Inwardly Rectifying K⁺ Channels and Volume-regulated Anion Channels in Multidrug-resistant Small Cell Lung Cancer Cells


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Abstract

Studies of multidrug-resistant H69AR cells which overexpress the multidrug resistance-associated protein, compared with drug-sensitive parental H69 cells and revertant H69PR cells, revealed an inwardly rectifying K⁺ channel current (conductance, 231 pS/pF) and increased volume-regulated anion current (limiting conductance, 2 nS/pF). The anion current was selective for Cl⁻ ions and sensitive to 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (0.1–1 mM) but ATP was not required for initial current activation even in excised patch experiments. K⁺ current reversal potential varied ±2 mV/10-fold change in the external K⁺ concentration and current was blocked by BaCl₂ (0.1–1 mM). The results indicate that overexpression of multidrug resistance-associated protein is accompanied by increases in both K⁺ channel and volume-regulated Cl⁻ channel current in the multidrug-resistant cell line H69AR.

Introduction

The multidrug-resistant SCLC cell line, H69AR, was derived from its parental cell line, H69, by stepwise selection in doxorubicin (1). H69AR cells display a drug cross-resistance pattern very similar to that of cells overexpressing P-glycoprotein, the drug efflux pump encoded by the MDR1 gene. However, the levels of P-glycoprotein in H69AR cells are extremely low and comparable with those found in parental H69 cells (1, 2). Furthermore, multidrug resistance in H69AR is poorly reversed by agents such as verapamil that are effective in other cell lines overexpressing P-glycoprotein (3, 4). Multidrug resistance in H69AR cells has recently been shown to be correlated with approximately a 100-fold increase in the levels of mRNA encoding a novel member of the ATP-binding cassette transmembrane transporter superfamily (5). The physiological function of this multidrug resistance-associated protein, MRP, and the mechanism by which it may contribute to drug resistance are currently unknown. MRP is distantly related to P-glycoprotein and CFTR (5). CFTR can function as a small-conductance cAMP-regulated Cl⁻ channel (6) and may also have the ability to modulate conductances such as outwardly rectifying Cl⁻ channels (7, 8). Recent evidence has been presented that P-glycoprotein, in addition to its role as an ATP-dependent efflux pump, may function as a volume-regulated chloride-selective channel (9) and that these two activities of P-glycoprotein may be mechanistically separable (10). In this report, we demonstrate that H69AR cells express markedly higher levels of a hypothetically activated, outwardly rectifying Cl⁻ channel and an inwardly rectifying K⁺ channel than parental H69 cells. In addition, we demonstrate that reversion to drug sensitivity is accompanied by loss of the K⁺ channel activity and a decrease in activity of the hypothetically activated Cl⁻ channel.

Materials and Methods

Cell Lines. The human SCLC cell lines NCI-H69 (H69), H69AR, and H69PR were used in the present experiments. The multidrug-resistant H69AR cell line was established by culturing the H69 cells in doxorubicin (1). The drug-sensitive revertant cell line H69PR was obtained by culturing H69AR cells in drug-free medium for 36 months (11). The cell lines were maintained in RPMI 1640 (GIBCO, Mississauga, Ontario, Canada) supplemented with 5% heat-inactivated supplemented calf serum (Hyyclone Laboratories, Logan, UT) and 4 mM L-glutamine. H69AR was challenged monthly with 0.8 μM doxorubicin. To obtain single cell suspensions, cells were gently syringed using a 22 G needle and then seeded onto glass coverslips coated with poly-o-lysine (0.01% w/v). Cells were maintained on the cover slips at 37°C in a 5% CO₂ incubator in 35-mm Petri dishes.

