress either the MDR protein (p-glycoprotein) or the homologue MRP (multidrug resistance related protein) (Endicott & Ling, 1989; McClean & Hill, 1992; Cole *et al.*, 1992) and exhibit substantially altered intracellular distribution of many hydrophobic compounds that are usually charged. This typically includes decreased cytoplasmic retention of a variety of chemotherapeutics. Both MDR and MRP are polytopic, integral membrane proteins that exhibit substantial homology to the cystic fibrosis transmembrane conductance regulator (CFTR) and are thus members of the recently described ABC family of transport proteins (Ames, 1986; Higgins *et al.*, 1990). Other homologues including CRP (chloroquine resistance protein) from *Plasmodium falciparum*, ltpgpA (Leishmania p-glycoprotein A) from *Leishmania tarentolae*, and PDR5 (pleiotropic drug resistance gene 5 protein) from *Saccharomyces cerevisiae* also appear to be important in drug resistance phenomena (Foote *et al.*, 1989; Ouellette *et al.*, 1990; Balzi *et al.*, 1994).

Several models for MDR protein-mediated tumor MDR currently receive experimental scrutiny. One model, based on gene homology, drug retention studies, and photolabeling experiments, hypothesizes that MDR protein actively transports out of the cell (directly pumps) the drugs to which MDR cells are resistant (Gerlach *et al.*, 1986; Gottesman & Pastan, 1988; Gottesman & Higgins, 1991), thereby lowering cytoplasmic levels of the drugs. The strongest support for this model comes from elegant drug uptake studies with inside-out plasma membrane or secretory vesicles made from *S. cerevisiae* harboring overexpressed mu (murine) MDR 3 protein[2] (Ruetz *et al.*, 1993; Ruetz & Gros, 1994).

An alternative model proposes that the altered intracellular retention (binding) of drugs [see Beck *et al.* (1983)] due to MDR protein overexpression is a consequence of perturbations in a variety of complex drug/cell interactions [see Roepe (1992) and Roepe *et al.* (1992, 1993)] that are essentially precipitated by alterations in the character and/or magnitude of the plasma membrane electrochemical potential ($\Delta\mu_{H^+}$). Consistent with this model are many intracellular pH (pH_i)

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* To whom correspondence should be addressed.

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[1] Abbreviations: MDR, multidrug resistance; AE2, anion exchanger 2 isoform; ATP, adenosine 5′-triphosphate; CCCP, carbonyl cyanide (*m*-chlorophenyl)hydrazone; $\Delta\mu_{H^+}$, plasma membrane electrochemical potential; pH_i, intracellular pH; $\Delta\Psi$, plasma membrane electrical potential; AE1, anion exchanger 1 isoform; NHE, Na^+/H^+ exchanger; PMT, photon multiplier tube; H_o, extracellular pH; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; DME, Dulbecco's modified eagles; BCECF-AM, acetoxy methyl ester form of BCECF; NMG, *N*-methyl-D-glucamine; VPL, verapamil.

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and plasma membrane electrical potential ($\Delta\Psi$) perturbations in MDR cells (Keizer & Joenje, 1989; Hasmann *et al.*, 1989; Thiebaut *et al.*, 1990; Roepe, 1992; Roepe *et al.*, 1993; Altenburg *et al.*, 1993; Wei & Roepe, 1994; Luz *et al.*, 1994) as well as anomalous Cl^- conductance in MDR cells (Valverde *et al.*, 1992; Gill *et al.*, 1992; Altenberg *et al.*, 1994; Bear, 1994), since Cl^- permeability and concentration of course impact greatly on the maintenance of $\Delta\mu_{H^+}$. Other factors that affect passive diffusion of hydrophobic molecules, such as bilayer characteristics and lipid composition (Wadkins & Houghton, 1993), are also important with regard to elucidation of this model, since they may be influenced by $\Delta\mu_{H^+}$.

A third model (Gill *et al.*, 1992) essentially marries the first two by proposing that MDR protein functions as both an active drug transporter and a Cl^- channel, implying that drug pumping and other bioenergetic phenomena may play a role in reducing cytoplasmic levels of chemotherapeutics.

Recent detailed kinetic investigations of drug efflux from MDR cells that distinguished between comparisons of drug efflux for cells preloaded to similar total intracellular levels of drug vs similar exchangeable levels of drug (Roepe, 1992; Bornmann & Roepe, 1994) are inconsistent with models that propose direct active drug transport by the MDR protein. On the other hand, another recent paper (Altenburg, 1993) shows that some MDR cells may not exhibit significant perturbations of pH_i, which is typically a hallmark of the phenotype. However, since both $\Delta\Psi$ and pH_i perturbations have been associated with the MDR phenotype (Roepe *et al.*, 1993), it is possible that an altered plasma membrane $\Delta\mu_{H^+}$ still exists for these cells even though steady state pH_i is not significantly perturbed. This is particularly germane in light of the recent observation that MDR cells may also exhibit significant overexpression of a variety of exchangers important for pH_i regulation, including AE1, AE2, and NHE (Roepe *et al.*, 1993; Luz *et al.*, 1994). Regardless, understanding the mechanisms by which pH_i homeostasis in MDR cells is altered, as well as the connections (if any) between $\Delta\Psi$ and pH_i perturbations, may prove important in developing better chemotherapy. The recent demonstration that low external pH (pH_o), a common growth condition of solid tumors (Wike-Hooley *et al.*, 1984), may induce MDR protein overexpression and the MDR phenotype in colon, kidney, adrenal, and liver tissue (Wei & Roepe, 1994; L.-Y.W., Sofia Yakren, and P.D.R., unpublished results) provides further impetus for this endeavor.

