Title
Swelling-activated chloride channels in multidrug-sensitive and -resistant cells

Permalink
https://escholarship.org/uc/item/31s3t5ns

Journal
Journal of General Physiology, 104(6)

ISSN
0022-1295

Authors
Ehring, GR
Osipchuk, YV
Cahalan, MD

Publication Date
1994-12-01

DOI
10.1085/jgp.104.6.1129

License
CC BY 4.0

Peer reviewed
Swelling-activated Chloride Channels in Multidrug-sensitive and -resistant Cells

GEORGE R. EHRING, YURI V. OSIPCHUK, and MICHAEL D. CAHALAN

From the Department of Physiology and Biophysics, University of California at Irvine, Irvine, California 92717

ABSTRACT Resistance to chemotherapeutic agents in neoplastic cells is often mediated by expression of P-glycoprotein, which functions as a drug-efflux pump for a broad range of substrates. We have used a combination of patch clamp and video-imaging techniques to examine the expression and drug-efflux function of P-glycoprotein and to determine the possible correlation with swelling-activated chloride channels in drug-sensitive and -resistant cell lines. Two pairs of cell lines were used in these experiments: (a) control NIH-3T3 cells and a corresponding MDR1-transfectant; and (b) control 8226 myeloma cells and a derivative cell line selected for resistance to chemotherapeutic agents. Control cells lacked detectable P-glycoprotein expression based on Western blotting, immunofluorescence staining with a specific monoclonal antibody, and a functional assay of rhodamine-123 (R123) efflux. Resistant cells expressed P-glycoprotein at high levels and rapidly exported R123. During whole-cell recording using either hyperosmotic pipette solution or hypoosmotic Ringer solution, cell swelling was accompanied by Cl– channel opening in all four cell lines. The rates of induction, biophysical properties and magnitudes of Cl– conductance (gCl) were indistinguishable between control and corresponding multidrug-resistant cells: gCl reached 0.96 ± 0.31 (n = 14) and 0.83 ± 0.31 nS/pF (mean ± SD; n = 31) in NIH-3T3 and NIH-3T3/MDR cells, respectively; and 0.31 ± 0.20 (n = 9) and 0.37 ± 0.22 nS/pF (n = 7) in 8226 and 8226/Dox40 cells, respectively. gCl exhibited moderate outward rectification in symmetrical Cl– solutions, with a rectification ratio of 1.4 at ± 50 mV. Cl– channels slowly closed during strong depolarization beyond +60 mV. Using video-imaging techniques with SPQ as a fluorescent probe, we monitored Cl–-channel opening in intact drug-sensitive and -resistant cells. gCl, measured either with whole-cell recording or SPQ imaging, was blocked by DIDS (voltage-dependent Kd < 50 μM at +40 mV), NPPB (Kd ~ 30 μM), and tamoxifen (complete and irreversible block ~10 μM). None of these blockers inhibited R123 efflux. NPPB accelerated R123 efflux, an effect that was mimicked by CCCP, a mitochondrial uncoupler. In contrast, verapamil selectively blocked R123 efflux (Kd = 0.3 to 0.5 μM); 10 μM left gCl unaltered. Induction of gCl was not affected by vincristine or doxorubicin in the pipette solution. Moreover, the rate of R123 efflux did not change during cell swelling. We conclude that P-glycoprotein and swelling-activated chloride channels function independently and are separable by expression and by pharmacological sensitivities.

Address correspondence to Michael D. Cahalan, Department of Physiology and Biophysics, University of California at Irvine, Irvine, CA 92717.
INTRODUCTION

When exposed to changes in extracellular osmolarity, many mammalian cells regulate their volume by coupling water flux to osmolyte transport across the cell membrane. K⁺ and Cl⁻ currents contribute a large fraction of the total osmolyte flux (Hoffmann and Simonsen, 1989). In particular, swelling-activated Cl⁻ channels may be important in triggering and maintaining the ionic flux required for volume regulation (Cahalan and Lewis, 1988, 1994). Although only cells within the kidney or digestive tract are likely to experience large changes in osmolarity, swelling-activated Cl⁻ channels are found in many epithelia and other tissues. Thus, swelling-activated Cl⁻ channels may serve alternative functions such as epithelial fluid transport or volume regulation during cell division or motility.

The molecular characterization of Cl⁻ channels provides significant insight into their structure and function. Unlike the families of homologous proteins that form K⁺, Ca²⁺, and Na⁺ channels, there is a wide diversity of molecules that form Cl⁻ channels (for review, see Frizzell and Cliff, 1992). Without highly conserved structural motifs to distinguish potential channel-forming molecules, and because there are no specific toxins or potent pharmacological agents to aid in their identification, each candidate Cl⁻ channel must be carefully evaluated for function and expression. Such a detailed analysis, including biophysical, biochemical, and molecular biological characterization, has been performed on the cystic fibrosis transmembrane conductance regulator (CFTR), indicating that the CFTR can form one type of Cl⁻ channel (reviewed by Welsh, Anderson, Rich, Berger, Denning, Ostedgaard, Sheppard, Cheng, Gregory, and Smith, 1992).

The CFTR belongs to the ABC (ATP-binding cassette) superfamily. A second member of this family, P-glycoprotein—the MDR1 gene product, is an ATP-dependent transporter responsible for multidrug resistance to a broad spectrum of chemotherapeutic agents (Higgins, 1992; Gottesman and Pastan, 1993). P-glycoprotein is endogenously expressed in several epithelial tissues, which may also express swelling-activated Cl⁻ channels (Hitchins, Harman, Davey, and Bell, 1988; Arceci, Baas, Raponi, Horwitz, Housman, and Croop, 1990; van Kalken, van der Valk, Hadisaputro, Pieters, Broxterman, Kuiper, Scheffer, Veerman, Meyer, and Scheper, 1991; van Kalken, Giaccone, van der Valk, Kuiper, Hadisaputro, Bosma, Scheper, Meijer, and Pinedo, 1992; Toth, Vaughan, Slocum, Arredondo, Takita, Baker, and Rustum, 1994). Valverde and co-workers (1992), based on evidence that transfection of the MDR1 gene into NIH-3T3 fibroblasts or into S1 lung carcinoma cells resulted in the expression of both P-glycoprotein and swelling-activated Cl⁻ channels, suggested that P-glycoprotein has two distinct functions: an ATP hydrolysis-dependent drug-transport pump or a swelling-activated Cl⁻ channel that requires ATP binding, but not hydrolysis. In their model (Gill, Hyde, Higgins, Valverde, Mintenig, and Sepulveda, 1992), cell swelling traps P-glycoprotein in its Cl⁻ channel conformation, thus making it unavailable to pump drugs. Conversely, drug binding and transport traps P-glycoprotein in its pump conformation, preventing the induction of its Cl⁻ conductance (gCl⁻) by subsequent cell swelling.

Recently, however, several studies have indicated a lack of correlation between P-glycoprotein expression and gCl⁻. T₈₄ colonic adenocarcinoma cells, which express
P-glycoprotein, showed similar rates of transepithelial secretion of vinblastine, an antineoplastic agent, whether or not they exhibited $g_{\text{cl}}$ (McEwan, Hunter, Hirst, and Simmons, 1992). Rasola, Galietta, Gruenert, and Romeo, (1994), using epithelial cell lines selected for drug resistance, found no correlation between P-glycoprotein expression and $g_{\text{cl}}$. In addition, hypoosmotic stress did not affect the accumulation of rhodamine-123 (R123), a substrate for P-glycoprotein transport, in mouse lymphoma cells transduced with the MDR1 gene (Weaver, Szabo, Pine, Gottesman, Goldenberg, and Aszalos, 1993). Altenberg, Vanoye, Han, Deitmer, and Reuss (1994b) found that multidrug-resistant Chinese hamster fibroblasts, which express high levels of P-glycoprotein, did not express swelling-activated Cl$^-$ channels. However, they also observed $g_{\text{cl}}$ in human breast cancer cells (BC19/3) rendered multidrug-resistant by transfection with the human MDR1 gene; such currents were not observed in the parental drug-sensitive cell line (MCF-7). Interestingly, neither BC19/3 nor MCF-7 cells volume-regulate or permit measurable amounts of Cl$^-$ efflux under hypoosmotic stress (Altenberg, Deitmer, Glass, and Reuss, 1994a).

We have further tested the hypothesis of Valverde et al. (1992) that swelling-activated chloride currents are associated with increased expression of human P-glycoprotein after (a) transfection with the MDR1 gene or (b) selection for multidrug resistance. Four cell lines were evaluated for both chloride conductance and P-glycoprotein-mediated substrate efflux: (a) the drug-sensitive 8226 human myeloma cell line; (b) its drug-resistant derivative line, 8226/Dox40 (Dalton, Grogan, Rybski, Scheppe, Richter, Kailey, Broxterm, Pinedo, and Salmon, 1989); (c) control NIH-3T3 mouse fibroblasts; and (d) NIH-3T3/MDR cells permanently transfected with the human MDR1 gene (Ueda, Cardarelli, Gottesman, and Pastan, 1987; Kioka, Tsubota, Kakehi, Komano, Gottesman, Pastan, and Ueda, 1989; Bruggemann, Currier, Gottesman, and Pastan, 1992). Initially, we measured $g_{\text{cl}}$ induced by osmotically driven cell swelling, using conventional whole-cell recording. In addition, we used video-imaging techniques to monitor both P-glycoprotein substrate efflux and swelling-activated chloride conductance in intact cells. We show that P-glycoprotein–mediated drug efflux and swelling-activated chloride conductance are separable functions, both pharmacologically and by mode of activation. Our results indicate that there is no correlation between P-glycoprotein and Cl$^-$ channel expression. These results have been presented in preliminary form (Ehring, Osipchuk, and Cahalan, 1994; Osipchuk, Ehring, and Cahalan, 1994).