Electrophysiology. The coverslips were placed in dishes containing the experimental solution at 23–25°C. Electrodes of 1–5 megohm resistance were pulled from TW150 glass (World Precision Instruments) on a horizontal Flaming-Brown micropipet puller, fire-polished, and filled with a standard internal solution for whole-cell recording that contained 120 mM potassium aspartate, 10 mM KCl; 4.0 mM Na₂ATP; 0.1 mM GTP; 5.0 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 10 mM MgCl₂; 10 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N'-'tetraacetic acid, pH 7.2, with KOH. Data were corrected for the −10 mV junction potential that arose from the different mobilities of K⁺ ions and aspartate ions in this solution. Variations in pipet Cl⁻ concentration were made by substituting Cl⁻ for aspartate ions. To block outward K⁺ currents, K⁺ was replaced by Cs⁺ in the pipet-filling solution or 4-AP was included in the pipet-filling solution at 1 mM (stock solution of 0.1 mM, pH 7.4, with 1 mM HCl). The osmolality of internal and external solutions was measured using freezing point depression (Model 3L, Advanced Instruments). An Axopatch 1D (Axon Instruments, Foster City, CA) was used for voltage clamp measurements and data were filtered at 5 kHz prior to digitization via a Labmaster DNA interface. The pClamp suite of programs was used for data acquisition and analysis. Analogue capacity compensation and 50–70% series resistance compensation were used during all whole cell measurements. The H69, H69AR, and H69PR cells used for electrophysiological analysis had mean (±SEM; n = 10) cell capacitances of 13.9 ± 1.3 pF, 20.0 ± 2.1 pF, and 15.5 ± 1.3 pF, respectively. Capacitance was measured by integration of the uncorrected capacity transient. The external solution contained 130 mM NaCl, 5.0 mM KCl, 2.6 mM sodium acetate, 1.0 mM MgCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mM glucose, 1.0 mM CaCl₂, pH 7.4, with 1 mM NaOH. Hypertonic solutions were obtained by the addition of sucrose to the standard iso-osmotic solutions. The 210 mosm hypertonic external was obtained by a reduction of the NaCl content to 90 mM. BaCl₂ was added to the bath solution to block inwardly directed K⁺ currents at concentrations of 50 μM to 1 mM. DIDS was used to block volume-activated chloride channels at 0.1–1 mM diluted in bathing solution. All chemicals unless otherwise stated were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Volume-activated Currents. The level of hypothetically activated anion current was determined in parental H69, multidrug-resistant H69AR, and revertant H69PR cells. In all cases, data were obtained in...
the absence of external K+ or with 1 mM BaCl2 in the bath solution, plus internal Cs+ replacement of K+ in the pipet-filling solution to prevent K+ channel currents. Anion currents could be elicited by exposure to hypotonic external solutions (below 250 mOsm) or large amplitude depolarizing voltage clamp pulses. Activation of anion currents was associated with cell swelling confirmed visually through the inverted microscope. Representative examples of volume-activated currents from the three cell lines are shown in Fig. 1 (A-C, lower panels) compared with control currents from the same cells (upper panels). The three cell lines displayed very different levels of volume-activated anion currents.

In H69 cells the maximal outward anion current was less than 700 pA at +100 mV. By contrast, in larger H69AR cells anion currents were observed of up to 10 nA in amplitude. Despite the use of low resistance electrodes and series resistance compensation (see "Materials and Methods") peak currents at the most positive potentials studied were subject to a voltage error. In the revertant H69PR cells, we also observed large anion currents relative to H69 cells (Fig. 1C). However, the currents were significantly less (P < 0.05) than in H69AR cells between -140 and +40 mV. Commonly, we observed instantaneous activation of anion currents in all three cell types. Some relaxation of current was detected at the most positive potentials studied as reported previously for outwardly rectifying chloride channels in epithelial cells. Mean I-V relations for the anion currents are shown in the lower panel. There was prominent outward rectification of the peak (Fig. 1D) and instantaneous I-V relations in both asymmetrical and equal bath and pipet chloride concentrations, which confirmed the existence of intrinsic channel outward rectification. In all three cell lines, currents reversed between -50 and -60 mV which was close to the calculated Cl− ion reversal potential for the bath and pipet solutions (-53 mV). Currents were not blocked by 4-AP (2 mM), by the replacement of pipet K+ with Cs+ ions or the inclusion of Ba2+ (1 mM) in the bathing medium, which indicated that currents were not dependent on transmembrane K+ fluxes. The mean current reversal potential changed by 40 mV for a 120 mM increase in pipet Cl− (replacing impermeant aspartate) which compared with a predicted shift of 46 mV for a Cl− selective channel. Currents were also blocked by the chloride channel inhibitor DIDS at concentrations between 0.1 and 1 mM. The mean current reduction was 88.10 ± 0.35% at 1 mM DIDS (n = 4) and was observed in all three cell lines.

