Volume-activated chloride currents are not correlated with P-glycoprotein expression

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It has been proposed that P-glycoprotein, the product of the human *MDR1* gene, may function not only as a drug transporter but, depending on the conditions, as a volume-activated Cl^- channel [Valverde, Diaz, Sepúlveda, Gill, Hyde and Higgins (1992) *Nature* (London) **355**, 830–833; Gill, Hyde, Higgins, Valverde, Mintenig and Sepúlveda (1992) *Cell* **71**, 23–32]. To verify this hypothesis, we have compared volume-activated Cl^- currents with the level of *MDR1* mRNA and its protein product in the human KB3 (epitheloid lung cancer) and HeLa cell lines. The related *MDR2* was also included to find out whether it could account for observed discrepancies between Cl^- current and *MDR1* expression. A 40% decrease in osmolarity evoked a Cl^- current in both cell types (at +80 mV: 50.3 ± 4.3 pA/pF in KB3, $n = 13$; 28.2 ± 3.3 pA/pF in HeLa, $n = 16$). The blocking of this

current in both cell types by 5-nitro-2-(3-phenylpropylamino)-benzoic acid and by 1,9-dideoxyforskolin is similar to that of the presumed P-glycoprotein associated Cl^- channel. As measured by reverse-transcriptase polymerase chain reaction, KB3 cells expressed only an extremely small amount of the messengers for *MDR1* and *MDR2*. The signal observed for *MDR1* in HeLa cells was at least an order of magnitude more intense than in KB3 cells, while *MDR2* mRNA was undetectable. A clear difference in *MDR1* expression between KB3 and HeLa was also observed at the protein level. These data are difficult to reconcile with the hypothesis that in HeLa and KB3 cells *MDR1*- or *MDR2*-encoded P-glycoproteins are associated with volume-activated Cl^- channels.

INTRODUCTION

Cl^- channels activated by cell swelling have been observed in several cell types including chromaffin cells [1], fibroblasts [2], Jurkat cells [3], epithelial cells [4,5] and endothelial cells [6]. All these volume-activated channels are outwardly rectifying, have a larger permeability for iodide than for chloride and seem to be independent of intracellular calcium. Data are still controversial concerning single-channel conductance (ranging between < 1 pS [7] and 80 pS [8,9]) and the requirement for intracellular ATP to maintain channel activity [9,10]. In all cell types tested so far, the amplitude of the macroscopic currents activated by swelling is very large (from 20 to as much as 2000 pA/pF [11]). It has been proposed that this current is involved in a number of other cell functions such as mechanosensitivity [12], intracellular pH regulation via modulation of the activity of $\text{Cl}^-/\text{HCO}_3^-$ or $\text{K}^+/\text{Na}^+/\text{2Cl}^-$ transporters [13], modulation of Ca^{2+} sequestration [14] and volume control during mitosis [15].

It has been proposed that the volume-activated Cl^- channel is identical with *MDR1*-encoded P-glycoprotein [2,16]. We have therefore made a detailed analysis of volume-activated Cl^- currents in the human KB3 (lung cancer epitheloid cell line) and HeLa cells and compared these with the relative expression levels of *MDR1* mRNA. Both cell types show very different levels of *MDR1* expression but the current density and the pharmacological properties of their volume-sensitive Cl^- currents are very similar. Since the *MDR2* member of this gene family is also expressed in humans, it could be argued that this related protein

could account for the observed channel activity. However, *MDR2* expression was very low (KB3) or undetectable (HeLa). Our interpretation of this lack of correlation is that *MDR1*- or *MDR2*-encoded P-glycoprotein and the volume-activated Cl^- channel are not related. Part of this work has been published in abstract form [17].

