P-glycoprotein is not a swelling-activated Cl^- channel; possible role as a Cl^- channel regulator

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1. The whole-cell configuration of the patch-clamp technique was used to determine if P-glycoprotein (Pgp) is a swelling-activated Cl^- channel.

2. Hamster *pgp1* cDNA was transfected into a mouse fibroblast cell line resulting in expression of functional Pgp in the plasma membrane. This cell line was obtained without exposure to chemotherapeutic agents.

3. Swelling-activated whole-cell Cl^- current ($I_{Cl,swell}$) was elicited by lowering the bath osmolality. $I_{Cl,swell}$ was characterized in detail in the *pgp1*-transfected mouse cell line and compared with that of its parental cell line. Expression of Pgp did not modify the magnitude or properties of $I_{Cl,swell}$, except that addition of the anti-Pgp antibody C219 to the pipette solution inhibited this current by 75% only in the Pgp-expressing cells.

4. $I_{Cl,swell}$ in the mouse Pgp-expressing cell line was compared with that in a Pgp-expressing hamster fibroblast cell line. The characteristics of $I_{Cl,swell}$ (voltage dependence, blocker sensitivity, anion selectivity sequence, requirement for hydrolysable ATP) in Pgp-expressing cells were different between the two cell lines. These results suggest that the channel(s) responsible for $I_{Cl,swell}$ are different between the two cell lines. In addition, C219 inhibited $I_{Cl,swell}$ in both Pgp-expressing cell lines, even though they seem to express different swelling-activated Cl^- channels.

5. We conclude that firstly, Pgp is not a swelling-activated Cl^- channel; secondly, it possibly functions as a Cl^- channel regulator; and thirdly, $I_{Cl,swell}$ is underlined by different Cl^- channels in different cells.

Following swelling by exposure to hyposmotic solutions, many cells regulate their volume by losing K^+ and Cl^- via ion channels (Hoffmann & Simonsen, 1989). Considerable information exists on K^+ channels, but the molecular identity and mechanism(s) of regulation of Cl^- channels activated by cell swelling are not completely understood. P-glycoprotein (Pgp) was originally suggested to be a swelling-activated Cl^- channel. It was reported that cells transfected with human Pgp cDNA (*MDR1*) displayed a swelling-activated Cl^- current ($I_{Cl,swell}$) while the non-transfected cells showed little or no $I_{Cl,swell}$ (Valverde, Díaz, Sepúlveda, Gill, Hyde & Higgins, 1992), but subsequent experiments demonstrated that $I_{Cl,swell}$ is independent of Pgp expression (e.g. McEwan, Hunter, Hirst & Simmons, 1992; Rasola, Galietta, Gruenert & Romeo, 1994; Han, Vanoye, Altenberg & Reuss, 1996). However, these observations do not rule out the possibility that Pgp is a swelling-activated Cl^- channel since Pgp expression could downregulate the expression of native Cl^- channels without affecting the magnitude of $I_{Cl,swell}$.

Although there is no correlation between Pgp expression and the magnitude of $I_{Cl,swell}$, two observations indicate a specific relationship between Pgp expression and $I_{Cl,swell}$: (1) the specific block of $I_{Cl,swell}$ by the anti-Pgp monoclonal antibody (mAb) C219 only in cells expressing Pgp (Han *et al.* 1996), and (2) inhibition of $I_{Cl,swell}$ by the phosphorylation of Pgp catalysed by protein kinase C (PKC) (Hardy, Goodfellow, Valverde, Gill, Sepúlveda & Higgins, 1994). In principle, the relationship between $I_{Cl,swell}$ and Pgp could be explained by Pgp being either a Cl^- channel or a Cl^- channel regulator. However, it was proposed that cells expressing Pgp display an altered threshold for activation of the swelling-activated Cl^- current (Luckie, Krouse, Harper, Law & Wine, 1994; Luckie, Krouse, Law, Sikic & Wine, 1996). The authors claimed that exposure of Pgp-expressing cells to chemotherapeutic agents, not Pgp expression *per se*, caused the altered threshold.

The aim of the present work was to determine the nature of the relationship between Pgp expression and $I_{Cl,swell}$. We

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attempted to answer the following questions. (1) Does Pgp expression *per se* modify $I_{Cl,swell}$, independently of cell exposure to chemotherapeutic agents? (2) Does Pgp function as a swelling-activated Cl^- channel or a Cl^- channel regulator? To answer these questions, inhibition of $I_{Cl,swell}$ by C219 was tested in cells induced to express Pgp without selection with chemotherapeutic agents, and $I_{Cl,swell}$ was characterized in detail in both *pgp1*-transfected (BALB–*pgp1*) and parental mouse fibroblast BALB/c-3T3 cells, and compared with $I_{Cl,swell}$ in the Pgp-expressing Chinese hamster fibroblast LZ-8 cell line. If Pgp is a channel, then the properties of $I_{Cl,swell}$ (i.e. blocker sensitivity, kinetics, ion selectivity, voltage dependence) exhibited by cells which express Pgp (LZ-8 cells) should be due, at least partially, to the Pgp channel. These properties should emerge in another cell line when it is induced to express the same gene (BALB–*pgp1* cells). On the other hand, if Pgp expressed in the plasma membrane modulates swelling-activated Cl^- channels, then $I_{Cl,swell}$ in different cell lines (underlaid by different native channels) could have different characteristics, regardless of the level of Pgp expression. Hence, the characteristics of $I_{Cl,swell}$ in a cell line transfected with Pgp cDNA would not differ from those of $I_{Cl,swell}$ in the parental cell line. Some of these results have been communicated in preliminary form (Vanoye, Altenberg & Reuss, 1996*a*, *b*).

METHODS

General procedures

We used the mouse fibroblast cell line BALB/c-3T3 (gift from Dr E. B. Mechetner), which does not express Pgp (Han *et al.* 1996), and the Pgp-expressing Chinese hamster lung fibroblast cell line LZ-8 (gift from Dr J. A. Belli), which expresses functional Pgp in the plasma membrane at very high levels (Sognier, Zhang, Ebrle & Belli, 1992; Altenberg, Young, Horton, Glass, Belli & Reuss, 1993). This cell line was originated from V79 cells by selection with doxorubicin (Adria Laboratories, Columbus, OH, USA). The mouse cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% (v/v) penicillin–streptomycin (P–S; 1 unit penicillin, 1 μg streptomycin; Gibco BRL). Cells transfected with *pgp1* cDNA or vector alone were grown in the presence of the antibiotic geneticin (G418; Gibco BRL). LZ-8 cells were grown in F-10 (Ham's) medium supplemented with 10% FBS, 1% P–S and 8 μg ml^{-1} doxorubicin. Doxorubicin and geneticin were removed from the culture medium 1 day prior to the experiments.

