Analysis of Ion Transport Perturbations Caused by hu MDR 1 Protein Overexpression†

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ABSTRACT: In previous work [Luz *et al.* (1994) *Biochemistry 33*, 7239−7249; Roepe *et al.* (1994) *Biochemistry 33*, 11008−11015] we measured changes in Cl^- and HCO_3^--dependent pH_i regulation for LR73 Chinese hamster ovary fibroblasts overexpressing mu MDR 1 protein. However, only one clonal cell line overexpressing the protein but not previously exposed to chemotherapeutic drug (i.e., a "true" transfectant) was examined, since very few MDR cell lines of this nature have been constructed. Recently [Hoffman *et al.* (1996) *J. Gen. Physiol. 108*, 295−313] we derived a series of true LR73/hu MDR 1 transfectants that are valuable for defining the MDR phenotype mediated by MDR protein alone, without the additional complexities introduced by exposing cells to chemotherapeutic drugs. Several independently derived clones from these and additional transfection experiments exhibit expression of MDR protein that is higher than that found in other true transfectants, and that is similar to the highest level of overexpression yet recorded for drug selected MDR cells. We examined altered Cl^--dependent pH_i regulation for these clones using improved single-cell photometry (SCP) techniques. Short-term isotonic Cl^- substitution experiments performed in the presence of CO_2/HCO_3^- reveal that mild overexpression of hu MDR 1 protein alters anion exchange (Cl^-/HCO_3^- exchange or AE) for LR73 cells, as expected on the basis of previous work [Luz *et al.* (1994) *Biochemistry 33*, 7239−7249]. Interestingly, we now find that several independently selected high-level MDR 1 overexpressing clones *acidify* quite extensively upon isotonic exchange of Cl^- and then rapidly alkalinize upon restoring normal [Cl^-]. These data suggest that MDR protein may effectively compete against AE. The MDR protein effect is not dependent on HCO_3^-/CO_2 or K^+, is partially inhibited by verapamil, is completely inhibited by substituting K^+ or *N*-methylglucamine (NMG^+) for Na^+ in the SCP perfusate but is not affected by 100 μM levels of amiloride, bumetanide, chlorothiazide, or stilbene. ATP depletion inhibits the MDR 1 effect. We are unable to restore normal AE activity for the MDR clones via manipulation of Cl^- or HCO_3^- gradients. We thus suggest that MDR protein overexpression provides a novel Na^+- and Cl^--dependent pathway for transmembrane H^+ transport. From analysis of ion dependency and inhibitor sensitivities, we conclude the transport is not via altered regulation of any known K^+/H^+, Na^+/H^+, or Cl^-/HCO_3^- antiporters, Na^+:K^+:$2Cl^-$, Na^+:K^+:$2HCO_3^-$, K^+:HCO_3^-, or Na^+:HCO_3^- co-transporters, or any combination of these. Thus, it appears to represent a novel ATP and Na^+-dependent Cl^-/H^+ antiport process that (1) may be directly mediated by the MDR protein, (2) may represent the modulation of one or more currently unidentified ion transport proteins by MDR protein, (3) may be due to some combination of direct ion transport and regulation of ion transport, or (4) may represent unusual passive H^+ movement in response to a novel Cl^--dependent electrical perturbation that occurs during our Cl^- substitution protocol. The results have important implications for understanding drug resistance mediated by MDR 1 overexpression, as well as the physiologic function of endogenously expressed MDR protein.

Review of the MDR[1] literature suggests a dizzying array of possible functions for the MDR protein (*1*−*6*), which is overexpressed in tumor cells in response to chemotherapeutic drug selection. Initially (*7*,*8*) MDR protein (also called P-glycoprotein or P-gp) was suggested to be the putative multisubstrate drug pump first hypothesized by Danø (*9*) to actively translocate chemotherapeutic drugs out of drug resistant tumor cells. It was envisioned that this hypothesized

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[1] Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein (MDR 1 protein); ATP, adenosine 5′-triphosphate; pH_i, intracellular pH; $\Delta\Psi$, plasma membrane electrical potential; CFTR, cystic fibrosis transmembrane conductance regulator; SUR, sulfonyl urea receptor; BCECF, 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; G418, geneticin; DME, Dulbecco's modified Eagle's medium; SCP, single-cell photometry; HBSS, Hank's balanced salts solution; β_i, intracellular buffering capacity; AE2, anion exchanger (Cl^-/HCO_3^- exchanger) isoform 2; pH_o, extracellular pH; NMG^+, *N*-methylglucamine; NHE, Na^+/H^+ exchanger; ABC, ATP-binding cassette; VPL, verapamil; RVI, regulatory volume increase; RVD, regulatory volume decrease; PT, proximal tubule.

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"drug pumping" lowered intracellular drug concentration, thereby conferring drug resistance. Some data that can be interpreted as support for this putative drug pumping function have been reported (*10*).

Other studies that have focused on the kinetic and thermodynamic details of drug translocation in cells expressing MDR protein (reviewed in refs *5*, *6*, and *11*) suggest revision of a drug pump model is necessary. For example, a kinetic capacity for the putative pump that could compete with inward passive diffusion of chemotherapeutic drugs under initial rate conditions such that the putative outward pumping process would then lower rates of intracellular drug accumulation as is observed (see refs 12−14) is not at all apparent (*15*,*16*). That is, rate constants for transmembraneous drug transport are not necessarily altered upon MDR protein overexpression (*6*). Also, MDR protein overexpression confers a similar percent reduction in the rate of cellular drug accumulation under initial rate conditions regardless of whether external drug concentrations are as low as 10^{-1} pM or as high as 10^2 μM (*12*, *17*, and P. D. Roepe and L. J. Robinson, unpublished), indicating that if it exists, the catalytic capacity of MDR protein "drug pumping" exceeds the enormous increase in the rate of passive diffusion of chemotherapeutic drugs that accompanies this 8−9 order of magnitude increase in drug concentration. That is, via a pump model, an "initial rate of pumping" vs "substrate concentration" plot for MDR protein would apparently have a steep and positive slope over at least 8 orders of magnitude of "substrate concentration"; behavior that would be extremely surprising and, to our knowledge, unprecedented. In addition, unusually high and widely variable estimates of ATP hydrolysis:drug translocation stoichiometries are extracted from analysis of recent MDR protein-catalyzed drug transport measurements, suggesting that the putative pump would also violate the coupling principle, another fundamental tenet of transport biochemistry (*5*). Along with the more well-known difficulties associated with trying to explain the lack of substrate specificity for the putative pump (MDR protein overexpression is believed to confer reduced cellular accumulation of well over 100 very structurally divergent compounds) these problems with direct drug pumping models have, for some time now, stimulated us to consider alternative models for MDR protein-mediated pleiotropic drug resistance.

One of these is the "altered partitioning model" (*11*,*18*) which proposes that MDR protein overexpression alters cellular retention of chemotherapeutic drugs and other compounds *indirectly* by modulating intracellular pH (pH_i), volume, and/or plasma membrane electrical potential ($\Delta\Psi$) but does not *directly* translocate drugs out of cells. Significant changes in one or more of these parameters are found for cells overexpressing MDR protein, are apparently due to MDR protein overexpression alone, and, importantly, are sufficient to confer the levels of drug resistance and altered drug retention that are unequivocally conferred by MDR protein overexpression alone (*6*,*11*,*19*). Presumably, MDR protein overexpression confers these perturbations in pH_i and $\Delta\Psi$ either directly (as some type of ion transporter) or indirectly (as a regulator of ion transport processes) translocating ions that are important for eukaryotic cell pH_i and/or $\Delta\Psi$ (namely, H^+, HCO_3^-, Na^+, K^+, or Cl^-). Many observations consistent with either the former or the latter have been reported (*3*,*15*,*18*−*23*), but some of the interpretations from these observations are in conflict with other recent work (see also *24* and *25*). Thus, it is not yet clear how MDR protein overexpression alters pH_i and $\Delta\Psi$. Elucidating this mechanism is now a key goal of cancer pharmacologists, membrane biochemists, and transport physiologists.

This pursuit is all the more relevant if one considers interesting possible parallels to the CFTR and SUR proteins, relatives of the MDR protein that when over- or underexpressed also perturb pH_i and $\Delta\Psi$ regulation (see *26*−*29*) and that directly translocate ions and apparently indirectly affect ion transport via other transporters (see refs *29*−*31*). Understanding the molecular similarities and differences between these homologous membrane transport proteins is important for many reasons.

We have studied pH_i regulation for true LR73/hu MDR 1 transfectants via single-cell photometry experiments with cells under constant perfusion. There are two key differences between the present work and almost all other previous studies, namely, (i) our experiments are performed with true transfectants not exposed in any way to chemotherapeutic drugs prior to analysis and (ii) cells are analyzed while they are perfused with media buffered with CO_2/HCO_3^-. Thus (i) complexities introduced into either the MDR phenotype (see *19*) or intracellular pH regulation (see ref *29*) via exposure to potent, complex chemotherapeutic drugs are avoided and (ii) a close-to-physiologic spectrum of pH_i regulatory processes is examined for these cells. The former is particularly important since exposure to drugs invariably alters the "pure" MDR protein-mediated phenotype (*19*). Our experiments uncover an attractive, yet curious, mechanism that explains how MDR protein overexpression perturbs pH_i (and conceivably $\Delta\Psi$) for eukaryotic cells, thereby leading to pleiotropic drug resistance via the predictions of the altered partitioning model (*6*). The data also suggest an interesting physiologic function for the protein.

MATERIALS AND METHODS

Materials. 2′,7′-Bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF−AM), the quinolinium probe SPQ, nigericin, and valinomycin were purchased from Molecular Probes (Eugene, OR) and used without further purification. Amiloride, ethylisopropyl amiloride (EIPA), furosemide, bumetanide, chlorothiazide, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), colchicine, and verapamil were from Sigma (St. Louis, MO). Doxorubicin, vincristine, and cyclosporin A were from the Memorial Sloan−Kettering pharmacy, and G418 was from Life Technologies (Grand Island, NY). All other chemicals were reagent grade or better, purchased from commercial sources, and used without further purification.

Cell Culture. Construction of the cell lines used in this work has been reported previously (*19*). In addition to this published two-plasmid co-transfection approach, we have also engineered an expression vector (Li Yong Wei and P. D. Roepe, unpublished) wherein the hu MDR 1 cDNA and a neomycin resistance marker are both contained within a single plasmid (pLYW1) by restricting pMDR1 (19) with *Eco*R1, isolating the 4.3 kbp hu MDR 1 cDNA and ligating to *Eco*R1 restricted pcDNAneo (Stratagene). In multiple transfection experiments with pLYW1 performed using the calcium phosphate precipitation method as described in (*19*),

we did not detect significantly greater transfection efficiency as measured by the appearance of G418 resistant (neomycin resistance marker-expressing) colonies, but we did notice a higher efficiency of high-level MDR 1 protein overexpression in these clones relative to the two-plasmid co-transfection method. Presumably, integration of full-length hu MDR 1 is more efficient when the cDNA is carried on the same vector as the neomycin resistance cDNA.

MDR 1 overexpressing clones and neomycin-selected control clones (LR73 transfected with pcDNAneo alone and selected in a similar concentration of G418, see ref 19) were grown in DME media supplemented with 10% fetal calf serum, 100 units of penicillin mL, 100 μg of streptomycin/mL, and 500 μg of active G418/mL. No chemotherapeutic drugs were included in any of the growth media at any time. Levels of MDR 1 overexpression for individual clones were analyzed vs the number of cell culture passages by western blot as described (*19*) and populations exhibiting any significant deviations in expression, relative to the original clonal isolate, were discarded.

For single-cell photometry (SCP) experiments, cells were grown as above, but on thin glass cover slips as described (see refs 23, 28, and 29). They were loaded with BCECF-AM and mounted in a specially designed perfusion cell as described (*28*) and immediately perfused with HBSS (118 mM NaCl/5 mM KCl/10 mM glucose/24.2 mM $NaHCO_3$/1.3 mM $CaCl_2$/0.5 mM Na_2HPO_4/0.5 mM $MgCl_2$/0.6 mM KH_2PO_4, equilibrated with 5% CO_2 and to 37 °C [pH = 7.33]).

