

Drug efflux mediated by the human multidrug resistance P-glycoprotein is inhibited by cell swelling

A. Sardini¹, G. M. Mintenig^{2,*}, M. A. Valverde^{2,†}, F. V. Sepúlveda^{2,‡}, D. R. Gill³, S. C. Hyde³, C. F. Higgins³ and P. A. McNaughton^{1,§}

¹Department of Physiology, Division of Biomedical Sciences, King's College London, The Strand, London WC2R 2LS, UK

²AFRC Babraham Institute, Babraham, Cambridge CB2 4AT, UK

³ICRF Laboratories, Institute of Molecular Medicine and Nuffield Department of Clinical Biochemistry, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK

*Present address: Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, E-25006 Lleida, Spain

†Present address: Nuffield Department of Clinical Biochemistry, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK

‡Present address: Centro de Estudios Científicos de Santiago, Casilla 16443, Santiago 9, Chile

§Author for correspondence

SUMMARY

P-glycoprotein (P-gp), the product of the human multidrug resistance (MDR1) gene, confers multidrug resistance on cells by acting as an ATP-dependent drug transporter. A method using confocal microscopy was developed to measure the transport activity of P-gp from the rate of movement of doxorubicin, a fluorescent substrate of P-gp, across the membrane of a single cell. Recent work has shown that expression of P-gp enhances the activation of chloride channels in response to cell swelling, suggesting that membrane stretch might switch P-gp from a drug-transporting mode to a mode in which it activates chloride channels. In agreement with this idea, we find that cell

swelling inhibits drug efflux in cells expressing P-gp but is without effect on the slower background efflux in cells not expressing P-gp and in cells transiently transfected with a mutated MDR1 in which the ATP hydrolysis sites had been inactivated. The identification of a novel means for inhibiting P-gp-mediated drug transport may have implications for the reversal of multidrug resistance during chemotherapy.

Key words: cell swelling, confocal microscopy, multidrug resistance, P-glycoprotein, membrane transport

INTRODUCTION

Multidrug resistance, a major problem in chemotherapy, results from the emergence of drug-resistant cells within the target population of neoplastic cells. A frequent cause of resistance to a wide spectrum of lipophilic drugs is the expression of P-glycoprotein (P-gp), the 170 kDa product of the MDR1 gene, in the membranes of drug-resistant cells. The P-glycoprotein, a member of the ABC family of transporters (Higgins, 1992), is thought to function as an ATP-dependent drug transporter which reduces the intracellular concentration, and therefore the effectiveness, of cytotoxic drugs of the MDR spectrum (reviewed by Gottesman and Pastan, 1993). Recent studies have shown that swelling cells that express P-gp causes the activation of a chloride conductance (Valverde et al., 1992; Gill et al., 1992), suggesting that cell swelling may switch P-gp from its transport mode to a mode in which it activates chloride channels.

In order to examine the possible connection between cell swelling and the regulation of drug transport activity we have used the same cell lines as Valverde et al. (1992), in which the

correlation between P-gp expression and the presence of volume-activated chloride currents has already been established. A new method, involving the use of confocal microscopy, has been developed to follow the movement of the drug between the cytoplasm of a single cell and the external medium. The confocal microscope produces an image of a single plane - effectively an optical section - within a three-dimensional tissue labelled with a fluorescent dye (White et al., 1987; Fine et al., 1989). Doxorubicin, a substrate for P-gp, is fluorescent and the confocal microscope can therefore be used to measure the movement of doxorubicin across the cell membrane and between subcellular compartments within the cell. The effects of treatments such as cell swelling that may alter drug transport across the cell membrane can be tested in a more direct and unequivocal way than by existing methods.

Previous studies have shown that verapamil and dideoxyforskolin (DDFSK) reduce cell survival in the presence of cytotoxic drugs and that they reduce the rate of loss of drug from cell populations (Tsuruo et al., 1981; Morris et al., 1991). In the present study we demonstrate more directly that the mode of action of these inhibitors is to inhibit drug efflux

across the surface membrane of single cells. The main objective of the study was to examine the effect of cell swelling on drug transport by P-gp. We find that cell swelling inhibits the drug efflux from cells expressing active P-gp, but has no effect on the slower "background" efflux rate in cells not expressing active P-gp. These results show that cell swelling can inhibit the transport activity of P-gp, and they provide support for the hypothesis that swelling causes a reversible interconversion between transporting and channel-regulating modes of the protein.

MATERIALS AND METHODS

Cell lines

Two different cell lines permanently transfected with the human MDR1 gene were used: the NIH3T3-MDR1 c/1 line, a derivative of the mouse fibroblast NIH3T3 line (Shen et al., 1986; Pastan et al., 1988); and the S1/1.1 derivative of the lung carcinoma S1 cell line (Baas et al., 1990). Both these permanently transfected cell lines have been demonstrated to express high levels of P-gp by western blotting (Valverde et al., 1992; Gill et al., 1992). In the present study the expression of high levels of P-gp in all cells on typical coverslips was confirmed by immunohistochemistry (see below). Cells were routinely cultured in Ham's F-10 medium (S1 cells and S1/1.1) or Dulbecco's modified Eagle's medium with 25 mM HEPES (NIH3T3 cells), supplemented with 1 µg ml⁻¹ colchicine in the case of NIH3T3 MDR1 cells. Both media were supplemented with 10% fetal calf serum. Cells were plated on glass coverslips and were used 1-2 days after subculturing. A coverslip plated with cells was mounted face down in an experimental chamber which was continuously perfused. Bath volume was 300 µl and solution changes were complete within 10 seconds. All experiments were carried out at room temperature (~22°C).

Confocal microscopy, imaging and analysis software

Confocal fluorescence images were taken with a Bio-Rad MRC-600 confocal laser scanning attachment to a Zeiss Standard microscope using a Nikon ×60 PlanoApo 1.4 NA oil immersion objective lens (see Sardini and McNaughton, 1994). Drug fluorescence was excited by the 514 nm laser line of an argon ion laser and was collected after passage through a long-pass dichroic filter with half-maximal cutoff at 540 nm and a barrier filter with a half-maximal cutoff at 550 nm. Cell autofluorescence was negligible at the usual gain setting and was not subtracted. Images of drug fluorescence were normally collected every 5 or 10 seconds by averaging 3-5 frames. Fluorescence changes in selected cellular areas were determined as a function of time using software written by P. McNaughton, or for later experiments using the Bio-Rad Time Course/Ratiometric Software Module (TCSM). Nomarski transmission and epifluorescence images could be acquired simultaneously.