In this study, we expand upon previous examinations of altered pH_i homeostasis in MDR cells (Luz *et al.*, 1994) performed with the more rigorously defined model system of Gros and colleagues (Azzaria *et al.*, 1989; Gros *et al.*, 1991), that is, MDR cells created by transfection that were not exposed to chemotherapeutic drugs, which likely induce other resistance mechanisms that may complicate interpretation of the effects of MDR protein overexpression. We substantiate our earlier conclusion that Na^+-independent anion exchange is significantly perturbed in wild-type MDR 1 transfectants but not transfectants harboring a MDR mutant that is unable to confer the MDR phenotype. Additionally, $Cl^-/^-HCO_3$ exchange appears to be more affected than the thermodynamic converse reaction ($^-HCO_3/Cl^-$ exchange). Perhaps in analogy to the recent finding that hypotonic conditions stimulate Cl^- conductance by MDR protein (Valverde *et al.*, 1992), we also find that hypotonic conditions stimulate the anomalous Cl^--dependent pH_i homeostasis behavior present in the MDR transfectants. Importantly, this anomalous behavior is (1) Na^+-independent, (2) $^-HCO_3$-dependent, (3) inhibited by verapamil, and (4) stimulated by hypotonicity.

MATERIALS AND METHODS

Materials. 2′,7′-Bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), carboxyseminaphthorhodafluor-1 (SNARF), nigericin, and valinomycin were purchased from Molecular Probes (Eugene, OR) and used without further purification. 4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and amiloride were from Sigma, and verapamil was a kind gift of Dr. James P. O'Brien, Sloan-Kettering Institute. The integrity of commercial 5% CO_2 (balance air) mixtures used in this work was checked using standard $^-HCO_3$ solutions and application of the Henderson–Hasselbach relation. All other chemicals were reagent grade or better, purchased from commercial sources, and used without further purification or analysis.

Tissue Culture. Construction of the cell lines used in this work has been described previously (Azzaria *et al.*, 1989; Devault & Gros, 1990; Gros *et al.*, 1991). All cell lines were initially created by transfecting two plasmid constructs, one harboring a neomycin resistance gene and the other the mu MDR 1 gene [mixed at a 1:10 ratio; see Devault and Gros (1990)]. Transfectants were initially selected with G418. In some cases (IF5/9 and 1-1), surviving colonies were further selected on vinblastine (25 and 50 ng/mL, respectively). The EX4N7 and 88-8 cell lines, harboring WT MDR 1 and K432R/K1074R MDR 1, respectively, were selected on G418 only. A detailed analysis of MDR protein and mRNA (murine and hamster) expression in these lines has been reported previously (Luz *et al.*, 1994).

Cells were grown at 37 °C in a 5% CO_2 atmosphere in DME medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. EX4N7 and 88-8 were grown in the presence of 500 μg/mL G418 (Sigma) and IF5/9 and 1-1 in the presence of 25 and 50 ng/mL vinblastine, respectively. Since we have documented that MDR cell lines may exhibit significant alterations in the level of MDR protein expression [or the expression of other transporters crucial for pH_i regulation, see Roepe *et al.* (1993) and Luz *et al.* (1994)]

[2] In Ruetz *et al.* (1993), [^{3}H]vinblastine uptake into vesicles harboring the MDR protein was found to be osmotically sensitive, ATP hydrolysis-dependent, and relatively CCCP-independent and to have a high temperature dependence, but fold accumulation of osmotically sensitive drug was not calculated, and the observed transport does not appear to be saturable. Nonetheless, the data support the possibility of active drug transport by the MDR protein. Further examination of the direct effects of additional ionophores and treatments that more completely collapse $\Delta\mu_{H^+}$ [see, in particular, Gradmann *et al.* (1978) and Ballarin-Denti *et al.* (1984)] for systems like *S. cerevisiae* plasma membrane that harbor non-negligible K^+ channel activity [see Bertl *et al.* (1993)] as well as a H^+-ATPase should resolve whether any transport observed in this system is due to direct active transport by MDR protein or to electrochemical membrane potential effects and/or volume perturbations. Since Cullis and colleagues (Mayer *et al.*, 1985; 1986; Bally *et al.*, 1985) as well as Praet *et al.* (1993) have clearly shown that $\Delta\Psi$ and ΔpH have huge and profound effects on the partitioning of doxorubicin, vinblastine, and other drugs, interpretation of other recent work (Reutz & Gros, 1994) that strongly concludes $\Delta\Psi$ has no effect whatsoever on the distribution of vinblastine in secretory vesicle preparations in puzzling. In this study, more data points (particularly at the origin and at early time) for presented transport curves, as well as addition of key volume and binding controls, would substantially strengthen the conclusions. As demonstrated in many instances, the effects of protonophores, ionophores, and ion composition on various potentials, and hence these partitioning phenomena, are complex and need to be carefully calibrated in different systems and different vesicle preparations, which may exhibit substantial variability under different conditions. Also, since MDR protein may function as a Cl^- channel (Valverde *et al.*, 1992) that may or may not be accessible to other anions, it is important to consider additional terms in expansions representing $\Delta\mu_{H^+}$ for MDR cells or vesicles harboring MDR protein.

even under continued "selective pressure" with G418 or chemotherapeutics, we have adopted the practice of discarding cell lines after six passages. Thus, unless quantitative Northern blots (probing for any and all conceivable AE, NHE, and MDR isoforms) and quantitative Western blots are again performed on the lines after six passages and evidence provided that levels of expression of these transporters have not been altered, we consider the line unsuitable for additional study.

For single-cell photometry analysis of pH_i, cells were grown as above on glass coverslips (Corning Glassworks, 18 mm^2/0.11 mm thick) that were immobilized in standard tissue culture plates with a dab of autoclaved silicon vacuum grease (Dow-Corning). They were kept in media at 37 °C and 5% CO_2 until immediately before mounting in a home-built perfusion chamber based on a design provided by Dr. Larry Palmer [see below and Luz *et al.* (1994)]. After mounting, cells were immediately perfused with HBSS (118 mM NaCl/24.2 mM $NaHCO_3$/5 mM KCl/1.3 mM $CaCl_2$/0.5 mM $MgCl_2$/0.6 mM Na_2HPO_4/0.5 mM KH_2PO_4/10 mM glucose) that had been equilibrated with 5% CO_2 and 37 °C (see single-cell photometry, below).