Cl^- Substitution Experiments. Intracellular pH was measured for single cells with our single cell photometry (SCP) apparatus described in detail elsewhere (*28*). In brief, a SCP apparatus constructed by interfacing a Nikon epifluorescence Diaphot to a PTI Alphascan fluorometer was used to monitor intracellularly trapped BCECF. Excitation was monitored at 439 and 490 nm by flipping a monochromator under computer control using PTI software. Excitation light was directed to the microscope stage with a fiber optic, and a 510 nm dichroic mirror under the stage reflected excitation and passed emission, which was further filtered with a 535 nm band-pass filter. Additional details may be found in (28). The pH_i-dependent response of trapped BCECF was calibrated via the K^+/nigericin approach (*32*) adapted to rapid-flow SCP experimental conditions as described (*23*,*29*). To produce Cl^--dependent changes in pH_i ("isotonic Cl^- substitution" experiments), Cl^- in the perfusate was rapidly replaced with equimolar glutamate essentially as described previously (*23*) but with several important modifications as described in brief below and in more detail elsewhere (*29*). These modifications included changing the flow rate of the buffer, switching between buffers manually instead of via a manifold, and additional equilibration time under serum-free conditions before starting the experiment (see below, Results, and the relevant figure captions). Unless noted, similar results were obtained via substitution with gluconate. After any observed pH_i perturbation clearly due to Cl^- removal reached a plateau, normal $[Cl^-]_o$ was restored by isotonic substitution of Cl^- for glutamate. In some cases, experiments were performed in the absence of HCO_3^-/CO_2 or Na^+, in the presence of various ion transport inhibitors, or after ATP-depletion (see appropriate figure captions for additional details). Intracellular BCECF response was calibrated for individual cells by the K^+/nigericin method (*32*) at the end of *every* H^+ flux experiment, as described (*29*) so that initial pH_i and the pH_i at which ion transport reactions were initiated could be easily calculated.

In contrast to earlier work (*23*,*33*) wherein cells were equilibrated with serum-free buffer for relatively short periods of time before beginning ion substitution experiments, and in which the flow rate of the perfusate was relatively fast and switched with a manifold that we have recently learned has a tendency to introduce slight pressure changes on the surface of cells during switching of buffers, we have modified our standard isotonic ion substitution protocol as recently described in more detail elsewhere (*29*). In brief, cells were equilibrated under serum-free conditions for longer time (at least 5 min prior to ion substitution) and the flow rate was reduced (to approximately 5 mL/min over a slightly larger elliptical surface area; see refs 28 and 29) relative to previous experiments, and switching between buffers was performed manually instead of via turning valves on a multichannel manifold. These modifications eliminate several transient, reversible perturbations in BCECF response noted in previous work (*23*) that are not directly due to Cl^- ion substitution (see Results and ref 29).

Intracellular ATP was depleted by perfusing with HBSS/50 nM rotenone/2 mM 2-deoxy-D-glucose (*14*). We found by HPLC analysis of cell extracts that continuous perfusion with this solution depleted ATP to less than 10% of original levels within 5−10 min and to <2% within 30 min (William Tong, M. M. Hoffman, and P. D. Roepe, unpublished).

Buffering Capacity. As reported previously (*33*) we have measured intracellular buffering capacity (β_i) for LR73 cells via the NH_4^+-pulse method. We assume $[NH_3]_o = [NH_3]_i$, that the pK_B of ammonium is 9.21, and that $[NH_4^+]_i$ is not substantially different in the absence of external HCO_3^- for a cell at a given pH_i (see ref 34). β_i is not a linear function of pH_i; for LR73 cells it is approximately 12.5, 11.2, and 22.3 mM at $pH_i = 7.0$, 7.5, and 8.0, respectively. β_i values at similar pH_i are only slightly different for pure MDR transfectants (see for example 33) and they follow a very similar parabolic relationship vs pH_i (not shown).

Analysis of Cl^- Flux Using SPQ. Verkman and colleagues (*35*,*36*) have developed methods to follow Cl^- transport in monolayer cultures using the quinolinium probe SPQ. We have adapted these approaches to monitor Cl^- transport for our LR73/hu MDR 1 and control LR73/neo-transfectants upon isotonic Cl^- substitution. Cells were mounted in our perfusion apparatus as described above for pH_i studies, and following the previously published protocol of Verkman and colleagues (*36*), we loaded transfectants with SPQ by exposing them for 5 min to a 20 mM solution that was 50% hypotonic (i.e., "solution 1" in 36). After re-equilibration of the cells by perfusing with normal HBSS for 3 min, rapid Cl^- substitution was performed as described above. Intracellularly trapped SPQ was excited at 360 nm and emitted light filtered through a 420 nm long-pass filter (Nikon). We did not notice significant differences in the rate of SPQ leak from the different cell types, and the efficiency of SPQ loading was essentially the same for the different cell types (see Results). Since this probe is not ratiometric, it is difficult to precisely quantitate $[Cl^-]_i$ or the Cl^- flux/unit time for an individual cell as can be conveniently done for H^+ using BCECF. Previously however, Verkman and colleagues (*36*) successfully calibrated SPQ response empirically either by using a combination of nigericin and 10 μM tributyltin or

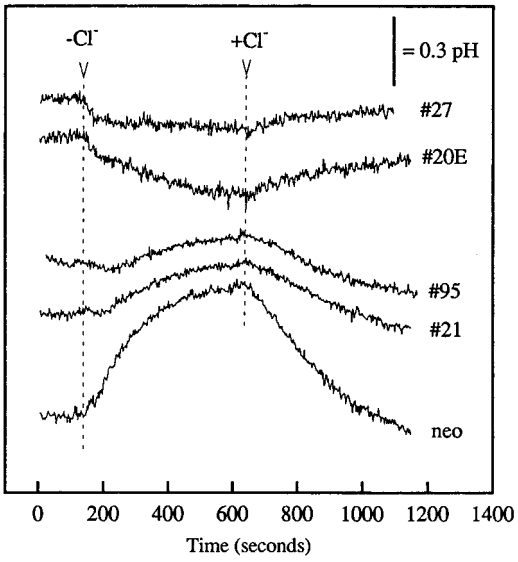

FIGURE 1: Results of isotonic Cl^- substitution experiments in the presence of CO_2/HCO_3^- for LR73/neo (bottom) and four representative LR73/hu MDR 1 clones selected on G418 only. LR73 AE activity (*23*) is inhibited in the MDR clones. Overexpression of hu MDR 1 to high levels (clones no. 27 and no. 20E, top two traces) leads to anomalous acidification upon Cl^- substitution. Traces shown are the average of at least six experiments performed with individual cells from separate cover slips. Note traces are offset for comparison purposes, resting pH_i (starting point for the traces) for LR73/neo, no. 21, no. 95, no. 20E, and no. 27 are 7.14, 7.38, 7.37, 7.51, and 7.53 $\pm$ 0.04 units, respectively. See refs 19 and 23 for demonstration that the BCECF 439/490 excitation vs pH_i response in MDR vs control transfectants is virtually identical. Thus, although calibration is required for accurate determination of steady state pH_i, a given *change* in 439/490 excitation represents a similar change in pH_i for the different cell lines; this is explicitly verified in each experiment by titrating with K^+/nigericin buffers at different pH (*32*) at the end of each experiment as described (not shown, see ref 29). Any minor variations in response are corrected by scaling individual scans to these K^+/nigericin points (see ref 29) which act as internal controls. The reference bar in the upper right-hand corner represents 0.3 pH units for each trace.

by equilibrating cells for extended periods of time (30−45 min) with buffer harboring high $[K^+]$ and variable $[Cl^-]$. We performed our calibration of trapped SPQ using the tributyltin/nigericin approach as described (ref 36 and see Results) and found this method to be quite reproducible and reliable.

RESULTS

Figure 1 presents the results of single-cell isotonic Cl^- substitution experiments performed using a modified protocol (see Materials and Methods and 29) relative to previous work (*23*,*33*) for control LR73/neo (bottom trace) and representative true LR73/hu MDR 1 transfectant clones exhibiting either high (no. 27, no. 20E, top two traces) or mild (no. 21, no. 95, middle two traces) levels of MDR 1 expression (see Figure 1 caption and ref 19). Southern blot analysis (not shown) reveals one full-length integrated copy of the hu MDR 1 cDNA in these clones; thus altered levels of expression in the different clones is not likely due to different cDNA copy number. Each trace in Figure 1 is the average of at least six experiments using six different cover slips and note that the traces are offset relative to each other for comparison purposes. In individual experiments the traces originate near the mean steady state pH_i for the cell lines, which is mildly (clones no. 21 and no. 95) or significantly (clones no. 27 and no. 20E) alkaline for the hu MDR 1 transfectants (see ref 19 and Figure 1 caption) relative to the control transfectant LR73/neo ($pH_i = 7.14 \pm 0.04$). Note that levels of MDR expression in clones no. 21 and no. 95 (see 19) are slightly lower than levels found in HCT15 or SKRC-15 colon and kidney carcinoma cell lines, respectively (see ref 37) and that levels of expression for no. 27 and no. 20E are analogous to those seen for Dox40, LR73/1-1, and DC3F-ADX, commonly used drug-selected human and hamster MDR cell lines (see ref 19). Thus, importantly, pH_i effects in the MDR clones are not due to overexpression of MDR protein to anomolously high levels.

Unless otherwise noted, results for clone no. 20E also hold for other individually isolated hu MDR 1 clones (no. 27, no. 24, no. 15E) expressing similar high levels of protein and results for one mild overexpressor (e.g., no. 95) hold for other individually isolated clones expressing similar mild levels of the protein (e.g., nos. 21, 71, and 8; see ref 19). We dilute out the possibility that behavior in these clones is due to a random recombination event during transfection by analyzing many different, individually isolated clones that exhibit similar levels of MDR protein overexpression. Also, we previously documented that BCECF loading efficiency was similar for the MDR 1 and control transfectants (*19*), and preliminary confocal microscopy data (not shown) does not reveal detectable differences in subcellular partitioning of BCECF during the 30 min time course of these experiments. Thus, altered character of the traces in Figure 1 is not due to different behavior of BCECF in the different cell lines. In addition, mock transfection with the neo resistance marker alone (bottom trace, Figure 1) or substantial overexpression of a mutant MDR protein unable to hydrolyze ATP or confer the MDR phenotype in LR73 cells (cell line 88-8, see refs 38 and 23) does not significantly affect the behavior measured for LR73 cells in these Cl^- substitution experiments (not shown), thus characteristics of the MDR clones are not likely due to selection for the presence of the neomycin resistance marker with G418 (see Materials and Methods) or to a nonspecific effect of overexpression of a large membrane protein in these cells. Considered together, these observations indicate changes in the BCECF traces upon overexpression of functional MDR protein represent ion-specific perturbations in pH_i regulation that is normally present in LR73 fibroblasts.

We previously documented that isotonic Cl^--substitution experiments monitored by SCP reveal apparent Na^+-independent Cl^-/HCO_3^- exchange activity (AE) for LR73/neo control cells that is likely catalyzed by the AE2 isoform.[2] Upon imposition of a Cl^- chemical gradient oriented outward, Cl^- effluxes out of the cell via the Cl^-/HCO_3^- exchanger and thus pH_i increases due to stoichiometric influx of HCO_3^- (first arrow, bottom trace in Figure1). The reverse is seen when Cl^- is re-introduced (Cl^- influx, HCO_3^- efflux, resulting in reacidification and return to baseline pHi; second arrow, bottom trace in Figure 1). Mild fold-overexpression of hu MDR 1 protein in LR73 cells (e.g., clones no. 21 and

[2] Cl^- susbtitution experiments are a convenient way to analyze Cl^-/HCO_3^- exchanger activities (*39*). In previous work, AE2 isoform-catalyzed Cl^-/HCO_3^- exchange activity was assigned to LR73 cells and control LR73/neo-transfectants based on inspection of ion dependencies, a K_i for SITS of 50 μM, characteristic pH_i vs H^+ flux behavior, and the presence of AE2 mRNA (but not AE1 or AE3 mRNA) via gene-specific northern blot analysis (*23*,*33*).