Cell transfection

In order to generate Pgp-expressing cells without exposure to chemotherapeutic agents, we transfected BALB/c-3T3 cells with either pLK212S or pLK444 (Devine, Hussain, Davide & Malera, 1991). The vector pLK212S contains the full-length *pgp1* cDNA, and the aminoglycosidephosphotransferase-3′(II) cDNA. The phosphotransferase inactivates the antibiotic G418 by phosphorylation. The vector pLK444 lacks the *pgp1* cDNA and was used to generate control transfectants. Transfections were performed using lipofectin (Gibco BRL). Approximately 10^6 cells were plated overnight in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% FBS and 1% P–S. After washing twice with FBS- and P–S-free medium, the cells were incubated for 6 h in 6 ml of the same medium containing a lipofectin–DNA mixture (10 μg lipofectin, 7 μg DNA). Then, FBS and P–S were added, and 48 h later, G418 (final concentration, 600 μg ml^{-1}) was added in order to select transfected cells. Approximately 2 weeks later, the surviving clones were assayed for functional expression of Pgp.

Rhodamine 123 accumulation and efflux

To determine which of the G418-resistant clones expressed Pgp, cells were loaded with 2 μM rhodamine 123 (R123), a fluorescent dye transported by Pgp (Neyfakh, 1988). At this concentration, R123 is not toxic to BALB/c-3T3 cells (C. G. Vanoye, G. A. Altenberg & L. Ruess, unpublished observations). The cells were loaded at room temperature in Hepes-buffered solution (HBS, mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 7·8 glucose and 5 Hepes–NaOH, pH 7·4, osmolality ~280 mosmol kg^{-1}) for ~1 h. Cell fluorescence (excitation at 495 nm, emission measured at wavelengths larger than 535 nm) was detected by confocal microscopy. Following loading, the wild-type cells are fluorescent (high intracellular R123 concentration), while the Pgp-expressing cells are dark (low intracellular R123 concentration). Dark clones were grown for subsequent studies. The Pgp-expressing clones were quantitatively tested for functional Pgp expression by measuring the unidirectional R123 efflux as described previously (Altenberg *et al.* 1993*b*). Briefly, the cells were loaded with 10 μM R123 for 1 h; then they were superfused with R123-free HBS, and the fall in intracellular R123 fluorescence (F_{R123}) was measured as a function of time. The decay in F_{R123} follows a single exponential; the rate constant (k) for the decay in F_{R123} was estimated from fits of the following equation to the data:

$$F_{R123} = F_0 + F_{R123}(0)\exp(-kt),$$

where F_0 is the background fluorescence, and $F_{R123}(0)$ is F_{R123} at time (t) 0.

Western blot analysis

Preparation of membranes and Western blot analysis were as described previously (Altenberg, Subramanyam, Bergmann, Johnson & Reuss, 1993). The anti-Pgp mAb C219 (Signet, Dedham, MD, USA) which recognizes two internal epitopes in human, hamster, and mouse Pgp (Georges, Bradley, Gariepy & Ling, 1990) was employed as described (Han *et al.* 1996).

Electrophysiology

Whole-cell currents were measured in the broken-patch configuration of the patch-clamp technique using an Axopatch 200A amplifier (Axon Instruments, Inc.). The compositions of the bath and pipette solutions (isosmotic (Iso) and 22% hyposmotic (Hypo)), designed to have Cl^- as the main conductive ion, were as follows: (a) Iso, mM: 140 NMDG-Cl (*N*-methyl-D-glucamine chloride), 1·3 $CaCl_2$, 0·5 $MgCl_2$, 10 Hepes, 7·8 glucose, pH 7·4, ~275 mosmol kg^{-1}; (b) Hypo: similar composition, except that NMDG-Cl was nominally reduced by 35 mM, lowering the osmolality to ~215 mosmol kg^{-1}; (c) NaCl–Hypo (used for determinations of anion selectivity): composition similar to Hypo, except that NMDG-Cl was replaced by NaCl; (d) pipette solution (mM): 140 NMDG-Cl, 1·2 $MgCl_2$, 10 Hepes, 2 Na_2ATP, 1 EGTA, pH ~7·0, ~270 mosmol kg^{-1}. Na_2ATP was purchased from Sigma. The pipette solution was diluted 5–10% with distilled water to prevent cell swelling and activation of swelling-activated Cl^- currents in Iso solution. The pipettes were pulled with a multistage P-87 Flaming–Brown micropipette puller (Sutter Instruments Co., San Rafael, CA, USA) and fire polished. Pipette resistances were 3–5 MΩ (NMDG-Cl in the pipette and bath solutions). The holding voltage was 0 mV. (As seen in the Iso traces, this holding potential *per se* did not activate membrane currents.) The Cl^- currents were measured from −80 to +80 mV (at 20 or 40 mV steps), 10 or 40 ms after the start of the voltage pulse, as indicated. The access resistance and apparent membrane

capacitance were estimated as described by Lindau & Neher (1988). Pulse generation, data collection and analyses were done with pCLAMP (Axon Instruments, Inc.). For the experiments in which ion channel blockers were tested, the osmolality of the Hypo bath solution was 89% of Iso. This allowed for stable $I_{Cl,swell}$ for long periods, hence permitting exposures of the same cells to several agents. Cells were exposed to each blocker concentration for 6 min. In some cells, the reversibility of the block was also examined at each concentration. To determine the ionic selectivity of $I_{Cl,swell}$, most bath NaCl was partially replaced (mole by mole) with NaI, NaBr, NaSCN, $NaNO_3$ or sodium gluconate. The liquid junction potentials generated upon changing the extracellular anion were calculated from the generalized Henderson liquid junction potential equation, using the program JPCalc (P. H. Barry, University of New South Wales, Sydney, Australia). The reversal potential (V_{rev}) was obtained by zero-current interpolation of a third order polynomial fit to the current–voltage relationships obtained for each anion. The anion permeabilities relative to Cl^- (P_X/P_{Cl}) were estimated from the changes in V_{rev} after partially substituting the extracellular Cl^- with another anion (X) by using the Hodgkin–Katz modification of the Goldman equation:

$$P_X/P_{Cl} = ([Cl]_i/(10^{V_{rev}/58.7})) - [Cl]_o/[X]_o,$$

where $[Cl]_i$ is the intracellular (pipette) Cl^- concentration and $[Cl]_o$ and $[X]_o$ are the extracellular Cl^- and anion concentrations (bath), respectively. In some experiments, the anti-Pgp antibody C219 was added to the pipette solution at a final concentration of 1 or 10 $\mu g\ ml^{-1}$. In these experiments, exposure to the Hypo solution was started ~30 min after breaking the patch to allow sufficient time for diffusion of the antibody into the cell. The access resistances varied by 7–10 MΩ. Only cells in which the access resistance did not increase by more than 2-fold were used.