Single-Cell Photometry and Measurement of pH_i. As described previously (Luz *et al.*, 1994), we have constructed a single-cell photometry apparatus by interfacing a Nikon diaphot epifluorescence microscope and associated optics to a Photon Technologies Inc. alphascan fluorometer. A 0.5 nm slit directed broad band excitation to a fiber optic and hence a 510 nm dichroic beneath the microscope stage. A 530 nm filter was placed beneath the dichroic. Signals from PMTs connected in T-format to the side port of the microscope were transferred to a Dell 433/L computer and analyzed with PTI software. ASCii output files were further analyzed with Sigmaplot software on a Macintosh IICi computer. Additional details may be found in the figure captions.

Cells were grown on sterile glass coverslips as described above and used >1.5 but <4 days after plating, before confluency but after several cell divisions. Coverslips were incubated with 5 μM BCECF-AM for 30 min before mounting on the microscope stage, and they were then continuously perfused at a constant rate (approximately 6 mL/min) with HBSS buffer equilibrated with 5% CO_2 and to 37 °C. We repeatedly verified that flow rate was very similar in different experiments. Uniform BCECF staining was verified visually and by monitoring the intensity of 490 nm excitation and was found to be very similar for the different cells. Buffers harboring $^-HCO_3$ were continuously purged with 5% CO_2, and a fine jet of 5% CO_2 was directed over the mounted coverslip. Buffer pH was monitored with a microelectrode. Several control experiments verified that leak of the esterified BCECF-AM was minimal in the time required to make a measurement and not any different for MDR cells vs their untransfected parent [see Luz *et al.* (1994) for BCECF calibration curves for these cell lines that verify pH_i-dependent behavior of BCECF is nearly identical for the different cell lines]. Exposure to excitation light was limited to the time of data collection to limit photobleaching.

To calculate steady state pH_i, calibration curves were obtained essentially using the K^+/nigericin titration approach of Thomas and colleagues (1979) as described previously (Roepe, 1992; Roepe *et al.*, 1993) but in a "single-cell mode" wherein buffer harboring nigericin was continuously flowed over the cells (Luz *et al.*, 1994).

To produce Cl^--dependent changes in pH_i ("Cl^- substitution pH_i transients"), Cl^- in the perfusate was rapidly replaced with equimolar glutamate or gluconate (we did not observe significant differences between experiments performed with the different impermeant anions). After the observed pH_i perturbation began to plateau (usually within 100–120 s; see Results), normal $[Cl^-]_o$ was restored. In some cases, for comparison purposes, ion composition was altered before an obvious plateau was reached; in these examples, we verified that the pH_i changes did indeed plateau upon continued perfusion with the appropriate buffer, and in all cases, presented behavior is very similar to the behavior witnessed with the same cells in similar experiments performed on the longer time scale (shortened protocols were in some cases necessary so that proper subtraction of pH_i transient curves could be performed; see Results). Perfusate flow was kept constant using a peristaltic pump (Pharmacia LKB Model P-1). In some cases, experiments were performed in the absence of $^-HCO_3$ (10 mM HEPES replacing the $^-HCO_3$) or Na^+ [either NMG^+ (*N*-methyl-D-glucamine) or K^+ replacing the Na^+] or in the presence of stilbene inhibitors or verapamil (see Results). We were able to obtain high-quality Na^+-independent $Cl^-/^-HCO_3$ exchange transients with K^+ replacing Na^+. Similar data were obtained with NMG^+, although in general the time necessary for equilibration was increased. A detailed comparison between the effects produced by changing to high K^+- or NMG^+-containing buffers will be presented elsewhere.

For experiments performed under hypotonic conditions, NaCl, NMG Cl, Na glutamate, K glutamate, or NMG glutamate concentrations were lowered in the perfusate; however, normal Ca^{2+}, Mg^{2+}, $^-HCO_3$, phosphate, and glucose levels were maintained. We verified visually and fluorometrically that the hypotonic conditions swelled the cells but did not rupture them or cause gross morphological changes. Since the regulatory volume decrease (RVD) response for many cells includes rapid changes in pH_i, we also monitored these closely by continuously ratioing 439/490 nm excitation of trapped BCECF during the hypotonic swelling. Further perturbation of the cells (*i.e.*, various ion substitutions) was not performed until pH_i returned to its normal steady state value (usually in <15 min). Additional details may be found in the figure captions.

Determination of Intracellular Buffering Capacity. Buffering capacity was determined in the absence of bicarbonate by monitoring pH_i alterations at the single-cell level upon diffusion of the weak base ammonium [see Roos and Boron (1981)]. Cells were loaded with BCECF as described, and pH_i was monitored by constantly ratioing 439 and 490 nm excitation. When a desired pH_i was attained, we switched rapidly to perfusate harboring NH_4Cl (2–10 mM), and the resultant jump in pH_i was measured (n = 4, SE < 10%). Knowing $[NH_3]_o$, assuming $[NH_3]_o = [NH_3]_i$, and using a pK_B for ammonium of 9.21, $[NH_4^+]_i$ was calculated using the Henderson–Hasselbach relation [see Roos and Boron (1981)]. Buffering capacity (β_i) was then calculated ($\beta_i = \Delta[NH_4^+]_i/\Delta[pH_i]$) for each of the cell lines clamped at a variety of pH_i between 7.0 and 8.0. β_i vs pH_i curves were well fit by quadratic equations and were similar for the different cell lines. β_i for LR73 was found to be approximately 12.5, 11.2, and 22.3 mM/pH_i at pH_i = 7.0, 7.5, and 8.0, respectively. Corresponding values for EX4N7 were 14.0, 12.6, and 18.0 mM/pH_i.