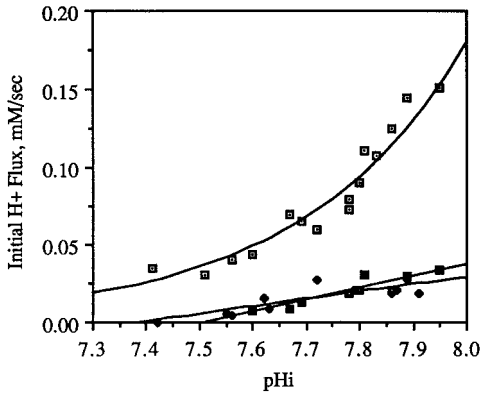

FIGURE 2: H^+ flux/s vs pH_i for control transfectants (open squares) and hu MDR 1/LR73 transfectants expressing modest amounts of MDR 1 protein (clones no. 21 and no. 95, closed diamonds, and squares, respectively) upon re-introduction of Cl^- in the SCP perfusate (e.g., rate of recovery at the second arrow, Figure 1, vs pH_i at which recovery is initiated). Each point represents a different experiment with a different cell on a different cover slip. H^+ flux/s is calculated by multiplying the pH_i change/s by β_i as described (*23*). Not only is residual AE activity delayed and damped in the MDR clones, but "forward" AE activity (i.e., Cl^- in, HCO_3^- out) appears to be anomalously regulated. Data are fit by either an exponential equation (for the controls) or a straight line (for the MDR 1 clones), $r^2 > 0.90$ in each case.

no. 95) leads to AE that exhibits three unique features relative to the control: (i) initiation of alkalinization upon Cl^- removal (first arrow) is delayed by 45−60 s or longer, (ii) the extent of the pH_i change produced upon Cl^- removal (height of the alkalinization curve) is damped, and (iii) the recovery upon replacing Cl^- in the perfusate is dysregulated, meaning that the rate of recovery of pH_i does not exhibit the same pH_i dependency as does Cl^-/HCO_3^- exchange for the control (see Figure 2). We will refer to this phenotype as "3-D" (delayed, damped, dysregulated) AE. The 3-D AE phenotype for these mildly overexpressing clones is somewhat similar, but not identical, to the altered Cl^--dependent pH_i regulation previously characterized for one LR73/mu MDR 1 clone via a somewhat different Cl^- substitution protocol (*23*). Differences between past and present data are likely due to (i) different levels of MDR protein expression in the different clones, (ii) perhaps expression of different MDR isoforms (mu vs hu), or (iii) modifications to our ion substitution protocol (see Materials and Methods and ref 29) or to some combination of these.

Overexpression of high levels of hu MDR 1 protein in LR73 cells (clones no. 20E and no. 27, as well as independently derived clones no. 24 and no. 15E that express similar levels of MDR 1, not shown) leads to even more dramatic perturbations of AE. Upon establishing an outward flux of Cl^- for these cells by isotonic Cl^- substitution, they acidify (top two traces, Figure 1). The acidification is reversed upon replacing Cl^- in the perfusate (second arrow, Figure 1). Over a 500 s time course of perfusion with Cl^--free perfusate buffered with CO_2/HCO_3^-, the acidification is relatively mild (<0.2 units). In some experiments, the acidification plateaued sharply (e.g., top trace, Figure 1, clone no. 27) whereas in others [particularly for clone no. 20E, which has the highest level of hu MDR 1 expression we have yet observed in a true transfectant (about 5% − 10% higher than expression for clone no. 27, shown in ref 19)] a mild, fast initial acidification is followed by a steady continued acidification that frequently did not appear to plateau within 500 s.

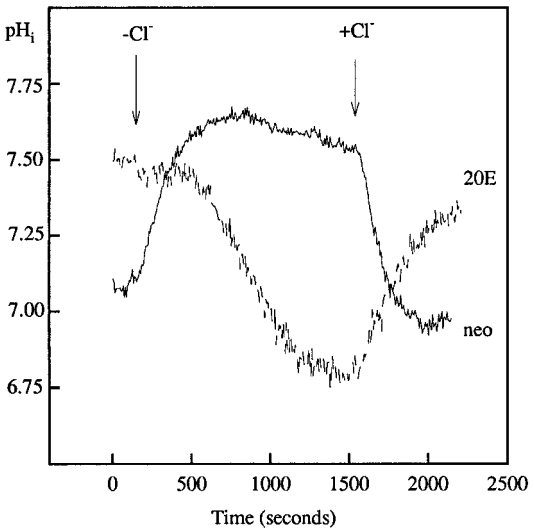

FIGURE 3: Longer-term isotonic Cl^- substitution experiment for control (top solid trace) and hu MDR 1 clone no. 20E (dashed trace). The first arrow indicates the substitution of glutamate for Cl^-, the second arrow denotes substitution of Cl^- for glutamate in the perfusate. The "base line" alkaline pH_i achieved upon Cl^- substitution for the control cells is relatively stable for 20 min in Cl^--free buffer, although there is a mild drift toward ΔpH equilibrium. In contrast, pH_i for the MDR 1 overexpressor is relatively stable for the first 350 s after Cl^- substitution but then begins to decrease dramatically. Upon return of Cl^- to the perfusate (second arrow), pH_i recovers for the MDR clone in a pH_o and $[Cl^-]_o$-dependent manner (see Figures 6 and 7). Data shown are representative of dozens of experiments with four independently selected MDR 1 clones.

To examine this behavior more thoroughly we performed isotonic Cl^- substitution experiments over longer (15−20 min) time periods. In the presence of CO_2 and HCO_3^- (Figure 3), a "long-term" Cl^- substitution experiment reveals even more remarkable behavior for the high-level MDR 1 overexpressors. Initial mild acidification is followed by a rather extensive acidification (about 0.7 ± 0.12 units in eight separate experiments for clone no. 20E; e.g., dashed trace, Figure 3). Acidification is quickly and fully reversed when Cl^- is replaced in the perfusate (second arrow, Figure 3). The rate of Cl^--dependent realkalinization upon reintroduction of Cl^- is highly dependent on pH_o and $[Cl^-]$ (see below) and is quite similar to the rate of acidification upon removal of Cl^-. Interestingly, extensive acidification in the presence of CO_2 and HCO_3^- is not initiated until about 350 s after Cl^- substitution. Conspicuously, this is the same time at which normal AE activity in the control cells plateaus, presumably because cytosolic $[Cl^-]$ has fallen significantly below K_m for the AE2 exchanger isoform present in these cells (*23*). Importantly, however, total $[Cl^-]_i$ is not zero at 350 s after isotonic Cl^- substitution. Similar Cl^- substitution experiments using the probe SPQ (see Materials and Methods) to monitor loss of intracellular Cl^- directly (Figure 4A)[3] reveals that under these conditions loss of Cl^-_{in} does not begin to plateau until at least 15 min after initial Cl^- substitution. Thus, extensive acidification in the MDR clones

[3] Upon switching to perfusate that is 30%−40% hypotonic, the leak rate of SPQ increases about 20-fold for both cell types; thus, although interesting differences between traces for the control and MDR transfectants are still observed, with our current protocols we were unable to unequivocally determine whether hypotonicity stimulates anomalous Cl^- transport in these transfectants as has been reported for other MDR cells (*3*,*20*).