MATERIALS AND METHODS

Culture of cells

The two cell types used in these experiments are HeLa cells (human epitheloid carcinoma) and KB3 cells (human epitheloid lung cancer cells), provided by Dr. M. Bootman, Cambridge, U.K. and Dr. B. Sarkadi, Budapest, Hungary respectively. Cultures were maintained at 37 °C in a fully humidified atmosphere of 10% CO_2 in air. The culture medium was exchanged every 48 h. Cells were detached by exposure to 0.05% trypsin in a Ca^{2+} - and Mg^{2+} -free solution for approximately 3 min and either harvested for RNA isolation or reseeded on gelatine-coated coverslips for further use in electrophysiological experiments. In the latter case, we kept the cells in culture for 2–4 days before use.

Current measurements

The electrophysiological methods used have been described in detail elsewhere [12]. Whole-cell membrane currents were

Abbreviations used: HTS, hypotonic extracellular solution; RT-PCR, reverse-transcriptase polymerase chain reaction; DDFSK, 1,9-dideoxyforskolin; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; M-MLV, Moloney-murine leukaemia virus; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting.

measured using nystatin-perforated patches to avoid disturbance of the intracellular Ca^{2+} buffering mechanisms [18]. Currents were monitored with an EPC-7 (List Electronic, Darmstadt, Germany) or an EPC-9 (Heka Electronics, Lambrecht, Germany) patch clamp amplifier and sampled at intervals of 4 ms (2048 points per record, filtered at 100 Hz). The following voltage protocol was applied every 10 s: from a holding potential of 0 mV a 1.2 s step was applied to -80 mV, thereafter the potential was stepped for 0.4 s to -150 mV, followed by a 5.2 s linear voltage ramp to $+100$ mV. Such a protocol has the advantage that the time course of the current as well as the reversal potential and voltage dependence of the volume-induced current can be studied.

Solutions

The standard bath solution was a modified Krebs' solution of the following composition (in mM): 132 NaCl/1.5 CaCl_2 /5.9 KCl/1.2 MgCl_2 /11.5 Hepes-NaOH/10 glucose, titrated to pH 7.3 with NaOH. Osmolarity was 290 mOsm/l. Osmolarity of all the solutions was measured with a vapour pressure osmometer, Wescor 5500 (Schlag Meßinstrumente, Gladbach, Germany). Hypotonic solutions (HTS) were obtained by diluting the normal Krebs' solution with distilled water to 185 mOsm/l.

The nystatin stock solution used in the 'perforated patch' technique was made up at 50 mg/ml in dimethyl sulphoxide and stored at -20°C . Immediately before use this stock solution was diluted 1000 times in the pipette solution and ultrasonicated. The tip of the pipette was filled with nystatin-free pipette solution by capillarity and the pipette was then backfilled with nystatin-containing solution. Access to the cells occurred within 5–10 min. The pipette (internal) solution was made up in Milli-Q water (Millipore) and contained (in mM): 100 potassium aspartate/40 KCl/4 $\text{Na}_2\text{-ATP}$ /10 Hepes/0.1 EGTA, adjusted at pH 7.2 with KOH, 290 mOsm/l.

Experiments were performed at room temperature between 20 and 22°C .

The following substances were used to modulate HTS-induced currents: 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), provided by Dr. Lang (Hoechst AG, Germany) and 1,9-dideoxy-forskolin (DDFSK) obtained from Sigma.

RNA isolation and reverse transcription

Total RNA was extracted from approximately 5×10^6 cells according to Chomzynski and Sacchi [19], followed by treatment with RNase-free DNase (from bovine pancreas, Boehringer Mannheim). Single-stranded cDNA was synthesized using 1 μg of total RNA and 200 ng of oligo(dT)₁₅ primer (Boehringer Mannheim) in 20 μl of solution containing 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl_2 , 1 mM DTT, 0.5 mM of each dNTP, 40 U rRNasin (Promega) and 200 U Moloney-murine leukemia virus (M-MLV) reverse transcriptase (RT) (Gibco BRL). Immediately before the first-strand cDNA synthesis, the RNA was denatured at 70°C for 5 min and immediately put on ice. Subsequently, primer-template annealing was done at room temperature for 10 min, followed by a 1 h incubation at 37°C and another 15 min incubation at 42°C . A blank RT reaction containing all the summed components except the M-MLV-RT was also performed in parallel.

