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ALTERATIONS OF IONIC MEMBRANE PERMEABILITIES IN MULTIDRUG-RESISTANT NEUROBLASTOMA × GLIOMA HYBRID CELLS

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Summary

A population of NG108-15 neuroblastoma cells resistant to doxorubicin (NG/DOXR) was established. The cells exhibited a multidrug resistance phenotype with cross-resistance to vinblastin and colchicine, overexpression of a 170 kDa membrane protein identified as P-glycoprotein and reversal of resistance by verapamil and quinine. Compared with NG108-15 cells, NG/DOXR cells showed an increase in Na^+ current density and a decrease in cyclic-AMP-activated Cl^- current density with no change in K^+- and volume-sensitive Cl^- current densities. As previously observed in NG108-15 cells, the vacuolar-type H^+-ATPase inhibitors bafilomycin A1 and nitrate induced membrane depolarizations in NG/DOXR cells. The resting potentials of sensitive and resistant cells were not significantly different, but the depolarizations evoked by these agents were significantly larger in NG/DOXR than in NG108-15 cells. The resting membrane potential of NG/DOXR cells, but not that of NG108-15 cells, was depolarized by verapamil, and this effect was abolished by bafilomycin. The volume-sensitive Cl^- currents of drug-sensitive and drug-resistant cells were inhibited by a decrease in intracellular pH from 7.3 to 6.8. Whereas bafilomycin prevents activation of Cl^- currents in both drug-sensitive and drug-resistant cells, verapamil inhibited the Cl^- current only in NG/DOXR cells. The results are discussed in terms of the roles of cytoplasmic pH and membrane potential in multidrug resistance.

Key words: multidrug resistance, neuroblastoma cells, H^+-ATPase, P-glycoprotein, doxorubicin, verapamil, bafilomycin, ion current.

Introduction

The resistance of tumour cell to cytotoxic drugs is one of the major obstacles to successful chemotherapy. Multidrug resistance (MDR) is the phenomenon observed in tumour cells *in vivo* and cultured cells *in vitro* involving cellular resistance to a wide range of chemically unrelated drugs. MDR is commonly associated with overexpression of a membrane protein, P-glycoprotein (P-gp) (for reviews, see Gottesman and Pastan, 1993; Higgins and Gottesman, 1992; Nielsen and Skovsgaard, 1992). This protein is hypothetized to be an active transporter that uses the energy from ATP to pump chemotherapeutic drugs out of cells, reducing their intracellular concentrations and, hence, toxicity. However, in most drug-selected MDR tumour cell lines, the activity of P-gp alone cannot account for the diversity of MDR phenotypes observed. Other cellular mechanisms seem to develop and contribute to resistance. In addition to P-gp overexpression, MDR is accompanied by multiple biochemical and morphological changes, including alterations to membrane ion transport which are thought to play a role in drug resistance. These alterations can be considered to be a direct consequence of P-gp expression when they appear in transfected cells that have not previously been exposed to chemotherapeutic drugs (Hoffman *et al.* 1996). In contrast, when they appear in drug-selected cells (which was the case in most MDR cells studied until now), they may be due to factors unrelated to P-gp expression, but induced by the drug selection. Whatever the origin of these alterations, it is interesting to know whether they play a role in multidrug resistance. It has been noted that Na^+, K^+ and Cl^- channel activities differ between MDR and parental cell lines (Gollapudi *et al.* 1992; Jirsch *et al.* 1993; Kunzelmann *et al.* 1994; Yamashita *et al.* 1987). Since 1992, several groups have focused on the relationship between the volume-sensitive Cl^- channel and MDR but conflicting results have been obtained. Although several independent studies confirm a relationship between P-gp expression and the magnitude of a cell's volume-sensitive current (Gill *et al.* 1992; Jirsch *et al.* 1993; Valverde *et al.* 1992), other studies have found no correlation (De Greef *et al.* 1995; Dong *et al.* 1994; Ehring *et al.* 1994; Kunzelmann *et al.* 1994; Morin *et al.* 1995; Rasola *et al.* 1994; Wang *et al.* 1994).

As a possible consequence of alterations to ion transport, a decrease in membrane potential has been observed in doxorubicin-resistant Friend leukaemia cells (Hasmann *et al.* 1989) and in Chinese hamster ovary fibroblasts transfected with hu MDR 1 cDNA and resistant to doxorubicin, vinblastin and colchicine (Hoffman *et al.* 1996). Since most anticancer drugs are positively charged at physiological pH, their accumulation

in cells should be influenced by membrane potential and should decrease as the membrane potential is made less negative. Hoffman *et al.* (1996) concluded that protein-mediated MDR could be the consequence of a less negative resting membrane potential. Another important cellular parameter that is modified by MDR acquisition is intracellular pH (pHi). Most MDR cells have a higher intracellular pH than their parental cells (Boscoboinik *et al.* 1990; Marquardt and Center, 1991; Roepe, 1995; Roepe *et al.* 1996). These latter observations led to recent investigations into the causal relationship between pHi and MDR. Agents that reverse MDR, such as verapamil, can also affect the elevated cytoplasmic pH (Keizer and Joenje, 1989), and an alkaline shift in pHi in sensitive cells is sufficient to prevent the accumulation of chemotherapeutic drugs and to decrease the drug sensitivity (Hoffman *et al.* 1996; Simon *et al.* 1994). Moreover, Marquardt and Center (1991) observed that bafilomycin A1, a specific inhibitor of the vacuolar-type proton pump (V-ATPase), induces a major increase in drug accumulation, suggesting a possible involvement of this ATPase in the pathway of drug efflux from MDR cell lines that is independent of P-gp expression. Since we recently advanced arguments in favour of the contribution of a plasma membrane proton pump in determining the resting potential of NG108-15 cells (Gérard *et al.* 1994), we wanted to know whether this proton pump could play a role in neuroblastoma cell drug resistance. We therefore selected NG108-15 neuroblastoma cells resistant to doxorubicin (NG/DOXR) that exhibited an MDR phenotype. A comparative analysis of electrophysiological properties was carried out on doxorubicin-sensitive and doxorubicin-resistant neuroblastoma cells with particular attention being paid to the V-ATPase and volume-sensitive Cl^- channel activities.