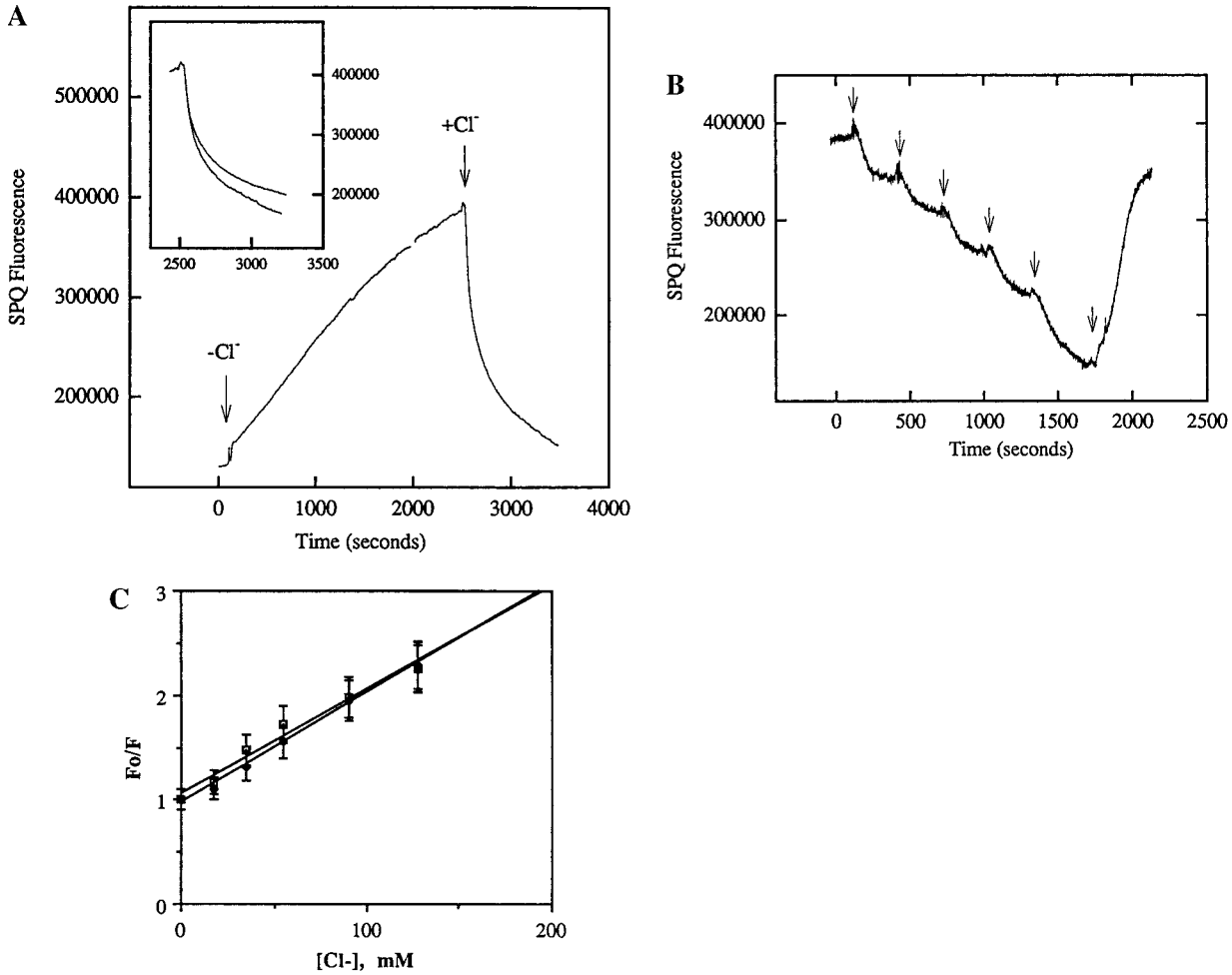

FIGURE 4: (A) Efflux and influx of Cl^- monitored directly via the increase and decrease, respectively, in fluorescence of intracellularly trapped SPQ. Upon replacing Cl^- in the perfusate with glutamate (first arrow), the fluorescence of trapped SPQ increases as Cl^- exits because emission from the probe is quenched by halide binding. Upon return of Cl^- to the perfusate intracellularly trapped SPQ is rapidly quenched due to Cl^- influx. Importantly, association/dissociation of Cl^- from SPQ is not rate-limiting in these experiments (*36*). The trace in A is representative of three SPQ Cl^- substitution traces for clone no. 20E; no dramatic differences in the shape of this trace were found for the control transfectants, although $F(2500\ s)/F(0)$ (an empirical measure of intracellular Cl^- concentration, see B and C) was always slightly higher for 20E relative to the control. Note the flat base line before Cl^- removal; we find that SPQ is loaded to nearly identical levels in both control and hu MDR 1 transfectants under controlled conditions and that the rate of leak under constant perfusion with buffer minus SPQ is slow (<8%/h) and virtually identical for the two cell types (similar results were obtained by Verkman and colleagues, see ref 36). In general, these results are similar to those previously reported for 3T3 fibroblasts by Verkman and colleagues (*36*); however, the perfusate flow rate in (*36*) was about 15 mL/min whereas our flow rate is 5 mL/min. Since calibration curves (see B and C) for control and MDR clones are very similar, a given change in SPQ fluorescence represents a similar change in $[Cl^-]_i$. (Inset) Influx of Cl^- into clone no. 20E in the absence (bottom trace) or presence (top trace) of 15 μM verapamil (see text). (B) Calibration of trapped intracellular SPQ response for LR73/hu MDR 1 (clone no. 20E) transfectants. Cells were perfused with HBSS for 10 min, loaded with SPQ as described, and then perfused with Cl^--free buffer (120 mM K^+ gluconate/100 mM mannitol/25 mM HEPES/10 mM Ca^{2+} gluconate/5 mM glucose, pH 7.4; see ref 36) containing 5 μM nigericin and 10 μM tributyltin to equilibrate $[Cl^-]_o$ with $[Cl^-]_i$. The change in trapped SPQ fluorescence was monitored during this equilibration period, and after a plateau was reached (in about 5 min) intracellular (equal to extracellular) Cl^- was titrated by changing to perfusate harboring various concentrations of Cl^- (substituting for equimolar gluconate, see ref 36). The arrows indicate titration to 17, 35, 55, 90, 157, and 0 mM Cl^-, respectively. The difference in trapped SPQ fluorescence at the two different 0 mM Cl^- points (0 and 2000 s) represents the leak in trapped SPQ over this 30 min period. (C) F_o (SPQ fluorescence at 0 mM Cl^- minus background scatter, that is, SPQ fluorescence at infinitely dilute Cl^-, see ref 36) over F values at a given $[Cl^-]_i$ are plotted vs $[Cl^-]_i$. Open squares are data for clone no. 20E, and closed diamonds are data for LR73/neo cells. Data shown are the average (±SE) of three titrations as shown in B performed with three separate cover slips. Each data set was fit by a straight line, $R^2 > 0.97$ in each case. Note that the two curves superimpose, indicating that the $[Cl^-]_i$-dependent response of trapped SPQ is essentially identical for the two cell types (see footnote 3).

(Figure 3) may be catalyzed by continued outward flux of residual reversibly bound Cl^-_{in}. Alternatively, we could hypothesize that it is catalyzed by unusual movement of another ion in response to loss of intracellular Cl^- for the MDR 1 clones (e.g., Na^+ or K^+; the efflux of which could then promote acidification via Na^+/H^+ or K^+/H^+ exchangers, respectively, see below).

Return of Cl^- to the perfusate in the SPQ experiment (Figure 4A) shows that the net cellular accumulation of Cl^- is faster than efflux under these conditions. Calibration of the SPQ response (Figure 4B,C) reveals that loss of total intracellular Cl corresponds to a net change of >40 mM. Similar results have been obtained by others in experiments with 3T3 fibroblasts (*36*); 40 mM is above most estimates of cytosolic $[Cl^-]$ for mammalian cells, therefore, a slower release of reversibly bound and sequestered Cl^-_{in} likely contributes to this larger than initially expected change in SPQ fluorescence, and also likely explains slower efflux

kinetics relative to influx (2nd arrow, Figure 4A). That is, at the first arrow in Figure 4A the SPQ experiment reveals the kinetics of dissociation of a pool of intracellularly bound Cl^- superimposed upon release of cytoplasmic Cl^-, whereas at the second arrow the faster kinetics of intracellular Cl^- binding (revealed in the kinetics of accumulation) are observed (see also *36*).

The novel acidification process tentatively linked to outward movement of Cl^- for the MDR 1 transfectants is quite remarkable and indicates that MDR 1 protein not only acts to inhibit endogenous AE activity as previously suggested (*18*, *23*, *33*) but that it competes against endogenous AE in these cells. That is, inward translocation of Cl^- via a pathway directly or indirectly mediated by hu MDR 1 protein appears to lead to cellular alkalinization (second arrow, dashed trace Figure 3), not to acidification as is catalyzed by the AE2 protein present in these cells (second arrow, solid trace in Figure 3).

We were not able in any way to mimic the effect found for the MDR 1 transfectants by manipulating the Cl^- or HCO_3^- gradients for the control cells or *vice versa.* That is, partially lowering $[Cl^-]_i$ or $[HCO_3^-]_i$ or raising $[Cl^-]_o$ or $[HCO_3^-]_o$ in these experiments via perfusion with buffers containing different concentrations of the ions (see Methods) did not produce "MDR" effects in the controls or reverse the behavior of the MDR transfectants such that they then behaved like the controls in Cl^- substitution experiments. For example, since the MDR clones are slightly alkaline (*19*) a larger outward-directed gradient in HCO_3^- exists relative to the controls. Producing a similarly larger HCO_3^- gradient for the controls by lowering $[HCO_3^-]_o$ during Cl^- substitution did not dramatically effect LR73 AE activity (not shown). Similarly, lowering $[Cl^-]_i$ for the MDR clones by perfusion with HBSS containing 50 mM Cl^- (balance glutamate to 120 mM) for 10 min prior to Cl^- substitution did not alter the effects seen in these clones (not shown). Thus, any mild alteration in Cl^- or HCO_3^- gradients (predicted by higher pH_i and lower membrane potential for the MDR clones, see ref 19) is not likely to be the explanation for the very unusual behavior shown in Figures 1−3 (see also section following).

HCO_3^-/CO_2 Dependency

We next tested whether the unique Cl^--dependent acidification process due to hu MDR 1 overexpression was dependent upon the presence of HCO_3^-/CO_2. Remarkably, the acidification that accompanies removal of extracellular Cl^- for the high-level overexpressors is even more dramatic (1.4−1.7 units; mean 1.57 ± 0.18 for nine experiments) when the substitution is performed in the absence of CO_2 and HCO_3^- (dashed trace, Figure 5). Also, acidification begins immediately upon Cl^- removal, not 350 s after removal as is the case in the presence of CO_2 and HCO_3^-, and is conspicuously faster than in the presence of CO_2 and HCO_3^-, presumably because AE2 is no longer competing against the MDR 1-catalyzed process. Similar Cl^- substitution experiments in the absence of CO_2 and HCO_3^- do not reveal significant changes in pH_i for the control transfectants (Figure 5, solid trace) or the untransfected LR73 cells (not shown).[4] Therefore, since it is apparently not dependent upon CO_2 and HCO_3^- we suggest that this process represents either a novel (directly catalyzed by hu MDR 1 protein) or anomalously regulated (endogenously expressed but regulated in some unusual fashion by hu MDR 1 protein overexpression)

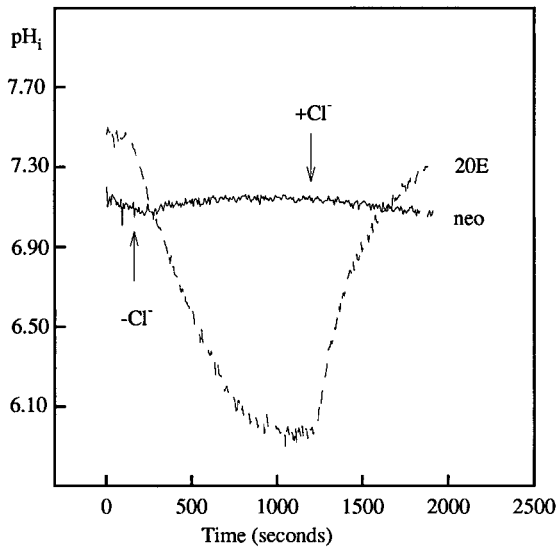

FIGURE 5: Long-term isotonic Cl^- substitution performed in the absence of CO_2/HCO_3^-. Cells were treated as described for experiments in Figure 3, except that they were perfused with HBS (140 mM NaCl/5 mM K Cl/20 mM HEPES/10 mM glucose/2 mM $CaCl_2$/1 mM $MgCl_2$, pH 7.35, 37 °C) for 10 min, and then perfusate was switched to Cl^--free HBS (glutamate isotonically replacing the Cl^-) at the first arrow and back again to normal HBS at the second arrow. No significant changes in pH_i occur for the control cells since HCO_3^- is absent and no AE2 activity is therefore possible (solid trace). In contrast, MDR 1 clones no. 20E (dashed trace) or no. 15E, no. 24, or no. 27 (not shown) acidify extensively upon Cl^- substitution in the absence of HCO_3^-, clearly suggesting acidification in Figure 3 is due to H^+ influx coupled to the Cl^- efflux instigated by the isotonic Cl^- substitution protocol. Note the symmetrical nature of the curve for no. 20E, i.e., recovery of pH_i upon replacing Cl^- is similar in rate to the initial acidification. This suggests the Cl^- and H^+ movements are coupled. Data shown is representative of dozens of experiments with four independently selected MDR 1 clones.

Cl^--dependent H^+ transport process. In analogy to the function of the MDR 1 homologue CFTR, which both directly translocates Cl^- and modulates the activities of other ion channels (*30*), it is also possible that the behavior represents the sum of two or more processes acting together, perhaps one directly catalyzed by hu MDR 1 protein and the other catalyzed by another protein that is regulated by MDR 1. Since the acidification process upon Cl^- removal apparently involves inward movement of H^+ instead of outward movement of HCO_3^- , this explains why acidification occurs immediately upon removal of Cl^- in the absence of CO_2 and HCO_3^- but is delayed by 5−6 min in their presence. That is, inward H^+ movement upon Cl^- substitution (via a Cl^--dependent process catalyzed directly or indirectly by MDR protein) competes with reverse AE mediated by endogenous AE2 (outward Cl^- flux coupled to an inward HCO_3^- flux upon Cl^- substitution) for 5−6 min when the experiment is performed in the presence of $CO_2/$

[4] In some previous experiments (*23*) we saw an instantaneous, small change in pH_i in the acid direction upon Cl^- substitution, with anion exchange behavior superimposed on top of the effect when experiments were performed in the presence of CO_2/HCO_3^-. Subsequent work and modification of our SCP protocol (28,29 and see methods) has revealed these acid "blips" were likely the result of incomplete equilibration with serum-free buffer, faster perfusate flow rates, switching perfusate with a manifold (leading to slight pressure changes on the cells), inconsistent CO_2 equilibration of buffers, or some combination of these. In contrast to the effects analyzed in the present work, they were not directly due to Cl^- movement.

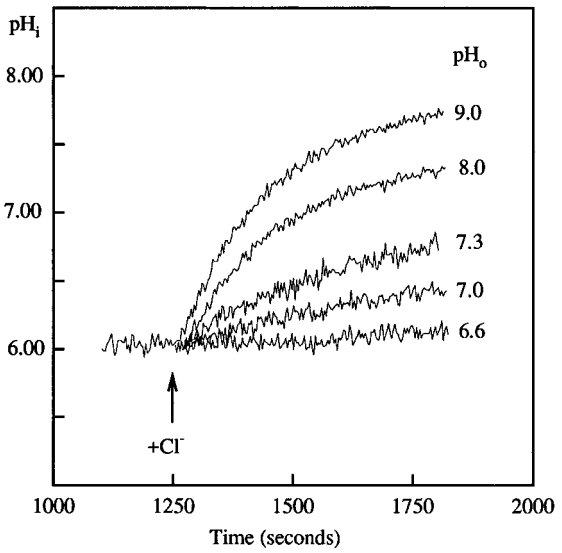

FIGURE 6: Effect of pH_o on Cl^--dependent realkalinization for clone no. 20E. We show recovery of pH_i upon reintroducing HBS containing 150 mM Cl^- but buffered at different pH (from bottom; 6.60, 7.00, 7.30, 8.00, 9.00). Clearly, recovery is highly pH_o dependent, as would be expected for a H^+ transport reaction. Cl^--efflux induced acidification was to the same pH_i in each experiment (e.g., to the same baseline before addition of Cl^-) as verified by K^+/nigericin titration after each experiment (not shown, see Materials and Methods and ref 29).