To investigate possible effects of pH on drug fluorescence within the cell the fluorescence emission spectrum of doxorubicin was measured at different pH values using a Hitachi F2000 fluorescence spectrophotometer with excitation at 514 nm (the excitation wavelength used for confocal microscopy). Emission peaks were observed at 509, 556 and 587 nm, of which the last contributes the major component of the signal recorded by the confocal microscope. No effect of pH on the entire emission spectrum was observed in the range pH 3.0-7.5 (<1% change). At more alkaline pH some change was observed, with emission at 587 nm reduced by 16% at pH 8.0 and by 34% at pH 9.0, but over the normal pH range within the cytoplasm or subcellular organelles there is unlikely to be a significant effect of pH on the fluorescence of doxorubicin.

Plasmids, mutagenesis and transient expression system

Plasmids pMDR7, expressing the human MDR1 gene, and pMDR712, expressing the human MDR1 gene mutated in both ATP binding domains (K433M and K1076M), were constructed as previously described (Valverde et al., 1992; Gill et al., 1992). Transient expression of the plasmids pMDR7 and pMDR712 in NIH3T3 fibroblasts was carried out using the vaccinia-T7 hybrid expression system (Fuerst et al., 1986; Gill et al., 1992).

Solutions

The isotonic bathing solution used for experiments with the S1 cell line and its derivative S1/1.1 contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, 5.5 mM D-glucose, pH 7.4. For the experiments with the NIH3T3 fibroblast cell line and its derivative NIH3T3-MDR1 the isotonic bathing solution contained 85 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.5 mM MgCl₂, 25 mM NaHCO₃, 25 mM HEPES, 5.5 mM D-glucose, bubbled with 5% CO₂ in air, pH 7.4. In the hypotonic solutions [NaCl] was reduced by 35 mM.

Immunohistochemistry

Expression of P-glycoprotein was detected by immunohistochemistry carried out on cells fixed at -20°C in methanol followed by acetone. Primary anti-P-gp antibody C219 mAb (Centocor) diluted to 10 mg ml⁻¹ in PBS with 2% goat serum was applied for 2 hours, and cells were then washed and incubated in the dark with rhodamine-conjugated goat anti-mouse IgG_{2a} in PBS with 2% goat serum for 1 hour. Cells were viewed and fluorescence was quantified using confocal microscopy as described above.

RESULTS

Measurement of drug efflux from single cells

Fig. 1A shows a view, taken with Nomarski optics, of an S1/1.1 cell permanently transfected with the MDR1 gene and expressing a high level of P-gp. Fig. 1B shows a series of images taken using confocal microscopy during an experiment on this cell. Fluorescence intensity is represented on a false-colour scale from red (high) to blue (low). The first image shows the fluorescence before addition of doxorubicin. In the second image addition of doxorubicin (25 µM) to the external medium bathing the cells caused an increase in external fluorescence. Fluorescence within the cell did not increase at early times, showing the high level of rejection by the confocal imaging method of the out-of-plane fluorescence signal from the large extracellular volume beneath the cell. At longer times drug accumulated within organelle-free regions of the cell (e.g. region labelled cyt.3 in Fig. 1A) to a level approximately equal to that in the extracellular solution. In organelle-rich regions of the cell cytoplasm (e.g. cyt.1 and cyt.2 in Fig. 1A) fluorescence intensity rose to levels substantially higher than that of the external medium, showing that the drug is accumulated in subcellular organelles. Accumulation within the nucleus was substantially slower than in the cytoplasm. The two final images show the cell fluorescence shortly after drug removal. Over the short times shown in Fig. 1B no decline in fluorescence is readily apparent, but at longer times fluorescence is observed to decline in all cellular compartments (see e.g. Fig. 2).

The time courses of the fluorescence changes in the cellular regions outlined in Fig. 1A are shown in Fig. 2A. The time course of exposure to doxorubicin is given by the trace labelled

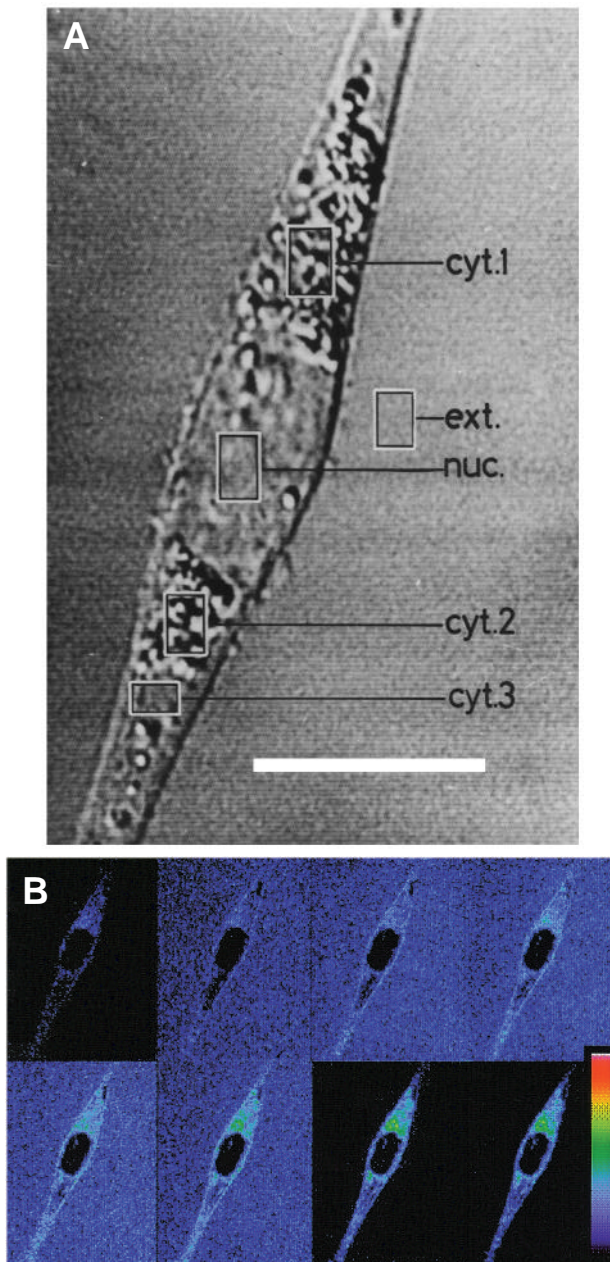


Fig. 1. (A) Transmitted-light image of an S1/1.1 cell taken with Nomarski optics implemented on the confocal microscope. The regions in the cell outlined and used for the analysis of the time course of fluorescence changes in Fig. 2 are: cyt.1 and cyt.2, containing abundant subcellular organelles; cyt.3, free of visible organelles; nucleus (nuc.); and external medium (ext.). Bar, 33 μm . (B) Sample images of fluorescence taken at 10 second intervals from a series in a typical experiment on the same cell. Fluorescence intensity is on a false-colour scale with blue representing low and red high fluorescence (see wedge to right). The cell had been exposed previously to doxorubicin, so the fluorescence level in the first frame is well above the usual level of cell autofluorescence. In subsequent frames the addition of 25 μM doxorubicin is shown by the increase in fluorescence in the external medium. Each frame is the average of 3 images.

