THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 2, Issue of January 14, pp. 1432-1436, 1994
Printed in U.S.A.

Volume-sensitive Chloride Currents in Four Epithelial Cell Lines Are Not Directly Correlated to the Expression of the MDR-1 Gene*

(Received for publication, March 25, 1993, and in revised form, August 18, 1993)

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It has been shown recently that heterologous expression of human MDR-1 gene, which is responsible for multidrug resistance during cancer therapy, causes appearance of volume-sensitive Cl^- currents, thus suggesting that the product of the MDR-1 gene (the P-glycoprotein) has a Cl^- channel activity (Valverde, M. A., Diaz, M., Sepulveda, M. A., Gill, D. R., Hyde, S. C., and Higgins, C. F. (1992) *Nature* 355, 830–833). In the present work, we have tested four epithelial cell lines both for the expression of MDR-1 gene and for the presence of volume-sensitive Cl^- currents. LoVo/H and LoVo/Dx cells derive from a human colon adenocarcinoma, the latter cell line being resistant to high concentrations of the antitumoral drug doxorubicin. 9HTEo$^-$ cells were obtained by transformation of human tracheal epithelium. The 9HTEo$^-$/Dx cell line was established from these cells by selection in doxorubicin. As expected, higher levels of P-glycoprotein expression were detected in LoVo/Dx and 9HTEo$^-$/Dx by means of reverse transcriptase polymerase chain reaction technique, indirect immunofluorescence, and Western immunoblot assays. In contrast with these data, the size of swelling-induced Cl^- current was the same in the sensitive cell line and in its drug-resistant counterpart. Actually, the Cl^- conductance of 9HTEo$^-$ and 9HTEo$^-$/Dx was 4-fold higher than that of either LoVo/H or LoVo/Dx cells. This indicates that the amplitude of this conductance is not directly related to the expression of the MDR-1 gene.

P-glycoprotein, the product of the MDR-1 gene, is a membrane-associated active transport protein which hydrolyzes ATP to pump out of the cell different chemotherapeutic agents, thus conferring multiple resistance in tumoral cells (the MDR phenotype; for a review, see Refs. 2 and 3). MDR-1 is part of a small gene family, which is composed of two genes in humans, MDR-1 and MDR-3 (also called MDR-2; Refs. 4 and 5), and by three genes in rodents (mdr-1a, mdr-1b, and mdr-2; Refs. 6–8). Only the products of the MDR-1 gene and of the mdr-1a/1b are responsible for the MDR phenotype (5, 7), even though a high sequence and structural similarity is displayed by all P-glycoproteins. MDR gene products are also part of the ABC (ATP-binding cassette) superfamily of active membrane transporters (9). Another important member of the ABC superfamily is the cystic fibrosis gene product, which is a Cl^- selective channel (10, 11). A similar ion channel function has been proposed recently for the P-glycoprotein itself following experiments in which a fibroblast and a lung epithelial cell line were transfected with the full-length MDR-1 cDNA (1). In that study, the lowering of extracellular osmolality caused the appearance of Cl^- currents only in MDR-1-expressing cells. Since volume-sensitive Cl^- currents ($I_{Cl(vol)}$) represent a large fraction of the membrane conductance in many epithelial cells (12–14), it can be inferred that such cells should also display P-glycoprotein expression to some extent. In the present study, we have examined the correlation between drug resistance and volume-sensitive Cl^- conductance in four epithelial cell lines.

MATERIALS AND METHODS

Cells and Culture Conditions—The drug-sensitive LoVo/H and the drug-resistant LoVo/Dx cells were previously obtained from a human colon adenocarcinoma (15–17). 9HTEo$^-$ are immortalized human tracheal epithelial cells (18) from which we selected the drug-resistant 9HTEo$^-$/Dx cell line by continuous exposition to increasing concentrations of doxorubicin (Adriamicina, Carlo Erba, Milano, Italy) up to 0.6 μg/ml.