BALB/c-3T3, BALB–444, and BALB–*pgp1* cells were detached the day of the experiment by exposure to Ca^{2+}-free phosphate-buffered saline (mM: 137 NaCl, 8 Na_2HPO_4, 2·7 KCl, 1·5 KH_2PO_4, pH ≈ 7·4) containing 0·5 mM EDTA. Before the experiments, the cells were allowed to recover for at least 30 min at room temperature (22–23 °C), in the Hepes-buffered saline. The mouse fibroblasts were detached because when grown at low confluency they spread out very thinly and are difficult to patch. Detachment of BALB–*pgp1* cells did not affect Pgp expression in the plasma membrane, i.e. R123 efflux was similar in attached and detached cells (data not shown). The LZ-8 cells were plated at low density and grown overnight on glass coverslips. Only isolated cells were patched. In these cell lines, $I_{Cl,swell}$ is similar in attached and detached cells (C. G. Vanoye, G. A. Altenberg & L. Reuss, unpublished observations).

Statistics

Data shown are means ± S.E.M. Statistical comparisons were done by one-way analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

Mouse cells transfected with Chinese hamster *pgp1* cDNA express functional Pgp in the plasma membrane

To obtain a cell line that expresses Pgp without selection with chemotherapeutic agents, we transfected BALB/c-3T3 cells with *pgp1* cDNA (BALB–*pgp1* cells). Western blot analysis of membranes from BALB–*pgp1* cells with the mAb C219 showed an immunoreactive band of ~170 kDa, as expected for Pgp (Fig. 1). Pgp was not detected in the control transfectants, BALB–444. In previous studies, Western blots of membranes of the parental BALB/c-3T3 cells were negative (Han *et al.* 1996). BALB–*pgp1* cells displayed significant R123 unidirectional efflux, whereas the R123 effluxes from the parental cells and the cells transfected with the vector alone were *ca* 10-fold slower than those of the Pgp-expressing BALB–*pgp1* cells (Fig. 2*A*). The R123 efflux in the Pgp-transfected cells was inhibited by the Pgp substrates verapamil (Sigma) and vinblastine (Lyphomed, Deerfield, IL, USA); these agents did not affect R123 efflux in BALB–444 cells. The data are summarized in Fig. 2*B*. These studies demonstrate that BALB–*pgp1* cells express functional Pgp in the plasma membrane. In addition, transfection of BALB/c-3T3 cells with the vector alone followed by selection with G418 did not induce Pgp expression.

The monoclonal anti-Pgp antibody C219 inhibits $I_{Cl,swell}$ only in Pgp-expressing cells

We have previously shown that C219 (added to the pipette solution) inhibits $I_{Cl,swell}$ in cells expressing Pgp, and has no effect in cells lacking Pgp expression (Han *et al.* 1996).

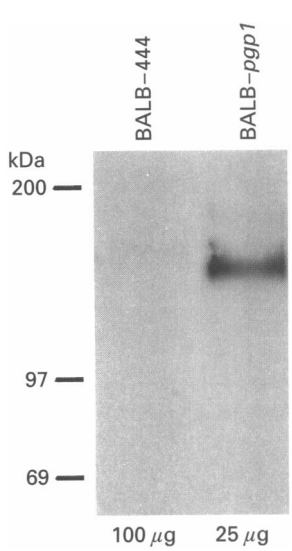

Figure 1. Pgp expression in BALB–*pgp1* cells

Western blot analysis of membrane proteins with the anti-Pgp mAb C219. The amount of protein loaded for each lane is indicated at the bottom of the figure. Molecular masses are indicated on the left.

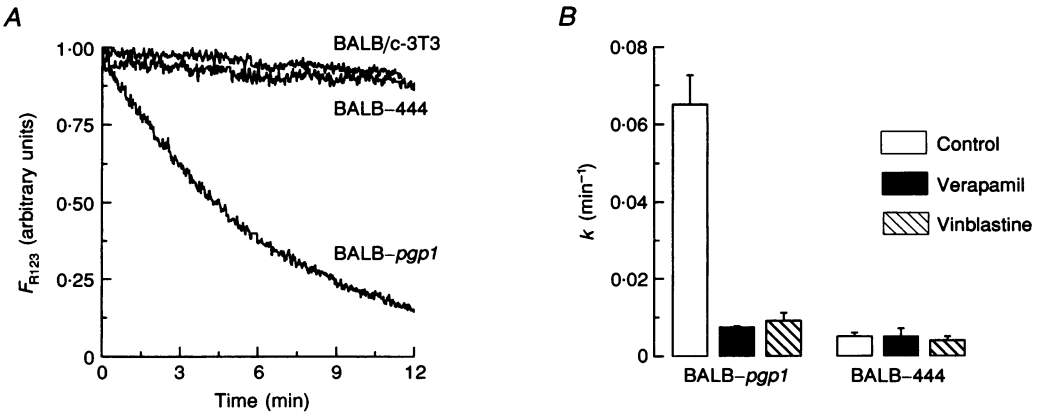

Figure 2. Functional expression of Pgp in BALB–*pgp1* cells

The decay in intracellular R123 fluorescence (F_{R123}) from R123-loaded cells was measured following superfusion with R123-free solution. *A*, mouse cells transfected with hamster *pgp1* cDNA (BALB–*pgp1*) display significant R123 efflux while the parental cells (BALB/c-3T3) and the control transfectants (BALB–444) had much slower efflux. The efflux rate constant (k) calculated for BALB–*pgp1* was $0{\cdot}065 \pm 0{\cdot}007$ min^{-1}, while the parental BALB/c-3T3 and control transfectant BALB–444 cells had efflux rate constants of $< 10\%$ of that of Pgp-expressing cells ($0{\cdot}004 \pm 0{\cdot}001$ min^{-1} and $0{\cdot}005 \pm 0{\cdot}001$ min^{-1}, respectively). *B*, Pgp substrates verapamil and vinblastine blocked R123 efflux in BALB–*pgp1* cells but had no effect in BALB–444 cells.