ext., taken from a region of the image outside the cell. The concentrations of doxorubicin in the other traces can be obtained by reference to the fluorescence level of 25 μM doxorubicin in

the ext. trace, as the fluorescence of doxorubicin is unaffected by pH over the normal pH range of the cytoplasm or subcellular organelles (see Materials and Methods). After removal of doxorubicin the fluorescence declined along a single exponential time course, from which the time constant of drug loss from the cell could be calculated from a logarithmic plot of the data (see Fig. 2B).

Drug movements between subcellular compartments

Figs 1 and 2 show that there is substantial accumulation of drug in subcellular organelles. From the distribution of maximal fluorescence around the nuclear region it is probable that drug is accumulated principally in the endoplasmic reticulum or Golgi apparatus. For the purpose of measuring drug transport across the surface membrane the time constant of decline of drug concentration in the cytoplasm is of most interest, but since it is rarely possible to distinguish an area of cytoplasm completely free of subcellular organelles the fluorescence signal will in general be the sum of fluorescence from both organelles and cytoplasm. If the time constant of drug movement from organelles to cytoplasm is substantially slower than the time constant of extrusion from the cytoplasm to the external medium then the decline of total fluorescence in organelle-rich regions of the cell will be slower than in organelle-free regions. On the other hand, if the equilibration between organelles and cytoplasm is rapid then the decline in fluorescence will be similar in all parts of the cell. In the experiment shown in Fig. 2A the time constant of decline of fluorescence in organelle-rich regions (cyt.1 and cyt.2) was not significantly different from that in an organelle-free region (cyt.3), showing that equilibration between organelles and cytoplasm is rapid on the time scale of experiments such as those reported in the present study. The signal from organelle-containing regions of the cell was generally used to measure the time constant of drug efflux mediated by P-gp as it was larger, and therefore less variable, than that from organelle-free regions.

Fig. 2A also shows the fluorescence signal from the nucleus (bottom trace). Accumulation of doxorubicin in the nucleus was considerably slower than in the cytoplasm, and the decline of the signal on removal of doxorubicin was also substantially slower in most experiments, showing that the nuclear compartment is not in equilibrium with the cytoplasmic compartment.

Drug efflux mediated by P-glycoprotein

Expression of P-gp substantially enhanced the ability of cells to extrude drug. In S1 cells, which do not express P-gp, the mean cell fluorescence after 90 minutes of incubation in the presence of 5 μM doxorubicin at 37°C was 40% higher than in S1/1.1 cells permanently transfected with the MDR1 gene and expressing high levels of P-gp. Similar results have been obtained in two other cell lines by Weaver et al. (1991). The mean time constant of decline of fluorescence from S1/1.1 cells at room temperature was $\tau=758\pm 114$ seconds (mean \pm s.e.m., $n=15$), compared with $\tau=1216\pm 77$ seconds from S1 cells ($n=14$). The almost twofold difference between the time constants of drug efflux from the two cell lines expressing and not expressing P-gp is highly significant ($P=2.87\times 10^{-3}$).

Application of verapamil, a known inhibitor of drug transport mediated by P-gp (Tsuruo et al., 1981) caused a sub-