### MATERIALS AND METHODS

#### Cells

The drug-sensitive 8226 myeloma cell line and the drug-resistant 8226/Dox40 cell line were obtained from Dr. William Dalton (Arizona Cancer Center, University of Arizona, Tuscon, AZ). Both cell lines were cultured in suspension at 37°C in 5% CO$_2$ and maintained in RPMI 1640 medium containing 25 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin (P-S) and 1% L-glutamine. 8226/Dox40 cells were also treated with $4 \times 10^{-7}$ M doxorubicin every two weeks. These cells were fed twice weekly and split weekly. For patch clamp experiments, the cells were plated on glass coverslips which had been pretreated with 1 mg/ml poly-L-lysine.

NIH-3T3 fibroblasts and NIH-3T3 fibroblasts transfected with the pHA MDR (Gly185) clone...
of MDRI were obtained from Dr. Michael Gottesmann (National Cancer Institute, National Institutes of Health, Bethesda, MD). Both lines were cultured as monolayers at 37°C in 5% CO₂ using DMEM containing 25 mM HEPES and supplemented with 4.5 g glucose per liter, 10% FBS, 1% P-S and 1% L-glutamine. The medium for the MDRI transfectant was supplemented with 60 ng/ml colchicine. We confirmed the increased expression of P-glycoprotein in the drug-resistant lines by immunoblotting. Membrane preparations from all four cell lines were run on SDS-PAGE gels, immunoblotted, and stained by an antibody (C219) to a cytoplasmic carboxy-terminal epitope of P-glycoprotein (Georges, Bradley, Gariepy, and Ling, 1990).

**Solutions and Reagents**

For most of the patch-clamp experiments, the cells were bathed in mammalian Ringer containing (in millimolar): 160 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 0.1 BaCl₂ and 10 HEPES, adjusted to pH 7.4 with NaOH and having an osmolarity of 310 mosm. Hyposmotic Ringer (67% Ringer) was prepared by diluting mammalian Ringer with deionized (MilliQ) H₂O (Millipore Corp., Milford, MA). Pipette (internal) solutions contained 160 Cs aspartate, 2 MgCl₂, 5 HEPES, and 10 ethylene glycol-bis (β-aminoethylether) N,N',N'-tetraacetic acid (EGTA), pH adjusted to 7.2 with KOH; 310 mosm. Hyperosmotic internal solution was prepared by adding 100 mM sucrose to the pipette solution. In some experiments, the cells were patch clamped with a pipette solution containing (in millimolar): 140 N-methyl-D-glucamine chloride (NMG-Cl), 1.2 MgCl₂, 1 mM EGTA and 10 mM HEPES with 2 mM NaATP adjusted to pH 7.4 (310 mosm). In these experiments, the cells were initially bathed in isosomotic NMG-Cl Ringer containing (in millimolar): 140 NMG-Cl, 1.3 mM CaCl₂, 0.5 MgCl₂ and 10 mM HEPES (pH 7.4, 310 mosm). Hypoosmotic (70%) Ringer was made by reducing NMGCl to 105 mM. Osmolarity was measured with a vapor pressure osmometer (Wescor Inc., Logan, UT). Bath solutions were exchanged by perfusing the chamber (~250 µl vol) with 2–5 ml of the test solution. Exchange was complete in <30 s. Chloride was replaced by either iodide or nitrate for video-imaging measurements of chloride conductance. Iodide solution: 135 NaI, 2.4 K₂HPO₄, 0.6 KH₂PO₄, 1 MgSO₄, 1 CaSO₄, 10 HEPES (pH 7.4, adjusted with NaOH). Nitrate solution: 135 NaNO₃, 2.4 K₂HPO₄, 0.6 KH₂PO₄, 1 MgSO₄, 1 CaSO₄, 10 HEPES (pH 7.4, adjusted with NaOH). Normal osmolarity was adjusted to 310 mosm. Hypoosmotic solutions (50–90% of normal osmolarity) were prepared by diluting solutions with MilliQ water.

Most salts and reagents (reagent grade or better) were obtained from Sigma Chemical Co. (St. Louis, MO). Doxorubicin, vincristine and verapamil were obtained from Sigma Chemical Co. and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) from Biomol Research Laboratories, Inc. (Philadelphia, PA). Rhodamine-123 (R123), 6-methoxy-N-((sulfopropyl)quinolinium (SPQ) and 4,4’-disothiocyanatostilbene-2,2'-disulfonic acid (DIDS) were obtained from Molecular Probes, Inc. (Eugene, OR). All drugs were dissolved in DMSO at a stock concentration at least 1,000 times greater than the final bath concentration. The 4E3 monoclonal IgG antibody specific to an extracellular epitope on P-glycoprotein, and C219 monoclonal antibody to an intracellular epitope on P-glycoprotein were obtained from Signet Laboratories (Dedham, MA). FITC-conjugated and Texas red-conjugated goat F(ab')₂ anti-mouse IgG were obtained from Caltag Laboratories (South San Francisco, CA).

**Flow Cytometry**

Aliquots of ~1 × 10⁶ cells were taken directly from 8226 myeloma or 8226/Dox₄₀ suspension cultures, or after trypsinization of NIH-3T3 control and NIH-3T3/MDR cell-adherent cultures, and resuspended in 100 µl phosphate buffered saline supplemented with 0.1% fetal calf serum (PBS/FCS). Primary antibodies (0.5 µg), either isotypic control IgG or monoclonal 4E3, were added to the cell suspension, vortexed for 3–5 s and incubated for 25 min at room
temperature. Unbound primary antibody was removed by dilution into 1 ml PBS/FCS followed by centrifugation at 300 g for 5 min. The wash procedure was repeated twice and the cells resuspended in 100 µl PBS/FCS containing ~0.5 µg FITC-conjugated goat F(ab')2 anti-mouse IgG. The cells were vortexed for 3–5 s and incubated in the dark for 25 min at room temperature. Unbound F(ab')2 fragments were removed as described for the primary antibody. Cell fluorescence was measured on a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA). FITC fluorescence was excited by 150 mW of 488-nm light from an argon laser, and the emitted light was passed to a photomultiplier tube through a 530 ± 15 nm band pass filter (FL-1). Frequency histograms of the logarithm of the fluorescence were calculated for ~5,000 cells from each cell line using LYSIS II software (Becton Dickinson).

Confocal Microscopy

To measure protein expression and functional drug transport, cells were simultaneously labeled for P-glycoprotein expression with 4E3 antibody and incubated with R123. In drug-sensitive cells, R123 diffuses across the cell membrane, enters the cytoplasm and gets trapped within the mitochondria because of the mitochondrial membrane potential. In drug-resistant cells, the P-glycoprotein pump keeps the cytoplasmic concentration of R123 low and prevents accumulation within mitochondria. For these experiments, cells were adhered to glass coverslips in normal culture medium overnight in the incubator. The following day, cells were washed with mammalian Ringer then incubated at room temperature for 1.5 h with 5 µM R123 in Ringer ± 10 µM verapamil. During the first half hour of incubation, the Ringer solution also contained 4E3 antibody. The cells were then washed twice with fresh Ringer containing 5 µM R123. During the last half hour of R123 incubation, the cells were labeled with Texas red-conjugated goat F(ab')2 anti-mouse IgG. Treated cells were placed on the confocal microscope and washed twice with normal medium.

The confocal microscope system consisted of a Nikon Labophot upright microscope with a Bio-Rad MRC-600 confocal laser scanner equipped with a krypton/argon laser (Bio-Rad Co., Hercules, CA). The light path included selectable excitation filters at 488 or 568 nm, a double dichroic mirror with near total reflectance for wavelengths of 488 and 568 nm and high transmission of wavelengths between 500 and 550 nm or wavelengths > 600 nm. Texas red emission was measured, after excitation at 568 nm, as the intensity of light transmitted through a second dichroic mirror (>560 nm) and through a 585-nm long-pass emission filter to a photomultiplier tube (PMT1). R123 emission was measured, following excitation at 488 nm, as the light reflected by the second dichroic mirror (<560 nm) and passing through a 522-nm band pass filter to a second photomultiplier tube (PMT2). Because of the wide band widths for both emission and excitation of R123, the fluorescence for the double-labeled cells was measured in two sequential images. The first image was taken using 488-nm excitation to collect R123 (green) fluorescence, and the second used 568-nm excitation to collect Texas red (red) fluorescence. This procedure eliminated spectral overlap between the two dyes. Transmitted light images were taken by replacing the filter cube for PMT2 with a fiber optic which collected the light passing directly through the specimen.