PCR

PCR was carried out on 3 μl aliquots of the first-strand reaction mixture (RT and blank RT) in a final volume of 50 μl consisting

of commercially supplied buffer (low Mg^{2+} , Amersham), 200 μM of each dNTP, 0.5 μM of each primer and 2.5 U Hot Tub DNA polymerase (Amersham). Samples were denatured at 94°C for 2 min, followed by 32 cycles each including 1 min of denaturation at 94°C , 1 min of primer annealing at 63°C and 1 min of synthesis at 72°C . PCR products were separated on a 6% non-denaturing polyacrylamide gel and visualized by ethidium bromide staining. To label the PCR products, 5 μl of the PCR reaction mixture was diluted into another 50 μl PCR mixture with the same composition as mentioned above, but supplied with 2.5 μCi [α - ^{32}P]dCTP (Amersham) and amplified for ten additional cycles. Aliquots of the ^{32}P -PCR products (5 μl) from the RT-PCR preparation were then digested with *Hinf* I in a total volume of 15 μl using NEBuffer 3 (New England Biolabs, Beverly, MA, U.S.A.). Cut and uncut samples (also 5 μl) from RT-PCR and blank RT-PCR were analysed on a 6% polyacrylamide gel and after drying of the gel, visualized on a PhosphorImager scanner and quantified using the Imagequant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Two sets of primers were used: a set of non-specific primers, amplifying fragments of equal length (265 bp) of both the *MDR1* and *MDR2* sequences, consisting of the sense-strand primer TGCCTATGGAGACAACAGCCGGT (residues 3817–3840 of *MDR1* [20] and residues 3422–3445 of *MDR2* [21]) and the antisense-strand primer GTCCAGGGCTTCTTGACAACCTT (residues 4058–4081 of *MDR1* and 3663–3686 of *MDR2*). Distinction between both genes was made by restriction analysis with *Hinf* I which cuts the PCR products derived from both genes, but in a different place: *Hinf* I cuts at position 3899 in *MDR1* (yielding fragments of 83 and 182 bp in length) and at 3548 in *MDR2* (fragments of 127 and 138 bp). The second set of primers amplifies only *MDR1* (463 bp fragment) by use of the same sense primer and TACAGTCAGAGTTCCTGCGCTT (residues 4256–4279) as antisense primer. There are two *Hinf* I restriction sites in this fragment at positions 3899 and 4173, resulting in fragments with lengths of 83, 274 and 106 bp.

To control the quality of the RNA preparation, the presence of actin mRNA was checked in another PCR reaction using the following isoform unspecific actin primers [selected to perfectly match the rat α (vascular)-, β - and γ (cytoplasmic)-actins]: sense-strand primer GCTGACAGGATGCAGAAGGAG and antisense-strand primer GATCCACATCTGCTGGAAGG, yielding a PCR product of 144 bp. Annealing was done at 55°C .

Immunocytochemistry

Cells grown on coverslips were fixed in 3% paraformaldehyde for 15 min, permeabilized in 0.5% Triton X-100 for 5 min and washed three times for 15 min with PBS. After blocking for 1 h with 10% goat serum (Sigma), the cells were incubated overnight with anti-P-glycoprotein monoclonal antibody MRK16 (Kamiya Biomedical Company, Thousand Oaks, CA, U.S.A.) at 50 $\mu\text{g}/\text{ml}$ or with rabbit polyclonal antibody 4007 (provided by Dr. M. Gottesman, NIH, Bethesda, MD, U.S.A.) at a 1:10 dilution. The cells were washed again with PBS (three times for 15 min) and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-(mouse IgG) (Fab specific) or goat anti-(rabbit IgG) (whole molecule) (both from Sigma) at a 1:50 dilution for 1 h. After another washing step with PBS, FITC-labelled cells were visualized by excitation at 490 nm. In control experiments, cells were incubated with the secondary antibody only.