Materials and methods

Cells and culture conditions

NG108-15 hybridoma cells are a clone of neuroblastoma × glioma hybrid cells formed by Sendai-virus-induced fusion of a subclone N 18TG-2 of mouse C1300 neuroblastoma cells with subclone C6Bu-1 of rat C6 glioma cells (Hamprecht, 1977). Doxorubicin-resistant (NG/DOXR) cells were selected by continuously exposing NG108-15 cells to gradually increasing concentrations (from 2 to 100 nmol l^{-1}) of doxorubicin. After 9 months in culture, NG/DOXR cells were able to grow uninhibited in 100 nmol l^{-1} doxorubicin and were thereafter cultured in that concentration of drug. The cells were grown at 37 °C in a humidified atmosphere (95 % air/5 % CO_2) in Dulbecco's modified Eagle's medium containing 5 % foetal calf serum, 100 µmol l^{-1} hypoxanthine, 0.4 µmol l^{-1} aminopterine, 16 µmol l^{-1} thymidine, 2 mmol l^{-1} glutamine, 3 µmol l^{-1} glycine, 100 µg ml^{-1} streptomycin and 50 µg ml^{-1} penicillin. NG/DOXR cells were continuously grown in the culture medium supplemented with 100 nmol l^{-1} doxorubicin, except for the last 5–7 days before all experiments.

Assay of drug sensitivity

Sensitive and resistant cells in the exponential phase of growth were used throughout. NG108-15 and NG/DOXR cells were seeded at 1.5×10^5 cells per 10 ml and were exposed to a range of concentrations of cytotoxic drugs in the absence or presence of chemosensitizers. After 48 h of continuous exposure, the cell number and volume distribution were determined using a Coulter counter (Coultronics). Each counting was made in quintuplicate.

Patch-clamp analysis

Membrane currents and potentials were measured using the whole-cell configuration of the patch-clamp technique (Hamill *et al.* 1981). Experiments were performed on non-confluent cells at room temperature. The compositions of the pipette and bathing solutions are given in Table 1. Pipette resistances ranged from 2 to 5 MΩ (external and pipette A media). Capacitance was measured by integrating the uncorrected capacity transient. The ACQUIS1 suite of programs (DIPSI) was used for data acquisition and analysis.

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

Crude membrane preparations were prepared from the various cultured cells. The human epidermoid carcinoma cells

Table 1. *Compositions of bathing and pipette solutions used in electrophysiological experiments*

	Concentration (mmol l^{-1})						
	External A	External B1	External B2	External C1	Pipette A	Pipette B	Pipette C
NaCl	140	–	–	–	–	–	–
NMGCl	–	140	60	105	–	–	140
$MgCl_2$	2	1	1	1	2	2	1
$CaCl_2$	1	1	1	1	–	–	–
KCl	5	–	–	–	140	–	–
CsCl	–	–	–	–	–	140	–
Sodium isethionate	–	–	80	–	–	–	–
Hepes	10	10	10	10	10	10	10

External and internal pH were adjusted to 7.3 using NaOH.
NMGCl, *N*-methyl-D-glucamine chloride.

KB3-1 were used as negative control and KBA-1 as a positive control for P-glycoprotein expression (Shen *et al.* 1986). Confluent cells were washed in 140 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 20 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 7.1, and then sedimented by centrifugation at 30 ***g*** for 5 min. The cells were then lysed and homogenized using a glass–Teflon tissue homogenizer in a hypotonic buffer (100 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EGTA, 0.5 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} phenylmethylsulphonylfluoride, pH 7.1). The undisrupted cells and nuclear debris were removed by centrifugation at 180 ***g*** for 10 min. The supernatants were centrifuged for 60 min at 14 000 ***g***, and membrane pellets were resuspended in 100 mmol l^{-1} KCl, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EGTA, 0.5 mmol l^{-1} dithiothreitol, pH 7.1. Protein contents were estimated according to Bradford (1976). SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The samples were treated with solubilization buffer [50 mmol l^{-1} Tris, 100 mmol l^{-1} sucrose, 5 % SDS (w/v), 2 % β-mercaptoethanol (v/v), 0.02 % Bromophenol Blue] and were resolved on a 7.5 % to 15 % acrylamide gel. After SDS–PAGE, proteins were electrophoretically transferred to nitrocellulose membranes. Non-specific binding to the blots was minimized by incubating them in a medium containing 3 % milk powder. The nitrocellulose membranes were then incubated overnight at 4 °C with C219, an anti-P-glycoprotein monoclonal antibody (0.5 μg ml^{-1}, final concentration in the milk-containing medium). Immunoreactive bands were visualized with peroxidase-conjugated anti-mouse IgG and an enhanced chemiluminescence detection system (Amersham France SA).

Reagents

Chemicals used for cell culture were purchased from GIBCO. Bafilomycin A1 was provided by Professor K. Altendorf (Universität Osnabrück, Germany). Anti-P-glycoprotein monoclonal antibodies were obtained from CIS Biointernational, and secondary antibodies were obtained from Amersham France SA. KB3-1 and KBA-1 cells were kindly provided by M. F. Poupon (Institut Curie, URA CNRS 620, Paris, France). All other products were purchased from Sigma.

Statistical analyses

All results are expressed as the mean value ± S.E.M. (*N*), where *N* represents the number of cells or experiments. Statistical comparisons were performed using Student's *t*-tests for paired or unpaired data as appropriate. Differences were considered significant at $P<0.05$.

Results

Characterization of neuroblastoma cell multidrug resistance

The sensitivity of the parental NG108-15 cells and NG/DOXR cells to doxorubicin is presented in Fig. 1A. The doxorubicin concentrations required for 50 % inhibition of cell growth (IC_{50}) were 10 and 1300 nmol l^{-1} for NG108-15 and NG/DOXR cells, respectively. The degree of resistance to doxorubicin (expressed as the ratio of IC_{50} values for resistant to sensitive cell lines) was 130. One should note that, as already observed in human and murine cells (Vichi and Tritton, 1989), growth of NG108-15 and NG/DOXR cells was stimulated by doxorubicin concentrations below 1 nmol l^{-1} and 100 nmol l^{-1}, respectively. The doubling time of NG108-15 cells, determined during the exponential growing phase, was 14.5 h (*N*=3). The rate of proliferation of NG/DOXR cells was not significantly different, with a doubling time of 17.8 h (*N*=3).

One property of MDR cells is their ability when exposed to a single drug to develop resistance, termed 'cross-resistance', to a broad range of cytotoxic compounds. NG/DOXR cells are cross-resistant to two other anticancer drugs: vinblastin and colchicine. Compared with NG108-15 cells, NG/DOXR cells were 87 times more resistant to colchicine (IC_{50} 1300 nmol l^{-1} *versus* 15 nmol l^{-1}) and 54 times more resistant to vinblastin (IC_{50} 540 nmol l^{-1} *versus* 10 nmol l^{-1}).