Patch Clamp Recording

Experiments were carried out using standard whole-cell patch clamp recording techniques (Lewis, Ross, and Cahalan, 1993). All experiments were done at room temperature (22–26°C). Patch pipettes were pulled from Accu-fill 90 Micropet (Becton, Dickinson, and Co., Parsippany, NJ) coated with Sylgard (Dow Corning Corp., Midland, MI) to within 50–100 µm of their tips, and fire polished to resistances between 1.2 and 3.0 MΩ when filled with internal solution and immersed in Ringer. Membrane voltages were corrected for the junction potentials arising
between the recording pipette and the bath solution. The pipette current was nulled before seal formation (Neher, 1992). Cell membrane and pipette capacitance were canceled using electronic feedback via the patch clamp amplifier (AxoPatch 1-D, Axon Instruments Inc., Foster City, CA). A PDP-11/73 computer and interface running programs written in BASIC-23 were used to generate voltage commands and digitize and analyze patch clamp current output (Indec Systems Inc., Sunnyvale, CA). Pipette access resistance and cell capacitance were measured by analyzing the current in response to a 5-ms, 20-mV test pulse. The capacitative current transient recorded immediately after break in was fitted to a single exponential function to determine resting membrane capacitance ($C_m$), and initial access resistance, $R_a$ (Lindau and Neher, 1988). Cells were selected for study if initial $R_a$ was <10 M$\Omega$. $C_m$ and $R_a$ were monitored throughout the experiment; if $R_a$ increased more than twofold, the experiment was terminated and data from that cell excluded. The holding potential for all experiments was −60 mV. Membrane currents were measured in response to 200-ms voltage ramps from −100 to 50 mV, or 200-ms voltage pulses from the holding potential in 20-mV increments from −100 to +100 mV. Grouped data are presented as mean ± SD. When appropriate, the difference between two means was statistically tested by a two-tailed t test.

Video Microfluorescence Measurements

NIH-3T3 cells or NIH-3T3/MDR cells grown on glass coverslips were placed on the stage of an inverted microscope (Zeiss Axiovert 35). 8226 or 8226/Dox$_{40}$ cells grown in suspension were seeded onto glass coverslips coated with poly-l-lysine (Sigma Chemical Co.). A 75 W Xenon arc lamp (Osram XBO-75), powered by a stable power supply (Model 1500, Opti-Quip Inc., Highland Mills, NY), was used as a light source. Excitation light was passed through an interference filter (470 nm, band width 20 nm for R123; and 340 nm, band width 10 nm for SPQ) and reflected by a dichroic mirror (cut-off wavelengths 505 and 400 nm, respectively, for R123 and SPQ) through a 60× plan-neofluar objective. Epifluorescence images were filtered by a barrier filter with cut-off wavelength 520/400 nm (R123/SPQ) and collected by an intensified CCD video camera (Hamamatsu, C2400). Images were digitized and averaged (8–16 frames), background-corrected, stored every 3–10 s, and analyzed by an image-processing system (VideoProbe, ETM Systems, Irvine, CA). Filters and dichroic mirrors were from Omega Optical (Brattleboro, VT). Fluorescence intensities of individual cells in the field of view (up to 512 per field) were determined by averaging the image intensity collected from regions of interest within each cell.

Imaging Plasma Membrane Chloride Conductance

Cells were loaded with SPQ by incubation in medium containing 10 mM SPQ for 2–6 h at 37°C. The intracellular SPQ concentration was ~100 μM. The fluorescence intensity of entrapped intracellular SPQ was used as a measure of halide concentration. Quenching of SPQ fluorescence by intracellular halides was determined according to the Stern-Volmer equation:

$$F = F_0/(1 + K[\text{Hal}^-]).$$

$F_0$ and $F$ are the SPQ fluorescence in the absence and presence of halide; $[\text{Hal}^-]$ the concentration of halide; and $K$ a quench constant. $K$ is equal to 118 M$^{-1}$ for Cl$^-$, 276 M$^{-1}$ for I$^-$, and is negligibly low for NO$_3^-$ (Illsley and Verkman, 1987). Cells loaded with SPQ were incubated in external solution containing 1$^-$ for 20 min to quench intracellular SPQ fluorescence. When NO$^-$ was exchanged for I$^-$ in the extracellular solution, intracellular I$^-$ ions escaped the cell through chloride channels and were replaced by NO$^-$, effectively unquenching SPQ and thereby increasing SPQ fluorescence. The rate of replacement and, hence, fluorescence increase is proportional to chloride conductance through the plasma membrane. To
study the time course of the chloride conductance, extracellular solution was toggled between solutions containing the permeant anions I⁻ and NO₃⁻, with a period of exchange ranging from 50 to 100 s. Solution flow was propelled by 10-psi air pressure from a multibarrel tube into the experimental chamber and controlled through electromagnetic valves (General Valve, Fairfield, NJ) by VideoProbe software. Solution exchange in the 200-μl chamber was complete within <10 s. Repetitive exchange of anions in extracellular solution led to repetitive exponential decreases and increases of intracellular SPQ fluorescence as I⁻ and NO₃⁻ ions moved in and out of the cells through chloride channels. Thus, the rate of fluorescence increase and decrease was proportional to the plasma membrane chloride conductance.

**Imaging P-Glycoprotein Substrate Efflux**

As a substrate for extrusion by the drug-efflux pump, R123 is particularly well suited for functional investigation of P-glycoprotein (Nefakh, 1988; Lampidis, Castello, del Gigho, Pressman, Viallet, Trevorrow, Valet, Tapiero, and Savaraj, 1989; Kessel, Beck, Kukuruga, and Schulz, 1991; Weaver, Pine, Aszalos, Schoenlein, Currier, Padmanabhan, and Gottesman, 1991; Ludescher, Thaler, Drach, Drach, Spitaler, Gattringer, Huber, and Hofmann, 1992; Aaltenberg et al., 1994b). Cells were loaded with R123 by incubation in medium containing 5 μM R123 for 20 min at room temperature. For drug-resistant cells (NIH-3T3/MDR and 8226/Dox₄₀), 5 μM verapamil was also added to block P-glycoprotein-mediated efflux of R123. After wash out of extracellular R123, the final concentration of R123 in the cells was measured by lysing ~10⁶ cells in 200 μl Ringer containing 1% Triton X-100 and comparing the fluorescence intensity of the lysate with standard concentrations of R123. The average intracellular concentration of R123 was ~50 μM. External dye was washed out by rinsing the cells with >10× excess Ringer. R123 efflux was assayed by the decrease in fluorescence intensity of individual cells, and fitted with a single exponential function using Igor software (WaveMetrics, OR) run on a Macintosh (Apple Computer, Sunnyvale, CA).

**RESULTS**

Results are presented below in four main sections: first, a determination of molecular and functional expression of P-glycoprotein; second, patch clamp experiments demonstrating Cl⁻ channel opening during osmotically induced cell swelling; third, SPQ fluorescence measurements of chloride conductance in intact cells; and fourth, measurements of R123 efflux mediated by the P-glycoprotein drug-efflux pump.

**Cell Phenotype**

We determined the increased expression of P-glycoprotein using flow cytometry, immunofluorescence, and Western blotting. For flow-cytometric analysis, we used monoclonal 4E3 (Arceci, Stieglitz, Bras, Schinkel, Baas, and Croop, 1993) and a secondary antibody conjugated to FITC. Control NIH-3T3 mouse fibroblasts (Fig. 1 A) and drug-sensitive 8226 myeloma cells (Fig. 1 C) had identical levels of fluorescence when stained with P-glycoprotein-specific 4E3 antibody or an isotypic control. No P-glycoprotein expression was measurable in these cell lines. In contrast, compared to control, fluorescent staining was >132-fold greater in MDR11-transfected NIH-3T3 fibroblasts (Fig. 1 B) and >85-fold greater in drug-resistant 8226/Dox₄₀ cells (Fig. 1 D) after staining with 4E3. Membranes from 8226/Dox₄₀ and NIH-3T3/MDR cells contained a ~170 kD protein which was recognized by monoclonal C219. This protein was not detected in the 8226 myeloma or NIH-3T3
control cell membranes. These data confirm previously published observations that P-glycoprotein was not detectable in the drug-sensitive cell lines, but gave a strong signal in the drug-resistant cell lines when analyzed by immunoblot (Dalton et al., 1989; Tanaka, Currier, Bruggemann, Ueda, Germann, Pastan, and Gottesman, 1990). Thus, by two different criteria, the drug-resistant cells showed high levels of P-glycoprotein expression, while the drug-sensitive cells failed to produce measurable quantities.