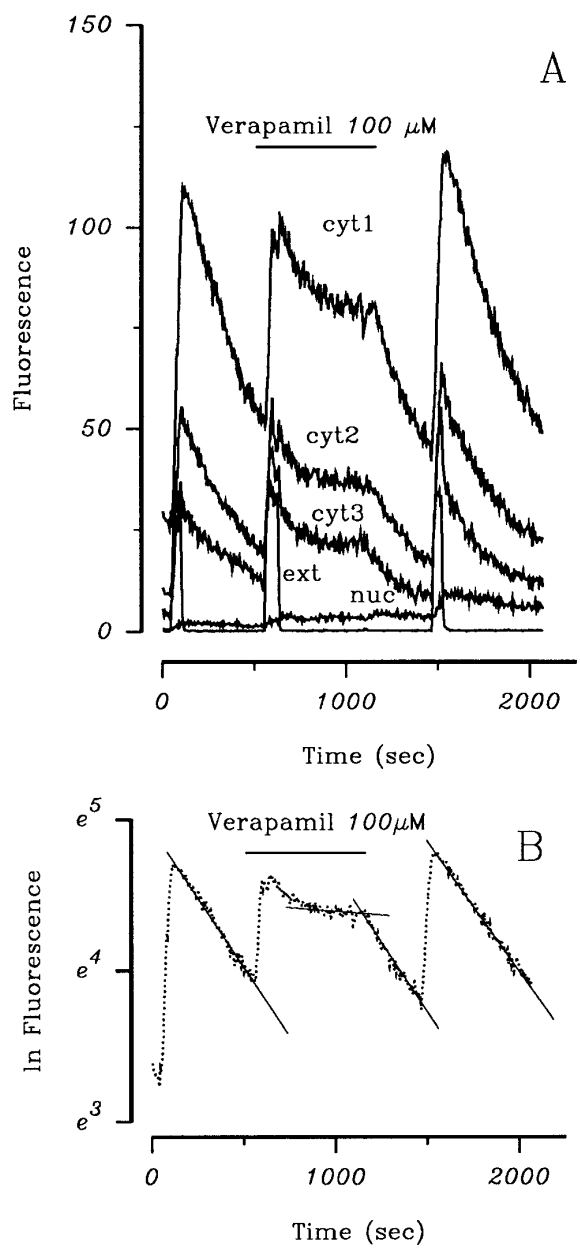


Fig. 2. (A) Fluorescence intensity (arbitrary units on a scale from 0 to 255) from the areas shown in Fig. 1A of a confocal image of an S1/1.1 cell during three exposures to doxorubicin (25 μM). Drug accumulated rapidly within the cell during the exposure periods. When doxorubicin was removed from the extracellular medium (trace labelled ext gives time course of application) fluorescence within the cell declined. The time constant of decline of fluorescence in the three cytoplasmic regions cyt.1, cyt.2 and cyt.3, obtained as shown in B, was 492 ± 27 seconds (mean \pm s.e.m.) in the absence of verapamil and was not significantly different among the three regions with the exception of the small rapid decline in fluorescence, visible on drug removal, in the organelle-free region cyt.3. Such rapid changes were visible in all regions if a thicker confocal section was taken, and can be attributed to contamination of the signal by extracellular concentration changes in thinner regions of the cell. Drug efflux was inhibited by application of 100 μM verapamil (exposure period shown by bar at top); mean time constant of decline in verapamil (in the steady state at the end of the application period) was 7531 ± 541 seconds. Percentage inhibition, calculated as $(1 - \tau_b/\tau_d) \times 100$, where τ_b is the time constant of efflux before application of verapamil and τ_d the time constant during application, was therefore 93.4% in this experiment. (B) Log plot of cyt.1 trace from upper panel (dotted) showing method of calculation of time constant of drug efflux. Continuous lines show least-squares fits to the indicated regions of data. Time constants of decline of fluorescence for this trace were 6834 seconds in verapamil and 558, 552 and 581 seconds during the three estimations in normal solution.

stantial increase in the time constant of efflux in cells expressing P-gp (from 492 seconds to 7531 seconds in the experiment shown in Figs 1 and 2), but was without effect in cells not expressing P-gp (Fig. 3). The inhibitory action of verapamil became apparent only after a delay substantially greater than the time required for the solution to be completely changed, which may indicate that the inhibition takes place at the internal face of the membrane and that verapamil must therefore enter the cell before it can act. Because of the delayed onset of inhibition we used the later part of the exposure period for calculating the time constant of drug efflux in the presence of verapamil and other inhibitors (see Fig. 2B).

The percentage inhibition of the efflux rate in experiments such as that shown in Fig. 2 was calculated as $(1 - \tau_b/\tau_d) \times 100$, where τ_b is the time constant of efflux before application of verapamil and τ_d the time constant during application. In this experiment the efflux rate was inhibited 93.4% by verapamil.

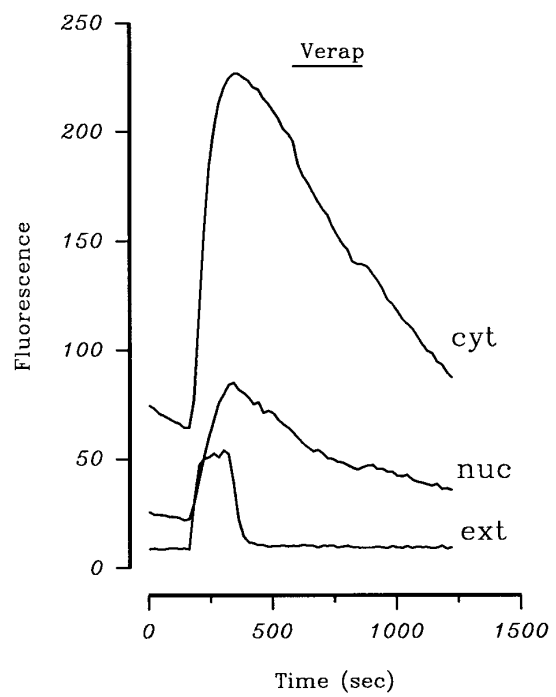


Fig. 3. Lack of effect of verapamil (100 μM) on drug efflux rate from an S1 cell not expressing P-gp. Time constant of drug efflux, 1158 seconds.

The mean inhibition in three experiments on S1/1.1 cells was 87.3% (see Table 1). Verapamil had no effect on the slower rate of drug efflux from S1 cells, which do not express P-gp (Table 1). In order to investigate the possibility that the effect was specific to the particular cell line we have also carried out similar experiments using the NIH3T3 fibroblast cell line.

Table 1. Percentage inhibition of doxorubicin efflux time constant

Cell type	Verapamil		DDFSK		Hypo	
S1	1.2±8.0 (n=5)	NS	—		-4.2±9.4 (n=5)	NS
S1/1.1	87.3±2.3 (n=3)	*	88.1 (n=1)		81.62±5.71 (n=6)	**
3T3	7.8±7.6 (n=6)	NS	5.4±3.8 (n=3)	NS	4.2±3.4 (n=11)	NS
3T3-MDR1	73.1 (n=1)		75.0 (n=1)		61.0±8.5 (n=7)	*
3T3 pMDR7	52.5±9.5 (n=6)	*	—		72.6±2.1 (n=3)	*
3T3 pMDR712	11.0±17 (n=4)	NS	—		25.8±18.8 (n=3)	NS

Mean percentage inhibition of doxorubicin efflux rate caused by verapamil, dideoxyforskolin (DDFSK) and by cell swelling (Hypo). Percentage inhibition expressed as $(1-\tau_b/\tau_d)\times 100$ where τ_b is the time constant of efflux before treatment and τ_d is the time constant during the given treatment, both measured as shown in Fig. 2B. Number of experiments given in brackets below each result; a dash indicates that the experiment was not performed. The s.e.m. of the percentage inhibition is given after each value. Significance of each result was tested by applying the *t*-test to the raw values of the efflux time constants. Verapamil and dideoxyforskolin concentrations 100 μ M; hypotonic solutions made by reducing [NaCl] to achieve 75% of the normal osmolarity (see Materials and Methods).

NS, No significant change in efflux rate was observed.

*Result was significant at the 5% level.

**Result was significant at the 1% level.

Verapamil had no significant effect on the rate of drug efflux from untransfected NIH3T3 cells, which were shown by immunohistochemistry not to express significant levels of P-gp, but substantially inhibited the efflux from an NIH3T3-MDR1 cell permanently transfected with the MDR1 gene and expressing high levels of P-gp (see Table 1).

Verapamil has effects other than blocking P-gp and we therefore investigated the block caused by dideoxyforskolin, another known inhibitor of drug transport mediated by P-gp (Morris et al., 1991). Fig. 4 shows the inhibition caused by dideoxyforskolin in an S1/1.1 cell. The magnitude of inhibition in both S1/1.1 and NIH3T3-MDR1 cells was similar to that caused by verapamil, and no effect was observed on the efflux rate from untransfected cells (see Table 1).