However, those experiments were carried out on cells grown continuously in the presence of chemotherapeutic agents. Hence the inhibition of $I_{Cl,swell}$ by C219 could be the result of exposure to chemotherapeutic agents, making it impossible to ascribe the properties of $I_{Cl,swell}$ to Pgp expression alone. Figure 3 shows that C219 (1–10 μg ml^{-1}) inhibited $I_{Cl,swell}$ in the Pgp-expressing BALB–*pgp1* and LZ-8 cells, but had no effect in BALB–444 cells. The inhibition of $I_{Cl,swell}$ by C219 in the two Pgp-expressing cell lines, one drug selected and the other transfected and never exposed to chemotherapeutic agents, denoted a specific interaction between Pgp and the protein(s) responsible for the swelling-activated Cl^- current. These results confirm and extend previous observations with this antibody (Han *et al.* 1996; Wu, Zhang, Koppel & Jacob, 1996).

The specific relationship between $I_{Cl,swell}$ and Pgp expression has at least two possible explanations: either Pgp is a swelling-activated Cl^- channel, or Pgp is a Cl^- channel regulator. If Pgp were a channel, then the properties of $I_{Cl,swell}$ exhibited by the Pgp-expressing BALB–*pgp1* and LZ-8 cells should be similar. However, if Pgp expressed in

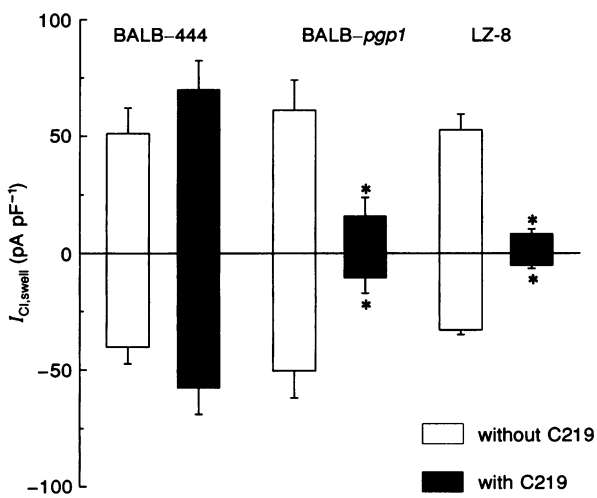

Figure 3. The anti-Pgp antibody C219 blocks $I_{Cl,swell}$ only in Pgp-expressing cells

The addition of C219 to the pipette solution (BALB–444 cells, 10 μg ml^{-1}; BALB–*pgp1* cells, 1 μg ml^{-1}; LZ-8 cells, 1–10 μg ml^{-1}) blocks $I_{Cl,swell}$ only in Pgp-expressing cells. $I_{Cl,swell}$ values were measured at −80 and +80 mV, 40 ms from the start of the voltage pulse, after a 10 min exposure to the Hypo solution. Data are means ± S.E.M. of currents normalized for the estimated cell membrane capacitance. * $P < 0{\cdot}05$ compared with values in the absence of C219.

the plasma membrane functions as a regulator of swelling-activated Cl^- channels, then $I_{Cl,swell}$ in BALB–*pgp1* and LZ-8 cells could be different. The following experiments were done to determine the role of Pgp in $I_{Cl,swell}$.

Expression of hamster Pgp does not alter the magnitude or *I–V* relationship of $I_{Cl,swell}$ in BALB/c-3T3 cells

Representative records of Cl^- currents under basal conditions and after activation by swelling of the mouse (Pgp-expressing and parental) and hamster cells are shown in Fig. 4. The apparent membrane capacitance values were: BALB/c-3T3, 31 ± 2 pF ($n = 31$), BALB–*pgp1*, 39 ± 2 pF ($n = 32$); and LZ-8 cells, 19 ± 1 pF ($n = 35$). Whole-cell Cl^- currents were measured after 8–10 min of exposure to Iso solution, and after 8–10 min of exposure to Hypo solution ($I_{Cl,swell}$). The three cell lines exhibited sizable swelling-activated Cl^- currents with different degrees of outward rectification. Although these three cell lines have different levels of Pgp expression, ranging from none to very high (Sognier *et al.* 1992; Han *et al.* 1996; and present results), the Cl^- currents elicited by a 22% reduction in bath osmolality were of similar magnitudes in all three. The data in Fig. 4 show that expression of Pgp did not increase the magnitude of $I_{Cl,swell}$. The rectification ratio (ratio of absolute currents measured at 10 ms, at +80 and −80 mV, respectively) was 1·26 ± 0·03 ($n = 11$) for the parental BALB/c-3T3 cells and 1·25 ± 0·03 ($n = 8$) for BALB–*pgp1* cells. In contrast, $I_{Cl,swell}$ in LZ-8 cells displayed a larger outward rectification ratio of 1·82 ± 0·12 ($n = 10$), statistically different from those in the mouse cell lines. These results show that Pgp expression did not alter the magnitude and *I–V* relationship of $I_{Cl,swell}$ in BALB/c-3T3 cells, and that the *I–V* relationship of $I_{Cl,swell}$ was dissimilar between the two Pgp-expressing cell lines.