To confirm that drug efflux was associated with P-glycoprotein expression in these cells, we performed dual-fluorescence measurements using a confocal microscope. R123 is a substrate for the MDR1-encoded drug-efflux pump (Lampidis et al., 1985; Neyfakh, 1988; Kessel et al., 1991) and mitochondrial accumulation of R123 has...
been shown to be inversely correlated with multidrug resistance (Neyfakh, 1988; Ludescher et al., 1992). We incubated drug-resistant and drug-sensitive cell lines in 5 μM R123 in the presence or absence of 10 μM verapamil (Fig. 2). Verapamil reverses multidrug resistance by inhibiting drug efflux (Tsuruo, Iida, Tsukagoshi, and Sakurai, 1981; Tsuruo, Iida, Naganuma, Tsukagoshi, and Sakurai, 1983). Indirect immunofluorescence with 4E3 antibody was used to test for expression of P-

**Figure 2.** P-glycoprotein expression correlates with R123 transport. NIH-3T3 cells (A–C) and NIH-3T3/MDR cells (D–I) in nonconfocal, transmitted light images (A,D,G); in confocal fluorescence images showing P-glycoprotein expression by immunofluorescence (B,E,H); and in confocal fluorescence images showing R123 accumulation (C,F,I). Only the NIH-3T3/MDR cells (E,H) were labeled by the anti-P-glycoprotein monoclonal antibody. NIH-3T3 cells accumulated intracellular R123 after 1.5 h incubation (C). Punctate staining indicates mitochondrial localization of R123. No dye accumulated in NIH-3T3/MDR cells incubated in 5 μM R123 alone (F); however, after incubation in 5 μM R123 plus 10 μM verapamil, NIH-3T3/MDR cells showed mitochondrial accumulation of R123 (I) identical to the NIH-3T3 control cells (C).

glycoprotein during these experiments. As expected from the FACS® experiments, the drug-sensitive NIH-3T3 control cells were not labeled by 4E3 (Fig. 2 B), but instead showed punctate staining with R123 (Fig. 2 C), indicating mitochondrial localization. In contrast, NIH-3T3/MDR cell membranes were distinctly labeled by 4E3, but the cells did not accumulate R123 (Fig. 2, E–F). When NIH-3T3/MDR cells were incubated with R123 plus 10 μM verapamil, both membrane staining for P-glycoprotein and mitochondrial staining with R123 were observed (Fig. 2, H–I).
Blocking the transport function allowed R123 to enter drug-resistant cells and accumulate in the mitochondria, as seen in drug-sensitive cell lines. Similar results were obtained using this labeling protocol on 8226 myeloma and 8226/Dox40 cells (data not shown). Thus, drug-sensitive and drug-resistant cell lines differ both in expression of P-glycoprotein and their ability to pump R123.

*Induction of g\(_{\text{Cl}}\) in Drug-sensitive and Drug-resistant Cell Lines*

In contrast to P-glycoprotein expression and drug transport, we observed no difference in the currents elicited by osmotic challenge between drug-sensitive and drug-resistant cell lines. Cell dialysis with hyperosmotic pipette solution (400 mosm) during whole-cell patch clamp experiments caused a gradual induction of an outwardly rectifying current for all four cell lines (Fig. 3). Two experiments identified this current as a chloride current. First, when the pipette solution contained cesium and aspartate as the principal ions and the bathing solution was mammalian Ringer, the reversal potential for the current was \(\sim -45\) mV. When most of the Ringer Cl\(^{-}\) was replaced by aspartate, the outward current was reduced and the reversal potential shifted to 0 mV, as expected for a chloride-selective channel (data not shown). Second, when the swelling-activated current was induced in solutions with...
equivalent intracellular and extracellular Cl\(^-\) concentrations, the reversal potential was near 0 mV (see Fig. 8 below).

Using voltage-ramp stimuli, we determined the time course of Cl\(^-\) current induction by calculating the slope conductance near the reversal potential after establishing the whole-cell patch clamp configuration (break in) (Fig. 3). When currents were induced by hyperosmotic pipette solution, the conductance remained relatively constant at < 1 nS for ~50 s and then increased over the next 200-300 s to a plateau (Fig. 4). There was no significant difference between drug-sensitive and -resistant cell lines in the maximum conductance, the rate of induction of \(g_{\text{Cl}}\), or the reversal potential (Table I, Fig. 4). In general, the currents elicited in both NIH-3T3 fibroblast cell lines were larger than those of the paired myeloma cell lines. This difference is accounted for, in part, by the larger size of NIH-3T3 cells as measured by their membrane capacitance (Table I).

Identical Cl\(^-\) currents were induced by using isoosmotic pipette solutions (310 mosm) and challenging the cells with hypoosmotic external solutions (210 mosm) (Table I). As with the hyperosmotic stimulus, there was no difference in the maximum conductance, the rate of induction of \(g_{\text{Cl}}\), or the reversal potential, comparing drug-sensitive with the corresponding drug-resistant cell line (Table I, Fig. 3). We also tested NMG-Cl solutions used in previous studies (Gill et al., 1992; Valverde et al., 1992), but found no difference in \(g_{\text{Cl}}\) obtained in drug-sensitive or -resistant cells. The rectification ratio \((I_{\text{Cl}} (+50 \text{ mV})/I_{\text{Cl}} (-50 \text{ mV})\) of the current in NMG-Cl solutions was 1.45 ± 0.04 \((n = 3)\) for NIH-3T3 control cells and 1.46 ± 0.16 \((n = 8)\) for NIH-3T3/MDR cells. These results suggest that the Cl\(^-\) channel has similar induction and permeation properties in both cell types.

Once induced, the outwardly rectifying Cl\(^-\) current was nearly constant during 200-ms voltage pulses from −100 to +100 mV, with no evidence of time-dependent channel opening or closing (Fig. 5). The current amplitudes recorded during voltage steps overlay those during voltage ramps, suggesting that outward rectification is due to permeation properties rather than voltage- or time-dependent gating. Channels slowly closed during prolonged depolarization with a time course that varied from cell to cell. During 200-ms pulses to voltages positive to +120 mV or during 10-s pulses to voltages positive to +60 mV, a slow relaxation indicating channel closure can be seen in Fig. 5. Thus, the biophysical properties of \(g_{\text{Cl}}\) did not differ between drug-sensitive and -resistant cells.

**Pharmacology.** The proposed link between P-glycoprotein expression and \(g_{\text{Cl}}\) was based, in part, on the argument that agents which block drug transport can also block \(g_{\text{Cl}}\). For example, Valverde et al. (1992) showed that 100 μM verapamil, a dose that reverses multidrug resistance and prevents drug transport, decreased \(g_{\text{Cl}}\) in MDR1-transfected NIH-3T3 fibroblasts.

We have shown that 10 μM verapamil completely blocks the transport of R123 from 8226/Dox40 cells and NIH-3T3/MDR cells (see Fig. 12 below); the \(K_d\) for verapamil block is 0.3 to 0.5 μM. However, as illustrated in Fig. 6, 10 μM verapamil had no effect on \(g_{\text{Cl}}\) in NIH-3T3 control or NIH-3T3/MDR cells. Higher doses of verapamil (100 μM) reduced \(g_{\text{Cl}}\) to 70 ± 15% \((n = 3)\) of control. The significantly higher concentrations required to block \(g_{\text{Cl}}\) suggest that this effect results from a different mechanism than the block of the MDR pump. In contrast, 50 μM NPPB, an anion-transport inhibitor, effectively blocked \(g_{\text{Cl}}\) (Fig. 6), but increased the rate of
FIGURE 4. Cl⁻ conductance in response to osmotic challenge. The slope conductance, $g_{Cl}$, plotted against time after break in. Currents were elicited as described in Fig. 3; $g_{Cl}$ was determined from a linear least-squares fit to the current-voltage relation at potentials between −35 and −55 mV. The maximal rate of increase of $g_{Cl}$ was determined by fitting a line to the maximum slope of $g_{Cl}$ vs time plot (e.g., straight line in B).

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Stimulus</th>
<th>$g_{Cl}$</th>
<th>$dg_{Cl}/dt$</th>
<th>$R_a$</th>
<th>$C_m$</th>
<th>$g_{Cl}/C_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-3T3</td>
<td>Hypertonic internal</td>
<td>33.8 ± 9.1</td>
<td>0.24 ± 0.11</td>
<td>3.8 ± 0.8</td>
<td>29.7 ± 6.3</td>
<td>1.30 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>Hypotonic external</td>
<td>25.2 ± 12.3</td>
<td>0.31 ± 0.30</td>
<td>5.3 ± 3.8</td>
<td>29.7 ± 6.0</td>
<td>0.83 ± 0.35</td>
</tr>
<tr>
<td>NIH-3T3/MDR</td>
<td>Hypertonic internal</td>
<td>39.4 ± 16.4</td>
<td>0.43 ± 0.13</td>
<td>4.7 ± 1.1</td>
<td>39.3 ± 9.2</td>
<td>0.99 ± 0.33</td>
</tr>
<tr>
<td>NIH-3T3/MDR</td>
<td>Hypotonic external</td>
<td>29.9 ± 10.9</td>
<td>0.52 ± 0.32</td>
<td>4.4 ± 4.2</td>
<td>36.6 ± 5.9</td>
<td>0.82 ± 0.30</td>
</tr>
<tr>
<td>8226</td>
<td>Hypertonic internal</td>
<td>6.1 ± 3.8</td>
<td>0.03 ± 0.02</td>
<td>4.9 ± 1.0</td>
<td>21.5 ± 4.9</td>
<td>0.31 ± 0.20</td>
</tr>
<tr>
<td>8226/Dox40</td>
<td>Hypertonic internal</td>
<td>7.7 ± 5.3</td>
<td>0.03 ± 0.02</td>
<td>4.6 ± 1.0</td>
<td>21.2 ± 7.2</td>
<td>0.37 ± 0.22</td>
</tr>
</tbody>
</table>

Cell swelling was induced by addition of 100 mM sucrose to the pipette solution or by exchange of the bathing solution with 67% Ringer. $g_{Cl}$ and $dg_{Cl}/dt$ were calculated from the slope of the $I-V$ relation near the reversal potential for currents elicited by ramp stimuli as in Fig. 5. $R_a$ and $C_m$ were calculated as described in the Methods. When $g_{Cl}$ was normalized for the size of the cell ($g_{Cl}/C_m$), there was no significance in the magnitude or rate of induction of $g_{Cl}$ in either drug-sensitive and -resistant cell pair. Data are presented as the mean ± SD.
R123 transport (see Fig. 13 below). The chloride-channel blocker, DIDS (50 μM) blocked \(g_{CI}\) in both NIH-3T3 control and NIH-3T3/MDR cells (Figs. 6 and 7), whereas doses as high as 1 mM had little effect on R123 transport (see Fig. 13 below). All three compounds had similar effects on \(g_{CI}\) in 8226 myeloma and 8226/Dox40 cells (data not shown). Thus, our results demonstrate that verapamil blocks drug transport at doses that have no effect on \(g_{CI}\). Conversely, DIDS blocks the current at doses which do not affect drug transport. Finally, NPPB can block the current at doses that increase drug transport.