One unexpected feature of these results is that the drug efflux rate from untransfected cells was substantially faster than that from transfected cells in which efflux had been inhibited by verapamil or dideoxyforskolin. The mean time constant of efflux from untransfected S1 cells was 1216 seconds (see above), while the mean time constant from the permanently transfected S1/1.1 cells in the presence of verapamil or dideoxyforskolin was 4737 seconds ($n=4$). Similar results were obtained for NIH3T3 cells. A possible explanation is that overexpression of P-gp may down-regulate the expression of other drug transport mechanisms not sensitive to verapamil or dideoxyforskolin.

Inhibition of drug efflux rate by cell swelling

Fig. 5A shows the effect of applying a hypotonic medium (tonicity reduced to 75% of normal) to an S1 cell not expressing P-gp. The reduction in tonicity caused a rapid initial decrease in fluorescence due to the swelling of the cell and the consequent dilution of the intracellular contents. The rapid

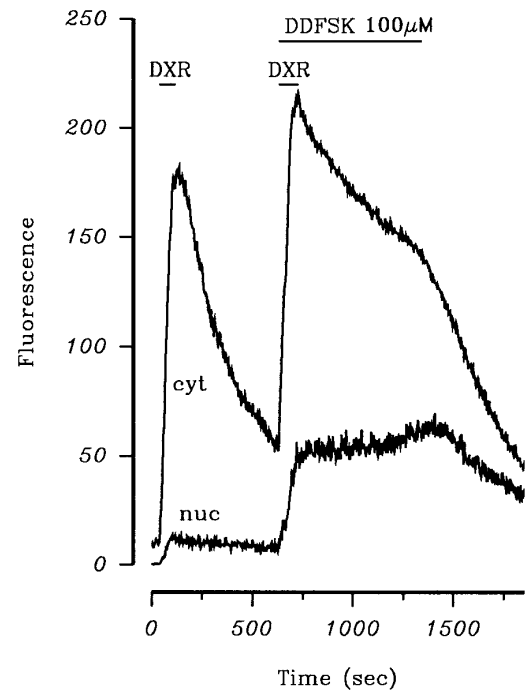


Fig. 4. Inhibition of drug efflux by dideoxyforskolin (DDFSK, 100 μ M; period of application shown by bar). Time constant of efflux 3587 seconds in the presence and 426 seconds in the absence of dideoxyforskolin; decrease in efflux rate in presence of dideoxyforskolin was therefore 88.1%. Data from an S1/1.1 cell. DXR, doxorubicin.

change in fluorescence provides a useful index of the speed of the change in cell volume, which in this and other cells was complete in 60 seconds. The time course of volume change has been confirmed by direct measurement (see Fig. 7, below). After the cell volume had reached a new steady state the rate of drug efflux was unchanged from its value before application of the hypotonic solution. Restoration of isotonic medium caused a rapid increase of fluorescence as the cell volume returned to normal, again with no significant effect on the drug efflux rate.

In a similar experiment on a permanently transfected S1/1.1 cell (Fig. 5B) application of hypotonic solution caused a substantial slowing of the efflux rate after the initial drop in fluorescence caused by the cell volume change. The efflux rate in this cell was slowed by 93% in the hypotonic solution. There is a clear delay between cell swelling and attainment of the reduced efflux rate, which may imply that the inhibitory effect depends on an intracellular mediator that takes some time to respond to the change in cell volume.

Similar experiments on NIH3T3 cells are shown in Fig. 6. No significant reduction in efflux rate was observed in untransfected NIH3T3 cells following cell swelling (A) while in all transfected NIH3T3-MDR1 cells a substantial reduction was seen. In NIH3T3-MDR1 cells, as in S1/1.1 cells, a significant delay occurred between the volume change, which reached steady state in less than 60 seconds, and the efflux inhibition.

The results of a number of similar experiments on transfected and untransfected cells are summarised in Table 1. In both S1/1.1 and NIH3T3-MDR1 cells the inhibition caused by swelling was highly significant and was similar in magnitude

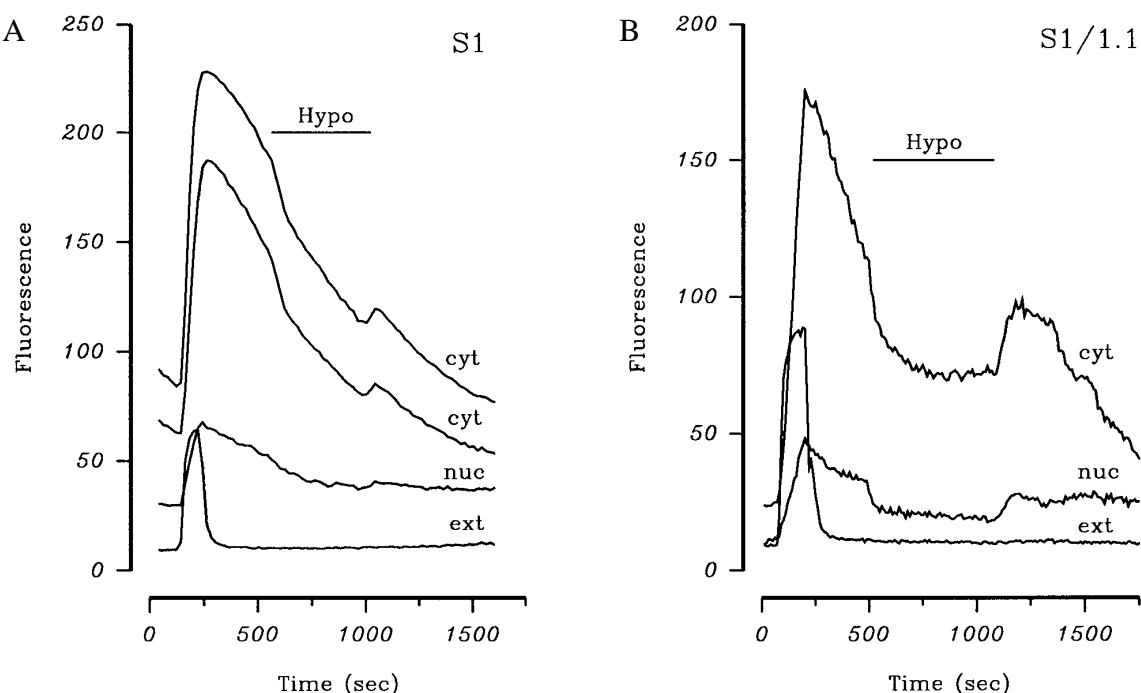


Fig. 5. Effect of cell swelling on doxorubicin efflux rate from S1 cells. (A) Untransfected S1 cell. Initial effect of hypotonic solution is to cause a rapid decrease in fluorescence because of the increase in cell volume; restoration of isotonic solution causes an opposite increase in fluorescence intensity. Efflux time constants, 1258 seconds in normal solution; no significant change observed in hypotonic solution. (B) Permanently transfected S1/1.1 cell. Delayed block of efflux is seen in hypotonic solution (osmolality decreased 25% by lowering [NaCl] from 140 μ M to 105 μ M; other constituents unchanged). During the application of hypotonic solution drug efflux rate is significantly slower ($\tau=658$ seconds before and 9955 seconds during hypotonic solution, so in this experiment hypotonic solution caused slowing of efflux rate by 93.4% of control).

to that produced by verapamil or dideoxyforskolin. No effect of swelling on drug efflux was observed in untransfected S1 or NIH3T3 cells.