Many compounds have been shown to antagonize MDR in a variety of cell lines and *in vivo* tumour models when co-administered with chemotherapeutic agents to which the cells are resistant (Ford and Hait, 1990). In general, agents used to antagonize MDR, termed 'chemosensitizers', increase intracellular drug accumulation. Among these agents are verapamil and quinine. The mechanism by which they antagonize MDR is still uncertain. While there is evidence that they interact directly with P-glycoprotein, they also have other

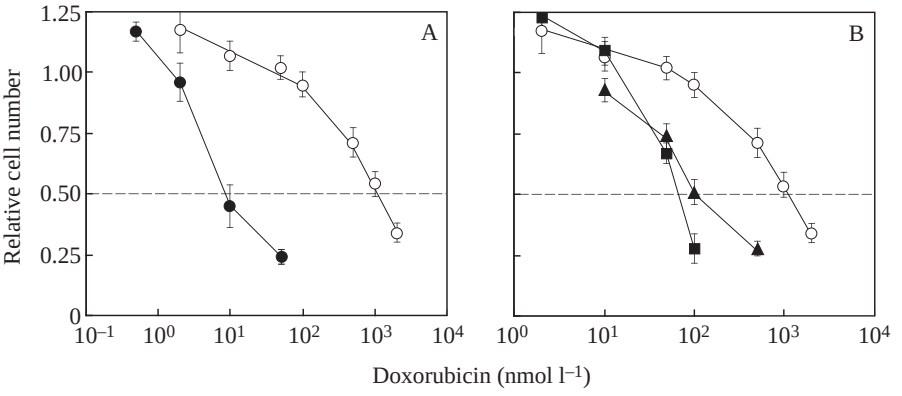

Fig. 1. Sensitivity to doxorubicin (DOX) of NG108-15 and NG/DOXR cells and reversal of DOX resistance by verapamil and quinine. (A) Cytotoxic effects of DOX on NG108-15 (●) and NG/DOXR (○) cells. (B) Effects of verapamil (VER) (■) and quinine (QUI) (▲) on the sensitivity of NG/DOXR cells to DOX. After 48 h of continuous exposure to DOX in the presence or absence of VER (1 μmol l^{-1}) and QUI (5 μmol l^{-1}), cells were counted as described in Materials and methods. In all cases, the number of cells is expressed relative to that in the absence of doxorubicin. Each value represents the mean ± S.E.M. (*N*=3).

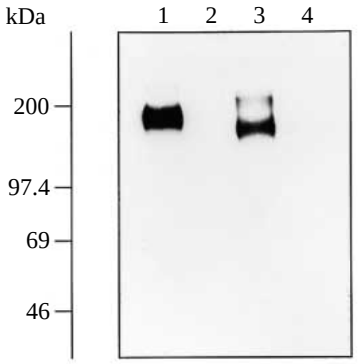

Fig. 2. Detection of P-glycoprotein by Western blot analysis. Membrane proteins (4 µg per lane) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with the C219 monoclonal antibody as described in Materials and methods. Lane 1, KBA-1 (positive control); lane 2, NG108-15; lane 3, NG/DOXR; lane 4, KB3-1 (negative control). Molecular mass markers (in kDa) are indicated on the left.

targets. In particular, verapamil is a classical Ca^{2+} channel blocker and to a lesser extent inhibits K^+ channels; the antimalarial agent quinine is a potent K^+ channel blocker. In NG/DOXR cells, 1 µmol l^{-1} verapamil and 5 µmol l^{-1} quinine produced respectively a 20- and 12-fold decrease in resistance to doxorubicin (Fig. 1B). It should be noted that, in contrast to quinine, verapamil also induced an apparent change in the slope of the dose–response curve.

P-glycoprotein expression was evaluated by Western blotting using the C219 monoclonal antibody, which recognizes a very well-conserved epitope in the C-terminal part of P-glycoprotein (Kartner *et al.* 1985). Immunoblotting analysis using KBA-1 cells as a positive control demonstrated the presence of P-glycoprotein in NG/DOXR cells (Fig. 2, lanes 1 and 3). A broad, heavily stained band was detected in NG/DOXR cells, but not in NG108-15 cells (Fig. 2, lanes 3 and 2). The labelled protein had an apparent molecular mass of 170 kDa, which is consistent with the known molecular mass of P-glycoprotein (Kartner *et al.* 1985).

Comparison of the properties of NG108-15 and NG/DOXR cells

Cell size

Electrophysiological techniques allow the estimation of cell size by measurement of membrane capacitance, which is assumed to be proportional to cell membrane area. Whether or not an alteration in cell capacitance reflects changes in membrane properties, it should be determined in order to quantify ionic currents and the number of membrane transporters per unit membrane surface area. The size of NG108-15 and NG/DOXR cells was determined by two methods: measurement of volume distribution using a Coulter counter and measurement of membrane capacitance under the whole-cell patch-clamp configuration. The volume distributions of both NG108-15 and NG/DOXR cells were unimodal, and the mean volume of NG/DOXR cells was significantly smaller (2.89±0.06 pl, N=5) than that of NG108-15 cells (3.48±0.09 pl, N=5). The ratio of resistant to sensitive mean cell volume was 0.89±0.04. The measurement of capacitance also revealed that NG/DOXR cells were smaller than NG108-15 cells. The mean capacitance of NG/DOXR was 43.3±1.2 pF (N=40) and that of NG108-15 cells was 56.5±1.6 pF (N=41) (ratio 0.77±0.04).