To determine whether activation of the anion channels was ATP dependent, experiments were carried out in the absence of ATP in both intact cells and excised membrane patches. We found that omission of ATP from the pipet-filling solution did not prevent activation of hypotonically activated current in H69AR cells. This finding was corroborated by studies using excised outside-out patches to ensure adequate dialysis of ATP. An example of such an experiment is illustrated in Fig. 2. The anion channels were activated during stan-

![Fig. 1. Anion current in H69, H69AR, and H69PR cells. A-C, volume-activated currents in the three cell types. Cells were held at -70 mV and pulsed for 750 ms to a range of potentials between -140 and +90 mV. In the upper portion of the panels are control currents obtained from the three cell types soon after the establishment of whole-cell recording mode. For each cell type the appearance of anion currents accompanied cell swelling induced by hypotonic swelling or large depolarizing voltage clamp pulses. Representative data are shown in the lower portion of the panels. In D are mean peak I-V relations for volume-activated conductance in the three cell types. Data were obtained from 7 H69 cells, 10 H69AR cells, and 5 H69PR cells. Data have been normalized to cell capacitance and are shown as mean ± SEM. Paired t tests between the H69AR and H69PR data revealed significant differences for data obtained at potentials between -140 mV and +40 mV (between -140 mV and +100 mV, P < 0.02; between -90 and +40 mV, P < 0.05, excluding data between -50 and -20 mV, between +50 and +80 mV, 0.05 < P < 0.1). Data at potentials -50 mV to -20 mV were excluded from analysis due to the convergence of all data near the Cl− reversal potential.](image)
dard whole-cell recording (Fig. 2A, control). The electrode was withdrawn to form an outside-out membrane patch in 280 mM K+ bath solution. Exposure of the membrane patch to hypertonic (320 mM) and hypotonic (210 mM) solutions resulted in suppression and activation of anion currents (Fig. 2A). I-V relations for these currents are shown in Fig. 2B. Patch currents were 10-fold smaller than whole cell currents (due to the reduced number of channels in the patch), but nevertheless there was clear activation and suppression of voltage-regulated Cl− currents in the absence of internal ATP. The reversal potential of current was unchanged from whole-cell to outside-out patch measurements at −43 mV, and the presence of macroscopic current in the patches attests to a high density of activated anion channels in the H69AR cell membranes in the absence of ATP and cellular constituents.

Inwardly Rectifying K+ Channel Current. Many epithelial cells possess basolateral K+ channels which are blocked by Ba2+ (12) and a delayed rectifier K+ channel current has been reported previously in H69 cells that activates positive to −30 mV (13, 14). We observed a delayed rectifier current in all three SCLC cell lines that activated over a similar potential range to that reported previously for H69 cells (13) and showed saturation at potentials positive to +40 mV (data not shown). Of more interest, was an inwardly rectifying K+ channel current that was essentially absent in H69 and H69PR cells (Fig. 3, A and C) but was present at high density in H69AR cells (Fig. 3B). In H69AR cells, hyperpolarizing voltage clamp pulses caused rapid activation of an inward current that inactivated partially at potentials negative to −100 mV. At 5 mm bath K+, the current almost completely rectified (Fig. 3D) and the passage of only small amounts of outward current was observed positive to the EK. However, at the resting potential of H69AR cells, a small net outward K+ flux through these inwardly rectifying channels is to be expected. The mean conductance of the inwardly rectifying channel was 231 pS/pF in H69AR cells in 5 mm external K+.

K+ Dependence. The potassium dependency of the inwardly rectifying current was established by varying external K+ concentrations from 5 to 100 mM. Increasing K+ concentrations resulted in amplification of the inwardly rectifying current only in H69AR cells (Fig. 4B). The slope conductance in 100 mM external K+ increased to 23 nS. This is consistent with the inwardly rectifying K+ channel conductance being proportional to the square of the bath K+ concentration. The zero current potentials for the inwardly rectifying currents in different bath K+ concentrations from a number of H69AR cells have been plotted against the log bath K+ concentration in Fig. 4D. The nonlinear, least-squares best fit to the data yielded a line (continuous line in Fig. 4D) with a slope of 52 mV. This value is close to that predicted for a pure K+ electrode (dotted line in Fig. 4D). The inwardly rectifying current was also sensitive to 50–200 µM BaCl2 present in the bath solution (15 cells; data not shown). Ba2+ produced a rapid, complete, reversible block of channels and provided further confirmation that the inward rectifier in H69AR cells was a K+ channel.