RESULTS

In previous experiments (Luz *et al.*, 1994), we determined that at least two Cl^--dependent pH_i homeostasis mechanisms (at least one Na^+-dependent and one Na^+-independent) exist

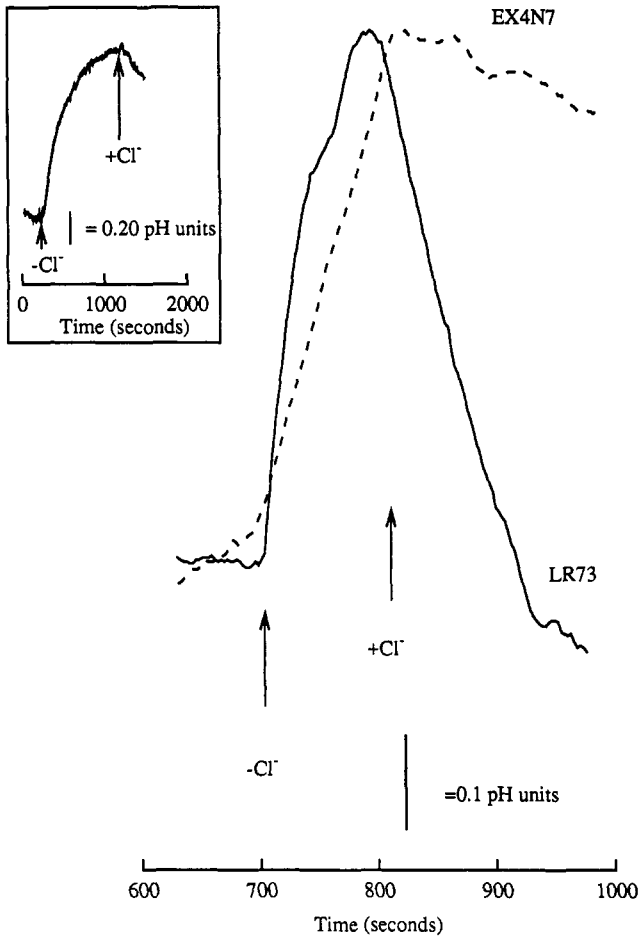

FIGURE 1: Comparison of Cl^--dependent pH_i transients for LR73 (solid line) and EX4N7 (dashed line) cells. Each trace is the average of six separate experiments performed with six different cells grown on six different coverslips. After averaging, the data were smoothed with the Savitsky–Golay algorithm (Savitsky & Golay, 1964). Visual inspection of the individual traces reveals the data are highly reproducible, assuming the cells are treated identically. In these experiments, cells loaded with BCECF (see Materials and Methods) were initially perfused with HBSS at 37 °C that was equilibrated with 5% CO_2. Within 5 min, a flat base line was achieved or the cell was discarded. Perfusate was then switched to Na^+-free HBSS (in these traces K^+ replaces Na^+; similar data are obtained with NMG^+ replacing Na^+, not shown; see methods), and the cells were allowed to equilibrate for another 10 min at which point a flat base line (within 0.15 pH unit of the original base line) was in general obtained. If a flat base line was not achieved or if perturbation of pH_i was too severe, the cell was discarded. At the first arrow, isotonic glutamate/Cl^- substitution was performed, and at the second arrow, Cl^-/glutamate substitution was performed 2 min later. Similar data were obtained using gluconate (not shown). Starting pH_i was near 7.1 for LR73 and 7.3 for EX4N7; thus note the EX4N7 trace is offset for display purposes (but see also Figure 2). Inset: Demonstrates that pH_i takes longer to equilibrate for EX4N7 upon Cl^- removal, relative to LR73, which plateaus in about 2 min (in this experiment, an EX4N7 cell was exposed to Cl^--free perfusate for 20 min). Regardless, even if allowed to plateau to very alkaline pH_i (calculated 8.4 pH_i) which should stimulate AE2 known to be present in these cells (Luz *et al.*, 1994), Cl-dependent reacidification is inhibited.

in LR73 Chinese hamster ovary fibroblasts. Similar observations have been made in a number of other cell lines, including Chinese hamster lung fibroblasts (Cassel *et al.*, 1988) and monkey kidney Vero cells (Madshus & Olsnes, 1987). To simplify analysis of Cl^--dependent pH_i regulation single-cell photometry experiments can be performed in the absence of Na^+ to eliminate the contributions of the Na^+-dependent system(s) (Figure 1). In the absence of Na^+, we find that LR73 cells (solid line, Figure 1) exhibit alkalinization upon

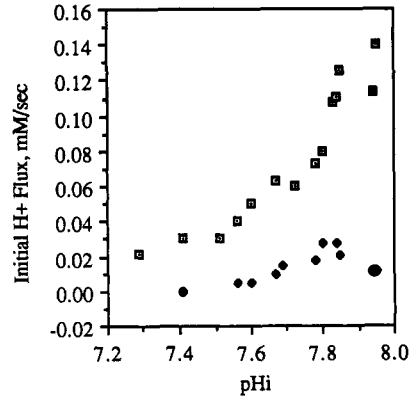

FIGURE 2: Summary of the rate of Cl^--dependent reacidification of pH_i for LR73 (open squares) and EX4N7 (closed diamonds) after transient alkalinization induced by glutamate/Cl^- substitution. Note the different shape of the curve for the LR73 cells as well as the dramatic increase in Cl^--dependent H^+ flux at higher pH_i. Alkalinization in Na^+-free HBSS was allowed to proceed to different levels before readdition of Cl^-. The measured slope of the first 25 s of the recovery (in 439/490 nm excitation units) was converted to $\Delta pH_i/s$ using a calibration curve obtained by the K^+/nigericin method [see Luz *et al.* (1994)]. After measuring the buffering capacity of the two cells at a variety of different pH_i (see methods), rates were converted to H^+/s [see Roos and Boron (1981) and Roepe *et al.* (1993)]. Each point represents a separate experiment with a fresh cell from a fresh coverslip.

Cl^- removal, most likely due to AE2-mediated $Cl^-/^-HCO_3$ exchange [see also Lee *et al.* (1991)] upon isotonic substitution of glutamate for extracellular Cl^- (glutamate/Cl^- substitution, first arrow). Subsequent return of Cl^- to the perfusate (Cl^-/glutamate substitution, second arrow) leads to reacidification as intracellular $^-HCO_3$ is presumably exchanged for extracellular Cl^-. Consistent with this interpretation [see Lee *et al.* (1991)] is a previously determined K_i of 45 μM for SITS (Luz *et al.*, 1994) as well as Northern blots with isoform specific probes that reveal the expression of the AE2 isoform but not AE1 in the LR73 cells (Luz *et al.*, 1994). Thus, previous results along with these data are consistent with AE2-mediated $Cl^-/^-HCO_3$ exchange in LR73 cells; however, it is possible that other Na^+-independent processes may contribute to the observed behavior.