\[\text{A} \quad \text{8226} \quad C_m = 15 \, \text{pF} \]
\[\text{B} \quad \text{8226 Dox40} \quad C_m = 22 \, \text{pF} \]
\[\text{C} \quad \text{NIH-3T3} \quad C_m = 34 \, \text{pF} \]
\[\text{D} \quad \text{NIH-3T3 MDR} \quad C_m = 40 \, \text{pF} \]
\[\text{E} \quad \text{NIH-3T3 MDR} \]

**Figure 5.** Cl⁻ currents elicited by a series of 200-ms voltage pulses from -100 to +100 mV. Internal solution: hyperosmotic Cs aspartate; external solution: mammalian Ringer. Currents were neither leak-subtracted nor normalized for cell size. For all cells, \(I_{Cl}\) had similar magnitude and kinetics. The larger currents in 8226/Dox40 and NIH-3T3/MDR cells (B and D) can be accounted for by cell size, as indicated by measured \(C_m\) values (see Table I). 200-ms pulses over this voltage range resulted in little or no channel closing. (E) During prolonged depolarizations up to +120 mV, slow channel closing may be seen for voltage pulses > +60 mV.

According to the dual-function model for P-glycoprotein, substrate binding to the drug transporter should inhibit drug transport (Gill et al., 1992). We tested this prediction during whole-cell patch clamp experiments by dialyzing the cell with 200 μM doxorubicin before exposure to hypoosmotic external solution. Since doxorubicin is fluorescent, we confirmed its intracellular dialysis by video imaging: half times for dialysis ranged from 150–300 s depending on \(R_s\). When fluorescence had reached a steady state, we exchanged the bath solution with 70% Ringer, effecting cell swelling
and the subsequent characteristic induction of outwardly rectifying Cl⁻ current (Fig. 8). In 17 similar experiments, neither the amplitude of g_{Cl} nor its rate of induction after hypoosmotic swelling was altered by 200 μM doxorubicin or 250 μM vincristine in the pipette solution (Fig. 9 and Table II).

**Figure 6.** Pharmacological effects on volume-activated g_{Cl}. Voltage ramps were used to test the effects of verapamil (A,B), NPPB (C,D) and DIDS (E,F) on g_{Cl} in NIH-3T3 (A,C,E) and NIH-3T3/MDR (B,D,E) cells. In each case, traces are labeled as 1 (control), 2 (drug), and 3 (wash out). Verapamil, at a dose (10 μM) that completely blocks R123 efflux (see text), did not affect g_{Cl}. Conversely, 50 μM NPPB and DIDS significantly reduced g_{Cl} in both drug-sensitive and drug-resistant cells. DIDS had no effect on R123 transport and NPPB actually increased the apparent rate of R123 transport.

**Swelling-activated Chloride Conductance in Intact Cells**

Our initial observations using whole-cell recording provided no support for the hypothesis that P-glycoprotein can function in a dual manner (Gill et al., 1992). To address this issue by an independent method, one that could be applied to intact cells, we used a video-imaging fluorescence-quench assay to monitor membrane chloride permeability (see Materials and Methods). Under isoosmotic conditions, there
FIGURE 7. DIDS blocks $g_{Cl}$. $Cl^-$ currents were recorded before, during, and after application of 50 μM DIDS. Currents were evoked by 200-ms voltage steps from −100 to +100 mV in 20-mV steps from a holding potential of −60 mV. Cells were first bathed in normal Ringer, then hypoosmotic Ringer to induce $g_{Cl}$ (not shown); the internal solution was isoosmotic CsAsp. DIDS was applied in hypoosmotic Ringer. DIDS blocked the current induced in NIH-3T3 control or NIH-3T3/MDR cells in a time- and voltage-dependent manner; block was partially reversed by a return to drug-free hypoosmotic Ringer. The degree of block and the recovery from block was variable for both the drug-sensitive and -resistant cell lines. Long incubations with higher concentrations of DIDS resulted in irreversible block.

was little $g_{Cl}$ activated, and little or no increase of SPQ fluorescence due to $I^-/NO_3^-$ exchange. Induction of $g_{Cl}$ by swelling the cells with hypoosmotic extracellular solution (67% of isoosmotic) led to a rise in SPQ fluorescence that could be fitted by a single-exponential function (Fig. 10 A). The rate of this rise was a measure of
chloride conductance at the time of osmotic challenge. Osmotically induced cell swelling increased $g_{Cl}$ in both drug-sensitive and -resistant cells (Fig. 10, B–E). Measurement of SPQ fluorescence from each individual cell in the microscopic field of view enabled a stimulus-response relationship between chloride conductance and cell swelling to be determined in separate experiments using drug-sensitive or -resistant cells. The threshold of the increase in the SPQ signal, and hence the chloride conductance, ranged from 80 to 70% of initial osmolarity. Although the

![Figure 8](image_url)

**Figure 8.** Swelling-activated chloride currents were not affected by doxorubicin. When 200 μM doxorubicin was included in the isotonic pipette solution, its entry into the cell could be monitored by measuring the fluorescence in a region (ROI) near the center of cell as shown in the inset diagram. (A) shows the increase in cellular fluorescence due to the dialysis of the cell with doxorubicin. In four experiments of this type, with pipette access resistances ranging from 2.8 to 9 MΩ, half times for dialysis ranged from 150 to 300 s. As the fluorescence intensity approached a steady state, the cell was swollen by exposure to hypoosmotic media (70% bar). A series of voltage ramps was applied to the cell before (1), during (2–4), and after (5) exposure to hypoosmotic Ringer. (B) The currents elicited by the voltage ramps show a clear induction of $g_{Cl}$ during exposure to hypoosmotic Ringer (1–4). This current was reversed upon return to isotonic Ringer (5). Pipette solution: isoosmotic NMGCl internal solution. Bath solutions: isoosmotic NMGCl Ringer followed by hypoosmotic NMGCl Ringer.

The maximal amplitude of swelling-activated chloride conductance was virtually indistinguishable in both cell types, the threshold for chloride channel opening varied from batch to batch, depending upon the time of cell passage, and between sensitive and resistant cell lines. Under identical conditions, drug-resistant cells (NIH-3T3/MDR) had a somewhat lower threshold (Fig. 10 F); this difference was more pronounced early after cell passage.
FIGURE 9. $g_{\text{Cl}}$ induction is not affected by cell dialysis with doxorubicin or vincristine. When NIH-3T3 (A) or NIH-3T3/MDR (B) cells were dialyzed with isotonic pipette solution, $g_{\text{Cl}}$ remained at a very low level for more than 200 s. At this time, dialysis directly monitored from the fluorescence of doxorubicin was roughly 50% complete. Exchanging isotonic Ringer solution bathing the cells with hypoosmotic Ringer (67% bar) increased $g_{\text{Cl}}$ with or without drug. Chloride conductance was measured as described in Fig. 4. Inclusion of 200 μM doxorubicin (C) or 250 μM vincristine (D) in the pipette solution did not affect the magnitude or the rate of increase of $g_{\text{Cl}}$ in NIH-3T3/MDR cells. The leakage conductance of the cell in isoosmotic solution was often increased during dialysis with either vincristine or doxorubicin.

### TABLE 11

| Substrates of the Drug-Efflux Pump Do Not Affect $g_{\text{Cl}}$ |
|-------------------|---------------|-----------|--------|-----------|
| Cell Type         | Drug          | $g_{\text{Cl}}$ | $dg_{\text{Cl}}/dt$ | $R_a$  | $C_m$     | $g_{\text{Cl}}/C_m$ |
| 3T3/MDR Control   | 29.9 ± 10.9   | 0.52 ± 0.32 | 4.4 ± 4.2 | 36.6 ± 5.9 | 0.83 ± 0.31 |
| 3T3/MDR 250 μM vincristine | 31.4 ± 9.7   | 0.62 ± 0.46 | 4.0 ± 1.4 | 30.6 ± 8.4 | 1.03 ± 0.16 |
| 3T3/MDR 200 μM doxorubicin | 33.4 ± 10.9   | 0.57 ± 0.28 | 5.3 ± 3.7 | 47.4 ± 14.4 | 0.73 ± 0.24 |
| 8226/Dox40 Control | 7.7 ± 5.3     | 0.08 ± 0.02 | 4.6 ± 1.0 | 21.2 ± 7.2 | 0.50 ± 0.34 |
| 8226/Dox40 200 μM doxorubicin | 6.0 ± 5.9     | 0.04 ± 0.05 | 4.7 ± 1.1 | 15.7 ± 3.8 | 0.34 ± 0.25 |

Drugs were included in the pipette solution and dialyzed into the cells for 200 s before $g_{\text{Cl}}$ was induced by cell swelling. Data are presented as the mean ± SD.
FIGURE 10. SPQ-fluorescence assay of transmembrane chloride conductance in intact cells. (A). Changes in SPQ fluorescence in NIH-3T3 cells. Initially, the fluorescence was quenched by preincubating the cells in extracellular solution containing I⁻. At first, chloride conductance is small, so I⁻/NO₃⁻ replacement does not lead to an immediate increase in fluorescence.
Pharmacology. The sensitivity of $g_{Cl}$ to various pharmacological agents in intact cells is illustrated in Fig. 11. Verapamil, which blocks P-glycoprotein-associated drug efflux at submicromolar concentration, had little or no effect on $g_{Cl}$. Among several agents tested, the most effective blocker was tamoxifen; 10 μM completely and irreversibly blocked the conductance after ~100 s. DIDS and NPPB quickly and reversibly blocked the conductance at concentrations of 1 mM and 30 μM, respectively. Our SPQ-quench measurements of $g_{Cl}$ corroborate the patch clamp data; in comparison with R123 efflux measurements (below), both methods of measuring $g_{Cl}$ demonstrate that the swelling-activated chloride channel and the P-glycoprotein-mediated drug efflux pump have very different pharmacological sensitivities.