The culture medium contained 45% Dulbecco's modified Eagle's medium, 45% Ham's F-12 medium, 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine. LoVo/Dx and 9HTEo$^-$/Dx were continuously grown in the medium supplemented with doxorubicin (1 and 0.6 μg/ml, respectively) except for the last 5–7 days before each experiment. Clonogenic assays were carried out by plating 100 cells per 60-mm Petri dish. After 24 h, the medium was supplemented with 0.01, 0.1, or 1 μg/ml doxorubicin, and, after 2 weeks, cell colonies were stained with methylene blue and counted. These assays were carried out in triplicate and with a control of cells grown without doxorubicin.

Patch-clamp Analysis—Membrane currents were measured with the whole-cell configuration of the patch-clamp technique (19). Experiments were performed on nonconfluent cells plated in 35-mm Petri dishes. The extracellular (bath) solution contained (in mM): 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 sodium Hepes, 10 glucose (pH = 7.3). Patch pipettes were instead filled with (in mM): 140 NaCl, 1 $MgCl_2$, 0.18 $CaCl_2$, 2 EGTA, 10 sodium Hepes (pH = 7.3). This pipette (intracellular) solution included also 100 μM ATP in part of the experiments. Osmolality was adjusted to 295–305 mosm/kg with mannitol. The hypotonic medium (210 mosm/kg) had the same composition of the extracellular medium but without mannitol and with only 90 mM NaCl instead of 130. 1,9-Dideoxyforskolin and verapamil (Sigma) were added to this medium to test their ability to block the swelling-induced Cl^- current.

Series resistance was measured in each experiment and used to correct the errors in the applied membrane potential. The voltage stimulation protocol consisted of 1-s-long voltage pulses to −100 mV every 10 s, starting from a holding voltage of 0 mV. To construct current-voltage relationships, voltage steps were applied to potentials in the range −80 to +80 mV.

RNA Analysis—Total RNAs were extracted from 10^7 cells with the guanidine-thiocyanate method (20), whereas poly(A$^+$) RNAs were extracted from 5×10^7 cells with Dynabeads M-280 Oligo(dT)$_{25}$ (Dynal A.S., Oslo, Norway) as described (21). To perform reverse transcriptase

* This work was supported with grants from Associazione Italiana per la Ricerca sul Cancro (AIRC). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by grants from the National Institute of Diabetes and Digestive and Kidney Disease and the National Heart, Lung and Blood Institute.

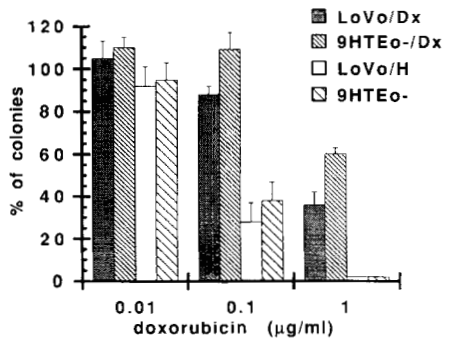

FIG. 1. **Inhibition of cell colony formation in the presence of doxorubicin.** The values are expressed as percent of control and are the mean of four experiments performed in triplicate. *Error bars* represent S.E.

PCR[1] experiments, 200 ng of mRNA or 2 μg of total RNA were converted in cDNA using the cDNA Synthesis System Plus kit (Amersham). Three-quarters of this cDNA were used for the detection of the MDR-1 mRNA. The remaining fraction was instead amplified to check for the presence of β_2-microglobulin mRNA, the positive control. The amplification was carried out in a final volume of 100 μl with 1 μM concentration of primers, 2.5 units of *Taq* polymerase and reaction kits (Perkin-Elmer Cetus Instruments). The primers sequence and PCR conditions were as reported by Noonan *et al.* (22). The expected PCR products were of 167 and 120 base pairs (for MDR-1 and β_2-microglobulin, respectively) and were separated on 2.5% agarose gels.