In dramatic contrast, EX4N7 cells, which harbor increased wild-type mu MDR 1 protein (Gros *et al.*, 1991; Luz *et al.*, 1994) but which were never exposed to chemotherapeutics, exhibit markedly different behavior in the Na^+-free Cl^- substitution experiment (dashed line, Figure 1). Alkalinization upon glutamate/Cl^- substitution is slower, and reacidification of pH_i upon Cl^-/glutamate substitution is virtually abolished. This general characteristic is strikingly reminiscent of behavior exhibited by MDR myeloma cells that were created by selection on doxorubicin (Roepe *et al.*, 1993). As shown in the inset to Figure 1, alkalinization of EX4N7 cells is more extensive upon removal of Cl^- and takes much longer to plateau. However, even if EX4N7 is allowed to completely plateau, recovery is severely inhibited.

Figure 2 summarizes Cl^--dependent recovery of pH_i for LR73 and EX4N7 cells measured at a variety of pH_i. It is clear that the efficiency of apparent Na^+-independent, AE2-mediated $Cl^-/^-HCO_3$ exchange as measured in this assay is severely reduced in the wild-type mu MDR 1 transfectants. In contrast to these data, transfectants (88-8 cells) that overexpress a mutant MDR 1 protein unable to confer the MDR phenotype (Gros *et al.*, 1991; Luz *et al.*, 1994) exhibit behavior nearly identical to that of the untransfected LR73 cells (not shown; see Figure 4 below).

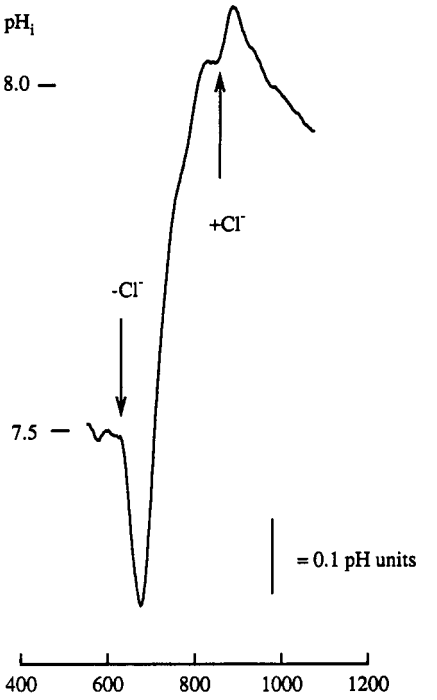

FIGURE 3: Cl$^-$-dependent pH$_i$ transient for 1-1 cells obtained under Na$^+$-free, isotonic conditions. Trace shown is the average of six separate experiments performed as in Figure 1, and the averaged trace was smoothed as in Figure 1. Note that 1-1 cells express nearly 10-fold more mu MDR 1 protein, relative to EX4N7 (Gros *et al.*, 1991; Luz *et al.*, 1994), and exhibit additional features including sharp acidification upon Cl$^-$ removal (first arrow) that spontaneously reverses and additional mild but fast alkalinization upon return of Cl$^-$ to the perfusate (second arrow).

1-1 cells, which are mu MDR 1/LR73 transfectants further selected on 50 ng/mL vinblastine, exhibit increased levels of mu MDR 1 protein and mRNA (Gros *et al.*, 1991; Luz *et al.*, 1994) and even more dramatic alterations in pH$_i$ upon Cl$^-$ substitution, relative to EX4N7 (Figure 3). In this case, upon removal of Cl$^-$, a sharp acidification that spontaneously reverses itself is seen. Also, upon return of Cl$^-$ to the perfusate, an additional mild yet fast alkalinization is observed, and upon continued incubation in the Cl$^-$-containing perfusate, recovery of pH$_i$ is poor, similar to the case for EX4N7. These results suggest that increased MDR protein expression (and/or perhaps vinblastine selection) may further perturb Cl$^-$-dependent pH$_i$ regulation; the interesting implications are entertained in the Discussion.

Another possible method for analyzing the contribution MDR protein might make to altered pH$_i$ regulation (other than comparing cells with different levels of MDR expression) would be to stimulate any ion translocation catalyzed by the MDR protein. Previous studies of Higgins and colleagues (Valverde *et al.*, 1992; Gill *et al.*, 1992) have suggested that hypotonicity-induced swelling of cells overexpressing MDR protein stimulates an anomalous Cl$^-$ conductance presumably mediated (directly or indirectly) by the transporter. In Figure 4, we present Cl$^-$ substitution pH$_i$ transients for LR73 (top solid line), 1-1 (middle solid line) and EX4N7 (bottom solid line) cells obtained essentially as in Figure 1–3, except under hypotonic conditions. Cells harboring inactive mu MDR 1 protein (88-8 cells, top dashed line) exhibit behavior very similar to that of LR73.