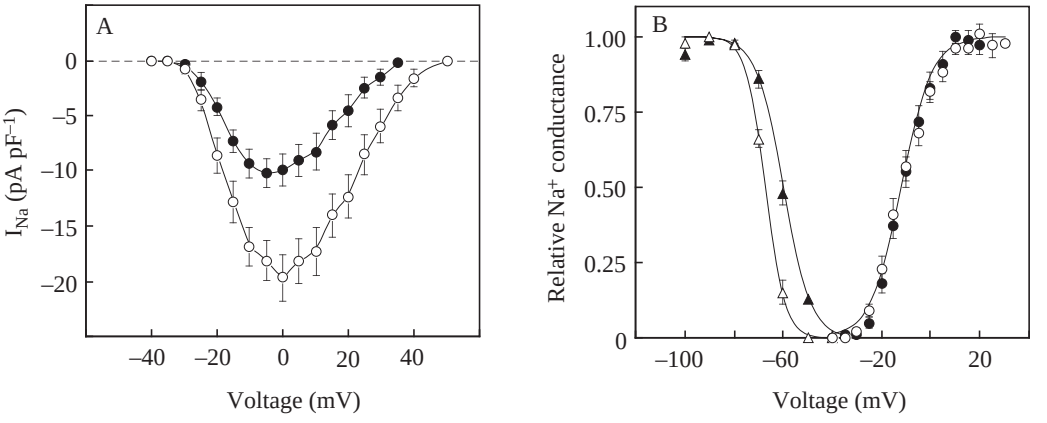

Fig. 3. Na^+ currents. (A) Current/voltage (relationships for the Na^+ current in NG108-15 (●) and NG/DOXR (○) cells. Peak Na^+ currents (I_{Na}) were recorded during depolarizations to a range of potentials between –40 and +40 mV from a holding potential of –80 mV. Data have been normalized to cell capacitance and are shown as means ± S.E.M. (N=7 for NG108-15 cells; N=8 for NG/DOXR cells). External A medium supplemented by 10 mmol l^{-1} TEA^+ and 0.5 mmol l^{-1} Cd^{2+} and pipette B medium were used. (B) Steady-state activation and inactivation of the Na^+ current in NG108-15 (▲, ●) and NG/DOXR (△, ○) cells. Activation/voltage relationships (circles) were calculated from peak Na^+ currents (I_{Na}) recorded during depolarizations to various potentials from a holding potential of –80 mV according to the following equation: $g_{Na}=I_{Na}/(V-V_{Na})$, where V is the membrane potential during test pulses and V_{Na} is the reversal potential of I_{Na}. g_{Na} was normalized to its maximum value ($g_{Na,max}$). The curve was calculated according to the equation $g_{Na}/g_{Na,max}=1/[1+e^{(V_{0.5}-V)/k}]$, where $V_{0.5}$ is the voltage at which $g_{Na}=g_{Na,max}/2$ and k is a steepness factor. $V_{0.5}$=–12 mV and k=6.2 for both cell types. Inactivation/voltage relationships (triangles) were calculated from peak I_{Na} recorded during depolarizations to 0 mV preceded by 100 ms prepulses of various amplitudes from a holding potential of –80 mV. The curves were calculated according to the equation: $I_{Na}/I_{Na,max}=1/[1+e^{(V-V_{0.5})/k}]$ where V is the membrane potential during prepulses and $V_{0.5}$ is the voltage for which $I_{Na}=I_{Na,max}/2$. $V_{0.5}$=–60 mV and k=5.3 for NG108-15 cells; $V_{0.5}$=–67 mV and k=3.8 for NG/DOXR cells.

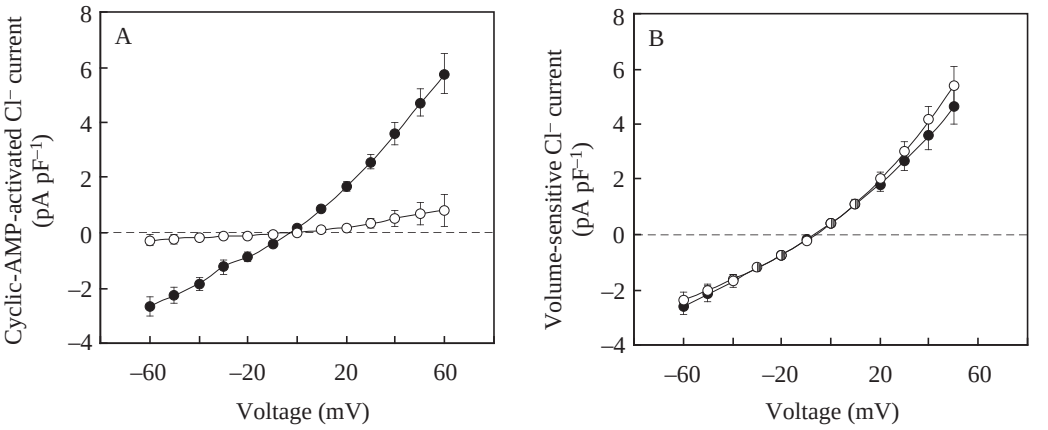

Fig. 4. Cl^- currents in NG108-15 and NG/DOXR cells. Ramp potentials from −60 to +50 or +60 mV (at 200 mV s^{-1}) were applied to NG108-15 (●) and NG/DOXR (○) cells. Recorded currents are normalized to cell capacitance and are shown as means ± S.E.M. (A) Cyclic-AMP-activated Cl^- currents. Pipette C medium was used in these experiments. The cAMP-activated current, plotted as a function of voltage, was calculated as the difference between currents recorded in the external B1 medium with and without 100 μmol l^{-1} dibutyryl-cAMP, 20 μmol l^{-1} forskolin and 100 μmol l^{-1} isobutylmethylxanthine (IBMX). *N*=11 for NG108-15 cells and *N*=4 for NG/DOXR cells. (B) Volume-sensitive Cl^- currents. Pipette C medium was supplemented with 2 mmol l^{-1} ATP. The activation of Cl^- currents was elicited by a change from external B1 to external C1 medium, which corresponds to a 24% reduction in tonicity. The volume-sensitive current, plotted as a function of voltage, was calculated as the difference between currents recorded with external C1 medium and with external B1 medium. *N*=14 for NG108-15 cells and *N*=20 for NG/DOXR cells.

Cell differentiation

While there is some evidence that drug-selected MDR cell lines are more differentiated than parental cells, contrasting evidence have been reported in other studies (Correale *et al.* 1994). Several chemical agents, including dimethylsulphoxide and dibutyryl-cAMP, induce NG108-15 cells to differentiate to neurones (Kimhi *et al.* 1976; Krystosek, 1985; Prasad, 1991). When cultured in the presence of 2% dimethylsulphoxide for 72 h and then exposed to 0.5 mmol l^{-1} dibutyryl-cAMP, NG108-15 cells stop dividing, produce extensive processes, become electrically excitable and, compared with undifferentiated cells, exhibit a 5- to 10-fold increase in voltage-dependent Na^+, Ca^{2+} and K^+ currents. Moreover, the voltage-dependent K^+ current of differentiated cells inactivates faster than that of undifferentiated cells (Rouzaire-Dubois and Dubois, 1991). By these criteria, NG/DOXR cells showed no sign of differentiation when cultured in the absence or in the presence of dimethylsulphoxide or dibutyryl-cAMP.