A possible artifact that could explain the results in Figs 5 and 6 without the need to invoke inhibition of P-gp is that a slow recovery of volume during the exposure to hypotonic solution may cause a steady increase in the intracellular fluorescence intensity and counteract the ongoing decline in fluorescence caused by the activity of P-gp. In the experiment of Fig. 7 the diameter of a NIH3T3-MDR1 cell observed in transmitted-light Nomarski images was measured. The effect of hypotonicity was to cause a rapid swelling of the cell. Over a similar time of exposure to that in the experiments of Figs 5 and 6 no recovery of volume was observed in response to changes of osmolality to 260 mosM and to 220 mosM, the hypotonic solution used in the present experiments. A small and rapid recovery of volume was observed in response to a more extreme tonicity change to 140 mosM, but the time course of this change was too rapid and the magnitude too small to account for the inhibition of drug efflux caused by cell swelling in experiments such as those shown in Figs 5 and 6. Similar results were obtained in four other experiments on both S1/1.1 and NIH3T3-MDR1 cells. These experiments do not rule out the possible existence of volume recovery in these cells, which may take place on a longer time scale than that shown, but they do demonstrate that the inhibition of drug efflux observed in Figs 5 and 6 cannot be attributed to volume recovery during the exposure to hypotonic solution. We

conclude that cell swelling causes a reversible inhibition of drug transport mediated by P-gp.

Inhibition of drug efflux from transiently transfected cells

The phenotype of permanently transfected cells may diverge from that of the parent cell line after many passages in cell culture, and a more unequivocal way of demonstrating a difference between transfected and untransfected cells is to observe drug efflux from cells transiently transfected with MDR1 genes coding for active and inactive P-gp. Using the plasmid-vaccinia expression system (Fuerst et al., 1986; Valverde et al., 1992; Gill et al., 1992), we have transiently expressed P-gp in NIH3T3 cells. This transfection method produced high levels of expression of P-gp, measured by immunohistochemistry using an antibody to P-gp (see Materials and Methods), in all cells on a given coverslip.

We confirmed that the drug efflux from transfected cells was dependent on the expression of active P-gp by observing the effects of verapamil. Fig. 8A shows an experiment using a protocol similar to that shown in Fig. 2A. Application of verapamil caused a small rapid drop in fluorescence in this cell, perhaps caused by quenching of fluorescence. Similar rapid changes in fluorescence were also observed in cells not expressing active P-gp (see for example Figs 3 and 8C). While the origin of such rapid changes is not clear, they are therefore not related to drug transport by P-gp. The inhibitory effect of verapamil can be seen as the increase in efflux rate observed

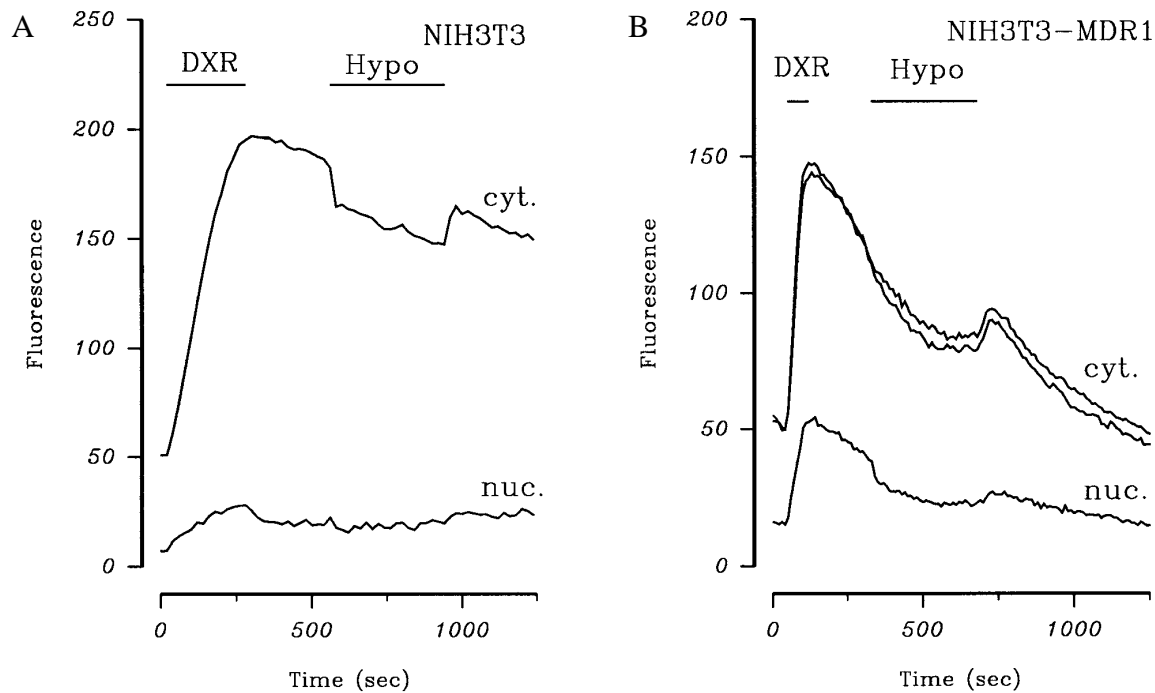


Fig. 6. Similar experiments to those shown in Fig. 5 carried out on NIH3T3 cells. (A) Untransfected cell. Efflux time constant 2744 seconds in normal solution; no significant change observed in hypotonic solution. (B) Permanently transfected NIH3T3-MDR1 cell. Signals from two cytoplasmic regions on either side of the nucleus are shown. Efflux time constant, 1328 seconds in normal solution, decreasing by 81% in hypotonic solution.

after removal of verapamil. In this experiment verapamil inhibited the efflux by 79.4%. Similar results were observed on six transiently transfected cells, with a mean inhibition of 52.5% in verapamil (Table 1).