Hypotonic conditions do not appear to significantly perturb the pH$_i$ transients induced in LR73 cells upon Cl$^-$ removal and subsequent return (compare Figure 1 to Figure 4, solid lines; note time in Cl$^-$-free buffer is increased to 5 min in

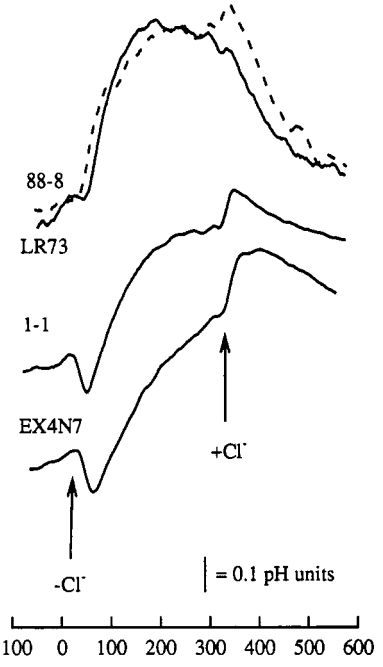

FIGURE 4: Cl$^-$-dependent pH$_i$ transients for LR73 (top solid line), 88-8 (top dashed line), 1-1 (middle solid line), and EX4N7 (bottom line) obtained under hypotonic conditions. Each trace is the average of at least three experiments performed over the course of at least 2–3 days with three different cells grown on three different coverslips. Data are smoothed. In these experiments, after equilibration in HBSS, we switched to Na$^+$-free HBSS (K$^+$ replacing Na$^+$) that was approximately 40% hypotonic (180 milliosmoles). Perfusate was then switched to similar buffer with glutamate replacing Cl$^-$ (first arrow) and then back to the hypotonic Na$^+$-free HBSS containing Cl (second arrow). Smaller effects for EX4N7 were seen at higher osmolality, but no additional effect was observed upon further decreasing osmolality. Cl$^-$/$^-$HCO$_3$ exchange in the LR73 cells is relatively unaffected by these hypotonic conditions and stilbene inhibitable (not shown), but anomalous effects seen in the EX4N7 cells are apparently stimulated, such that the cells more closely mimic 1-1, which expresses about 10-fold more MDR protein.

Figure 4 to illustrate the stability of pH$_i$ under these conditions). Dramatically, however, EX4N7 cells exhibit even more significantly different behavior. Glutamate/Cl$^-$ substitution causes fast acidification which spontaneously reverses itself, and return of Cl$^-$ to the perfusate causes additional fast alkalinization, similar to what occurs for the 1-1 cell line under isoosmotic conditions. Also, net alkalinization for EX4N7 upon Cl$^-$ removal appears to be even slower than is the case under isoosmotic conditions. Under hypotonic conditions, 1-1 cells exhibit behavior very similar to that of EX4N7 cells. The results suggest that hypotonic stimulation of cells expressing relatively low levels of MDR protein leads to behavior that mimics cell lines harboring much higher levels and that at some level of MDR protein expression these effects saturate. Interestingly, a small, fast alkalinization is also seen for 88-8 cells harboring inactive MDR protein upon readdition of Cl$^-$ under hypotonic conditions (top dashed line); however, this is not followed by an inability to reacidify (compare top solid line to top dashed line), since the 88-8 cells reacidify nearly as well as the LR73 cells. This small alkalinization perhaps indicates remaining partial activity in the mutant that is only apparent under hypotonic stimulation.

To additionally test whether the unusual features in the pH$_i$ transients for the MDR 1 transfectants are due to the expression of MDR protein, similar experiments were performed in the presence of verapamil, known to be a potent inhibitor of the ability of MDR protein to confer the MDR phenotype and to conduct Cl$^-$ (Valverde *et al.*, 1992). As

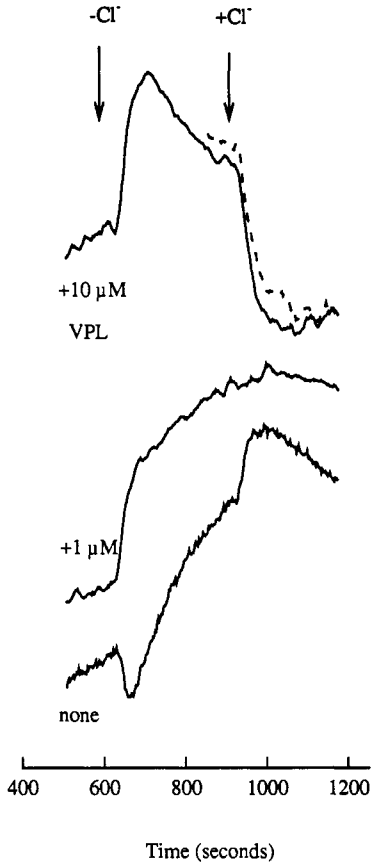

FIGURE 5: Comparison between Cl^--dependent pH_i transients obtained for EX4N7 cells under hypotonic conditions (see Figure 4) in the absence (bottom line) or presence of 1 μM (middle line) or 10 μM (top solid line) verapamil. Also shown (dashed line) is part of a similar transient obtained for LR73 in the absence of verapamil to illustrate similar kinetics of reacidification. Note the presence of verapamil reduces the anomalous behavior exhibited by the MDR cells such that they more closely resemble the parental untransfected line, in a dose-dependent manner (apparent K_i approximately 3.5 μM, as calculated by analyzing the rate of acidification upon replacing Cl^-, data not shown). Data shown are unsmoothed single traces that are representative of at least three experiments.

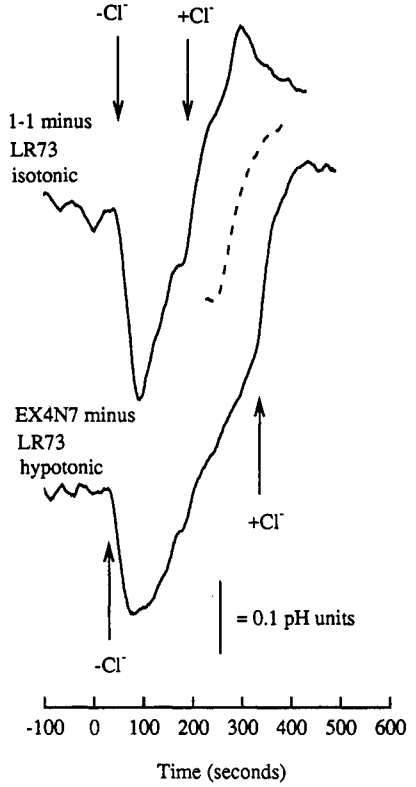

FIGURE 6: Different traces obtained by subtracting the LR73 Cl^--dependent pH_i transient obtained under isotonic conditions (see Figure 4) from similar data obtained using the 1-1 cells (top trace), as well as a similar subtraction using LR73 and EX4N7 traces obtained under hypotonic conditions (bottom trace). Note the direction of the pH_i changes upon removal (first arrow) or replacement (second arrow) of Cl^-, as well as the negative slope that reverses initial acidification, and the additional alkalinization upon readdition of Cl^-. Note also the kinetic similarity of this alkalinization to the alkalinization observed in LR73 cells upon *removing* Cl^- (dashed line), *i.e.*, the alkalinization catalyzed by AE2 upon Cl^- exit coupled to $^-HCO_3$ entry into the cell.