Resting potential

Resting potentials of NG108-15 and NG/DOXR cells were measured under current-clamp mode with KCl or potassium aspartate in the pipette. The results (Table 2) indicate that the resting potential of NG/DOXR cells is not significantly different (P>0.1) from that of NG108-15 cells. When internal Cl^- was replaced by aspartate, the resting potential was significantly reduced in both NG108-15 and NG/DOXR cells and by a similar amount. Since, under these conditions, the equilibrium potential of Cl^- was shifted towards negative values, the reduction in membrane potential was unlikely to have been due to changes in the Cl^- current.

K^+ current

The K^+ current of undifferentiated NG108-15 cells is essentially a slowly inactivating delayed rectifier current (Rouzaire-Dubois and Dubois, 1991). No significant differences were observed in the physiological properties of this current recorded in NG108-15 and NG/DOXR cells (Table 3). Moreover, tetraethylammonium (TEA^+) and verapamil had similar effects on the K^+ currents of both NG108-15 and NG/DOXR cells. At a depolarizing potential of +50 mV, 10 mmol l^{-1} TEA^+ reduced the K^+ current to 27±2% of the control value in NG108-15 cells (*N*=5) and to 22±1% (*N*=5) in NG/DOXR cells, and 10 μmol l^{-1} verapamil reduced the peak K^+ current to 50±2% (*N*=3) in NG108-15 cells and to 59±4% (*N*=4) in NG/DOXR cells. In contrast to results previously described in multidrug-resistant small cell lung cancer cells expressing MRP (a multidrug-resistance-associated protein distantly related to P-glycoprotein) (Jirsch *et al.* 1993), an inwardly rectifying K^+ current could not be detected in NG/DOXR cells.

Na^+ current

Current–voltage relationships for the peak Na^+ current (I_{Na}) in NG108-15 and NG/DOXR cells are shown in Fig. 3A. Current density estimates revealed that the amplitude of the maximum peak I_{Na} in NG/DOXR cells (−19.68±2.07 pA pF^{-1}, *N*=8) was significantly greater than that in NG108-15 cells (−9.96±1.41 pA pF^{-1}, *N*=7) (P<0.001). Na^+ conductance was increased in NG/DOXR cells by 1.66-fold without significant

Table 2. *NG108-15 and NG/DOXR cell resting potentials*

Experimental conditions	NG108-15	NG/DOXR
140 mmol l^{-1} $[KCl]_i$	−77±3 (26)	−85±2 (26)
140 mmol l^{-1} $[KAsp]_i$	−61± 4(44)	−61±3 (57)

Resting membrane potentials (in mV) were measured under current- clamp mode with KCl or potassium aspartate (KAsp) in the pipette and external A medium.

Values are means ± S.E.M. (*N*).

Table 3. *Ionic current parameters in NG108-15 and NG/DOXR cells*

K^+ current	$g_{K,max}$ (nS)	$g_{K,max}$ (pS pF^{-1})	$V_{m,0.5}$ (mV)	$V_{h,0.5}$ (mV)
NG108-15	4.03±0.31 (11)	67.55±7.58 (5)	2±1 (11)	–30±1 (3)
NG/DOXR	3.14±0.33 (10)	67.67±4.57 (3)	1±1 (10)	–30±1 (4)
Na^+ current	$g_{Na,max}$ (nS)	$g_{Na,max}$ (nS pF^{-1})	$V_{m,0.5}$ (mV)	$V_{h,0.5}$ (mV)
NG108-15	18.97±2.49 (7)	0.37±0.04 (7)	–12±1 (7)	–61±1 (4)
NG/DOXR	23.16±1.49 (8)	0.61±0.05 (8)	–11±2 (8)	–68±1 (5)

	g_{Cl} (pS pF^{-1})	
Cyclic-AMP-activated Cl^- current	At –60 mV	At +60 mV
NG108-15	44.54±5.45 (11)	97.93±11.09 (11)
NG/DOXR	4.45±3.54 (4)	13.58±9.42 (4)

	g_{Cl} (pS pF^{-1})	
Volume-sensitive Cl^- current	At –60 mV	At +50 mV
NG108-15	47.86±6.20 (14)	82.25±11.25 (14)
NG/DOXR	43.86±5.26 (20)	96.59±11.85 (20)

$V_{m,0.5}$ and $V_{h,0.5}$ are voltages corresponding to half-maximal activation and inactivation, respectively.
Results are expressed as means ± S.E.M. (*N*).

changes in its voltage sensitivity, except for a 7 mV shift in the steady-state inactivation curve towards negative potentials (Fig. 3B; Table 3). The addition of 1 μmol l^{-1} tetrodotoxin to the external medium suppressed the Na^+ current in NG108-15 and NG/DOXR cells but did not alter the sensitivity of either cell type to doxorubicin (results not shown).

Cl^- currents

To our knowledge, no Cl^- currents have yet been described in NG108-15 cells. Under different experimental conditions, two types of Cl^- current could be observed in both NG108-15 and NG/DOXR cells: a cAMP-activated current and a volume-sensitive current. A cAMP-activated Cl^- current could be revealed by the addition of dibutyryl-cAMP, forskolin and isobutylmethylxanthine (IBMX) to the solution bathing the cells. Fig. 4A shows that the cAMP-activated Cl^- current is 7–10 times larger in NG108-15 cells than in NG/DOXR cells (see also Table 3). The ionic nature of this current was confirmed by replacement of 80 mmol l^{-1} external Cl^- with isethionate. In these conditions, the cAMP-activated current observed in NG108-15 cells reversed at +18 mV (*N*=3), a value closed to the Nernst equilibrium potential for Cl^- (+20 mV). A volume-sensitive Cl^- current could be elicited by exposing the cells to hypo-osmotic external solutions (24 % reduction in osmolarity). In NG108-15 cells, this current was sustained for prolonged periods when the cells remained swollen and were dialysed with a pipette solution containing ATP. In contrast, as already observed by Jirsch *et al.* (1994) in multidrug-resistant lung cancer cells, ATP was not required for the activation and maintenance of the current in NG/DOXR cells. In both cell types, the current was Ca^{2+}-independent since the addition of 2 mmol l^{-1} EGTA to the pipette solution did not affect its activation. When recorded during square voltage steps of various amplitudes from a holding potential of 0 mV, the current showed a pronounced outward rectification; no difference in its density was found between cell types (Fig. 4B).