Fluorescence-activated cell sorting (FACS) analysis

Cells used for FACS analysis (about 5×10^5 cells) were gently detached by exposure to 1.5 mM (HeLa) or 3.0 mM (KB3)

EDTA for approximately 5 min before harvesting. Cells were washed once with PBS, pelleted and resuspended in blocking buffer (0.5% BSA, 5% normal mouse serum and 5% normal human serum in PBS). MRK16 was added to a final concentration of 10 $\mu\text{g/ml}$ and the cell suspension was incubated at 4 °C for 30 min. The cells were washed twice with PBS and incubated with biotinylated horse anti-(mouse IgG) (Vector Laboratories Inc.) at a 1:500 dilution in the presence of 5% normal horse serum. After another two washing steps with PBS, the cells were incubated with the streptavidine phycoerythrin conjugate (Beckton Dickinson) followed by two washes with PBS. Finally, cell labelling was analysed on a FACS flow cytometer (Beckton Dickinson). At least 10^4 events were counted. In parallel, control experiments were performed in which the incubation step with MRK16 was omitted.

Statistics

Pooled data are given by mean $\pm$ S.E.M. For significance, Student's *t*-test was used (level of significance, $P < 0.05$). Fitting routines were used from the software package Origin (Microcal Software, Northampton, MA, U.S.A.).

RESULTS

Volume-activated Cl^- currents in HeLa and KB3 cells

We have measured transmembrane currents evoked by a challenge with HTS in HeLa and KB3 cells using the voltage protocol described in the Materials and methods section. In order to compare the data obtained in both cell types, we have expressed them per unit membrane capacitance of the cell. This parameter was automatically measured with the EPC-9 amplifier between two successive voltage protocols.

The time course of the HTS-induced current at any potential was reconstructed from the average currents in a small voltage window during successively applied voltage ramps. Figure 1(a) shows this time course for each cell type in a voltage window between +75 and +85 mV. From the current amplitudes collected from a number of cells, we calculated a current density which was significantly higher in KB3 cells than in HeLa cells [50.3 ± 4.3 pA/pF in KB3 cells ($n = 13$), compared with 28.2 ± 3.3 pA/pF in HeLa cells ($n = 16$), $P < 0.05$].

Representative traces, showing the voltage dependence of the HTS-induced current as obtained from the difference of current traces recorded during voltage ramps applied before and at the maximum of the HTS-induced current, are shown in Figure 1(b) for each cell type. The reversal potential of -19 mV was close to the equilibrium potential for Cl^- (-18 mV), indicating that the current is mainly carried by Cl^- ions.

In another series of experiments we have tested the pharmacological modulation of the volume-activated Cl^- current in both cell types. Figures 2(a)–2(d) show examples of the blocking of the volume-activated current by NPPB and DDFSK. The following data were obtained: 50 μM NPPB blocked the volume-activated current in KB3 cells by $95 \pm 2\%$ ($n = 26$) and in HeLa cells by $91 \pm 6\%$ ($n = 22$). The blocker of the P-glycoprotein-mediated transport, DDFSK, exerted only a weak block in both cell types. DDFSK (50 μM) blocked the volume-activated current in KB3 cells by $38 \pm 12\%$ ($n = 10$). In HeLa cells, where blocking of the current has already been shown [4], we measured inhibition by $31 \pm 4\%$ in 18 cells. Both cell types thus show virtually identical features of blocking by NPPB and DDFSK. The latter compound also blocks P-glycoprotein-mediated transport, a fact that has been used as an important argument

Figure 1 Volume-activated Cl^- currents in HeLa and KB3 cells

(a) Time course of the HTS-activated current at +80 mV in HeLa (●) and KB3 (○) cells. Data points were calculated as the average current in a voltage window from +75 to +85 mV during voltage ramps. The horizontal bar indicates the period of hypotonicity (HTS). (b) Current–voltage relationships derived from voltage ramps (see the Materials and methods section) applied before and just before the end of the HTS stimulations in (a). Note the reversal potential of the HTS-activated current at -19 mV, which is close to the Cl^- equilibrium potential ($E_{\text{Cl}} = -18$ mV). The currents in both (a) and (b) were normalized to the membrane capacitance measured before HTS.

for an association between volume-activated Cl^- channels and P-glycoprotein [2,4].