shown in Figure 5, when 10 μM verapamil is added to the perfusate, the pH_i transient for the EX4N7 cells obtained under hypotonic conditions exhibits character very similar to that for the transient obtained for LR73 cells under hypotonic conditions (compare top solid traces in Figures 4 and 5). Although the EX4N7 cells now alkalinize by nearly the same amount seen for LR73, pH_i is apparently less stable for the transfectants in the absence of Cl^- (note negative slope from 600 to 900 s that is steeper than the negative slope for LR73 in the absence of Cl^-). However, reacidification of pH_i upon readdition of Cl^- is seen for EX4N7, and it is kinetically similar to the reacidification exhibited by LR73 at similar pH_i (compare top dashed trace, Figure 5, to top solid). A slight overshoot in pH_i to below the initial value is also observed, which slowly reverses over 10–15 min (not shown).

A lower concentration of verapamil (1 μM) produces more subtle inhibition of the contribution made by MDR protein, essentially inducing better alkalinization upon removal of Cl^-. However, reacidification upon return of Cl is still poor (Figure 5, middle solid trace).

To more closely analyze the features in these transients due to overexpression of MDR protein, we first verified that relative expression of AE2 was similar in all cells by quantitative Northern blot (Luz *et al.*, 1994). Knowing this allows us to qualitatively examine the features due to MDR protein overexpression by subtraction of the transients shown in Figures 1 and 4. However, note that 1-1 cells overexpress AE1 (Luz *et al.*, 1994) which may complicate analysis in this case. In Figure 6, we show the results obtained upon subtracting the LR73 transient from the EX4N7 transient obtained under hypotonic conditions (bottom solid trace), as well as the result from subtracting LR73 and 1-1 transients obtained under isotonic conditions (top solid trace; note the different time under Cl^--free conditions). In both cases, the difference trace represents behavior that exists in the MDR cells but not the untransfected parental cells. Note similar difference traces are obtained upon subtracting the corresponding transients for 88-8 cells from the traces for the MDR cells (not shown).

Note the overall general similarity between the two difference traces in Figure 6. A similar subtraction between EX4N7 and LR73 traces obtained under isotonic conditions produces a trace with shape similar to these but dramatically reduced height (not shown). The difference trace illustrates Cl^--dependent pH_i changes specific to the MDR cells that are kinetically very similar to the changes mediated by AE2 (dashed line) except that they go in the opposite direction (*i.e.*, Cl^- *removal* leads to alkalinization via AE2, Cl^- return to acidification; the converse appears to be true for the MDR protein effect). However, the decrease in pH_i specific to the MDR cells that is caused by Cl^- removal is not as stable in the absence of Cl^- as the increase in pH_i catalyzed by AE2 (compare the positive slope from 100 to 400 s for the difference

trace to the flatter region between 100 and 400 s for the LR73 trace in Figure 4), suggesting a $^-HCO_3$ or H^+ "leak" specific to the MDR cells.

Importantly, control experiments wherein CO_2 is rapidly removed and then restored (P.D.R., J.G.L., and J.W., unpublished results) reveal this behavior is not likely due to significantly different membrane CO_2 permeability or carbonic anhydrase activity (although the latter point does require additional detailed study). Experiments wherein $^-HCO_3$ is rapidly removed/replaced (not shown) reveal that effects on pH_i are larger for the MDR cells, which is consistent with our overall conclusions (see Discussion).

DISCUSSION

Several recent studies have suggested that MDR protein overexpression mediates an anomalous Cl^- conductance across eukaryotic plasma membrane (Valverde *et al.*, 1992; Gill *et al.*, 1992; Altenberg *et al.*, 1994; Bear, 1994). Whether the conductance is directly mediated by MDR protein or some other protein regulated by MDR protein [see Rasola *et al.*, (1994)] is currently a topic of debate, as are possible gating mechanisms. Regardless, it is also apparent that pH_i homeostasis is altered in most, if not all, MDR cells when it is clear that MDR is due to overexpression of the MDR protein (Keizer & Joenje, 1989; Thiebaut *et al.*, 1990; Roepe, 1992; Roepe *et al.*, 1993; Altenberg *et al.*, 1993; Luz *et al.*, 1994; Wei & Roepe, 1994). Thus, recalling the fundamental importance of $Cl^-/^-HCO_3$ exchange for the regulation of pH_i in a variety of cells [see Knauf (1986), Grinstein *et al.* (1984), and Boyarsky *et al.* (1988)], along with consideration of membrane bioenergetics ($\Delta\mu_{H^+} = \Delta\Psi + \Delta pH$), suggests that in MDR cells anomalous Cl^- flux and altered pH_i regulation might be connected. We have investigated this possibility by performing Cl^- substitution experiments and monitoring the accompanying changes in pH_i that occur for transfected cells harboring different levels of mu MDR 1 protein. These studies of pH_i dynamics are only the first step in deducing the role of Cl-dependent processes in regulating anomalous steady state (equilibrium) pH_i; however, they provide insight. In general, the results are similar to previous data obtained via different methods with a series of MDR cells (human myeloma cells) created not by transfection but by selection with doxorubicin (Roepe *et al.*, 1993). The data may be summarized as follows:

(1) AE2-mediated [see Luz *et al.* (1994)], Na^+-independent $Cl^-/^-HCO_3$ exchange is inhibited for MDR cells overexpressing MDR protein to high (1-1) or low (EX4N7) levels that were or were not, respectively, exposed to vinblastine. Reacidification ($Cl^-/^-HCO_3$ exchange) is more affected than alkalinization ($^-HCO_3/Cl^-$ exchange), and 1-1 cells expressing approximately 10-fold more MDR protein exhibit additional alkalinization upon return of Cl^-. We could speculate that different pH_i for the MDR cells [see Luz *et al.* (1994)] causes the anomalous behavior; however, the MDR cells are alkaline, and alkaline pH_i is known to *stimulate* AE activity not inhibit it.