HCO_3^-, such that little net change in pH_i occurs until intracellular Cl^- is depleted sufficiently below the AE2 isoform K_m for Cl^-_i. After the reverse AE2 activity plateaus, the MDR-catalyzed process then dominates and acidification coupled to loss of remaining intracellular Cl^- is evident. In the absence of CO_2/HCO_3^-, AE2 activity is abolished, and thus only the MDR 1-catalyzed process is observed. Hence, acidification is faster and more extensive. Return of Cl^- to the perfusate in the absence of HCO_3^-/CO_2 (second arrow, Figure 5) then drives H^+ efflux as Cl^- influx occurs. This symmetrical reversibility, as well as Cl^-/H^+ stoichiometry and other characteristics (see below), suggests some type of reasonably well-coupled ion antiport process is catalyzing these effects.

Extracellular pH and [Cl$^-$] Dependencies

To test this idea further, we measured the pH_o and $[Cl^-]_o$ dependencies of the Cl^--induced realkalinization process for clone no. 20E (Figures 6 and 7). As shown, the realkalinization process is highly dependent on the extracellular concentration of H^+, being very slow at $pH_o \leq 6.6$ but accelerated nearly 10-fold at $pH_o = 9.0$ relative to $pH_o = 7.3$ (Figure 6). In addition, the rate of realkalinization is also dependent upon the magnitude of the initial Cl^- gradient (Figure 7), with an apparent K_m for extracellular Cl^- near 75 mM (see Figure7B). The rate of alkalinization is maximal at 100−150 mM Cl^-_o. The results indicate that the rate of the Cl^--dependent alkalinization process in the MDR 1 transfectants is dependent on the magnitude of both the H^+ and Cl^- gradients and suggests that the novel movement of H^+ in the MDR clones may be coupled to movement of Cl^-. Although it is quite surprising, the simplest interpretation of these observations is that MDR 1 somehow (directly or indirectly) catalyzes Cl^-/H^+ antiport. This would be an electrogenic process involving net movement of −2 charge (assuming 1:1 stoichiometry, see below), thus it should be sensitive to membrane potential.

Na$^+$ and K$^+$ Dependencies and Sensitivity to Amiloride and Other Compounds

Figure 8 shows that replacing extracellular Na^+ with K^+ does not significantly affect the AE activity measured for LR73/neo upon Cl^- substitution in the presence of CO_2/HCO_3^- (solid trace) but does reverse the hu MDR 1 effect found in the high-level overexpressors (dashed trace). Although clone no. 20E (as well as clones no. 15E, no. 24, and no. 27, not shown) now alkalinizes upon Cl^- substitution instead of acidifying, Cl^- dependent recovery from alkalinization (apparent "forward" AE) is still somewhat impaired in the MDR 1 clones (second arrow, top trace in Figure 8).

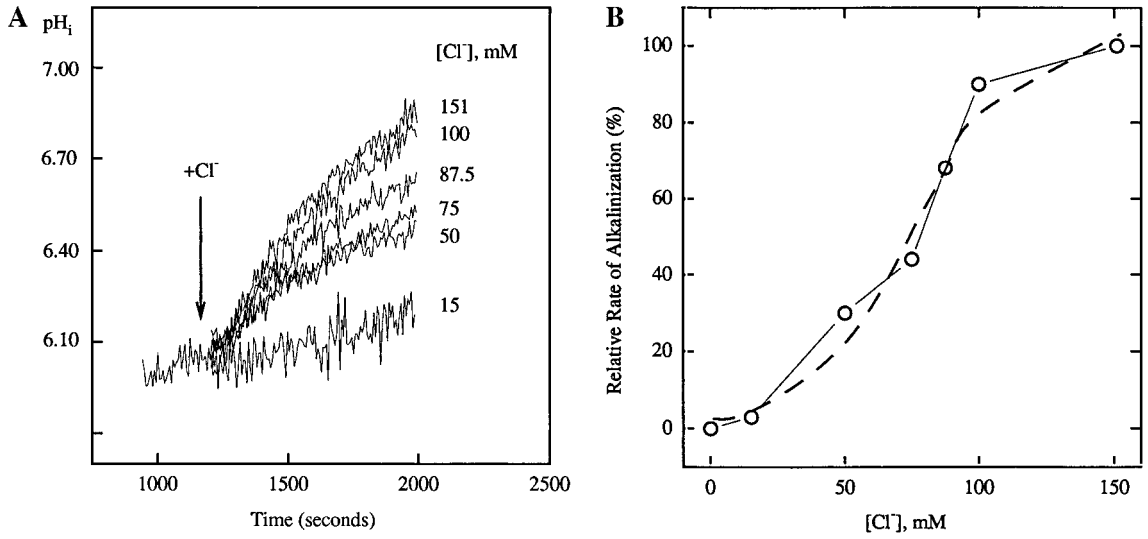

FIGURE 7: Effect of $[Cl^-]_o$ on the Cl^--dependent realkalinization for clone no. 15E measured in the absence of CO_2/HCO_3^-. (A) Actual recovery of pH_i upon changing HBS/glutamate to HBS containing different concentrations of Cl^- (balance to 150 mM with glutamate); from bottom, 15, 50, 75, 87.5, 100, and 151 mM. External pH is 7.5 during recovery of pH_i. Cl^--efflux induced acidification prior to restoration of Cl^- was to the same pH_i in each experiment. (B) Plot of the rate vs $[Cl^-]$ data and fit of an equation of the form $Y = a/(1+\exp(b(x-c)))$ using an iterative least-squares procedure (SigmaPlot software). After convergence in nine iterations, $a = 104.4$, $b = -0.05224$, and $c = 74.71$.

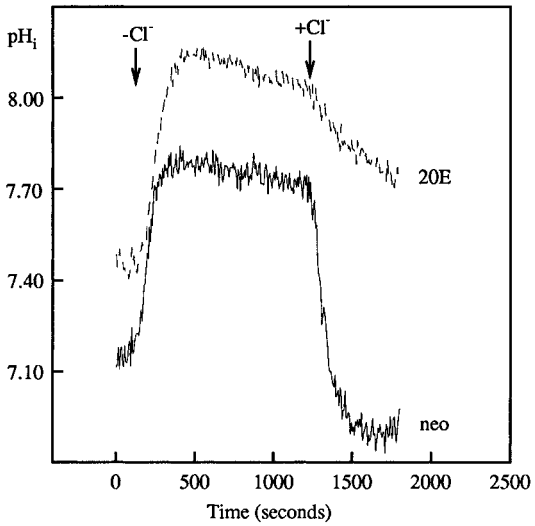

FIGURE 8: Effect of replacing perfusate Na^+ with K^+ (149 mM K^+ replacing 144 mM Na^+/5 mM K^+ in normal HBSS) on the pH_i changes promoted by isotonic Cl^- substitution for control (solid trace) and clone no. 20E (dashed trace) cells. Data shown are representative of many experiments performed with different cover slips. Interestingly, high extracellular K^+ reverses the anomalous behavior for clone no. 20E (as well as clones no. 15E, no. 24, no. 27, not shown) shown in Figure 3. Similar experiments with NMG^+ replacing Na^+ instead of K^+ also reverse the MDR effect, thus the effect is due to removal of Na^+, and not necessarily any depolarization caused by K^+ substitution (see ref 19) or to altering the K^+ chemical gradient. Recovery from alkalinization upon readdition of Cl^- is still somewhat impaired for clone no. 20E, but the rate of alkalinization upon Cl^- removal is similar to that of the control.

Similar reversal of the MDR 1 effect is also observed upon substituting *N*-methylglucamine (NMG^+) for Na^+ (not shown); thus the effect is not due to the presence of high extracellular K^+ but rather to removal of Na^+.

We also depleted clone no. 20E of intracellular K^+ via perfusion with K^+-free (balance Na^+) perfusate for 30 min and then performed isotonic Cl^- substitution. Although these K^+-free perfusion conditions likely do not lower $[K^+]_{in}$ to zero, since we do not observe any restoration of AE or decrease in the MDR 1 effect at all (not shown), outward movement of K^+ is likely not obligate for the acidification catalyzed by MDR 1 upon Cl^- substitution. In addition, after intracellular acidification plateaued for the MDR 1 clones upon Cl^- substitution in either the presence or absence of HCO_3^-, we re-introduced Cl^- using completely K^+ free buffers. Virtually identical Cl^--dependent realkalinization was observed (not shown); thus the novel transport process is not K^+ dependent.

The Na^+ dependency and HCO_3^- independency suggest that acidification could, in theory, represent some significant dysregulation of the Na^+/H^+ exchanger (NHE) that is somehow connected to efflux of Cl^- in the MDR 1 clones. Such an effect via overexpression of an ABC transporter would not even be unprecedented, since we have recently shown that CFTR may modulate NHE activity (*29*). To directly test this possibility, Cl^- substitution experiments were performed in either the presence or the absence of HCO_3^- and CO_2 and in the presence of the well-known NHE inhibitors amiloride (100 μM) or ethylisopropyl amiloride (EIPA), a more potent amiloride analogue (50 μM). No effect on the MDR 1-catalyzed process was observed (not shown). Similarly, 100 μM concentrations of other inhibitors of Na^+-dependent Cl^- transport processes, such as bumetanide, furosemide, and chlorothiazide, did not have any significant effect upon the Na^+-dependent Cl^-/H^+ transport process catalyzed directly or indirectly by MDR 1 protein nor did 100 μM levels of SITS or DIDS (not shown). Taken together, these inhibitor and ion-dependency data suggest that the pH_i effects due to MDR protein are not due to altered regulation of known Cl^-/HCO_3^-, K^+/H^+, or Na^+/H^+ exchangers, Na^+:HCO_3^-, K^+:HCO_3^-, Na^+:Cl^-:2 HCO_3^-, Na^+:K^+:2Cl^-, or Na^+:Cl^- co-transporters, or any combination of these.

However, the apparent Cl^-/H^+ exchange process is indeed Na^+ dependent. To determine whether this indicates that movement of Cl^- and H^+ is also coupled in some fashion to movement of Na^+, we analyzed realkalinization in the presence or absence of an inward-directed sodium gradient and in the presence of 50 μM EIPA to inhibit any possible NHE activity that might be promoted under these conditions. A Na^+ gradient was produced after intracellular Cl^- depletion by additional perfusion with Na^+ free HBS (NMG^+ substituting for Na^+) for 5 min, and then restoration of Na^+ in the perfusate when Cl^- was also restored (e.g., at a time corresponding to the second arrow in Figure 5). We did not measure any enhancement in the rate of Cl^--induced intracellular alkalinization in the presence of an induced Na^+ gradient at a range of pH_o (not shown). Thus, although the process appears to be Na^+ dependent (Figure 8), we find no evidence to suggest that it can be accelerated by a Na^+ gradient.

Verapamil and Apparent Cl^-/H^+ Antiport Stoichiometry

We examined the effects of a known inhibitor of MDR 1 protein, verapamil (VPL). Importantly, inhibition of hu MDR 1 protein effects by nontoxic levels of VPL (as measured by relative drug resistance for pure MDR 1 transfectants, see ref 19) are not complete. Nonetheless we performed long-term Cl^- substitution experiments in the presence of VPL (Figure 9). Interestingly, 15 μM VPL strongly inhibited Cl^--dependent realkalinization (presumably, the "forward" or "physiologically relevant" reaction) but actually appeared to enhance the "reverse" (Cl^- out, H^+ in) reaction catalyzed directly or indirectly by MDR 1 protein, causing extensive acidification to occur earlier upon withdrawal of Cl^- in the presence of CO_2/HCO_3^-. This suggests to us that VPL might accelerate release of extracellular Cl^- in a putative catalytic cycle that could be used to explain the data in this paper (see Discussion).