Proton pump

The resting potential of most eukaryotic cells is generally assumed to be essentially controlled by a selective membrane permeability to K^+. However, we recently proposed arguments in favour of the idea that the resting potential of differentiated neuroblastoma cells is maintained at negative values by both voltage-dependent K^+ channels and an electrogenic vacuolar-type H^+-ATPase (V-ATPase) (Gérard *et al.* 1994). This conclusion was mainly based on the observations that the V-ATPase inhibitors bafilomycin A1 (BFA) (Bowman *et al.* 1988), NO_3^- and *N*-ethylmaleimide and the K^+ channel blocker Cs^+ induced additive depolarizations. In most cells, V-ATPases function in a variety of intracellular systems where they contribute to decreasing intraorganellar pH. V-ATPases are also functionally expressed in the plasma membrane of several animal cells, including tumour cells (Gluck, 1992; Harvey and Wieczorek, 1997; Nanda and Grinstein, 1991), and evidence has been provided for the involvement of a V-ATPase in MDR (Ma and Center, 1992; Marquardt and Center, 1991). Taking all these factors into consideration, the effects of BFA, NO_3^- and Cs^+ on the resting potential of NG108-15 and NG/DOXR cells were studied. Fig. 5 shows that these three agents depolarized both cell types by 20–30 mV. Although the depolarizations induced by Cs^+ in sensitive and resistant cells were not significantly different, the depolarizations induced by BFA and NO_3^- were

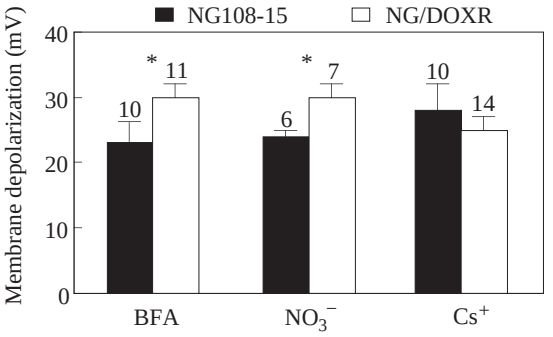

Fig. 5. Effects of bafilomycin, nitrate and caesium on the resting potential of NG108-15 and NG/DOXR cells. The resting potential was measured 3–5 min after establishing the whole-cell configuration with 10 μmol l^{-1} bafilomycin A (BFA) in the pipette A medium, or with 140 mmol l^{-1} KNO_3 or 140 mmol l^{-1} CsCl replacing 140 mmol l^{-1} KCl in the pipette A medium. In all cases, external A medium was used. Values are means + S.E.M. of *N* experiments. *Significant difference between cell types, $P<0.05$.

significantly ($P<0.05$) larger in resistant (BFA, 30±2 mV, $N=11$; NO_3^-, 30±2 mV, $N=7$) than in sensitive (BFA, 23±3 mV, $N=10$; NO_3^-, 24±1 mV, $N=6$) cells.

Effects of chemosensitizing agents on the membrane potential and volume-sensitive Cl^- current

Since cytoplasmic pH and/or V-ATPase activities are thought to play a role in MDR and the resting potential of NG108-15 and NG/DOXR cells seems to be partly determined by a H^+ pump (see below), we analyzed the effects of the chemosensitizing agents verapamil and quinine on the membrane potential. Before the application of external verapamil (10 μmol l^{-1}) or quinine (100 μmol l^{-1}), the membrane potential was held at different levels between −40 and −90 mV using constant injected currents. Verapamil and quinine induced instantaneous reversible depolarizations in NG/DOXR cells whatever the initial potential. In NG108-15 cells, verapamil and quinine induced depolarizations when the initial potential was more positive than −60 mV, but had no effect for initial potentials near or more negative than the resting potential (−70 mV) (Fig. 6A). In NG/DOXR cells, the depolarization induced by verapamil from an initial potential of −70 mV was inhibited by BFA but was not significantly modified by Cs^+. When the initial potential was −40 mV, the verapamil-induced depolarization was not significantly modified by BFA but was decreased by Cs^+ in both NG108-15 and NG/DOXR cells (Fig. 6B).

The preceding observations suggest that the V-ATPase of NG/DOXR cells (but not that of NG108-15 cells) was inhibited by verapamil and quinine. Given that such inhibition should induce a decrease in cytoplasmic pH and, as shown in kidney cells (Mori *et al.* 1994), that the volume-sensitive Cl^- current may be highly sensitive to intracellular pH, we examined the effects of intracellular pH, BFA and verapamil on the activation of this Cl^- current. Fig. 7A,B shows that, in both NG108-15 and NG/DOXR cells, the volume-sensitive Cl^- current was greatly

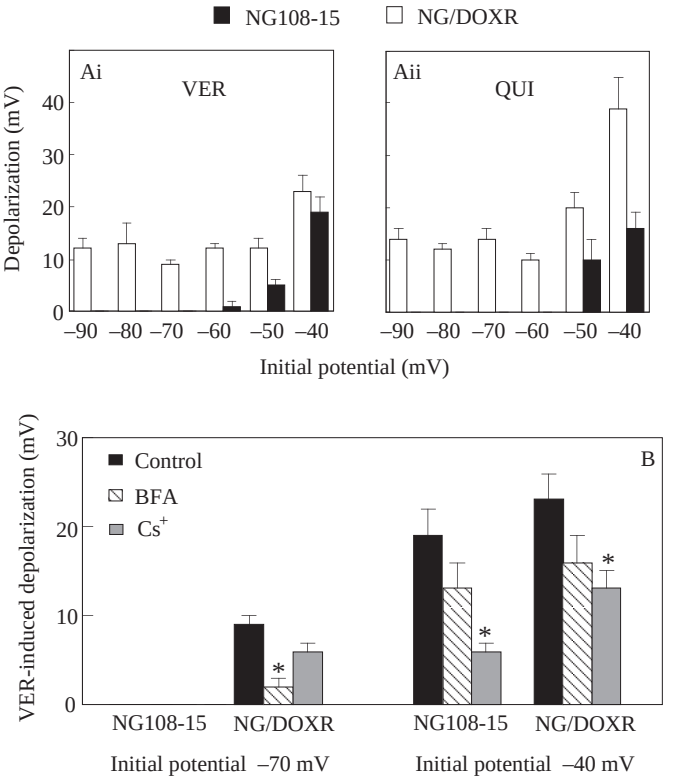

Fig. 6. Effects of verapamil (VER) and quinine (QUI) on the membrane potential of NG108-15 and NG/DOXR cells. (A) Depolarization induced by 10 μmol l^{-1} verapamil (Ai) or 100 μmol l^{-1} quinine (Aii) as a function of membrane polarization. External A and pipette A media were used. (B) Effects of bafilomycin A (BFA) and Cs^+ on the depolarization induced by 10 μmol l^{-1} verapamil from an initial potential of −70 mV (left) or −40 mV (right). Results are expressed as means + S.E.M. (*N*=4–9). *Significantly different from the control value, $P<0.05$.

reduced when the pH of the pipette solution was lowered from 7.3 to 6.8. Similar effects were observed with BFA (Fig. 7C,D). In contrast, verapamil affected the volume-sensitive Cl^- current differentially in NG108-15 and NG/DOXR cells. Whereas verapamil had a minor effect on NG108-15 cells, it reduced the volume-sensitive Cl^- current of NG/DOXR cells to less than 20 % of the control value (Fig. 7E,F).