Ionic selectivity of $I_{Cl,swell}$ in mouse and hamster cell lines

To characterize $I_{Cl,swell}$ in BALB/c-3T3 and in the Pgp-expressing BALB–*pgp1* and LZ-8 cells further, the relative anion permeabilities (P_X/P_{Cl}) of $I_{Cl,swell}$ in each of the three cell lines were determined (see Table 1). $I_{Cl,swell}$ in the two

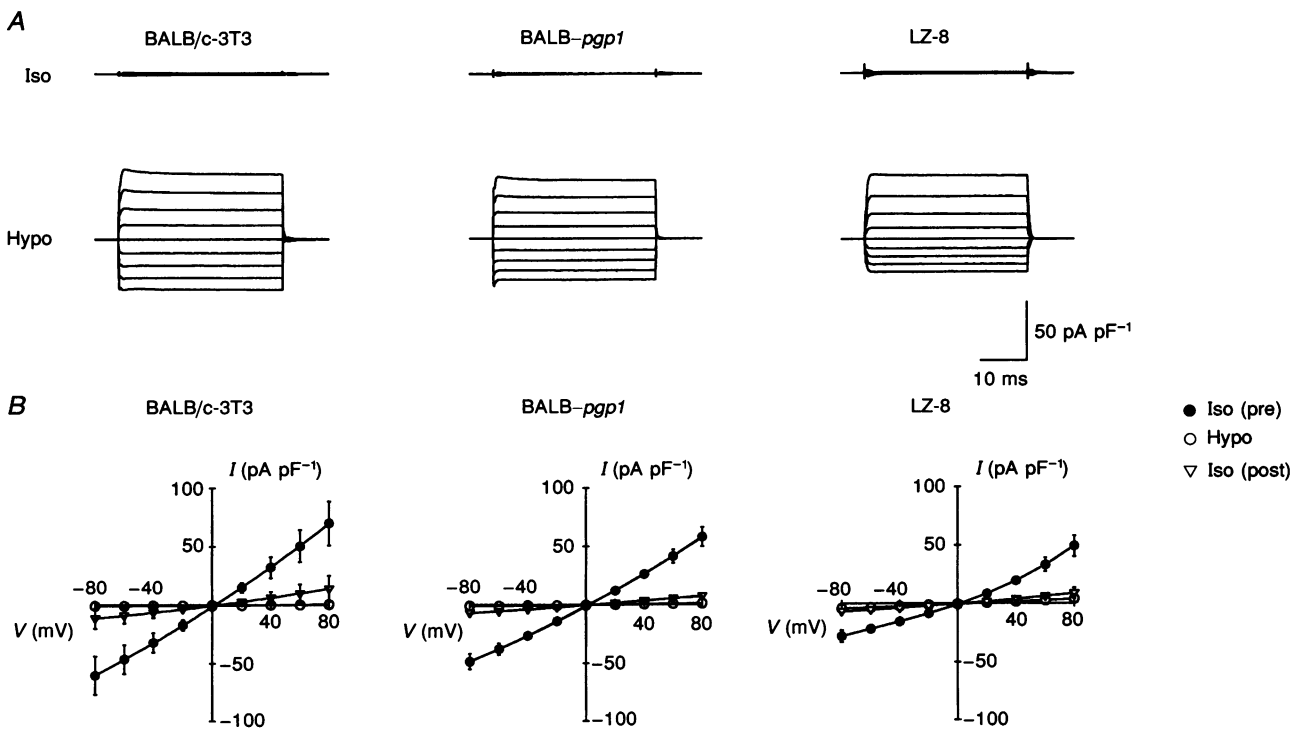

Figure 4. Whole-cell swelling-activated Cl^- conductance in mouse and hamster cells

A, representative traces of the Cl^- current measured in Iso and Hypo solutions for the three cell lines. Currents, normalized for apparent cell membrane capacitance, were measured in the whole-cell configuration of the patch-clamp technique. The holding voltage was 0 mV (as seen in the Iso traces, this holding potential *per se* did not activate membrane currents). Currents were measured at voltages ranging from −80 to +80 mV in 20 mV steps, 10 and 40 ms from the start of the voltage pulse. After obtaining basal currents (~10 min in Iso bath), the extracellular solution was changed to the Hypo bath. $I_{Cl,swell}$ was measured 8–10 min after lowering the bath osmolality. *B*, *I–V* relationships measured for BALB/c-3T3, BALB–*pgp1* and LZ-8 cells. Data are means ± S.E.M. of currents normalized for the estimated cell membrane capacitance plotted as a function of the membrane potential (V). These results demonstrate that Pgp expression does not alter the magnitude and *I–V* relationship of $I_{Cl,swell}$ in BALB/c-3T3 cells, and that the *I–V* relationships of the two Pgp-expressing cells are different.

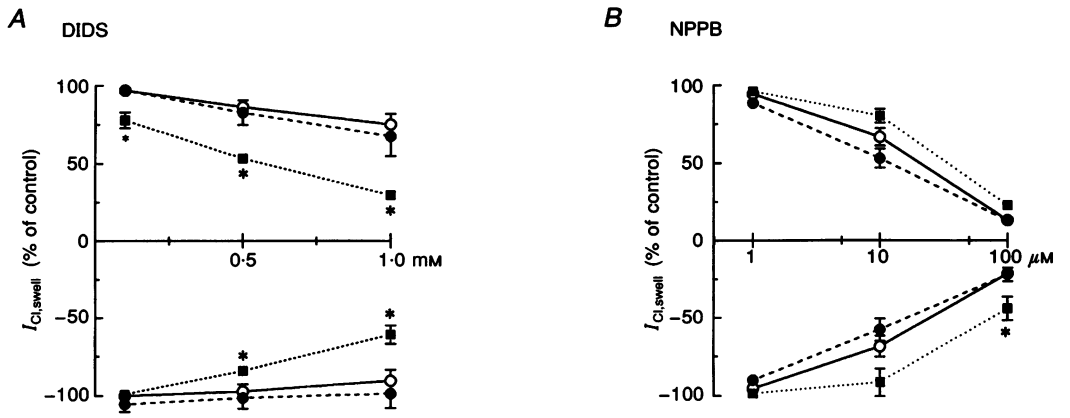

Figure 5. Blocker sensitivity of $I_{Cl,swell}$

$I_{Cl,swell}$ was measured at a V_m of −80 and +80 mV, 6 min after initiating exposure to blocker. Data are expressed as the percentage of $I_{Cl,swell}$ (means ± S.E.M.) measured at the same voltages ~30 s prior to the first exposure to blocker. $I_{Cl,swell}$ was inhibited by Cl^- channel blockers DIDS (*A*) and NPPB (*B*) with similar strength in both Pgp-expressing and parental BALB/c-3T3 cells, but the blocker sensitivity profile is different in LZ-8 cells. DIDS was a stronger inhibitor of $I_{Cl,swell}$ in LZ-8 cells, while at high concentrations, NPPB had a stronger block in BALB–*pgp1* cells than in LZ-8 cells. Symbols: LZ-8 cells (■), BALB/c-3T3 (○), and BALB–*pgp1* (●). * $P < 0.05$ compared with values in BALB/c-3T3 and BALB–*pgp1* cells.