Expression of *MDR1* and *MDR2* in KB3 and HeLa cells

To test the hypothesis of a relation between the volume-activated Cl^- current and the expression of P-glycoprotein, relative levels of mRNA encoding P-glycoprotein were estimated by RT-PCR. Two different fragments of the *MDR1* sequence were amplified using two different pairs of primers. One primer pair was specific for *MDR1*, whereas the annealing sites of the other pair were identical in *MDR1* and *MDR2*, resulting in the co-amplification of both sequences with equal efficiency. In the latter case, the *MDR1*- and *MDR2*-derived PCR products were of equal length but could be discriminated by their *Hinf* I restriction patterns. The RT-PCR for KB3 and HeLa cells was started from the same amount of total RNA. In parallel with the RT reaction, an equal amount of total RNA was treated in the same way except that no RT enzyme was added. In these controls no PCR products were formed.

When the PCR products were analysed on ethidium bromide-stained polyacrylamide gels, a PCR fragment of the predicted

Figure 2 Pharmacological properties of HTS-induced currents in HeLa and KB3 cells

(a), (c) Blocking of the HTS-induced currents measured at +80 mV by a short application of 50 μ M NPPB. (a) HeLa; (c) KB3. (b), (d) In both cell types 50 μ M DDFS induced a smaller effect than NPPB. Currents were measured at +80 mV. (b) HeLa; (d) KB3.

length for each primer pair could be visualized in HeLa cells but not in KB3 cells (Figures 3a and 3b). To increase the sensitivity of our detection method, the PCR was repeated in the presence of [α - 32 P]dCTP and radioactive bands were detected and quantified using the PhosphorImager system. Using the non-specific primers, a clear signal was detected in HeLa cells, whereas in KB3 cells it was difficult to visualize the PCR product (Figure 4a). In HeLa cells, digestion of the PCR product by *Hinf* I yielded only *MDR1*-specific fragments (Figure 4a). No trace of an *MDR2* product was found, although a clear *MDR2* signal was obtained using the non-discriminating primers under similar conditions in other cells (e.g. Jurkat cells contained 10% mRNA encoding *MDR1* and 90% encoding *MDR2*; results not shown). The product amplified in KB3 cells using the non-discriminating primers was derived from both *MDR1* and *MDR2*. The amount of radioactivity in the PCR product of KB3 cells and in its restriction fragments was too low to be measured accurately (in the experiment shown, the restriction fragments corresponding to each isoform were too weak for photographic reproduction).

Figure 4 Analysis of radioactively labelled PCR products separated by PAGE and visualized using the PhosphorImager

(a) Coamplification of *MDR1* and *MDR2* using non-specific primers. (b) Specific amplification of *MDR1* only. The image for KB3 cells has been enhanced relative to that for HeLa to allow photographic visualization of the *MDR1* band. Lane 1, PCR on a control RNA sample incubated in the absence of RT; lane 2, PCR product obtained on a reverse-transcribed sample, undigested; lane 3, PCR product digested with *Hinf* I.

Using the *MDR1*-specific primers, the PCR products had the expected length and their identity was further confirmed by restriction analysis with three different enzymes, of which only the *Hinf* I digest is shown in Figure 4. The amount of PCR product in KB3 cells was also very small and difficult to visualize and quantify. In contrast, a clear signal was present in HeLa cells (Figure 4b).

The minimum values estimated for the ratio of mRNA encoding *MDR1* in HeLa/KB3 was about 20 using the non-discriminating primers and 120 using the *MDR1*-specific primers. The extremely low expression level of *MDR1* in the KB3 cells used in our experiments is in agreement with the results of Fojo et al. [22] and Noonan et al. [23]. These authors found, out of a great number of tissues and cell lines tested, the lowest level of *MDR1* in KB3 cells.