Effect of BFA on doxorubicin resistance

The electrophysiological studies presented above show that drug resistance is accompanied by several alterations in ionic membrane permeabilities. Among these, modifications to the pharmacological properties of a plasma membrane V-ATPase seem to be particularly interesting. Taking into account the suggested role of intracellular pH in drug resistance (see Introduction), one can ask whether the V-ATPase contributes in some way to doxorubicin resistance. To answer this question, we examined the effects of BFA (0.5 or 1 μmol l^{-1}) on the sensitivity of NG/DOXR cells to doxorubicin (100 or 200 nmol l^{-1}). BFA alone was cytotoxic and decreased the cell number to approximately 40 % of the control value. When BFA

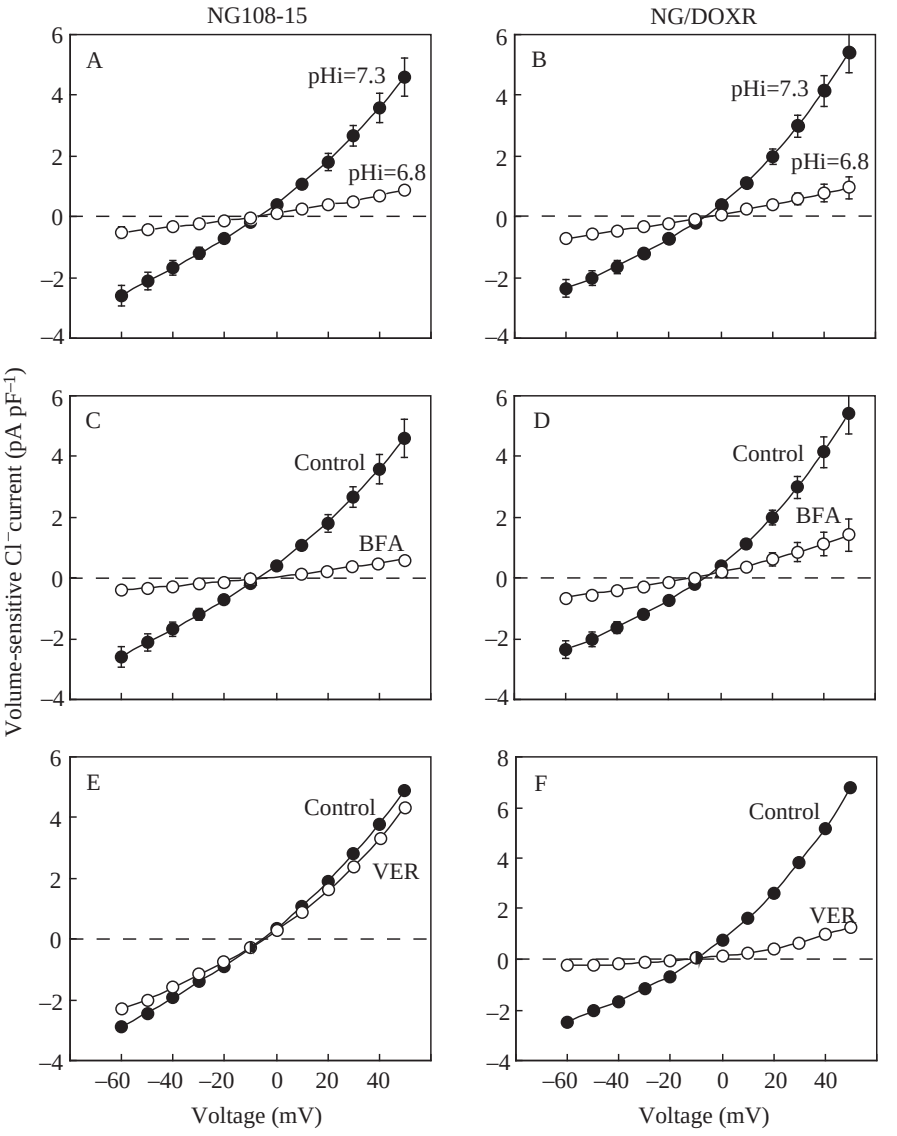

Fig. 7. Effects of intracellular pH (pHi), bafilomycin A (BFA) and verapamil (VER) on the volume-sensitive Cl^- current of NG108-15 and NG/DOXR cells. The volume-sensitive Cl^- current, plotted as a function of voltage, was recorded as described in Fig. 4B under control conditions and with a pipette medium buffered at pH 6.8 (A,B), with 10 μmol l^{-1} bafilomycin A added to the pipette medium (C,D) or with 10 μmol l^{-1} verapamil added to the external medium (E,F) (*N*=3–6). At –60 mV, the volume-sensitive Cl^- current of NG108-15 cells was reduced to 20±10 % (pH 6.8), 15±6 % (bafilomycin A) and 84±3 % (verapamil) and that of NG/DOXR cells was reduced to 30±9 % (pH 6.8), 26±9 % (bafilomycin A) and 11±5 % (verapamil) of the control values.

was applied in the presence of doxorubicin, the effect was not significantly different from that of BFA alone (Table 4).

Discussion

We have established that NG108-15 neuroblastoma × glioma hybrid cells resistant to doxorubicin exhibit an MDR phenotype. They are cross-resistant to two other anticancer drugs: vinblastin and colchicine, express a 170 kDa membrane protein identified as the P-glycoprotein and their drug resistance is antagonized by the classical chemosensitizers verapamil and quinine. The development of MDR in neuroblastoma cells is accompanied by several alterations in the expression levels or properties of ionic channels or transporters. These alterations include increased expression or activity of Na^+ channels, decreased expression or activity of cAMP-activated Cl^- channels and acquired sensitivity to verapamil and quinine of an electrogenic plasma membrane system which has the pharmacological properties of a vacuolar-type H^+-ATPase.