Protein Analysis—For the immunofluorescent detection of P-glycoprotein, cells were cultured on glass coverslips, washed in PBS, and fixed in a cold (−20 °C) methanol/acetone (4:1) solution. They were then incubated for 30 min at room temperature with the P-glycoCHEK C219 monoclonal antibody (Centocor Inc., Malvern, PA) diluted 1:10 in PBS. After washing, the cells were incubated for 45 min at room temperature with a sheep anti-mouse IgG, fluorescein-linked second antibody (Amersham International, England) diluted 1:20 in PBS.

For Western blot immunoassays, cells were detached from 100-mm Petri dishes with a cell scraper, washed in PBS, lysed in an ice-cold buffer (10 mM Tris-HCl, 1% Triton X-100, pH 8.0) and spun in a Microfuge at 13,000 rpm for 10 min. An amount of the supernatant containing 200 μg of proteins was run on a 6.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose filter with a transfer buffer composed of 25 mM Tris, 192 mM glycine, 20% methanol, and 0.04% SDS (pH 8.3). The filter was then blocked with 5% skim milk powder in Tris-buffered saline (TBS; 200 mM Tris, 5 M NaCl, pH 7.5), washed in TTBS (TBS with 0.05% Tween 20), and incubated for 4 h with 4 μg/ml mdr(Ab-1), a rabbit polyclonal antibody against P-glycoprotein (Oncogene Science), or, alternatively, with 1 μg/ml P-glycoCHEK C219 monoclonal antibody. After washing, the filter was incubated with an anti-rabbit or with an anti-mouse IgG second antibody conjugated to alkaline phosphatase. Protein detection was performed with both colorimetric and chemiluminescent techniques. Second antibodies and the colorimetric detection system were purchased from Bio-Rad (Munich, Germany). The chemiluminescent revelation kit was from New England Biolabs.

Determination of band intensities in reverse transcriptase PCR and Western blot experiments was performed with a LKB Ultroscan XL densitometer.

Statistics—Data are presented as representative experiments or as arithmetic means ± S.E. Significance is calculated according to the Student's *t*-test.

RESULTS

To check the drug resistance of cells, we have performed clonogenic assays. As shown in Fig. 1, only LoVo/Dx and 9HTEo$^-$/Dx cells are able to form colonies in 1 μg/ml doxorubicin. A significant higher resistance of LoVo/Dx and 9HTEo$^-$/Dx is also observed at 0.1 μg/ml ($p < 0.01$ with respect to both LoVo/H and 9HTEo$^-$ cells). This suggests a higher expression of P-glycoprotein in the two drug-resistant cell lines.

To investigate the correlation between the MDR phenotype and the volume-sensitive Cl$^-$ currents, we used the whole-cell configuration of the patch-clamp technique. After breaking the patch to obtain the whole-cell configuration, a Cl$^-$ current began to appear in isotonic conditions (Fig. 2). This spontaneous activation was previously observed by Worrell *et al.* (12) in T84 cells and subsequently found also in 9HTEo$^-$ and other epithelial cells (23) and attributed to an osmotic disequilibrium occurring in the whole-cell configuration. As it is shown in Fig. 2, an additional conductance increase was obtained by applying a hypotonic shock (210 mosm/kg). To demonstrate the Cl$^-$ selectivity of this current, extracellular Cl$^-$ (140 mM) was almost totally replaced (130 mM) with gluconate (Fig. 3, *A* and *B*). This intervention shifted the reversal potential from 0 to +43.9 ± 4.5 mV in 9HTEo$^-$ ($n = 5$), +36.7 ± 1.7 in 9HTEo$^-$/Dx ($n = 3$), +42.2 ± 5.7 mV in LoVo/H ($n = 5$), and +46.5 ± 5.2 mV in LoVo/Dx ($n = 8$) as expected for a volume-sensitive Cl$^-$ selective channel (14). The swelling-induced Cl$^-$ current found in all four cell lines had characteristics similar to those constitutively present in other epithelial cells (12–14, 23) or induced by heterologous expression of P-glycoprotein (1). In fact, the current showed outward rectification, and a marked time-dependent inactivation was observed at positive membrane potentials (Fig. 3*C*). At +80 mV, the current completely inactivated in 10 s. Another characteristic was the sensitivity to 1,9-dideoxyforskolin (Fig. 2) and verapamil: both compounds are able to revert the multidrug resistance by blocking the ATP-dependent drug efflux through P-glycoprotein. 1,9-Dideoxyforskolin at 100 μM caused in our experiments a strong and reversible current inhibition (9HTEo$^-$: 79.4 ± 9.6%, $n = 3$; 9HTEo$^-$/Dx: 81.2 ± 5.6%, $n = 4$; LoVo/H: 92.1 ± 1.1%, $n = 3$; LoVo/Dx: 87.0 ± 3.7%, $n = 4$) as also reported by Valverde *et al.* (1). Verapamil was instead a poor blocker of the current, the inhibition being less than 70% at 500 μM concentration in the four cell lines ($n = 3$ for each one; not shown). Doxorubicin (180 μM) in the pipette solution was instead unable to inhibit the volume-sensitive Cl$^-$ current in LoVo/Dx ($n = 3$) and 9HTEo$^-$ ($n = 3$) cells.