As a control, it was ascertained that actin could be amplified from the same RT mixtures used for the amplification of *MDR*. Equal amounts of starting RNA yielded somewhat more actin product in KB3 cells than in HeLa cells (Figure 3c). As a consequence, the difference in *MDR* content between HeLa and KB3 cells would be even greater if the amount of *MDR* product were expressed as the ratio of *MDR* product to actin product.

Figure 3 Analysis of *MDR1/2* PCR products from HeLa and KB3 cells on ethidium bromide-stained polyacrylamide gels

(a) Amplification using the non-discriminating primer pair, designed to amplify both *MDR1* and *MDR2*. (b) Specific amplification of *MDR1* only. (c) Amplification of actin. Lane 1, PCR on a control RNA sample incubated in the absence of reverse transcriptase; lane 2, PCR product obtained on samples incubated in the presence of reverse transcriptase; M, markers of defined length (indicated as number of bp).

Figure 5 Evaluation of *MDR1* P-glycoprotein content in HeLa and KB3 cells

(a) Fluorescent staining using FITC-labelled secondary antibodies. For each condition the corresponding bright-field (BF) image is shown. Cells were incubated with or without (controls) monoclonal MRK16 primary antibody. Identical photographic exposure conditions were used. (b) FACS analysis of cells labelled with antibody MRK16 and control cells without primary antibody. Note that for KB3 cells the shift of the MRK16 curve relative to the control curve is small, whereas in HeLa cells the MRK16 curve is shifted to a higher fluorescence, indicating significant expression of P-glycoprotein.

To ascertain that the difference in mRNA level reflects a similar difference at the protein level, amounts of P-glycoprotein were evaluated by immunocytochemistry and by FACS analysis. In both cases, cell surface expression of P-glycoprotein was measured by labelling the cells with MRK16, a monoclonal antibody directed against an extracellular epitope of the *MDR1* gene product [24]. Immunocytochemistry reveals a clear fluorescence above background for HeLa cells (Figure 5a). In KB3 cells, the signal seen in presence of the MRK16 antibody was almost indistinguishable from the background, which was however considerably higher than in HeLa cells. Similar results are obtained by FACS analysis. The profiles in Figure 5(b) clearly show that HeLa cells have a higher level of P-glycoprotein, since the shift to higher fluorescence of the MRK16 curve relative to the control curve is more pronounced for HeLa than for KB3. Also here, KB3 cells show a higher background signal. Similar immunocytochemical results were obtained with the polyclonal antibody 4007 which recognizes both *MDR1*- and *MDR2*-encoded proteins (results not shown).

DISCUSSION

All mammalian cells tested so far express volume-activated Cl^- channels with several common properties: outward rectification, a higher permeability for iodide than for chloride and blocking by NPPB [1–6,25]. However, the molecular identity of the channel or family of channels carrying this current is not known. From our experiments we conclude that in both KB3 and HeLa cells, volume-activated Cl^- channels have a low conductance ([25], compare also [3,7]) rather than a high conductance [8,9]. Since

this would imply a large number of channels, channel protein expression would be large. It has recently been proposed that P-glycoprotein, the product of the *MDR1* gene, may function as a volume-activated Cl^- channel. According to the results of Valverde et al. [2] and Gill et al. [16], P-glycoprotein can exist in two different, mutually exclusive functional states: it functions either as a transporter of hydrophobic substances or it is associated with a volume-activated Cl^- channel. The interconversion between the two states is induced by changes in cell volume or by the availability of substrate for transport. The properties of this proposed P-glycoprotein associated Cl^- current are similar to the ubiquitously expressed volume-activated current. This hypothesis has been challenged recently [26–30], but also supported [31,32]. However, the latter authors also argued against the bimodal function of P-glycoprotein [33]. Therefore, the controversy is still unsolved.