mouse fibroblast cell lines had similar ionic selectivity sequences: $SCN^- \approx I^- > NO_3^- \approx Br^- > Cl^- \gg$ gluconate, independently of Pgp expression. On the other hand, comparison of the P_X/P_{Cl} values obtained for both Pgp-expressing cell lines (BALB–*pgp1* and LZ-8) yielded slightly different ionic selectivity sequences between the two. The ionic selectivity sequence in BALB–*pgp1* cells was $SCN^- \approx I^- > NO_3^- \approx Br^- > Cl^- \gg$ gluconate, while it was $SCN^- > I^- > NO_3^- \approx Br^- > Cl^- \gg$ gluconate in LZ-8 cells. In addition, the relative permeabilities estimated for Br^-, I^- and gluconate in the LZ-8 cells were different from those calculated in BALB–*pgp1* cells. These results suggest that the swelling-activated anion permeation pathways are identical between the two mouse cell lines (BALB/c-3T3 and BALB–*pgp1*), and different between the two Pgp-expressing cell lines (BALB–*pgp1* and LZ-8). Hence, Pgp does not appear to be a swelling-activated Cl^- channel for two reasons. First, its expression did not alter the ionic selectivity of $I_{Cl,swell}$ in BALB/c-3T3 cells and second, the two Pgp-expressing cells have different $I_{Cl,swell}$ ionic selectivities.

Channel blocker sensitivity of $I_{Cl,swell}$ in mouse and hamster cell lines

Channel blockers can be used to distinguish between subtypes of ion channels (Hille, 1992). We studied the Cl^- channel blockers 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; Sigma) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; Research Biochemicals International). Figure 5 summarizes $I_{Cl,swell}$ values measured at V_m values of −80 and +80 mV, plotted as a percentage of $I_{Cl,swell}$ measured before exposure to the blocker. As shown in Fig. 5*A*, DIDS produced a small, voltage-dependent block in both Pgp-expressing and parental BALB/c-3T3 cells (e.g. ~25% at +80 mV and ~10% at −80 mV at 1 mM DIDS). In LZ-8 cells, DIDS reduced $I_{Cl,swell}$ with similar voltage dependency, but the effect was larger (i.e. ~70% block at +80 and ~40% block at −80 mV at 1 mM DIDS). At high concentrations, NPPB had a significantly greater effect on the murine cells than on the LZ-8 cells (Fig. 5*B*). At 100 μM, NPPB blocked $I_{Cl,swell}$ in Pgp-expressing and parental BALB/c-3T3 cells, by ~90%

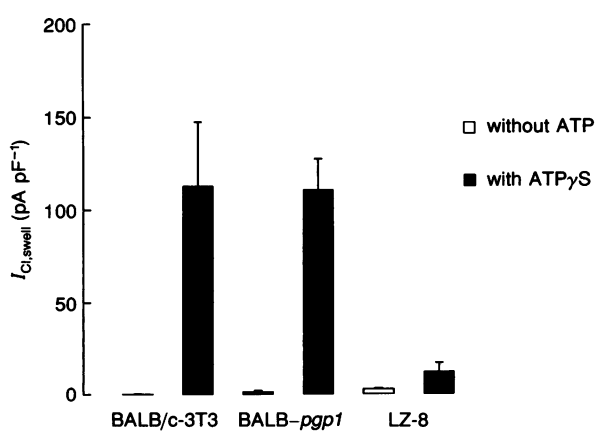

Figure 6. Hydrolysable ATP is required for activation of $I_{Cl,swell}$ in LZ-8 cells, but ATP binding is sufficient in BALB/c-3T3 and BALB–*pgp1* cells

In the absence of ATP in the pipette solution, there was no measurable $I_{Cl,swell}$ in any cell line. Intracellular ATPγS was enough to sustain $I_{Cl,swell}$ in both BALB/c-3T3 and BALB–*pgp1* cells, i.e. independently of Pgp expression. Data shown are $I_{Cl,swell}$ values measured at +80 mV, 40 ms after the start of the voltage pulse.

Table 1. Relative anion permeability of $I_{Cl,swell}$

Anion	P_X/P_{Cl} BALB/c-3T3	BALB–*pgp1*	LZ-8
Thiocyanate	1·63 ± 0·03	1·60 ± 0·07	1·55 ± 0·05*
Iodide	1·58 ± 0·03*	1·57 ± 0·04*	1·43 ± 0·03*†
Bromide	1·43 ± 0·02	1·40 ± 0·01	1·29 ± 0·02†
Nitrate	1·43 ± 0·03*	1·38 ± 0·01*	1·32 ± 0·03*
Gluconate	0·06 ± 0·00	0·05 ± 0·00	0·12 ± 0·02†

Reversal potential (V_{rev}) was obtained by interpolation of a third order polynomial fitted to the *I–V* relationships, corrected for junction potentials. Anionic permeabilities relative to Cl^- were estimated from changes in V_{rev} using the equation:

$$P_X/P_{Cl} = \{([Cl]_i/(10^{V_{rev}/58.7})) - [Cl]_o\}/[X]_o.$$

Data are means ± S.E.M. and the number of cells ranged from 3 to 8. * denotes statistical significance between calculated relative anion permeabilities (compared with next anion) for each cell line. † denotes statistical significance between the relative anion permeabilities calculated for LZ-8 cells *vs.* the other 2 cell lines.

at +80 and ~80% at −80 mV. At the same concentration, NPPB blocked $I_{Cl,swell}$ in LZ-8 cells by ~80% at +80 and ~60% at −80 mV. The blocks of $I_{Cl,swell}$ by DIDS and NPPB in all three cell lines were fully reversible at the low concentrations, and partially reversible at the highest concentrations (data not shown). Pgp expression did not alter the blocker sensitivities to DIDS and NPPB in the mouse BALB/c-3T3 cells. DIDS inhibited with greater efficacy in LZ-8 cells than in both mouse cell lines, while NPPB had a stronger block in the mouse cells than in LZ-8 cells.