Swelling the cell in a hypotonic solution was also effective in inhibiting efflux from transiently transfected NIH3T3 cells. Fig. 8B shows an experiment similar to that shown in Figs 5 and 6, in which cell swelling in a hypotonic medium caused an inhibition of 72.8% in the drug efflux rate. The mean inhibition in three transiently transfected cells expressing active P-gp was 72.6% (Table 1), similar to the inhibition of 61% obtained in the permanently transfected 3T3-MDR cells.

Effect of expression of mutant P-gp on drug efflux rate

The experiments described so far show that the drug efflux from cells either permanently or transiently transfected with P-gp is sensitive to inhibitors and to cell swelling. The possibility remains that this sensitivity could be an indirect effect of the expression of P-gp in transfected cells, perhaps due to an increased density of protein in the membrane causing an alteration in the properties of the lipid bilayer, and not a direct action on the transport activity of the protein. This possibility can be investigated by testing the sensitivity of drug efflux in cells expressing an inactive form of P-gp.

P-gp contains two ATP-binding domains, each containing a Walker A sequence characterised by several spaced glycine residues and a lysine residue. Mutation of the lysine in either domain abolishes ATP hydrolysis and drug resistance (Azzaria et al., 1989) but does not prevent binding of ATP or non-hydrolysable ATP analogues or activation of a chloride conductance in response to cell swelling (Gill et al., 1992).

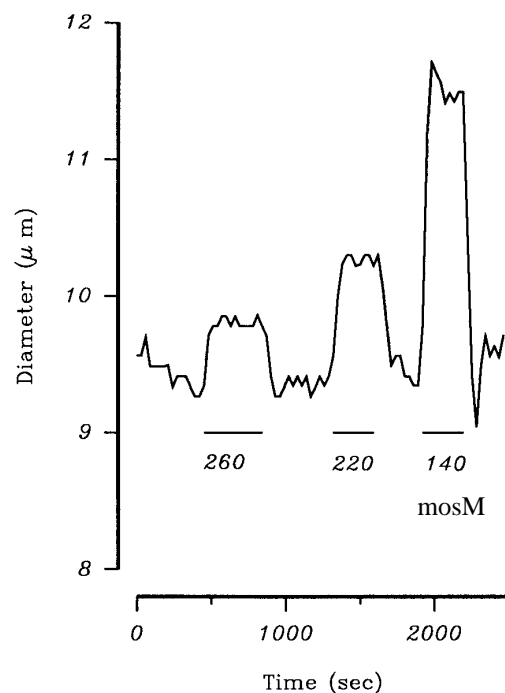


Fig. 7. Lack of volume recovery on the time scale of experiments such as that shown in Figs 4, 5 and 7. Three exposures to hypotonic solution of different osmolarities are shown; the hypotonic solution used to inhibit drug efflux (Figs 4, 5 and 7 and Table 1) was 220 mosM. Cell imaged using Nomarski optics, and edge detection improved by convolving image with a two-dimensional difference-of-Gaussians filter before measuring diameter along a line passing through the cell axis showing maximal swelling. Results shown from a NIH3T3-MDR1 cell; similar results obtained with S1/1.1 cells.