Fig. 4 shows the values of the conductance activated by the hypotonic shock. The chord conductance at −100 mV was corrected for the series resistance and normalized for the membrane capacitance to compensate for the larger size of 9HTEo$^-$ and 9HTEo$^-$/Dx with respect to LoVo cells. The volume-sensitive conductance of LoVo/Dx cells was almost identical with that of LoVo/H but 4-fold smaller than that of both 9HTEo$^-$ and 9HTEo$^-$/Dx ($p < 0.001$).

Most of the experiments were performed using an ATP-free pipette (intracellular) solution. ATP (100 μM) was added in the pipette solution to investigate its possible role in the activation of the $I_{Cl(vol)}$. The Cl$^-$ current measured under this condition was not significantly different from that obtained with ATP-free solution. For example, the mean normalized conductance in LoVo/H was 1.27 ± 0.11 nS/pF ($n = 7$) with ATP and 1.51 ± 0.19 ($n = 20$) without ATP ($p > 0.05$). Similarly, in LoVo/Dx, the conductance was 1.49 ± 0.37 nS/pF ($n = 6$) with ATP and 1.50 ± 0.17 nS/pF ($n = 18$) without it. Therefore, the results obtained under these two experimental conditions were pooled in Fig. 4.

In order to account for the smaller conductance detected in LoVo cells, we asked whether a greater swelling stimulus might be required to induce a current comparable to that seen in 9HTEo$^-$ and 9HTEo$^-$/Dx. This was not the case since a decrease of extracellular osmolality from 210 to 150 mosm/kg did not further activate the chloride conductance in LoVo/Dx cells ($n = 3$; not shown).

As it is apparent from these electrophysiological data, the hypotonic shock is able to elicit a Cl$^-$ current with similar features both in doxorubicin-resistant (LoVo/Dx and 9HTEo$^-$/Dx) and in doxorubicin-sensitive (9HTEo$^-$ and LoVo/H) cells. Unexpectedly, the size of this current was not correlated with

[1] The abbreviations used are: PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

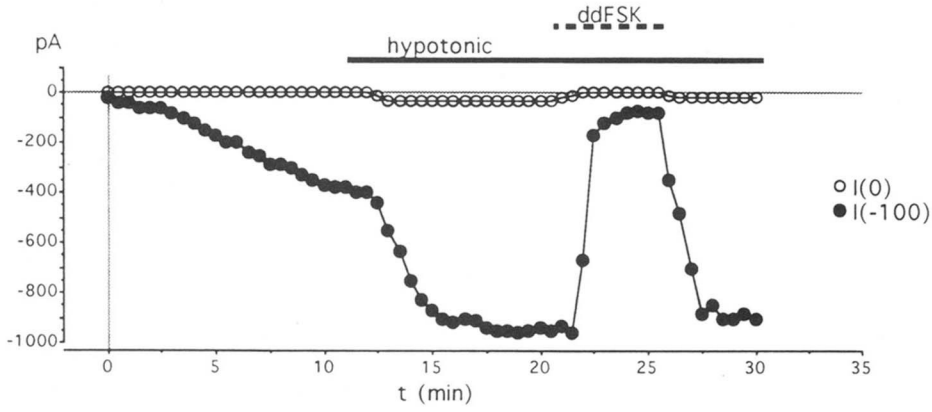

FIG. 2. **Effect of the hypotonic shock and of 100 μM extracellular 1,9-dideoxyforskolin (*ddFSK*) on membrane currents.** The figure depicts a representative whole-cell experiment performed on a LoVo/H cell. Data show the membrane currents recorded at 0 and −100 mV.