This VPL effect, along with the ability to calibrate the Cl^- dependent response of intracellular SPQ, suggests a method for estimating the stoichiometry of Cl^- movement vs H^+ movement via the MDR 1 protein-mediated pathway. Thus, calibration of the VPL inhibitable component of Cl^- influx that occurs upon restoration of perfusate Cl^- after Cl^- depletion (Figure 4A, inset), using tributyltin and nigericin calibration curves as described by Verkman and colleagues (Figure 4B,C), along with parallel calibration of the VPL inhibitable initial H^+ flux that occurs in similar time under similar conditions [Figure 3 (dashed trace, second arrow) vs Figure 9 (bottom trace, second arrow)] via multiplication of the pH_i unit change per second by the intracellular

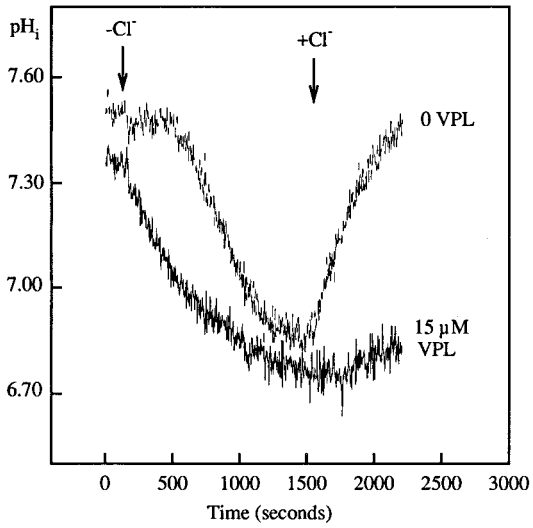

FIGURE 9: Isotonic Cl^- substitution for clone no. 20E performed in the presence of CO_2 and HCO_3^- and the absence (top trace) or presence (bottom trace) of 15 μM verapamil. The calcium channel blocker was present throughout the entire experiment. Note how verapamil accelerates the "reverse" reactions (Cl^- out/H^+ in) but strongly inhibits the "forward" (presumably physiologic) reaction (Cl^- in/H^+ out). Similar experiments with control cells reveals acceleration of the "reverse" reaction is not due to inhibition of endogenous AE2 by verapamil (not shown). If we assume a simple "ping pong" scheme for the apparent coupled Cl^-/H^+ antiport, then these effects suggest that verapamil may reduce the affinity of binding of extracellular Cl^- that is relevant for the translocation reaction. This would stimulate release of Cl^- from the external binding site during the "reverse" reaction, thereby accelerating the reverse process, but inhibit binding of external Cl^- in the "forward" reaction, thereby slowing the physiologically relevant reaction. This may be the mechanistic basis for how verapamil reverses the multidrug resistance mediated by overexpression of MDR 1 (*19*), and likely explains how verapamil normalizes pH_i for MDR cells exhibiting alkaline pH_i (*15*). 15 μM VPL is slightly toxic to these cells in long-term growth inhibition assays (see ref 19), but is not measurably cytotoxic over the 30 min exposures as performed in this experiment (M. M. Hoffman, LiYong Wei, and P. D. Roepe, unpublished).

buffering capacity, yields a stoichiometry of apparent Cl^-/H^+ exchange near unity.[5] Thus the apparent Cl^-/H^+ antiport appears to be well coupled.

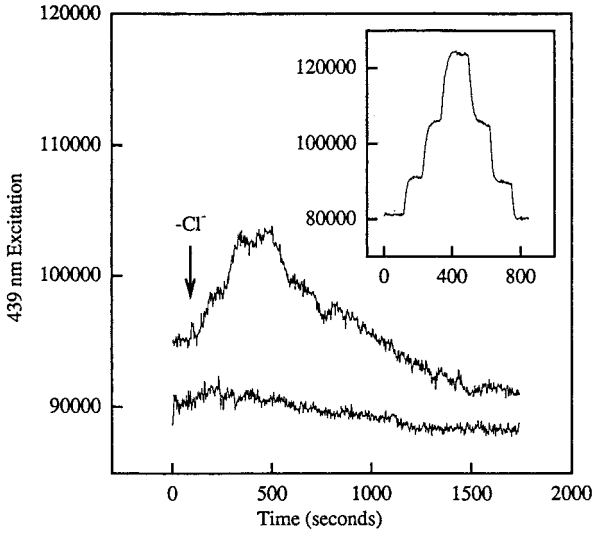

FIGURE 10: Changes in intracellular volume for single cells upon isotonic Cl^- substitution in the absence of CO_2/HCO_3^-. Bottom trace is control cell, top trace is clone no. 20E. Single-cell volume was measured using the method of Muallem and colleagues (*41*) by following the 439 nm BCECF excitation. Since 439 nm is isosbestic with regard to pH changes, the change in excitation is an empirical measure of internal volume; as a cell shrinks the dye becomes more concentrated, leading to enhanced excitation, the reverse occurs when a cell is swollen and the dye is diluted. Perfusion with solutions of various osmolarity (150, 300, 450, 600, 450, 300, and 150 mOs), as shown in the inset, verifies this to be the case and verifies that the 439 nm response vs solution osmolarity is surprisingly linear (see ref 41). Although this method does not allow precise quantitation of volume changes, we verified that for cells loaded to exactly the same level of BCECF (as measured by 439 nm excitation), the perturbation in 439 nm excitation was nearly identical for control and hu MDR 1 clones upon perfusion with solutions of various tonicity as shown in the inset. The cells analyzed in this experiment are loaded to virtually identical levels of BCECF (hence the similar initial fluorescence); thus, although we cannot quantitate the precise magnitude of the volume perturbation, the data suggest that rapid Cl^- substitution causes a greater, yet spontaneously reversible, perturbation in cell volume for MDR 1 transfectants relative to controls. Data shown are representative of many experiments.

Volume Pertubations and RVD-Associated Acidification

Since many pH_i regulatory processes also contribute to volume regulation (*40*), we analyzed the volume perturbations that accompany ion substitution using the isosbestic method of Muallem and collegues (*41*). Figure 10 shows that upon Cl^- substitution in the absence of HCO_3^-, no significant change in intracellular volume occurs for the control cells (bottom trace, Figure 10), whereas an interesting decrease and then spontaneous increase in volume occur for the high-level MDR 1 transfectants[6] (top trace, Figure 10). Initially, compensatory cation transport is presumably unable to adequately balance the electrogenic ion transport catalyzed directly or indirectly by MDR 1 when Cl^- is substituted, but this transport is then triggered within 5−7 min of cell shrinkage in order to complement an appropriate regulatory volume increase (RVI) response (see *42*). Importantly the kinetics of this volume perturbation do not match the kinetics

[5] The rate of Cl^--dependent H^+ translocation out of the MDR clones following intracellular Cl^- depletion is easily calculated from data like that shown in Figure 5, using KNH calibration of the BCECF response and the buffering capacity (β_i) determined as described (*33*,*39*). For example, in one experiment at $pH_o = 7.35$, $pH_i = 6.51$, we find that restoration of 150 mM Cl^- in the perfusate induces a rate of alkalinization that corresponds to transport of about 2.1×10^7 H^+/s for a single cell. The presence of verapamil completely inhibits alkalinization under these conditions. The rate of alkalinization is clearly dependent on pH_o and likely also dependent on pH_i; however, inspection of many Cl^- substitution experiments performed under identical conditions reveals the extent of the acidification upon perfusion with Cl^--free buffer for a given period of time is highly reproducible (1.11 ± 0.23 pH units at 1200 s after Cl^- removal, $n = 9$). Similar experiments performed for cells loaded with SPQ instead of BCECF reveal a component of Cl^- influx that is similarly inhibited by verapamil (Figure 4A inset). Using a calibration curve for the SPQ response as shown in Figure 4B,C we calculate that the verapamil-inhibitable Cl^- flux under these initial rate conditions is 2.9×10^7 Cl^-/s for a single cell. Thus, although we are not completely certain that pH_i is precisely the same in both the SPQ and BCECF experiments (which is a concern considering the process may be pH_i-dependent), we can tentatively conclude that the apparent stoichiometry of the process is near unity (0.74 ± 0.19 for three determinations). More precise calculation of Cl^-/H^+ transport stoichiometry will require detailed work with vesicles.

[6] The isosbestic 439 nm method does not allow for precise calibration of the magnitude of the change in V_i (see caption to Figure 10) but the calibration curve shown in the inset to Figure 10, along with other measures of tonicity induced changes in volume (D. Carlson and P. D. Roepe, unpublished) suggest the change in volume for the MDR 1 transfectants is <10% of the original cell volume.

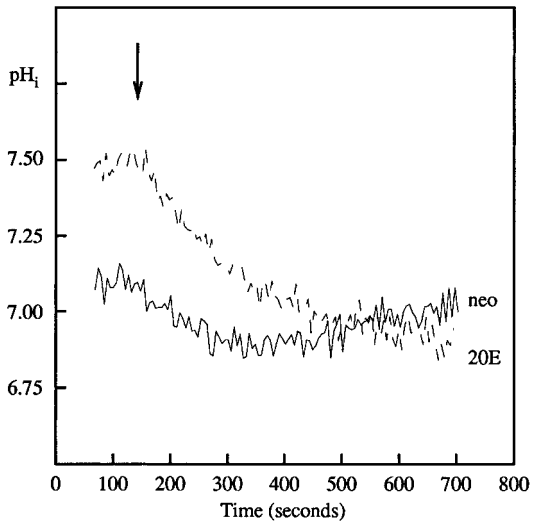

FIGURE 11: Changes in pH_i upon perfusion with 40% hypotonic HBSS (HBSS NaCl reduced to 70.8 mM) for control (solid trace) and hu MDR 1 (clone no. 20E, dashed trace) transfectants. The arrow denotes the switch from normal HBSS perfusate to hypotonic HBSS. Similar results are found for other control and other hu MDR 1 (e.g., nos. 24 and 27) clones. When cells swell, they generally react by effluxing Cl^- and K^+/Na^+ (the typical "RVD response", see ref 42). The results for the MDR clones are consistent with the presence of an additional process for Cl^- and/or cation exit coupled to intracellular acidification. Data shown are representative of dozens of experiments.

of the pH_i changes observed under similar conditions and they spontaneously reverse before Cl^- is reintroduced. We could propose that this volume perturbation might lead, secondarily, to the pH_i changes we observe in the Cl^- substitution experiments (Figure 3, etc.); however, the instantaneous change in pH_i shown in Figure 5 (pH_i changes associated with RVD or RVI usually occur over a longer time period), as well as the fully reversible, symmetrical nature of the pH_i changes and other features (see Discussion) argue that this is not the case.

When control or MDR 1 transfectants are swollen via perfusion with 40% hypotonic media, their intracellular volume increases by nearly the same extent (not shown, see caption to Figure 10), but changes in pH_i that accompany this volume perturbation are distinctly different (Figure 11). Control cells initially acidify to a mild extent but then reverse this acidification as normal RVD is presumably initiated (solid trace, Figure 11). The high-level MDR 1 overexpressors, however, acidify more extensively and continue to acidify even after RVD has presumably been initiated. The increased and continued acidification due to MDR 1 overexpression is not dependent on the presence of HCO_3^- or CO_2 (not shown), suggesting it is due to H^+ influx. This suggests that the apparent Cl^-/H^+ exchange process revealed in Figures 1 and 3 may also respond to volume perturbations under some conditions. That is, the pathway may contribute to hypotonicity-induced RVD in some cells by performing entirely called for Cl^- efflux, but a consequence of this is unusual influx of H^+ (coupled to the outward movement of Cl^- in response to the volume perturbation) resulting in abnormal intracellular acidification (see caption to Figure 11). If this pathway is a contributor to volume regulation for some cells, it might explain why certain volume perturbations lead to MDR 1 overexpression in colon, kidney, and liver tissue as recently described (*37*) and why similar overexpression is also seen upon perturbations in pH_o (*37*).