It is generally assumed that an increased expression of P-gp is sufficient for the development of an MDR phenotype. However, although overexpression of P-gp is associated with cellular changes that could be directly responsible for MDR, a number of forms of drug resistance not associated with P-gp have been observed. Moreover, P-gp overexpression alone has not been associated with levels of drug resistance above approximately 10-fold, and high levels of drug resistance are related more to drug selection than to P-gp overexpression (Hoffman *et al.* 1996). The observation that NG/DOXR cells are 130 times more resistant to doxorubicin than NG 108-15 cells suggests that additional changes have been induced in these cells by the doxorubicin selection. Independently of P-gp overexpression, these events may be responsible for at least part of the observed alterations in ionic membrane permeabilities.

The enhancement of Na^+ current density in NG/DOXR cells is comparable with that observed by Yamashita *et al.* (1987) in MDR human leukaemia cells. As suggested by these authors, it may be a consequence of either an increased

Table 4. *Effects of bafilomycin on the sensitivity of NG108-15 and NGDOX/R cells to doxorubicin*

			Relative cell number		
	DOX (nmol l^{-1})	BFA (μmol l^{-1})	In DOX	In BFA	In DOX + BFA
NG108-15	2	0.5	0.92±0.03	0.34±0.01	0.36±0.01
	2	1	0.90±0.02	0.25±0.01	0.24±0.01
NGDOX/R	100	0.5	0.94±0.02	0.47±0.02	0.59±0.02
	100	1	0.96±0.05	0.42±0.05	0.46±0.03
	200	1	0.92±0.02	0.39±0.02	0.39±0.01

Cells were counted as described in Materials and methods after 48 h of exposure to doxorubicin (DOX) or bafilomycin A (BFA) alone or to DOX + BFA.

Cell number is expressed relative to its control value in the absence of drug.

Values are means ± S.E.M., *N*=3–5.

expression of genes encoding Na^+ channels or an alteration in the lipid structural order of the membrane.

The decrease in cAMP-activated Cl^- current density observed in NG/DOXR cells may be due to a decreased expression of genes encoding these channels. Another possibility is that cyclic AMP is pumped out of the cells by P-gp, which would explain the absence of NG/DOXR cell differentiation in the presence of dibutyryl-cAMP.

An altered expression or activity of Cl^- channels has been thought to play a role in MDR. According to Gill *et al.* (1992) and Valverde *et al.* (1992), the appearance of P-gp is associated with the appearance of Cl^- currents activated by cell swelling, suggesting a dual role for P-gp as a drug transporter and a Cl^- channel. In constrast, several recent studies have indicated a lack of correlation between P-glycoprotein expression and volume-sensitive Cl^- channel activity (De Greef *et al.* 1995; Dong *et al.* 1994; Ehring *et al.* 1994; Kunzelmann *et al.* 1994; Morin *et al.* 1995; Rasola *et al.* 1994; Wang *et al.* 1994). Our results, showing the presence of volume-sensitive Cl^- channels with similar activity in both drug-sensitive and drug-resistant cells, led us to conclude that, in neuroblastoma cells, there is no relationship between P-glycoprotein expression and the Cl^- channel activated by cell swelling.

Another phenomenon observed in highly MDR cells is an alkaline shift in intracellular pH, suggesting that protons may be extruded from the cells by the action of P-gp. This could be due to a direct effect of P-gp (which would be a proton pump) or to an indirect effect (either the cytosolic substrate of P-gp is protonated or P-gp activity is coupled with proton transport, which provides the motive force for drug transport) (Gottesman and Pastan, 1993; Thiebaut *et al.* 1990). Another possibility is that P-gp affects pHi by altering the transport of HCO_3^- (Hoffman *et al.* 1996). We have previously presented arguments in favour of the contribution of an electrogenic V-ATPase to the genesis of NG108-15 cell membrane potential (Gérard *et al.* 1994). This conclusion is confirmed here in both sensitive and resistant cells by the observation that their resting potentials are decreased by BFA and by replacement of internal Cl^- with NO_3^- or aspartate (Fig. 5; Table 2). The observation that resting potentials of sensitive and resistant cells are not significantly different may indicate that MDR is not associated with an increased expression of V-ATPase. However, we suggest that the properties of the V-ATPase are modified in MDR cells since (1) depolarizations induced by BFA and NO_3^- are larger in NG/DOXR than in NG108-15 cells, and (2) verapamil induces membrane depolarizations in NG/DOXR cells that are abolished when the cells are pretreated with BFA. Alterations to the properties of the V-ATPase of MDR cells were confirmed by the observations that the volume-sensitive Cl^- current was inhibited by intracellular acidification and BFA in both NG108-15 and NG/DOXR cells, whereas it was inhibited by verapamil in NG/DOXR cells only. We thus conclude that MDR is accompanied by an acquired sensitivity to verapamil of a vacuolar-type H^+-ATPase. As a consequence, verapamil should induce a decrease in intracellular pH (Keizer and Joenje, 1989), which results in an inhibition of the volume-sensitive Cl^- current. The vacuolar H^+-ATPases of plants have been shown to be inhibited by verapamil (Pfeiffer, 1995). Given the lack of information on the sensitivity to verapamil of V-ATPases in animal cells, the origin of the differential sensitivity to verapamil of drug-sensitive and drug-resistant neuroblastoma cells cannot be precisely identified. It may be due to the existence of two isoforms of one subunit of the ATPase, differentially sensitive to verapamil and differentially expressed in NG108-15 and ND/DOXR cells. Other possibilities are MDR-associated changes in the membrane lipid composition or interactions between V-ATPase and P-glycoprotein. It is important to determine whether this difference is the cause of MDR *via* an alteration in intracellular pH. Our results showing that BFA did not reverse the resistance of NG/DOXR cells to doxorubicin argue against a role of pHi in multidrug resistance of drug-selected MDR cells (see also Altenberg *et al.* 1993). However, in addition to its action on pHi, it has been shown in Swiss 3T3 fibroblasts that BFA inhibits DNA synthesis and induces a rounding up of cells (Saurin *et al.* 1996). Moreover, we have shown that BFA depolarizes neuroblastoma cells. These effects may induce a decrease in chemotherapeutic drug accumulation or efficacy and thus may annul a possible reversing action of BFA mediated by a decrease in pHi.

In conclusion, our results show that, in addition to P-glycoprotein overexpression, MDR in neuroblastoma cells is accompanied by alterations in ionic membrane permeabilities with, in particular, modifications in the properties of a plasma membrane V-ATPase. This latter point may be an important factor which should be examined further.

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