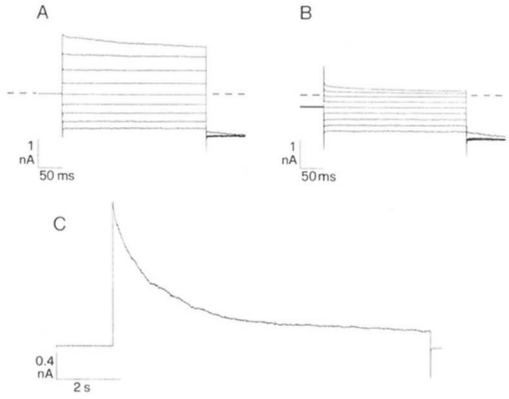

FIG. 3. **Cl^- currents activated by the hypotonic shock.** *Plots A* and *B* show superimposed membrane currents (*A* and *B*) elicited by voltage pulses to membrane potentials in the range −80 to +80 mV in 20-mV steps. The holding potential was at 0 mV, and a 1-s step to −100 was applied after each test pulse to allow recovery of currents from inactivation. Data were obtained from a LoVo/H cell with 140 mM Cl^- (*A*) or 10 mM Cl^- plus 130 mM gluconate (*B*) in the extracellular solution. *Plot C* shows the inactivation produced by a long lasting step to +80 mV on the same cell.

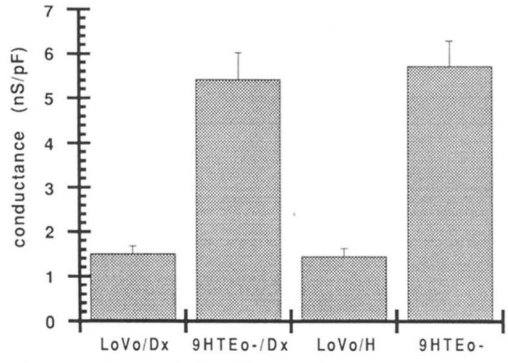

FIG. 4. **Comparison of the Cl^- conductance activated by the hypotonic shock.** The values of the chord conductance at −100 mV are corrected for the series resistance and normalized for the membrane capacitance. The conductance is significantly higher in $9HTEo^-$ ($n = 12$) and $9HTEo^-$/Dx ($n = 11$) than in LoVo/H ($n = 27$) or LoVo/Dx ($n = 24$) cells ($p < 0.001$ for both cases). *Error bars* represent S.E.

the degree of doxorubicin resistance. A more subtle analysis was therefore needed both at the RNA and at the protein level to look for the presence of P-glycoprotein also in $9HTEo^-$ and LoVo/H cells.

Due to its high sensitivity (24), we used the reverse transcriptase PCR technique to study the MDR-1 mRNA expression in our cell lines. After 46 cycles of cDNA amplification, a marked band was found in LoVo/Dx and $9HTEo^-$/Dx lanes (Fig. 5). A weaker band was also detected in the other two cell lines.

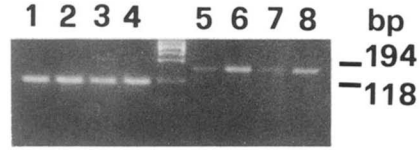

FIG. 5. **Gel electrophoresis of the products obtained by 46 cycles of cDNA amplification in $9HTEo^-$ (*lanes 1* and *5*), $9HTEo^-$/Dx (*lanes 2* and *6*), LoVo/H (*lanes 3* and *7*), and LoVo/Dx (*lanes 4* and *8*).** *Lanes 1, 2, 3,* and *4* show the results obtained with two primers from the β_2-microglobulin gene. *Lanes 5, 6, 7,* and *8* show instead amplification performed with two MDR-1 primers. The marker in the middle is ϕX174 *Hae*III. Relative intensities of the bands were assessed by densitometry (see Table I).