Discussion

There have been a number of reports of changes in ion channel activities associated with the development of multidrug resistance in human tumor cell lines. For example, a reduction in DIDS-sensitive chloride channels and in cAMP-regulated Cl− currents has been reported in the non-P-glycoprotein mediated, multidrug-resistant, HL60 leukemia model system (15). On the other hand, overexpression of P-glycoprotein in transfected epithelial cells is associated with increased activity of an ATP-dependent, volume-activated Cl− channel (9, 10). It is presently unknown whether the ability of P-glycoprotein to act as an ion channel is of functional significance with respect to multidrug resistance. However, such currents can affect intracellular ion equilibria and alter the activity of transmembrane ion exchangers such as Cl−/HCO3− or Na+ /H+ exchange (16, 17). Such changes may secondarily alter intracellular pH and thus influence the sensitivity of tumor cells to a number of cytotoxic agents (16).

The development of multidrug resistance in the SCLC model system we have used does not involve increased expression of P-glycoprotein. However, it is accompanied by the overexpression of MRP, a novel member of the ATP-binding cassette transporter gene family (5). In view of structural similarities between MRP, P-glycoprotein, and CFTR, we initially examined Cl− channel activity in the parental, drug-resistant and revertant SCLC cell lines. In the H69AR cells, we have noted the appearance of an extremely large volume-activated anion current (Fig. 1) that displays a high selectivity for Cl− ions. In common with Cl− channels described previously in epithelial cells, the current showed prominent outward rectification. It was sensitive to DIDS and verapamil and the reversal potential showed shifts appropriate to a channel with high Cl− and low aspartate selectivities. All of the aforementioned characteristics are similar to those of the ion channel activity described in P-glycoprotein-transfected cells (9). However, unlike the P-glycoprotein-associated channel, initial activation or deactivation of this outwardly rectifying channel in H69AR cells was not ATP dependent (Fig. 2). Most important, although the volume-activated Cl− channel was reduced in the drug-sensitive revertant H69PR cell line, the activity was still much higher than in the parental H69 cell line. This suggests that increases in volume-regulated Cl− channels are not sufficient to confer multidrug resistance in the H69AR cell system. However, they may be essential if it is necessary for Cl− and K+ channels to function cooperatively. Com-
We have defined it as an inwardly rectifying K+ channel on the basis of its voltage dependence, the dependence of the slope conductance and reversal potential on the extracellular K+ concentration and its sensitivity to low concentrations of BaCl2.

Although we cannot exclude the possibility that MRP is directly responsible for the inwardly rectifying K+ currents, its structure is not similar to any previously characterized K+ channels. An alternative possibility is that it could play a role in regulating such channels. In this regard, it is interesting to note that CFTR expressed in Xenopus oocytes has been shown to regulate Ba2+-sensitive K+ conductances (20). Inwardly rectifying K+ channels provide important control of the cell resting potential in numerous cell types (21). They also allow for oscillatory changes in resting membrane potential (22) and can be modulated by cAMP (23). The presence of the current described in H69AR cells is expected to allow K+ efflux at potentials positive to the K+ equilibrium potential (Ek) and could keep the membrane potential negative to the Cl− equilibrium potential in these cells. This would allow Cl− efflux from the cell on activation of the volume-regulated conductance. The coupled extrusion of K+ and Cl− from the cell is important in regulating cell volume in a number of epithelial cell systems (24). The coexistence of markedly elevated K+ and Cl− channels in the H69AR cells suggests that solute export from these cells may differ significantly from the parental and revertant cell lines. Experiments are in progress to determine whether modulation of the activity of these channels has any bearing on the efficacy of cytotoxic drugs in multidrug-resistant cells which overexpress MRP.

References


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