R123 Efflux in Drug-sensitive and -resistant Cells

The first section of Results used steady state levels of R123 accumulation to document P-glycoprotein levels in drug-resistant cell lines. Here, we describe the rates of R123 efflux measured from the decrease in R123 fluorescence after removing extracellular dye. The time course demonstrates that the rate of R123 efflux in 8226/Dox40 cells is nearly 100 times higher than in the drug-sensitive counterpart, 8226 (Fig. 12). Verapamil is a highly effective inhibitor of P-glycoprotein–mediated multidrug resistance, and blocked P-glycoprotein–mediated R123 efflux (Fig. 12 A). In the control cells, verapamil had no effect (Fig. 12 B). To study the dose dependence of verapamil block, we measured the decrease in R123 fluorescence in the presence of varying concentrations of verapamil (Fig. 12 C). The concentration dependence was fitted with the equation:

$$\frac{1}{\tau} = \left(\frac{1}{\tau_0}\right) * \frac{1}{1 + \left(\frac{[\text{ver}]}{K_d}\right)} + \frac{1}{\tau_v},$$

where $\tau$ is a time constant for the decrease in R123 fluorescence, $\tau_0$ the time constant in the absence of verapamil, $\tau_v$ the time constant at saturating concentrations of verapamil, $[\text{ver}]$ the verapamil concentration, and $K_d$ the binding constant for

Hypoosmotic challenge (67%) activates $g_{Cl}$ leading to an increase in the rate of SPQ fluorescence rise. NO$_3^−$/I$^−$ pulsing with a period of 100/100 s. Quenching and unquenching of SPQ fluorescence was fitted with a single exponential function. There was also a slow, irreversible decrease in SPQ fluorescence (indicated by arrows in A), apparently due to leak of SPQ from the cell, which was further facilitated by osmotic challenge. The time constant of this leak was ~600 s and was taken into account during prolonged experiments. During the first 100 s after solution exchange, the time dependence of SPQ fluorescence was close to linear and the simple ratio of maximal fluorescence over initial fluorescence ($\Delta F/F_0$) could be used as a measure of membrane chloride conductance. Therefore, we used short pulses (50/50 s) of solution to measure $g_{Cl}$. Arrows indicate upper limits of SPQ fluorescence that would be reached after NO$_3^-$ application. Fluorescence measurements for three cells are shown in each of panels (B–E). The bar indicates hypoosmotic/isoosmotic Ringer exchange. Reducing osmolarity to 80% (B,D) caused a small increase of $g_{Cl}$ in NIH-3T3 control cells and a slightly larger increase in NIH-3T3 MDR cells. 60% Ringer (C,E) induces a larger change in $g_{Cl}$ in NIH-3T3 control cells and in NIH-3T3 MDR cells. (F) Stimulus-response of Cl$^−$ conductance vs osmolarity. Values represent mean ± SD for $g_{Cl}$ for NIH-3T3 control ($n = 37$) and NIH-3T3 MDR ($n = 42$) cells. Thus, drug-resistant and -sensitive cells had the same maximal $g_{Cl}$, but drug-resistant cells had a slightly higher sensitivity to osmotic challenge.
verapamil. The $K_d$ for blocking R123 efflux from 8226/Dox40 cells was 0.54 $\mu$M. Similar results were obtained in NIH-3T3/MDR cells ($K_d$ of 0.39 $\mu$M, data not shown).

$\text{Cl}^-$-channel blockers do not inhibit R123 efflux. Patch clamp studies presented above show that swelling-activated chloride channels in drug-resistant and drug-sensitive 3T3 fibroblasts and 8226 myeloma cells are blocked by DIDS, tamoxifen, and NPPB. Inhibition was confirmed in intact cells using the SPQ fluorescence assay (Fig. 11). None of these blockers inhibited P-glycoprotein-mediated R123 efflux (Fig. 13, A–C). These results demonstrate that pumping can occur normally even though $\text{Cl}^-$ channel function is completely blocked.

Mitochondrial uncouplers accelerate R123 efflux. The chloride-channel blocker, NPPB, markedly increased the rate of R123 efflux in drug-resistant cells (Fig. 13 D).
Rhodamine-123 efflux differs between 8226/Dox40 (A) and 8226 myeloma cells (B). Cells were loaded by incubation with 5 μM R123 and 5 μM verapamil (ver) for 20 min. The cells were washed with Ringer containing the indicated concentration of verapamil at 0 s, and were washed with drug-free Ringer at 200 s. Fluorescence intensity was used as a measure of intracellular R123 concentration. Each panel is an average of the traces from the indicated number of cells. (A) The rate of R123 efflux was determined by fitting exponential functions to the decrease of fluorescence with time. In the absence of verapamil (A, top), the trace was fitted with a single-exponential function. For the remaining panels, the trace was divided into two parts (from 0 to 200 s in the presence of verapamil; and from 200 s to 850 s after washout of verapamil), and each part was separately fitted by a single exponential function. This analysis gave the rate of efflux of R123 in presence and absence of verapamil. Verapamil caused a dose-dependent decrease in the rate of R123 efflux. After washout of verapamil, the rate of R123 efflux did not differ significantly from control. (B) In control, 8226 cells, the decline of R123 fluorescence is extremely slow and unaffected by verapamil added either early or late in the experiment. (C) The rate of R123 efflux is plotted against the log of the dose of verapamil for both cell types.
This result was initially surprising, but can be explained by the ability of NPPB to increase the cytoplasmic concentration of R123 by redistributing the substrate inside the cells. R123 is positively charged and accumulates primarily in mitochondria (Fig. 14); the cytoplasmic concentration is much lower because the mitochondrial membrane potential allows the positively charged dye to be sequestered (Neyfakh, 1988; Lampidis et al., 1989; Weaver et al., 1991). It has been shown that NPPB, besides being a Cl⁻ channel blocker, uncouples oxidative phosphorylation in mitochondria and increases the proton permeability of the plasma membrane in phagocytic cells.

(Lukacs, Nanda, Rotstein, and Grinstein, 1991). An uncoupler would decrease the electrochemical gradient across the mitochondrial membrane, causing R123 release to the cytoplasm, thus making the dye more accessible to the P-glycoprotein-associated pump in the plasma membrane.

We confirmed the uncoupling action of NPPB by demonstrating that NPPB causes changes in the pattern of R123 fluorescence in NIH-3T3 cells. Confocal microscopy images taken before adding NPPB revealed R123 fluorescence within mitochondria, with little or no nuclear staining; after adding NPPB, the fluorescence became evenly...
FIGURE 14. NPPB releases R123 from mitochondria. (A) Confocal images of NIH-3T3/MDR cells loaded with R123 in the presence of 10 μM verapamil display bright, punctate staining of mitochondria. (B) Images taken 100 s after adding NPPB show more diffuse staining with R123. The integral of the fluorescence intensity over each individual cell was not significantly different before or after addition of NPPB. Similar results were obtained with NIH-3T3 control cells and with both cell lines using CCCP instead of NPPB (data not shown).

FIGURE 15. Cell swelling does not affect R123 efflux. Cells were swollen by applying 60% Ringer, causing a large increase in g_C (see Fig. 10). R123 efflux was measured as described in Fig. 12. Rates of efflux were not significantly different in control experiments (A) and when NIH-3T3 MDR cells were swollen with 60% Ringer (B). Traces in A and B are averages from 37 and 32 cells, respectively.
distributed throughout the cytoplasm, with considerable nuclear staining (Fig. 14). As expected from results with NPPB, extracellular application of another protonophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP) causes redistribution of mitochondrial R123, along with a rapid collapse of the mitochondrial electrochemical gradient. In cells expressing P-glycoprotein, CCCP causes a dramatic increase in the rate of R123 efflux (Fig. 14 E). We conclude that the normal rate of R123 efflux observed in the absence of uncoupler is limited by the rate of release from mitochondria.

R123 efflux is not blocked by cell swelling. It has been proposed that the swelling-activated chloride conductance is mediated by P-glycoprotein, with P-glycoprotein functioning either as a chloride channel during cell swelling or a drug-efflux pump (Valverde et al., 1992). This model implies that activation of \( g_{\text{Cl}} \) would impair drug pumping by drug-resistant cells. However, the measured time constants for R123 efflux were 314 ± 19 s (\( n = 37 \)) in isoosmotic Ringer and 328 ± 23 s (\( n = 32 \)) in 60% Ringer in NIH-3T3/MDR cells (Fig. 15). Similar results were obtained in three additional experiments on 3T3/MDR cells and two experiments on 8226/Dox40 cells. Thus, R123 extrusion was not significantly different in cells swollen by hyposmotic extracellular solution.