ATPγS can sustain $I_{Cl,swell}$ in both Pgp-expressing and parental BALB/c-3T3 cells, but hydrolysable ATP is required for activation of $I_{Cl,swell}$ in Pgp-expressing LZ-8 cells

To determine whether intracellular ATP was required for the activation of $I_{Cl,swell}$, BALB/c-3T3, BALB–444 and LZ-8 cells were dialysed for ~10 min in Iso with either an ATP-free pipette solution, or a 2 mM ATPγS-containing pipette solution (Sigma), before reducing the bath osmolality. Figure 6 depicts the $I_{Cl,swell}$ values measured at +80 mV, 40 ms after the start of the voltage pulse, 8–10 min after changing the bath solution to Hypo. Without intracellular ATP, no $I_{Cl,swell}$ was elicited in any of three cell lines. The non-hydrolysable ATP analogue ATPγS could sustain $I_{Cl,swell}$ in BALB/c-3T3 and BALB–*pgp1* cells, i.e. independently of Pgp expression. However, ATPγS was not sufficient for activation of $I_{Cl,swell}$ in LZ-8 cells. These results show that: (1) the ATP requirement of $I_{Cl,swell}$ in BALB/c-3T3 cells is not modified by Pgp expression, and (2) the ATP requirements of $I_{Cl,swell}$ in two Pgp-expressing cell lines are different. In conclusion, nucleotide binding without hydrolysis is sufficient for $I_{Cl,swell}$ activation in BALB/c-3T3 and BALB–*pgp1* cells, while ATP hydrolysis appears to be required for $I_{Cl,swell}$ activation in LZ-8 cells.

DISCUSSION

The relationships between Pgp expression and $I_{Cl,swell}$ are consistent with Pgp being either a swelling-activated channel or a channel regulator. In addition, it has been proposed that the putative Pgp–$I_{Cl,swell}$ relationship is due to alterations induced by cell exposure to chemotherapeutic agents, rather than to Pgp expression *per se* (Luckie *et al.* 1994, 1996). After the initial report that Pgp expression induced the appearance of $I_{Cl,swell}$ (Valverde *et al.* 1992), subsequent experiments showed that Pgp expression is not necessary for $I_{Cl,swell}$ and does not augment it (e.g. Ehring, Osipchuk & Cahalan, 1994; Rasola *et al.* 1994; Han *et al.* 1996). Moreover, $I_{Cl,swell}$ in both Pgp-expressing and non-expressing parental cells exhibited similar sensitivities to Cl^- channel blockers (e.g. Ehring *et al.* 1994). We found no differences in magnitude, rectification and inactivation at positive voltages of $I_{Cl,swell}$ between Pgp-expressing and their parental non-Pgp-expressing cells, but the voltage dependence and kinetics of $I_{Cl,swell}$ among different Pgp-expressing cell lines did vary (Han *et al.* 1996). Although these observations strongly suggest that Pgp is not a swelling-activated Cl^- channel, they do not rule out this possibility. Pgp expression could confer channel activity and downregulate the expression of native Cl^- channels, so that the magnitude of $I_{Cl,swell}$ would not be affected by Pgp expression. In this context, it has been proposed that Pgp expression downregulates the expression of the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel (Kunzelmann *et al.* 1994). The downregulation of native Cl^- channels could account for the lack of difference in the magnitude of $I_{Cl,swell}$ found between Pgp-expressing cells and parental cells lacking Pgp expression. Although possible, this scenario is unlikely since it would require the properties of the Pgp channel to be identical to those of the

native channels, or for Pgp to mimic these properties which, as shown here, vary from cell to cell.

Pgp is not a swelling-activated Cl^- channel

To resolve whether or not Pgp is a swelling-activated Cl^- channel, we employed a cell line obtained by transfection of *pgp1* cDNA without selection with chemotherapeutic agents (BALB–*pgp1*). We avoided exposure to chemotherapeutic agents to be able to attribute any changes in the properties of $I_{Cl,swell}$ to Pgp expression alone.

$I_{Cl,swell}$ in both *pgp1*-transfected and parental BALB/c-3T3 cells were characterized and compared with $I_{Cl,swell}$ in the Pgp-expressing hamster LZ-8 cell line. If Pgp were a channel, then the following expectations would be fulfilled: (a) the properties of $I_{Cl,swell}$ in Pgp-expressing LZ-8 cells would be, at least partially, due to Pgp. (b) These properties would also emerge in cells transfected with *pgp1* cDNA (BALB–*pgp1*), differentiating them from the parental cell line. (c) Pgp would be expected to confer similar $I_{Cl,swell}$ properties regardless of the recipient cells. This is the case when CFTR, a cAMP-activated Cl^- channel (Bear *et al.* 1992) is expressed in a variety of recipient cells (e.g. Kartner *et al.* 1991; Rich *et al.* 1992; Cliff, Schoumacher & Frizzell, 1992). In these and other studies, the biophysical characteristics (i.e. ionic selectivity, voltage dependence, blocker sensitivity) of the CFTR Cl^- channel are constant, regardless of the recipient cell.

Based on this expectation, we asked whether or not Pgp *per se* is a swelling-activated Cl^- channel by transfecting Pgp into a cell line and comparing $I_{Cl,swell}$ in wild-type and transfected cells. We found that the magnitude, blocker sensitivity, requirement for hydrolysable ATP, ionic selectivity sequence and *I–V* relationship of $I_{Cl,swell}$ displayed by BALB/c-3T3 cells were not modified by Pgp expression. All but one of these $I_{Cl,swell}$ characteristics (i.e. current magnitude) were significantly different from those in the Pgp-expressing hamster cell line (LZ-8). These observations strongly suggest that the ion channel(s) responsible for $I_{Cl,swell}$ in both BALB/c-3T3 cell lines (parental and Pgp-transfected) are the same, but different from the channel(s) present in the LZ-8 cell line. In summary, Pgp expression in BALB/c-3T3 cells did not cause $I_{Cl,swell}$ to resemble that of the Pgp-expressing LZ-8 cell line, a result consistent with the notion that Pgp is not a swelling-activated Cl^- channel. Another argument in support of this conclusion is that the $I_{Cl,swell}$ elicited in LZ-8 cells and the parental cell line V79 by an identical reduction in bath osmolality (22% of Iso) is similar (data not shown), although LZ-8 cells express >10-fold more Pgp than V79 cells (Sognier *et al.* 1992). If Pgp were a swelling-activated Cl^- channel, then the LZ-8 cells would be expected to display larger $I_{Cl,swell}$ values. We and others have also failed to demonstrate a correlation between $I_{Cl,swell}$ and the level of Pgp expression (e.g. McEwan *et al.* 1992; Ehring *et al.* 1994; Rasola *et al.* 1994; Han *et al.* 1996). From these studies, we conclude that Pgp is not a swelling-activated Cl^- channel.