ATP Dependency

Since MDR 1 protein harbors two ATP binding sites and appears to hydrolyze ATP at a reasonable rate (500−1000 ATP/min, see ref 4), we performed Cl^- substitution experiments for controls and clone no. 20E under conditions that deplete intracellular ATP (Figure 12). ATP depletion does not (as expected) appear to significantly affect apparent AE2 activity for the control (bottom panel between second and third arrows, Figure 12). In contrast, after perfusion with HBSS minus glucose (plus 2 mM 2-deoxy-D-glucose and 50 nM rotenone) for 30 min in the presence of HCO_3^-/CO_2, the MDR 1 acidification effect is abolished (top panel Figure 12). Interestingly, the high-level overexpressors reproducibly acidify by a much larger extent (0.9 ± 0.2 units in four experiments) relative to the controls (0.35 ± 0.08 units in three experiments) upon ATP starvation (0−1000 s, Figure 12). Since these clones have alkaline steady state pH_i (19) this suggests that ATP is required for the "forward", presumably physiologic (Cl^- in, H^+ out) reaction catalyzed in some fashion by MDR 1 protein. Alternatively, note that after glucose starvation pH_i decreases in the MDR clone to near 6.5. Perhaps at this pH_i substantial intracellular acidification upon Cl^- substitution is not possible for any number of possible reasons (see Discussion). Notably, although it was possible in repeated Cl^- substitutions with the same cover slip (see caption to Figure 12), we had difficulty restoring apparent AE2 activity to the MDR 1 clones via ATP depletion, in contrast to the case for Na^+ depletion (Figure 8), where the MDR effect was reversed and some AE activity restored much more easily.

DISCUSSION

In the present study, detailed single-cell photometry analysis of "pure" hu MDR 1 transfectants reveals an apparent ATP-, Na^+-, and Cl^--dependent H^+ transport process catalyzed (directly or indirectly) by MDR 1 protein. This process is likely responsible for altering steady state pH_i and membrane potential in MDR cells. Inhibitor effects and ion dependencies reveal it is a novel process; however, note that some caution should be used in interpreting these data. Interesting as these effects are, and as helpful as they may be for modeling the function of MDR protein, they are measured using cell lines engineered to overexpress a rather large, polytopic integral membrane protein. Effects found for clones no. 27, no. 20E, etc. may not be exactly analogous to the effects expected for lower levels of endogenous hu MDR 1.

Several other reports have described unusual ion transport, pH_i, or volume regulation in MDR cells overexpressing mu or hu MDR proteins (reviewed in refs 6 and 11). However, many previous studies have investigated cells that were selected or maintained on chemotherapeutic drugs to promote high-level expression of MDR protein. In contrast, in this work and other recent studies (e.g., refs 12, 19, and 43) we have analyzed "pure" MDR transfectants that have not ever been exposed to chemotherapeutic drugs prior to analysis (see also refs 38 and 44 for data obtained with non-drug-selected MDR cells). This distinction is important, since exposure to potent chemotherapeutic drugs alters the behavior

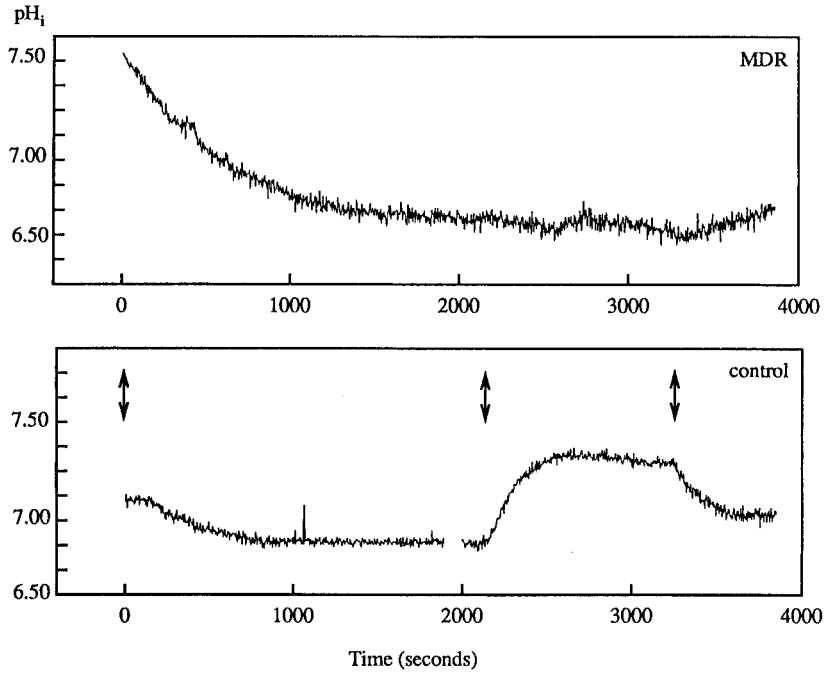

FIGURE 12: Effect of ATP depletion on the pH_i changes associated with isotonic Cl^- substitution for clone no. 20E (top panel) or control clones (bottom). The first arrow denotes the switch to HBSS perfusate without glucose and with 2 mM 2-deoxy-D-glucose and 50 nM rotenone to deplete intracellular ATP as described (*14*). Note that the MDR overexpressors acidify much more extensively during ATP depletion, relative to the controls, suggesting ATP is required for high pH_i in the MDR clones. Loss of intracellular ATP does not (as expected) appear to significantly affect apparent AE2 activity (bottom trace, revealed between second and third double arrows which denote removal and restoration of Cl^-), but abolishes the MDR 1-catalyzed Cl^-/H^+ antiport reaction (top trace). Interestingly, we are not able to easily recover normal AE2 activity for the MDR 1 overepressors. In some experiments, if a second or third Cl^- substitution was attempted with the same cover slip, mild apparent AE2 activity was observed for 20E (not shown). Data shown are representative of many experiments.

of cells and almost certainly induces additional drug resistance mechanisms that operate in addition to MDR protein (*19*, *29*). It is possible that as exposure to these potent drugs induces other drug resistance mechanisms, perhaps MDR protein effects become less necessary and are thus even down-regulated or modified. Indeed, we have recently shown (*29*) that the chemotherapeutic drug doxorubicin has effects on Na^+/H^+ exchange that complicate analysis of pH_i effects due to MDR 1 expression in doxorubicin-selected cells. Parenthetically, this doxorubicin effect could be the basis for unusual Na^+/H^+ overexpression in some members of a series of doxorubicin-selected MDR myeloma cells (*18*). The point is, aspects of novel pH_i regulation in our "pure" transfectants clearly due to MDR 1 protein overexpression may be modified in chemotherapeutic drug-exposed cell lines, and the possibilities should be considered when comparing published data on pH_i regulation in MDR cells. Similarly, apparent contradictions in the literature surrounding other aspects of altered ion transport in MDR cells may also be due, in part, to a variety of possible "superimposed" drug exposure effects in various model MDR cell lines.

In any case, since $[Cl^-]_o$ is lower than $[Cl^-]_i$ within 1−2 s in our rapid ion-substitution experiments and since this outward-directed Cl^- gradient is larger than any outward directed HCO_3^- gradient that exists for these MDR clones, "reverse" AE (alkalinization due to influx of HCO_3^- coupled to efflux of Cl^-) should begin upon Cl^- substitution, since the MDR 1 transfectants do indeed express levels of AE2 that are actually slightly higher than those for the control (not shown). However, this is not what is observed in the presence of physiologic levels of Na^+ and K^+. In the absence of Na^+, AE2 appears to be functional in the MDR clones, thus lack of activity in the presence of Na^+ is apparently not due to decreased expression of functional exchanger or to interaction between hu MDR 1 and AE2 unless that interaction is Na^+ dependent.

We can draw a phenomenological model consistent with the present data (Figure 13). We stress that this model is phenomenological; we do not yet rigorously know if the diagrammed ATP- and Na^+-dependent Cl^-/H^+ antiport process is directly catalyzed by MDR protein (i.e., does MDR protein directly move Cl^- and H^+?), indirectly catalyzed (does MDR protein modulate/regulate other endogenously expressed proteins in some unusual way that then catalyzes these reactions?), or some combination of the two. Importantly, however, our analysis of ion dependencies and inhibitor sensitivities eliminates the involvement of Cl^-/HCO_3^-, K^+/H^+, or Na^+/H^+ exchangers, Na^+:HCO_3^-, K^+:HCO_3^-, Na^+:Cl^-, Na^+:Cl^-:$2HCO_3^-$, or Na^+:K^+:$2Cl^-$ cotransporters in this diagrammed process. Thus we favor the interpretation that we have isolated a novel process that is at least in part directly catalyzed by MDR 1 protein, since (neglecting certain channels) the above list of ion transporters represents the common participants in cell pH_i and volume regulation.

However, detailed measurements with purified protein will be required to completely distinguish between the direct vs indirect ion transport possibilities. With regard to indirect possibilities, it is formally possible that an unusual volume perturbation in the MDR clones (Figure 10) caused by fast Cl^- efflux in our experiments is indirectly responsible for causing the pH_i changes we measure (see also Figure 11) since volume and pH_i regulation are linked. However, we cannot in any way mimic the pH_i effects by perfusing with

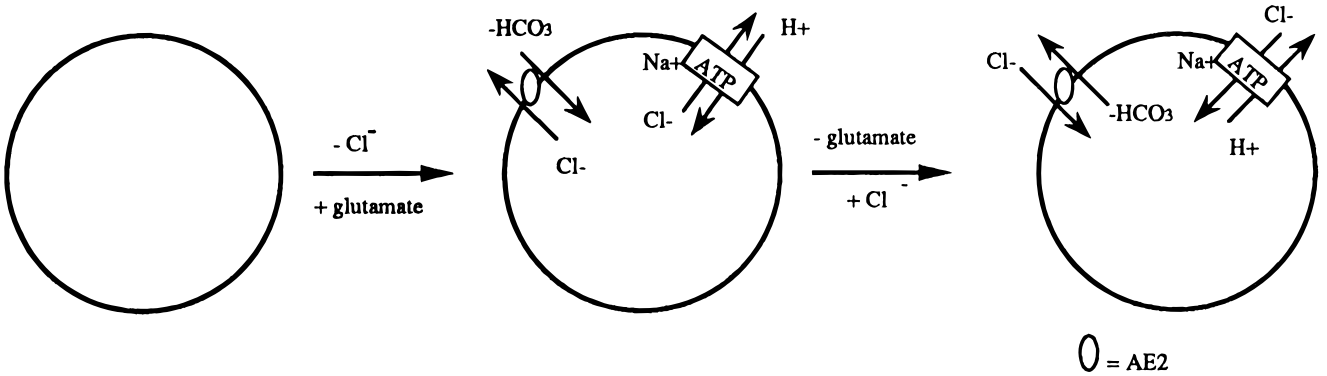

FIGURE 13: Phenomenological model that represents one possible explanation for the data in this paper. *We stress* that we have not shown whether the net ion transport reaction depicted is directly or indirectly catalyzed by MDR 1 protein. However, the dramatic and symmetrical changes in pH_i upon induced Cl^- flux, the stoichiometric relationship between the unique verapamil-inhibitable Cl^- and H^+ flux, and the Na^+ and ATP dependencies are consistent with this scheme. We propose that energy for the uphill movement of Cl^- and H^+ is provided either by movement of Na^+ or hydrolysis of ATP. Although it is a negative result, our inability to accelerate apparent Cl^-/H^+ antiport with a Na^+ gradient (see text) argues for the latter.

a variety of hypertonic or hypotonic buffers under a variety of conditions (not shown). Also, the symmetrical, reversible nature of the Cl^- flux-driven pH_i changes, the magnitude of these pH_i changes relative to those seen upon volume perturbations, and the apparent stoichiometric relationship between the Cl^- flux and H^+ flux mediated by MDR 1 (see footnote 5) further argue that this explanation is less attractive. Alternatively, pH_i changes in the MDR clones might be the result of passive H^+ transport in response to a perturbation in membrane potential caused by unusual Cl^- flux during our Cl^- substitution protocol, but if this is the case, the symmetrical nature of the Cl^- substitution pH_i transient for the MDR clones obtained in the absence of CO_2/HCO_3^- (Figure 5) argues that the perturbation is fully reversible under these conditions and that the passive H^+ movement occurs with the same rate in each direction. Thus this possibility also appears less likely to us.