DISCUSSION

In this study, we examined the hypothesis that P-glycoprotein forms swelling-activated Cl⁻ channels. Our principal aim was to determine if P-glycoprotein expression affected the magnitude or biophysical properties of \( g_{\text{Cl}} \) in drug-sensitive and -resistant cells. Indirect immunofluorescence and confocal microscopy demonstrated that membrane localization of P-glycoprotein accompanied the multidrug-resistant phenotype. Video imaging showed that unidirectional R123 efflux, a measure of P-glycoprotein function, was significantly higher in drug-resistant cells. Using two independent and complementary assays of \( g_{\text{Cl}} \), whole-cell current recording and SPQ-fluorescence quench, we showed that cell swelling induced the same level of chloride conductance in the drug-sensitive and -resistant cells. In addition, comparing the dose dependence for inhibition of \( g_{\text{Cl}} \) and R123 transport clearly dissociates the pharmacology of block for drug transport and chloride conductance. Furthermore, we tested two predictions of a model proposing that P-glycoprotein acts exclusively as a transport pump or an ion channel (Gill et al., 1992). Contrary to the model's predictions, we find that induction of swelling-activated chloride channels does not inhibit R123 efflux, and R123 efflux does not inhibit \( g_{\text{Cl}} \) in drug-resistant cells. Thus, these two processes appear to occur independently.

Biophysical Properties of \( g_{\text{Cl}} \) in Drug-sensitive and -resistant Cells

In drug-sensitive and -resistant NIH-3T3 fibroblasts or 8226 myeloma cells, \( I_{\text{Cl}} \) exhibited moderate outward rectification in symmetrical chloride solutions, slow and somewhat variable closing kinetics during long pulses to voltages positive to +60 mV, and similar \( K_D \) values for block by DIDS, NPPB, and tamoxifen. The rectification ratio of \( I_{\text{Cl}} \) and the relative permeabilities of chloride, aspartate, and glutamate were identical in all four cell lines. The induction of \( g_{\text{Cl}} \) in response to cell dialysis with
hyperosmotic pipette solution, or bath exchange with hypoosmotic Ringer, occurred over the same time course for both drug-sensitive and -resistant cells. During whole-cell recording, the maximum \( g_{Cl} \) varied with the size of the cell, but not with the level of expression of P-glycoprotein. Thus, electrophysiological measurements do not distinguish between \( g_{Cl} \) in these cells.

Swelling-activated Cl\(^-\) channels have now been observed in a wide variety of cell types (reviewed in Cahalan and Lewis, 1994). Indeed, in our experience it has been difficult to find a cell type in which \( g_{Cl} \) does not activate when swollen during whole-cell recording by an imposed osmotic gradient. The control drug-sensitive NIH-3T3, myeloma, and MCF-7 cell lines lacking P-glycoprotein are no exception. Based on biophysical characteristics, swelling-activated Cl\(^-\) channels may be divided into two classes: a small conductance channel that closes very slowly during strong depolarization, and a larger conductance channel that closes more rapidly during strong depolarization. Lymphocytes, chromaffin cells, neuroblastoma cells, neutrophils, fibroblasts, osteoclasts, cardiac cells, endothelial cells and *Xenopus* oocytes have channels that are activated by swelling, show moderate outward rectification in symmetrical chloride solutions, and lack voltage-dependent gating (Estacion, 1991; Tseng, 1992; Falke and Misler, 1989; Lewis et al., 1993; Doroshenko and Neher, 1992; Pollard, 1993; Stoddard, Steinbach, and Simchowitz, 1993; Zhang, Rasmusson, Hall, and Lieberman, 1993; Kelly, Dixon, and Sims, 1994; Nilius, Oike, Zahradnik, and Droogmans, 1994; Ackerman, Wickman, and Clapham, 1994). A single-channel conductance of 1–2 pS was estimated by fluctuation analysis of whole-cell currents in T lymphocytes, neutrophils and endothelial cells (Cahalan and Lewis, 1988; Lewis et al., 1993; Stoddard et al., 1993; Nilius et al., 1994). In epithelial cells from the airway and trachea, intestinal or colon cells, and Ehrlich ascites tumor cells, single-channel conductances of 28–75 pS have been measured (Worrell, Butt, Cliff, and Frizzell, 1989; Solc and Wine, 1991; Kubo and Okada, 1992; Christensen and Hoffmann, 1992). Several epithelial cell lines have volume-sensitive Cl\(^-\) channels that close at voltages positive to +80 mV (Worrell et al., 1989; Solc and Wine, 1991; Kubo and Okada, 1992; Rasola, Galietta, Gruenert, and Romeo, 1992). The time course of closing appears to be one of the most variable properties of these channels. For example, in T84 cells, closing time constants range from several hundred ms (Solc and Wine, 1991) to several seconds (Worrell et al., 1989). Slow channel closing was observed with variable kinetics in our experiments on NIH-3T3 cells (e.g., Fig. 5 E). The widespread expression of swelling-activated chloride currents and the variability in their biophysical properties (single-channel conductance, permeability ratios, and closing) suggest that these channels comprise a large and complex family of proteins or have multiple regulatory mechanisms.

To this point, swelling-activated chloride conductances have mainly been studied using the whole-cell patch clamp configuration after induction of cell swelling by an applied transmembrane osmotic gradient. Cell swelling can be initiated during whole-cell recording either by increasing the osmolarity of the pipette solution or by lowering the osmolarity of the extracellular solution. In either case, the amount of cell swelling is difficult to control, because it depends not only on the osmotic gradient (as in intact cells), but also on the size of the pipette opening, on membrane permeability to water, and on mechanical properties of the cell membrane. These
variables have been modeled recently (Ross, Garber, and Cahalan, 1994). Because the cell interior under whole-cell recording is dialyzed by the infinite reservoir of solution in the patch pipette, the model predicts the ultimate disruption of the cell membrane due to uncontrolled swelling if a high enough osmotic gradient is applied. This prediction has been confirmed experimentally (Ross et al., 1994). Because of problems in controlling cell swelling during whole-cell recording, it is important to control for access resistance when comparing \( g_{Cl} \) obtained by patch clamp experiments on different cell lines. By monitoring SPQ fluorescence with video imaging, we developed a technique to assay \( g_{Cl} \) in intact cells under controlled conditions. The SPQ-quench experiments corroborated our patch clamp data on the time course and pharmacological sensitivity of \( g_{Cl} \) and permitted a fairly accurate assessment of the threshold for channel activation by cell swelling.

**Does P-Glycoprotein Form a Chloride Channel?**

The model of P-glycoprotein as a swelling-activated \( Cl^- \) channel rests on the observation that transfection of P-glycoprotein cDNA into drug-sensitive cells resulted in the expression of swelling-activated \( Cl^- \) channels (Valverde et al., 1992). This observation was supported by experiments showing overlap in the pharmacological inhibition of drug transport.

\( g_{Cl} \) is not correlated with P-glycoprotein expression. During our patch clamp experiments, osmotic challenge with either hypoosmotic external solution or hyperosmotic internal solution resulted in cell swelling, which was invariably followed by the induction of \( g_{Cl} \) in both control or drug-resistant cell lines. We found that cell swelling induced \( g_{Cl} \) of similar magnitudes, rates of induction and biophysical properties, irrespective of P-glycoprotein expression or drug-transport activity (Figs. 3–5, Table I). After we observed large chloride conductances in the control NIH-3T3 fibroblasts, which were not reported by Valverde et al. (1992), we first ruled out the possibility of anomalous P-glycoprotein expression or drug-efflux activity in our cells. Direct assays of P-glycoprotein content and functional assays showed that our control cells do not express measurable amounts of P-glycoprotein. FACS analysis and confocal microscopy showed (Fig. 1) that P-glycoprotein was abundantly expressed in the drug-resistant cells (8226/Dox40 and NIH-3T3/MDR) but not in drug-sensitive cells (8226 myeloma and NIH-3T3 control). The transport function of P-glycoprotein in these cells was confirmed using R123-accumulation assays (Fig. 3). R123 accumulation was blocked by verapamil, a known inhibitor of P-glycoprotein-mediated transport. Thus, drug resistance was correlated with expression of P-glycoprotein in our cell lines. These results were confirmed and extended by measuring efflux rates for R123 (Figs. 12, 13, 15); rates were > 60-fold faster in 8226/Dox40 than in 8226 myeloma cells, and > 100-fold faster in 3T3/MDR cells compared with NIH-3T3 control cells. In control cells, verapamil had no effect on the rate of R123 loss, indicating undetectable functional expression of R123 (Fig. 12). Weaver and co-workers (1994, and personal communication) found more than 55,000 binding sites for the P-glycoprotein-specific monoclonal antibody, MRK-16, on NIH-3T3/MDR cells and fewer than 400 antibody binding sites on NIH-3T3 control cells (the limits of resolution of the measurement). In comparison, how many chloride channels are present? The
single-channel conductance has not been determined in these cells. If we take an estimate from noise analysis of 2 pS for similar, outwardly rectifying, swelling-activated Cl⁻ channels in lymphocytes (Cahalan and Lewis, 1994), the average number of channels per cell would be 14,000 and 16,000 channels per cell in drug-sensitive and -resistant NIH-3T3 cells, respectively. Thus, there appears to be no correspondence between the number of chloride channels and the number of P-glycoprotein molecules per cell. Regardless of the exact numbers, the near equality of $g_{\text{Cl}}$ combined with the disparity in P-glycoprotein expression strongly argues that drug efflux and chloride channel functions are mediated by different proteins.