(2) Hypotonic conditions do not significantly effect measured $Cl^-/^-HCO_3$ exchange for LR73 cells but further perturb Na^+-independent, Cl^-- and $^-HCO_3$-dependent pH_i homeostasis for the MDR cells. In addition, hypotonic conditions appear to stimulate the anomalous behavior exhibited by EX4N7 cells such that they then more closely resemble the 1-1 cells.

(3) Verapamil inhibits anomalous pH_i homeostasis exhibited by MDR cells and restores apparent AE2 activity, at concentrations previously found to completely or partially reverse MDR in cell models or clinical settings, respectively [see Sikic (1993)]. Notably, similar concentrations lower elevated steady state pH_i for some MDR cells (Keizer & Joenje, 1989; Roepe *et al.*, 1993).

(4) Subtraction of pH_i transients reveals a component specific to MDR cells, and its contribution increases upon further overexpression of MDR protein or hypotonic stimulation.

Under isotonic, Na^+-free conditions (Figure 1), $^-HCO_3/Cl^-$ exchange stimulated by removal of extracellular Cl^- is slower for EX4N7 cells, relative to LR73 cells, and $Cl^-/^-HCO_3$ exchange upon replacing extracellular Cl^- is more strongly inhibited. These observations suggest that the presence of the MDR protein in the transfectants inhibits AE2-catalyzed anion exchange as previously suggested (Luz *et al.*, 1994) and that this inhibition does not necessarily involve Na^+-dependent processes. Furthermore, it appears that the forward (physiologic) AE reaction is more severely inhibited by the presence of MDR protein than the reverse reaction. We speculate that the decrease in $\Delta\Psi$ previously noted for EX4N7 perturbs a step in the AE catalytic cycle that is more important for $Cl^-/^-HCO_3$ exchange (*i.e.*, association of extracellular Cl^- to the extracellular AE Cl^- binding site). This would be somewhat consistent with the work of Jennings and colleagues (Jennings *et al.*, 1990) which showed that extracellular Cl^- binding to AE is reduced upon lowering $\Delta\Psi$.

Not only is AE2 inhibited but any residual activity remaining in the EX4N7 cells apparently does not exhibit a significant pH_i dependency (Figure 2). This is in contrast to AE activity observed in many other cells, which typically increases as pH_i is elevated (see shape of the curve for LR73 in Figure 2). Since EX4N7 cells still express AE2, and may even overexpress a 4.4 kb AE1 transcript (Luz *et al.*, 1994), these data are curious. They suggest that inhibition of AE by the MDR protein may be pH-dependent or that regulation of endogenous AE2 activity is perturbed in the transfectants.

Relatedly, when examining the trace for 1-1 obtained under isotonic conditions, or the trace for EX4N7 under hypotonic conditions, it is clear that there are additional features in the pH_i transients that are not easily explained by simple inhibition of a step in the AE2 catalytic cycle. These features are more clearly revealed by subtraction (Figure 6) of the LR73 and EX4N7 or 1-1 transients. The shape of the MDR cell specific component revealed by subtraction is similar to an anion exchanger transient that is upside down and "leaky". That is, in many cells, when "reverse" AE is stimulated by removing extracellular Cl^- (*i.e.*, promote $^-HCO_3/Cl^-$ exchange), the alkalinization of pH_i due to influx of $^-HCO_3$ remains reasonably stable (see Figure 4). If the experiment is performed in the presence of Na^+ and the cells also harbor a $Na^+/Cl^-/2^-HCO_3$ cotransporter, the plateau in pH_i may be less stable, due to possible alternate routes of Cl^- and/or $^-HCO_3$ translocation. Thus, although more exotic scenarios can be envisioned, this "leaky" character in the MDR cell specific feature suggests an alternate route for slower $^-HCO_3$ entry or H^+ exit that is somehow linked to the activation or overexpression of MDR protein. Importantly, this is apparently Na^+-independent.

Since it has been shown that MDR protein may function as a Cl^- channel (Valverde *et al.*, 1992), sudden removal of extracellular Cl^- for the MDR cells might lead to substantial Cl^- flux through MDR protein that may be faster than the flux mediated by AE2. Thus, the MDR cells may require additional ion translocation to electrically balance increased Cl^- movement; this could perhaps manifest itself as H^+ exit to electrically balance Cl^- exit, leading to the features revealed

in Figure 6. Somewhat similar H^+ flux that outpaces $Cl^-/^-HCO_3$ exchange is observed during anomalous loss of salt during the RVD response associated with cell swelling (Hoffman & Simonsen, 1989). Alternatively, perhaps MDR protein also mediates translocation of $^-HCO_3$ (Luz *et al.*, 1994). Another possibility could be altered carbonic anhydrase activity in the MDR cells, although our initial cursory experiments with CO_2 removal argue against this. A much more extensive analysis of carbonic anhydrase activity in MDR cells is likely warranted.

In any case, since we have previously documented that plasma membrane electrical potential ($\Delta\Psi$) is different for the different lines, we cannot completely rule out the possibility that the presence of high $[K^+]$ in these experiments (which may at least transiently depolarize the cells to different extents) affects some cells differently. However, additional studies using oubain to lower $\Delta\Psi$ before addition of K^+ argue against this (P.D.R., unpublished data). Continued work that more closely examines connections to altered $\Delta\Psi$ and pH_i regulation in MDR cells will likely prove beneficial to our understanding of this complex phenotype, and further inhibitor- and ion-dependency measurements may help to identify any additional proteins involved in anomalous pH_i regulation in the MDR cells.

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