We have tested the hypothesis of a relation between P-glycoprotein and the volume-activated Cl^- current by comparing cells expressing different levels of this protein. Since the untreated human KB3 carcinoma cell line is known to express extremely low levels of the *MDR1* gene [22,23], we expected this cell type to express no or very little volume-activated Cl^- current. Our RT-PCR, immunocytochemical and FACS experiments confirm the very low expression of *MDR1* reported in the literature for the same batches of KB3 cells that were used for the functional measurements. We found, however, that these cells express a large volume-activated Cl^- current. Indeed, we found that out of 15 different cell types tested, the current density was highest in KB3 cells, together with Jurkat lymphocyte cells [25].

For comparison with the KB3 cells we have used the HeLa cell

line. The permeation properties of the volume-activated Cl^- channel of HeLa cells have recently been described and were found to be similar to the current ascribed to P-glycoprotein [4]. The amount of *MDR1* expressed in HeLa cells was much higher than in KB3 cells, both at the mRNA level (at least one order of magnitude) and at the protein level. Nevertheless, the measured current density was lower in HeLa cells than in KB3 cells.

Although the volume-activated Cl^- current described above presents very similar biophysical and pharmacological properties in a great variety of cell types, there is a quantitative variation in some of these properties. Discrepancies still exist in the single-channel conductance which scatters between < 1 pS and 80 pS [7–9]. This observation could suggest that these currents are mediated by more than one type of channel. However, we have no evidence that in both cell types different populations of Cl^- channels were activated or that in the same cell another channel population is involved which might be related to *MDR1* and open at a lower threshold, as discussed previously in BC19/3 cells [31]. In our experiments on both cell types, the Cl^- current started to activate at a hypotonicity of 10 % and the hypotonicity-response relation was monophasic (F. Viana and B. Nilius, unpublished work). Furthermore, it would be difficult to explain how these different channel types share so many common properties, unless they belonged to one gene family. In humans, only two members of the *MDR* gene family have been detected [34]. Since the sequence of *MDR2* is closely related to *MDR1*, the *MDR2* gene product may be another candidate for volume-activated Cl^- conductance. However, this hypothesis could also be excluded. The amount of RT-PCR product for *MDR2* mRNA was extremely small (KB3) or undetectable (HeLa), in line with the fact that the immunocytochemical signal obtained with an antibody recognizing both *MDR1* and *MDR2* proteins was similar to that seen with an *MDR1*-specific antibody. In addition, it has been shown that the major defect caused by disruption of the homologous murine *mdr2* gene is an impairment of secretion of phosphatidylcholine into bile, suggesting that *MDR2*-encoded P-glycoprotein mediates phospholipid translocation [35].

Rasola et al. [29] described a lack of correlation between swelling-induced Cl^- current and expression of *MDR1* in cells in which overexpression of P-glycoprotein was induced by prolonged culture in the presence of cytotoxic drugs. It can be argued, however, that in these cells a large fraction of the P-glycoprotein molecules may be locked in their transport mode, e.g. by selection of a mutant channel, and that the absence of additional Cl^- channel activity is due to the inability to activate the channel. This argument does not apply to our results because no overexpression was induced and the cells had not been exposed to substrates of the multidrug transporter.

In conclusion, the data are difficult to reconcile with the hypothesis that a current through P-glycoprotein-associated channels is the major component of an outward-rectifying swelling-activated Cl^- current which is present in a variety of cell types. This current is more likely to be due to permeation through a channel or a number of related channels not yet identified at the molecular level.

C. De G. and B. N. were supported by the Max Planck Gesellschaft, Germany. J. S. was supported by the 'Studienstiftung des Deutschen Volkes', Germany. F. V. was supported by a CICYT fellowship from the Spanish Government. K.V.A. was

supported by I.W.T. J.E. is a Postdoctoral Researcher and L.M. is a Research Assistant at the Belgian National Fund for Scientific Research (NFWO, Belgium).

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