After densitometric analysis, relative intensities of MDR-1 lanes normalized to those of the β_2-microglobulin were: LoVo/H, 1.00; $9HTEo^-$, 1.0; LoVo/Dx, 3.6; and $9HTEo^-$/Dx, 4.3.

These results confirm the higher expression of the MDR-1 gene in the drug-resistant cells. We asked whether this was also seen at the protein level. Therefore, we performed both immunofluorescence and Western blot assays.

Indirect immunofluorescence was carried out using the C219 monoclonal antibody against P-glycoprotein. As shown in Fig. 6, a clear difference exists between drug-resistant and drug-sensitive cells. LoVo/Dx displayed a fluorescent signal much stronger than that observed in LoVo/H. Similarly, a significant fluorescence was detected in $9HTEo^-$/Dx, whereas in $9HTEo^-$ it was hardly distinguishable from the background.

Fig. 7 illustrates the result of a Western blot experiment performed using the polyclonal antibody mdr(Ab-1). A diffuse band of apparent molecular weight of 141,000–172,000 was clearly detectable in the LoVo/Dx and $9HTEo^-$/Dx lanes. A similar band was visible in LoVo/H and $9HTEo^-$ cells, but with a relative intensity 7-fold lower as resulted from densitometric analysis. Same results were obtained using the C219 monoclonal antibody (data not shown). The smeared shape of the band is typical of P-glycoprotein as observed by Schinkel *et al.* (25) and is probably due to alternative forms of the protein caused by differential glycosylation or phosphorylation (25–27).

The results obtained with the different techniques are summarized in Table I.

DISCUSSION

The present work describes a swelling-induced Cl^- conductance ($I_{Cl(vol)}$) which shows similar biophysical and pharmacological features in the four cell lines that we have examined. The current-voltage relationship is outwardly rectifying, and a marked time-dependent inactivation is observed at the most positive membrane potentials (*i.e.* +80 mV). This cell volume-dependent channel is almost completely inhibited by 100 μM 1,9-dideoxyforskolin and partially inhibited by verapamil. The normalized conductance is comparable between LoVo/H and