Inhibition of $I_{Cl,swell}$ by C219 requires Pgp expression

In most published studies, Pgp-expressing cells are maintained in medium containing chemotherapeutic agents to prevent reversal of the multi-drug resistance (MDR) phenotype. Accordingly, it has been proposed that the putative relationship between Pgp and $I_{Cl,swell}$ is due to the exposure to chemotherapeutic agents, and not to Pgp expression *per se*. By some unknown mechanism, the chemotherapeutic agents could alter the threshold for swelling activation of the Cl^- current (Luckie *et al.* 1994, 1996). Our results indicate that C219 inhibits $I_{Cl,swell}$ only in cells expressing Pgp, regardless of (a) origin of cDNA (human or hamster; Han *et al.* 1996 and present results, respectively), (b) procedure employed to elicit Pgp expression, i.e. drug selection, transfection or transfection plus drug selection (Han *et al.* 1996 and present results), and (c) recipient cell (Han *et al.* 1996 and present results). Therefore, we conclude that the relationship between Pgp and $I_{Cl,swell}$ (i.e. inhibition of $I_{Cl,swell}$ by C219) is specific for Pgp expression.

The antibody C219 has been reported to cross-react with other proteins and block other Cl^- conductances. A Cl^- conductance in pancreatic zymogen granule membranes was inhibited by the anti-Pgp antibodies C219 and JSB-1, and immunoblot studies with these antibodies showed a C219-reactive band at ~65 kDa (Thévenod, Anderie & Schulz, 1994). Although this protein seems to regulate a Cl^- conductance in zymogen granules, it is not present in our cells. It has also been reported that in one instance addition of internal C219 to Pgp-expressing cells did not inhibit $I_{Cl,swell}$ (Tominaga, Tominaga, Miwa & Okada, 1996). Although the authors showed the presence of endogenous Pgp in the plasma membrane of attached (most probably polarized) human Intestine 407 cells by immunofluorescence, the cells were detached prior to the electrophysiology experiments. Upon isolation, many epithelial cells internalize regions of their plasma membrane (Vega-Salas, Salas & Rodríguez-Boulan, 1987). In this context, Pgp expression is found in the canalicular membrane of rat hepatic cells (Arias, 1990). Upon isolation, rat hepatocytes lose polarity, the canalicular membrane is internalized, there is no functional expression of Pgp in the plasma membrane, and C219 does not inhibit $I_{Cl,swell}$ (Han, Altenberg & Reuss, 1994). Nevertheless, immunofluorescence of isolated hepatocytes with C219 showed intracellular labelling (Han *et al.* 1994). These results suggest that for C219 to inhibit $I_{Cl,swell}$, Pgp must be expressed in the plasma membrane, but expression of Pgp in the plasma membrane was not demonstrated in detached Intestine 407 cells (Fig. 4 of Tominaga *et al.* 1996). Furthermore, Intestine 407 cells were exposed to the hypotonic solution ~2 min after starting intracellular dialysis with C219-containing pipette solution (Tominaga *et al.* 1996, Fig. 4). At this time, it is highly unlikely that C219 would have equilibrated between the pipette and the cell interior (Han *et al.* 1996).

Pgp in the plasma membrane regulates native swelling-activated Cl^- channels

$I_{Cl,swell}$ is inhibited by the anti-Pgp mAb C219 in cells expressing Pgp (Han *et al.* 1996; Wu *et al.* 1996 and present results), and by the phosphorylation of Pgp catalysed by PKC (Hardy *et al.* 1994). These effects indicate a relationship between Pgp expression and $I_{Cl,swell}$. Since Pgp is not a swelling-activated Cl^- channel, our results suggest that Pgp might regulate this Cl^- conductance. The precise mechanism of this regulation is not known. P-glycoprotein-dependent changes in intracellular or extracellular concentration of Cl^- channel agonists or antagonists is not likely, because both Pgp-expressing cells and parental cells have identical $I_{Cl,swell}$ (Ehring *et al.* 1994; Rasola *et al.* 1994; Han *et al.* 1996). In addition, our studies ruled out extracellular ATP as a mediator in these cells (Vanoye *et al.* 1996*b*). An attractive hypothesis is that Pgp regulation of swelling-activated Cl^- channels is via protein–protein interactions. Phosphorylation of the mini-linker domain of Pgp may cause a conformational change and thus affect the interaction with the Cl^- channel, modulating channel activity. Such an interaction has been demonstrated to exist between purified human CFTR and recombinant human R domain (Ma *et al.* 1996).

Membrane transport regulation by ATP binding cassette (ABC) transporters.

Pgp is a membrane ATPase thought to actively extrude drugs from cells (Doige & Sharom, 1992). Besides lowering the intracellular levels of hydrophobic substrates, this protein may serve other functions. Multiple differences, when compared with the non-Pgp-expressing, parental cells, have been observed in Pgp-expressing cells. Examples include increase or decrease in steady-state intracellular pH (e.g. Altenberg *et al.* 1993*a*; Roepe, Wei, Cruz & Carlson, 1993), cell membrane depolarization (Hasmann, Valet, Tapiero, Trevorrow & Lampidis, 1989), and increase in Na^+ channel activity (Yamashita, Hamada, Tsuruo & Ogata, 1987). Other members of the ATP superfamily of membrane proteins, to which Pgp belongs, are ion channels and/or channel regulators. Besides being a low-conductance Cl^- channel, CFTR has been proposed to regulate outward-rectifying Cl^- channels (ORCC) (e.g. Schwiebert *et al.* 1995), Na^+ channels (Stutts *et al.* 1995) and K^+ channels (McNicholas, Guggino, Schwiebert, Hebert, Giebish & Egan, 1996). The sulphonylurea receptor (SUR) has been implicated in the regulation of ATP-sensitive K^+ channels (Aguilar-Bryan *et al.* 1995). The mechanisms by which these three ABC proteins may regulate the activity of other membrane transporters are not fully understood. ATP secretion could account for the regulation of ORCC by CFTR (Schwiebert *et al.* 1995), but ATP secretion mediated by CFTR (directly or indirectly) is disputed (e.g. Reddy *et al.* 1996). Understanding how some ABC proteins modulate the activity of other transporters is important not only by itself but also because defective regulation by these proteins, e.g. CFTR and SUR, may be clinically significant (i.e. in cystic fibrosis and diabetes, respectively). The information gained by studying how Pgp modulates swelling-activated Cl^- channels may provide insights about the general regulatory mechanisms of ABC proteins other than Pgp.

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