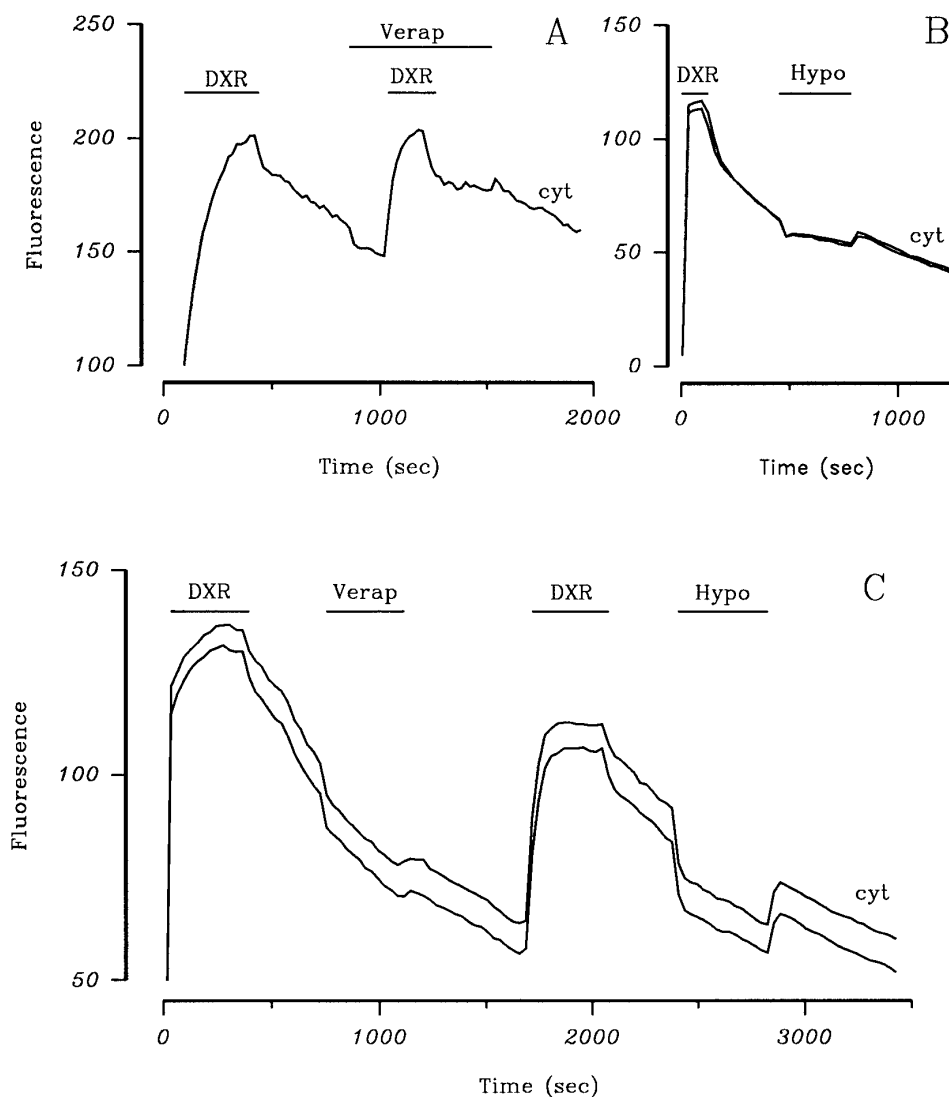


Fig. 8. Effect of verapamil and hypotonic solution on doxorubicin efflux from transiently transfected NIH3T3 cells. (A,B) Cells transfected with pMDR7 plasmid coding for active P-gp. Verapamil applied in A before loading the cell with doxorubicin in order to allow the inhibition to reach steady state (cf. Fig. 2). The rapid reduction in fluorescence observed in both A and B on removing doxorubicin (DXR) was due to slight contamination of the signal by fluorescence from doxorubicin in extracellular space in these cells, which were unusually thin, and does not represent efflux from the cell. Reduction in efflux rate in these experiments were 79.4% in verapamil and 72.8% in hypotonic solution. (C) Effect of verapamil and hypotonic solution on efflux from a NIH3T3 cell transfected with pMDR712 plasmid coding for P-gp defective in both putative ATP hydrolysis domains (Gill et al., 1992). Verapamil and hypotonic solution were without significant effect on efflux rate.

Fig. 8C shows the effects of transfecting NIH3T3 cells with a plasmid coding for P-gp mutated in both intracellular ATP binding sites. The time constant of drug efflux was unaffected by application of either verapamil or hypotonic solution. Similar results were obtained in several other cells from three independent transfection experiments (Table 1). In similar experiments on separate coverslips a high level of expression of P-gp in all cells was demonstrated by immunohistochemistry (see Materials and Methods). These observations rule out the possibility that the results could be explained by a failure of transfection in the particular cells used for the efflux experiments. We conclude, in agreement with Azzaria et al. (1989), that mutation of the ATP-binding domains abolishes the transport activity of P-gp. The experiments also make it clear that sensitivity of the drug efflux to cell swelling depends on the expression of P-gp that is capable of active transport.

DISCUSSION

In this paper we describe a method that employs confocal

microscopy to measure drug transport by P-glycoprotein from the cytoplasm of single cells. We have used this method to demonstrate that verapamil and dideoxyforskolin inhibit drug transport across the surface membrane of single cells expressing P-gp. We also find that swelling cells that express active P-gp cause an inhibition of drug transport, consistent with the hypothesis that cell swelling causes a reversible interconversion between transport and channel-regulating modes of P-gp.

Advantages of the method

There are several advantages in measuring drug efflux using the method described in this paper compared with conventional studies of efflux of radiolabelled drug: (i) time resolution is better by at least two orders of magnitude; (ii) drug transport is measured from a single cell, while in studies using labelled drug the efflux from a large and possibly variable population is measured; (iii) influx as well as efflux is readily measured in the same cell; (iv) the high spatial and temporal resolution of the confocal microscope means that transport across the cell surface membrane can be distinguished from the accumulation of drug in subcellular compartments such as the nucleus. With

a sufficiently thin confocal section the signal is uncontaminated by fluorescence from the extracellular medium or from cellular compartments lying above or below the image plane.

Inhibition of P-gp-mediated drug transport

Verapamil and dideoxyforskolin are known to inhibit drug loss from cell populations expressing P-gp. The present study extends these observations by showing that, at the level of a single cell, the action of these inhibitors is to abolish P-gp-dependent drug transport across the cell membrane, ruling out other explanations such as the possibility that the action of the inhibitors may be through effects on drug sequestration within the cell. Drug loss from the cell continues at a much reduced rate in the presence of inhibitors, probably by passive diffusion of the lipophilic drugs through the cell membrane.

Expression of P-gp mutated in the ATP-binding domains has been shown to have no effect on cell survival in the presence of cytotoxic drugs, in contrast to the effect of expression of wild-type P-gp (Azzaria et al., 1989). In the present study we show more directly that cells transfected with mutated P-gp perform no verapamil-sensitive drug transport.

Effect of cell swelling on drug transport by P-gp

The main reason for carrying out the present study was to investigate the effect of cell swelling on drug transport by P-gp. Recent work has shown that in cells expressing P-gp a chloride conductance can be induced by cell swelling (Valverde et al., 1992; Gill et al., 1992). One possible explanation of this observation is that P-gp could be both a drug transporter and a chloride channel, and that cell swelling switches the protein between the two modes. An alternative explanation is that P-gp and the chloride channel may be separate entities, but that P-gp regulates the activity of the chloride channel in response to membrane stretch. Consistent with either of these hypotheses are the findings that CFTR, a member of the same superfamily of transporters as P-gp, is both a chloride channel (see Welsh et al., 1992) and a regulator of the activity of other chloride channels (Egan et al., 1992; Gabriel et al., 1993).

The results presented here show that cell swelling inhibits drug transport mediated by P-gp, and that the inhibition was similar in magnitude to that caused by known blockers of P-gp such as verapamil. Drug transport in cells not expressing P-gp, or expressing non-functional P-gp, was not affected either by cell swelling or by verapamil. In contrast, a recent report found that swelling human breast cancer cells transfected with MDR1 cDNA did not reduce the rate of efflux of rhodamine 123, a substrate for P-gp (Altenberg et al., 1994). The reason for this discrepancy is unclear, but a clue may lie in the observation (see Figs 5 and 6) that the inhibition of drug transport caused by cell swelling occurred with a delay substantially greater than that required for the cell to attain a new steady-state volume. The delay suggests the involvement of a signalling pathway linking the volume change to P-gp inhibition. The present study was carried out using cell lines in which a link between P-gp expression and the regulation of chloride channels by cell volume had been established, and in which all elements of the putative signalling pathway must therefore be functional. If elements of this pathway are missing in the breast carcinoma cells studied by Altenberg et al. (1994) then the sen-

sitivity of drug transport to changes in cell volume may be absent.

The results in the present study are consistent with a direct link between P-gp and volume-activated chloride channels. In the case of an indirect link - such as might occur if P-gp expression enhanced the sensitivity of the cell membrane to stretch - there would be no reason to expect an effect of cell swelling on drug transport. The results support instead the hypothesis (Gill et al., 1992) that changes in cell volume cause P-gp to undergo a reversible interconversion between a transporter and a channel-activating mode.

The finding that cell swelling can inhibit P-gp-mediated drug transport raises the possibility of developing new strategies for reversing multidrug resistance. A compound that is able to mimic the effects of cell swelling and switch P-gp from its drug transporting mode to the channel-regulating mode could potentially inhibit drug transport and reverse multidrug resistance with fewer side effects than existing blockers. An important area for future research will be to elucidate the mechanism by which cell swelling brings about this switch in P-gp function.

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