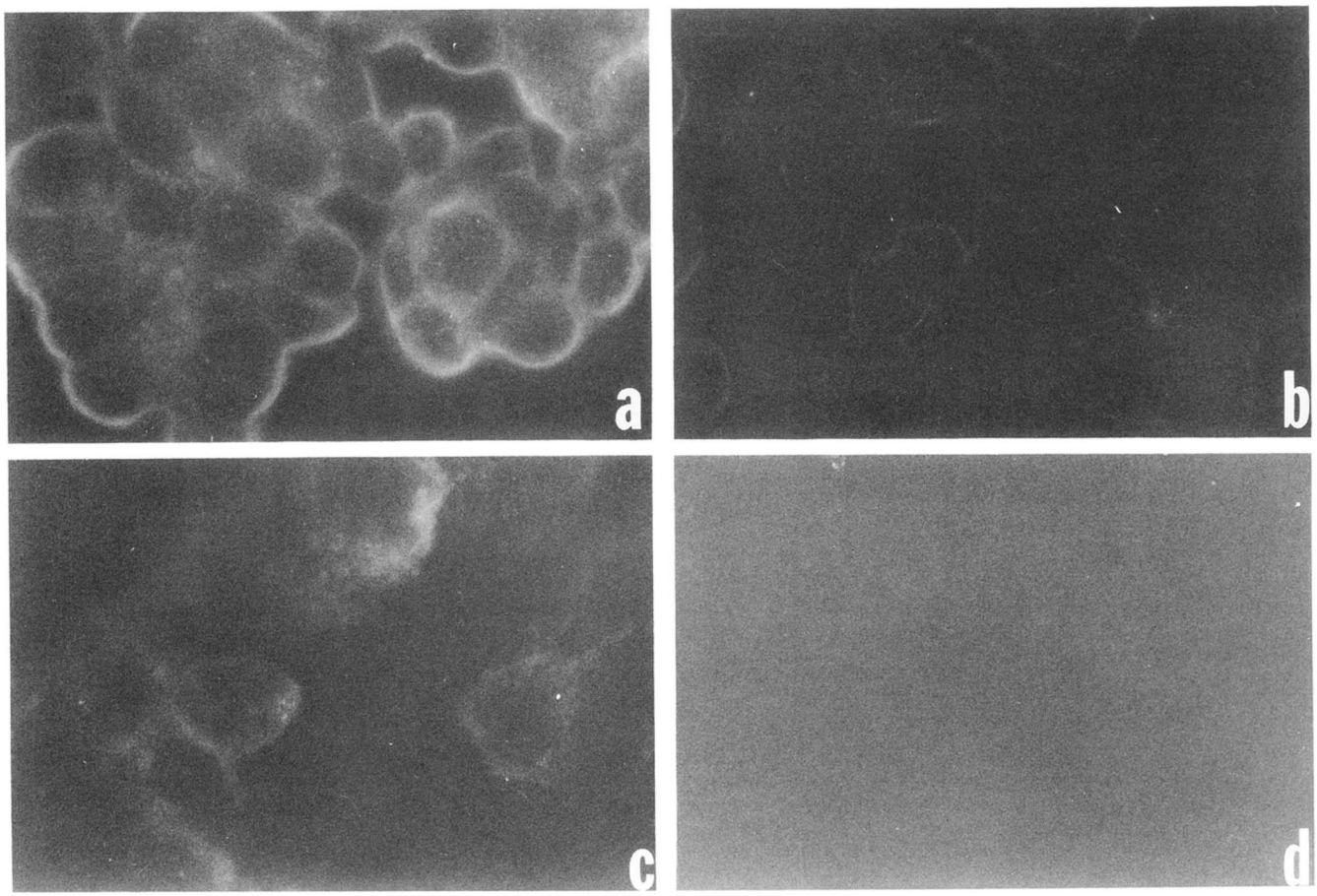

FIG. 6. **Indirect immunofluorescence assay performed with the monoclonal antibody P-glycoCHEK C219 on: LoVo/Dx (*a*), LoVo H (*b*), 9HTEo⁻/Dx (*c*), and 9HTEo⁻ (*d*).**

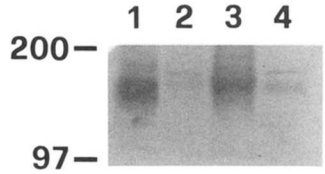

FIG. 7. **Western immunoblot performed with the polyclonal antibody mdr(Ab-1).** The anti-rabbit second antibody was conjugated to alkaline phosphatase and revealed by chemiluminescent analysis. *Lanes 1, 2, 3,* and *4* are LoVo/Dx, 9HTEo⁻, 9HTEo⁻/Dx, and LoVo/H cells, respectively. On the *left* are the markers expressed × 10^3.

TABLE I

Summary of the results obtained with the various techniques

Quantitative results have been normalized with respect to those of LoVo/H cells.

	LoVo/H	LoVo/Dx	9HTEo⁻	9HTEo⁻/Dx
Drug resistance	No	Yes	No	Yes
Reverse transcriptase PCR	1	3.6	1	4.3
Immunofluorescence	+	+++	–	+++
Western blot	1	7.7	1.1	6.5
Cl^- conductance	1	1	3.9	3.8

LoVo/Dx cells, while it is about 4 times greater in 9HTEo⁻ cells and 9HTEo⁻/Dx. Summing up, this volume-sensitive Cl^- current has several characteristics similar to that observed by Valverde *et al.* (1) after heterologous expression of P-glycoprotein. A striking difference lies instead in the ATP dependence. Indeed, we have obtained activation of $I_{Cl(vol)}$ also with ATP-free pipette solutions. Nevertheless, it is not possible to be sure of a complete wash out of endogenous ATP in our cells. Another important difference is that a high concentration of doxorubicin in the pipette solution was not able to inhibit the $I_{Cl(vol)}$ in contrast with the results obtained by Gill *et al.* (28).