Whatever the precise molecular explanation, outward flux of intracellular Cl^- upon Cl^- substitution results in intracellular acidification for pure LR73/hu MDR 1 transfectants, apparently due to some type of Cl^--coupled H^+ influx (Figure 13). In the presence of HCO_3^- the acidification predicted to accompany H^+ influx is "damped" for 5 min or so (Figure 3) because "reverse" AE2 activity (which results in influx of HCO_3^- coupled to efflux of Cl^-) is also functioning. Thus, a prediction of the model is that cell functions catalyzed by Cl^-/HCO_3^- exchangers may be compromised or modulated by overexpression of MDR 1 protein. This may explain why cells frequently overexpress AE isoform mRNA upon overexpression of MDR 1 (*18*,*23*,*29*): they may be attempting to compensate for the predicted AE competition. The ratio of relative MDR 1:AE activity should determine the net result (acidification or alkalinization) in a Cl^- substitution experiment for a given cell. Thus, an attractive explanation for the behavior of clones no. 21 and no. 95 (which express a lower amount of MDR 1 protein) is that Cl^--dependent acidification due to MDR 1 is also superimposed on AE2-catalyzed alkalinization in these clones, but because of the lower MDR 1:AE2 protein ratio a "hybrid" 3-D AE phenotype is observed.

In the absence of CO_2 and HCO_3^- the acidification process catalyzed directly or indirectly by MDR 1 protein upon efflux of Cl^- is not delayed and is more extensive, presumably because competing influx of HCO_3^- via the AE2 is obviously not possible. The high levels of overexpression in these clones, as well as our signficantly improved SCP protocol, make the putative Cl^-/H^+ antiport process relatively easy to isolate under these conditions.

The more important implications of the Cl^--dependent H^+ transport are revealed upon return of Cl^- to the perfusate. As Cl^- rushes back into the transfectants under these conditions, H^+ efflux results in intracellular alkalinization. This process presumably represents the physiologically relevant reaction catalyzed directly or indirectly by MDR 1 protein, since the MDR transfectants are alkaline relative to controls (*19*). Importantly, however, although under physiologic conditions the Cl^- chemical gradient is pointed inward, the driving force for transport of Cl^- in eukaryotic cells is also determined by the membrane potential. Thus, for most eukaryotic cells, inward movement of Cl^- is uphill electrochemically. Therefore, under physiologic conditions alkalinization of MDR clones by this putative Cl^-/H^+ antiport process would require energy. This could be provided by ATP, which may also be required for the reaction (Figure 12), or by movement of Na^+, since the reaction is also Na^+ dependent. However, importantly, we could not measure any acceleration in Cl^- flux-driven H^+ movement by imposing a Na^+ gradient. Thus, another possibility is that the ATPase activity of MDR 1 protein is Na^+ dependent in some fashion, but movement of Cl^- and H^+ is not explicitly coupled to movement of Na^+.

In either case, whether or not Na^+ is co-transported upon movement of Cl^-, the reaction may be electrogenic (unless there is a strict $2Na^+$:Cl^-/ H^+ stoichiometry to the process). Thus, another simple prediction of the model is that this transport is likely sensitive to the magnitude of the membrane potential. The fact that the process is likely electrogenic and that it results in altered permeability of the membrane to Cl^- is also consistent with the transport process affecting membrane potential for eukaryotic cells. We have indeed proposed that MDR 1 protein overexpression alters both pH_i and membrane potential regulation (*18*,*19*,*23*). However, MDR overexpressors are in general depolarized but net inward movement of Cl^- and OH^- (if that is indeed the physiologic reaction) might in and of itself, simplistically, be predicted to hyperpolarize some cells. On the other hand, the long-term cell biological consequences of elevated intracellular Cl^- in these cells (a possible consequence of

the putative transport that is also suggested by our SCP data; see caption to Figure 4) might be up-regulation of other (outward) Cl^- transport pathways (e.g., perhaps Cl^- channels), leading to an increased permeability of the membrane to Cl^-. Since the Cl^- permeability term in the Goldman/Hodgkin/Katz equation would then become more significant, depolarization would be predicted. Description of precisely how membrane potential is affected by the novel ion transport that we have uncovered will require more detailed analysis of ATP and ion dependencies than is possible with living whole cells and these methodologies.

In any case, if the model is reasonable, this putative ion transport process should also be sensitive to the magnitude of the transmembraneous Cl^- and H^+ gradients. Indeed, SCP experiments wherein different concentrations of Cl^- are reintroduced into the perfusate, as well as experiments wherein Cl^- is returned at different pH_o, show this to be the case. One parameter we have not yet examined is the pH_i dependency of the Cl^- flux catalyzed (directly or indirectly) by MDR 1 protein. This will be important for understanding the more detailed physiologic implications of this model and for understanding function in different cell types.

The apparent ATP dependency of the Cl^-/H^+ antiport process is interesting. This opens up the possibility that MDR 1 protein catalyzes ATP hydrolysis driven uphill (inward) Cl^- transport. The concept of whether electrogenic Cl^- translocating ATPases even exist, as well what physiologic function they might have, has been debated for quite some time (*45*−*47*). A popular opinion is that Cl^- translocating ATPases are unnecessary for Cl^- uptake in Cl^- absorbing epithelia (site of hu MDR 1 endogenous expression), since known Na^+:Cl^- co-transport processes and/or Cl^-/HCO_3^- exchangers presumably accomplish the task adequately. An electrochemical gradient in Na^+ would presumably provide adequate energy for Cl^- uptake. This is why even though we cannot find evidence for Na^+ gradient stimulation of the putative Cl^-/H^+ antiport, we consider a Na^+:Cl^-/H^+ phenomenological model more intuitively satisfying. Nonetheless, good evidence in favor of ATP-driven Cl^- pumps has been obtained from analysis of *Aplysia californica* foregut absorptive cells (*48*), and data supporting Cl^--dependent ATPase activity in the rat brain (*49*) have also been published. However, note in these cases the Cl^- pumps are moving Cl^- *out* of the cell. Thus, although it is consistent with the data, until these reactions are more thoroughly examined with vesicle and proteoliposome studies, any model that includes ATP hydrolysis-driven Cl^- pumping should be viewed cautiously.

Alternatively, via another model, MDR protein overexpression could catalyze (directly or indirectly) some type of Na^+:Cl^-/H^+ co-transport (Na^+ in, Cl^- in, H^+ out), and ATPase activity (in analogy to the CFTR) might play some role in regulating this transport, but energy for any uphill Cl^-/H^+ movement would be provided by a Na^+ gradient. Important sites of endogenous expression of hu MDR 1 protein include Cl^--absorbing epithelia such as proximal tubule (PT) cells of the kidney. Thus it is worthwhile to examine the present data with regard to possible physiologic consquences. A principle function of the PT is of course re-absorption of Na^+, Cl^-, and HCO_3^-. A variety of transporters present in the PT work together to accomplish this. Thus, a Cl^-/HCO_3^- exchanger on the basolateral side of the PT cell promotes Cl^- transport toward the interstitium, while a Na^+:HCO_3^- co-transporter directs transport of HCO_3^-. A protein acting in some fashion to move Cl^- in and H^+ out on the luminal side of the PT would indeed assist both processes by trapping HCO_3^- inside (CO_2 diffusing into the cell is rapidly converted to H^+ and HCO_3^-; thus net movement of H^+ out of the cell traps the HCO_3^-) as well as transporting Cl^- from the lumen into the cell. Thus, the proposed Cl^-/H^+ antiport model does make sense with regard to PT cell biology. A Na^+:Cl^-/H^+ model regulated by ATP hydrolysis for the PT is a bit more intuitively satisfying to us than a Cl^-/H^+ process driven by Na^+-dependent ATPase activity, since the former would take advantage of the existing PT electrochemical gradient in Na^+ and thus not "waste" ATP, and also assist Na^+ uptake in the PT.

The possible models also raise interesting questions for other sites of endogenous MDR protein isoform expression. One of these is the cells lining the bile canaliculus. The chloride concentration of bile is conspicuously lower than in serum, so Cl^- uptake systems in canalicular cells would make sense, but bile is also alkaline, thus any Cl^- uptake strictly coupled to H^+ efflux into the bile is initially curious. However, a very precise balance in bile electrolyte composition and pH is absolutely critical for bile acid/cholesterol micelle formation and hence proper solubilization of phosphotidyl choline and other lipids, and other net base-excreting mechanisms exist in canalicular cells. Thus, a variety of ion transport proteins in cells lining the bile canaliculus act together to tightly regulate bile ion composition. We suggest MDR protein(s) is one of these and that the apparent Cl^-/H^+ antiport function we describe in this paper may have an important role in balancing bile electrolytes and pH, which are such critical parameters for proper lipid transport. Altered lipid transport in the bile of MDR gene knockout mice (50) could conceivably be a manifestation of improper electrolyte composition of the bile due to a disruption in the ion transport normally catalyzed (directly or indirectly) by MDR protein.

Other points are also intriguing. Several Na^+:Cl^- co-transporters (either K^+ dependent, as the NKCCs, or K^+ independent, as the polythiazide sensitive Na^+:Cl^- co-transporter) have recently been cloned (*51*−*54*). None of these are currently known to translocate H^+ directly, although some interesting pH effects on NKCC activity have been described (*55*). Although as mentioned the presence of a Na^+:Cl^- co-transporter in the kidney PT would make physiologic sense, interestingly, the recently cloned Na^+:Cl^- co-transporter (*51*) is not expressed in the PT (Steven C. Hebert, personal communication) although it is expressed elsewhere in kidney tissue. Thus, since MDR protein is expressed in the PT, perhaps MDR protein represents a PT-specific contributor to net Na^+:Cl^- co-transport. Therefore, for a variety of reasons, resolving the exact nature of the apparent Na^+ and ATP dependencies for the unique Cl^-/H^+ transport we have observed is a key issue for future work.

Finally, a variety of other studies have also provided evidence for novel Cl^- transport mediated by the MDR 1 protein (*3*,*20*,*56*,*57*), but the magnitude and regulation of this flux has remained a major topic of debate (*3*,*24*,*58*) and co-transport or exchange mechanisms and various ion dependencies have not been completely explored. Perhaps importantly, with two exceptions (see ref 20 and the present work), Cl^- flux has usually been measured for MDR cells selected for long periods of time with potent chemotherapeutic drugs

after "stimulation" via hypotonic shock. Thus the systems that have been studied are complex (due to drug exposure), and the mechanism for stimulating the conductance (hypotonic shock) in these examples is likely indirect. We find that, in our cells, hypotonic shock results in prolonged and magnified acidification of our clones. If, as our data suggest, the flux of Cl^- mediated directly or indirectly by MDR protein is coupled in some fashion to H^+ movement, then this observation is important. Depending on the mechanism for RVD in a given cell type, these changes in pH_i might affect the ability to measure a Cl^- flux mediated by MDR 1 protein under some conditions. Alternatively, since the data we present in this paper are more consistent with MDR 1 protein directly or indirectly translocating Cl^- *and* H^+ with "carrier-like" or "pump-like" kinetics (not "channel-like" kinetics) perhaps the Cl^- channel behavior noted in some cells overexpressing MDR protein is the result of somewhat variable and cell-specific up-regulation of channel activity *in response* to the behavior mediated by MDR 1. In any case, the present data suggest several possible ways to explain the discrepencies in data pertaining to Cl^- channel activity linked to MDR 1 protein overexpression (e.g., *20* vs *24*).

Further testing and refinement of various possible models will require more detailed studies with vesicles and reconsituted proteoliposomes harboring purified MDR 1 protein. Regardless, the data in a general way very strongly support the altered partitioning model for MDR protein-mediated drug resistance and argue against other models that envision direct drug transport by MDR protein (see ref 5). They also suggest reasonable explanations for a variety of unexplained effects frequently seen in MDR cells [e.g., their response to certain ionophores, overexpression of AE mRNA (*18*,*23*), and the pH_o and volume regulated expression of MDR 1 in colon and kidney cells (*37*)] and predict a number of important factors to consider in further analysis of the physiologic function of hu MDR 1 protein. Our model predicts that the physiologic function of the protein may include modulation of pH-dependent Cl^- transport (and/or perhaps volume and pH_i regulation) in proximal tubule cells of the kidney and at other sites.

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