Activation of $g_{\text{Cl}}$ consistently occurred with several combinations of pipette and bath solutions, whenever the cells were swollen and access resistance maintained. Most of our experiments were performed with solutions containing asymmetric Cl⁻ concentrations, providing a negative reversal potential to distinguish Cl⁻ currents from nonspecific leak currents; solutions containing symmetrical Cl⁻ were equally effective. Using NIH-3T3 cells obtained from the same source, and duplicating the solutions and voltage-clamp protocols used in previous studies (Gill et al., 1992; Valverde et al., 1992; Diaz, Valverde, Higgins, Rucareanu, and Sepulveda, 1993), we found equivalent $g_{\text{Cl}}$ in both control and MDR-transfected cells. We also varied the stimulus for cell swelling by using Ringer solutions which were 90, 80, 70, or 67% hyposmotic. In all cases, $g_{\text{Cl}}$ induction occurred to the same extent in both drug-sensitive and drug-resistant cell lines varying in P-glycoprotein expression by at least two orders of magnitude.

Our SPQ-quench measurements of swelling-activated $g_{\text{Cl}}$ in intact cells suggest that P-glycoprotein expressing cells may be somewhat more sensitive to the effects of osmotic gradients than control cells (Fig. 10). We have shown previously (Ross et al., 1994) that the extent of cell swelling and $g_{\text{Cl}}$ induction during whole-cell recording depends critically on several variables including pipette access resistance, as well as osmolyte concentration. We routinely monitored access resistance during whole-cell recording, and typically obtained values less than 5 MΩ. The use of large osmotic gradients (33% difference between intracellular and extracellular osmolarity) ensured maximal swelling and induction of $g_{\text{Cl}}$. In some patch clamp experiments, we observed spontaneous increases in access resistance, accompanied by artifactual decreases in $g_{\text{Cl}}$ (data not shown). In general, $g_{\text{Cl}}$ values reported previously on NIH-3T3/MDR cells were severalfold smaller than the currents we observed in control or transfected NIH-3T3 cells. The differences in experimental outcomes from previous work (Valverde et al., 1992) might be explained by the extent of cell swelling, the threshold for activation of $g_{\text{Cl}}$, the access resistance, or all three factors together. The opening of Cl⁻ channels may involve a cascade of events following cell swelling. P-glycoprotein expression may lead to pleiotropic changes that result in upregulation of a component in the pathway that links the cell's volume increase to channel activation. We have observed that control NIH-3T3 cells tend to be flatter than transfected NIH-3T3/MDR cells (data not shown), possibly making low access resistance more difficult to achieve, or reducing the degree of stretch in the surface membrane for a given increase in volume. An indirect role for P-glycoprotein might also explain a previous report that antisense inhibition in NIH-3T3/MDR cells
reduced $g_{CI}$ (Valverde et al., 1992). Recently, Altenberg et al., (1994b) reported finding volume-sensitive Cl$^-$ channels in human breast cancer cells (BC19/3) after transfection with P-glycoprotein cDNA, but not in the parental cells (MCF-7). However, using our standard protocol, we found large outwardly rectifying Cl$^-$ conductances in MCF-7 cells following cell swelling induced by hypotonic Ringer (data not shown). We also confirmed that the MCF-7 cells showed no evidence of P-glycoprotein expression, but have not pursued this comparison further (data not shown). $g_{CI}$ magnitudes reported by Altenberg and colleagues (1994a, b) in the drug-resistant BC19/3 cells were smaller than we observed in control MCF-7 cells, again suggesting suboptimal activation of $g_{CI}$. We conclude that expression of P-glycoprotein does not alter $g_{CI}$ with the caveat that the cells may have to be optimally swollen to reveal similar levels of $g_{CI}$.

Drug efflux and $g_{CI}$ are pharmacologically separable. A second line of evidence used previously to link $g_{CI}$ to P-glycoprotein was the report that some agents block both drug efflux and $g_{CI}$ (Valverde et al., 1992; Diaz et al., 1993; Nilius et al., 1994). We have compared the ability of several agents to inhibit R123 transport and $g_{CI}$. Valverde and colleagues (1992) reported that 100 μM verapamil blocked $g_{CI}$ and drug transport in NIH-3T3 cells transfected with P-glycoprotein; this was seen as evidence that P-glycoprotein forms both chloride channels and the drug transport pump. In 3T3/MDR cells, we determined a $K_d$ of 0.39 μM for verapamil block of unidirectional R123 efflux, while 100 μM verapamil had little or no effect on $g_{CI}$ determined by whole-cell recording or by SPQ imaging in NIH-3T3/MDR cells (Figs. 6, 11, 12). We also found agents that blocked $g_{CI}$, but not R123 efflux. For example, DIDS, tamoxifen, and NPPB blocked $g_{CI}$ at doses which did not block R123 efflux. Certain organic blockers of K$^+$, Na$^+$, and Ca$^{2+}$, but not Cl$^-$, channels have been shown to inhibit drug transport in mouse lymphoma cells, most likely not by virtue of channel block, but by interacting directly with P-glycoprotein (Weaver et al., 1993). Thus, there appears to be a clear dissociation between the pharmacology of $g_{CI}$ and the drug-efflux pump.

Not only was NPPB ineffective in blocking R123 efflux, but the rate of R123 efflux increased in the presence of NPPB. We propose that the enhanced efflux rate is due to greater access of the dye substrate to the P-glycoprotein efflux pump after collapse of the mitochondrial membrane potential. Using confocal microscopy, we determined that the cytoplasmic concentration of R123 increases after exposure to NPPB, an effect that is most likely related to its known protonophore activity (Lukacs et al., 1991), because the more effective protonophore CCCP further enhances the R123 efflux rate. CCCP also increases the cytoplasmic concentration of R123, but does not significantly affect R123 efflux in control NIH-3T3 cells. The results with mitochondrial uncouplers suggest that R123 efflux is limited by substrate access to P-glycoprotein in the surface membrane, and is rate limited by release from mitochondria. We checked the ability of verapamil to inhibit R123 efflux from NIH-3T3/MDR cells in the presence or absence of CCCP, and obtained $K_d$ values of 0.26 μM and 0.39 μM, respectively (data not shown). The close agreement of these $K_d$ values confirms that verapamil inhibits substrate efflux by affecting the pump rather than substrate availability.
Drug Efflux and \( g_{Cl} \) Are Not Mutually Exclusive

Gill et al. (1992) proposed a specific model in which a molecule of P-glycoprotein can function either as a pump or a channel, but not as both at the same time. According to this pump/channel model, substrate binding to P-glycoprotein drives the molecule into the pump conformation and channel activation is inhibited. In support, they reported: (a) during whole-cell recording, pipette addition of P-glycoprotein substrates inhibited the activation of \( g_{Cl} \); (b) this inhibition could be reversed by substituting nonhydrolyzable analogues of ATP in the pipette solution; and (c) mutations in P-glycoprotein's nucleotide binding domain also made \( g_{Cl} \) insensitive to inhibition by substrates for the pump. Other investigators, using human colon adenocarcinoma cells (Rasola et al., 1994) and breast cancer cells (Altenberg et al., 1994b; Han, Altenberg, and Reuss, 1994), have had difficulty in reproducing these results. Han et al. (1994) found that of cytotoxic agents tested only vinblastine prevented the induction of \( g_{Cl} \) in BC19/3 cells, and suggested an indirect effect on the \( Cl^- \) channel. We have also been unable to replicate the inhibition of \( g_{Cl} \) by drug substrates of P-glycoprotein. Including doxorubicin or vincristine in the pipette solution did not affect \( g_{Cl} \) induction (Figs. 8, 9, Table II); dialysis of doxorubicin was confirmed by monitoring intracellular fluorescence. Thus, our results support the conclusion that \( g_{Cl} \) is not inhibited by substrate binding to P-glycoprotein.

A second prediction of the pump/channel model is that drug efflux should be inhibited if the molecule is driven to function as a \( Cl^- \) channel during cell swelling. Sardini, Mintenig, Valverde, Sepulveda, Gill, Hyde, Higgins, and McNaughton (1994) reported that cell swelling reduced the rate of doxorubicin efflux by 69% and 87.2% from NIH-3T3/MDR and S1/1.1 cells, respectively. However, in our experiments, cell swelling by 67% Ringer had no effect on R123 efflux (Fig. 15); the same stimulus activated \( g_{Cl} \) maximally in intact cells monitored with the SPQ-quench assay (Fig. 10). Similar results were reported by Altenberg et al., (1994b) in BC19/3 cells. In conclusion, our experiments are not consistent with the dual-function pump/channel hypothesis.

The authors gratefully acknowledge Dr. Lu Forrest for culturing and maintaining the cell lines used in this study and Ms. Sue DeMaggio for assistance with the FACS® analysis. Ms. Ruth Davis provided helpful suggestions on the manuscript. We thank Dr. Michael Gottesman and Dr. William Dalton for generously supplying the cell lines used in this study. We thank Dr. Jose Lutzky of the Department of Hematology-Oncology, UCI and Dr. John Fruehauf and Mr. Larry Eck of Oncotoech, Irvine, CA for helpful technical discussions. Supported by NIH grants NS14609 and GM41514.

Original version received 28 June 1994 and accepted version received 7 September 1994.

REFERENCES