Our data argue against a quantitative correlation between drug sensitivity, P-glycoprotein expression, and activity of volume-sensitive currents. Despite LoVo/H and LoVo/Dx having quite different levels of P-glycoprotein expression and drug resistance, they display almost identical amplitudes of swelling-induced Cl^- conductance. On the other hand, the 9HTEo⁻ cell line has P-glycoprotein expression comparable to that of LoVo/H but a Cl^- conductance 4-fold higher than both LoVo cell lines. Furthermore, the generation of the 9HTEo⁻/Dx cell line by continuous selection in doxorubicin was successful in increasing the P-glycoprotein expression to a level similar to that of LoVo/Dx, but did not affect the amount of the Cl^- conductance.

A lack of correlation between swelling-induced Cl^- secretion and transport of vinblastine has also recently been observed in T84 cells (29). To explain this discrepancy, different hypotheses can be postulated. The simplest one is that P-glycoprotein is not a Cl^- channel. While this is not very probable due to existing experimental evidences (1, 28), it must be considered in light of our data.

An alternative explanation could be that the hypotonic shock is able to activate only a fraction of the volume-sensitive channels present in the cell. LoVo cells could have a reduced ability to activate Cl^- channels due to a less efficient regulatory mechanism linking swelling to current activation. However, this would explain the smaller conductance of LoVo cells with respect to 9HTEo⁻, but not the lack of difference between drug-resistant and drug-sensitive cells. Furthermore, an insufficient

swelling stimulus can also be excluded since our results demonstrate that a more hypotonic solution is not able to activate more channels.

Also to be considered is the model proposed by Gill *et al.* (28). These authors hypothesize that the ion transport and drug transport functions of P-glycoprotein exist exclusive of one another. In other words, when P-glycoprotein is "locked" in the pump mode, it is not able to function as a volume-sensitive Cl^- channel. In accord with this model, LoVo cells could have a higher fraction of P-glycoprotein locked in drug transport mode and therefore have a smaller Cl^- conductance. Nevertheless it is difficult to explain why the generation of the 9HTEo$^-$/Dx cell line by selection has not caused any change in the volume-sensitive conductance with respect to its drug-sensitive counterpart.

Another interesting possibility is that, in addition to or as an alternative to P-glycoprotein, other membrane proteins are responsible for the volume-dependent Cl^- conductance in our cells. As an example, a Cl^- channel that shows voltage-dependent inactivation at positive potentials and outward rectification has been cloned recently in MDCK cells (30). Moreover, one of the features displayed by $I_{Cl(vol)}$ and P-glycoprotein, *i.e.* the sensitivity to 1,9-dideoxyforskolin and verapamil, is not a very specific characteristic, since it is also shared by other ion channels. For instance, a voltage-dependent K^+ current described in the same 9HTEo$^-$ cells is inhibited by these two compounds at even lower concentrations (31). In addition, the product of the MDR-3 gene, which shows a very high degree of sequence homology with the MDR-1 P-glycoprotein and whose function is unknown, could be responsible for a Cl^- conductance. Finally, a ubiquitous Cl^- channel termed ClC-2 is also activated by osmotic disequilibria (32, 33). Thus, volume regulation seems to be a crucial and complex mechanism controlled by different anion channels.

In conclusion, it appears from our data that the $I_{Cl(vol)}$ is quantitatively unrelated with the P-glycoprotein expression. This discrepancy needs further investigation since it could provide interesting information on the regulatory mechanisms and the membrane proteins responsible for the multidrug resistance and the volume-activated conductances.

Acknowledgments—We acknowledge Monica Scaranari for expert technical assistance and Drs. V. Corrias and G. P. Tonini for providing the C219 antibody and for helpful discussion. We are also grateful to Dr. C. Lo Cunsolo for help with densitometric analysis.

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