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Regulation of volume-sensitive Cl^- channels in multi-drug resistant MCF7 cells

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Abstract

The P-glycoprotein (P-gp) is thought to be involved in the regulation of volume-sensitive chloride channels. In this study, the possible coupling between P-gp and swelling-activated chloride channels has been examined in MCF7 cells with sensitive (MDR^-), resistant (MDR^+), and reversed resistant (MDR^{REV}) phenotypes. Western blot analysis showed that incubation of cells with doxorubicin induced P-gp expression in a reversible manner. Exposure of MDR^+ cells to hypotonicity resulted in an inhibition of P-gp activity while hypotonic challenges induced swelling-activated chloride currents ($I_{Cl\text{-}swell}$) in MDR^-, MDR^+, and MDR^{REV} MCF7 cells. While verapamil inhibited $I_{Cl\text{-}swell}$ in all cell types, doxorubicin and vincristine rapidly and reversibly inhibited $I_{Cl\text{-}swell}$ uniquely in MDR^+. Intracellular dialysis of MDR^+ cells with C219 anti-P-gp antibody abolished the sensitivity of $I_{Cl\text{-}swell}$ to doxorubicin and led to a response pattern very close to that of MDR^- cells. Taken together, these results strongly suggest that the P-glycoprotein regulates $I_{Cl\text{-}swell}$ in resistant MCF7.

Keywords: Multi-drug resistance; P-glycoprotein; Volume-sensitive chloride channels; Cell volume; MCF7; Patch-clamp; Whole-cell configuration; Regulatory volume decrease; MTT; Calcein-AM

Multi-drug resistance (MDR)[1] is a phenotype associated to breakdown of cancer treatment and characterized by resistance to a broad spectrum of chemotherapeutic agents belonging to different pharmacological classes [1–3]. The major cause of MDR phenotype is the overexpression of a transmembrane phosphoglycoprotein, the P-glycoprotein (P-gp) [4]. The human P-gp, a 170 kDa product of the human *mdr1* gene, is a member of the ATP-binding cassette (ABC) superfamily of transporters. This family encompasses many other proteins, for instance, a chloride channel such as the cystic fibrosis transmembrane regulator (CFTR), regulatory subunits associated to ion channels such as the sulfonylurea receptor SUR1, and even a bacterial histidine transporter [5,6]. The P-gp acts as a weakly specific carrier responsible for the energy-dependent efflux of a number of structurally and functionally unrelated hydrophobic compounds, including anti-cancer agents (doxorubicin, vincristine) and fluorescent dyes (calcein-AM or rhodamine B) [7,8], causing a drastic reduction in accumulation of the chemicals in the cytosol.

One of the most crucial regulatory processes in living cells is volume regulation. A large number of cellular mechanisms are influenced by changes in cell volume such as receptor recycling, excitability and contraction,

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[1] *Abbreviations:* MDR, multi-drug resistance; P-gp, P-glycoprotein; ABC, ATP binding cassette; CFTR, cystic fibrosis transmembrane regulator; SUR1, sulfonylurea receptor 1; RVD, regulatory volume decrease; AVD, apoptotic volume decrease; VSOR, volume-sensitive outward rectifying; MXR, multi-xenobiotic resistance; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; VRP, verapamil; Doxo, doxorubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; CSP A, cyclosporine A; $I_{Cl\text{-}swell}$, swelling-activated chloride current; MRP, multi-drug resistance-associated protein; BCRP, breast cancer resistance protein.

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cell proliferation, migration, and programmed cell death [9,10]. This may explain why most cell types, if not all, are able to actively regulate their own volume. Especially, under hypoosmotic conditions, cells are able to readjust their volume after swelling and, therefore, to escape from outburst by activation of a mechanism known as regulatory volume decrease (RVD). This phenomenon is underlain by KCl efflux through parallely gated potassium and chloride channels [9]. Interestingly, in the early phase of apoptosis, a normotonic decrease in cell volume (called apoptotic volume decrease, AVD) without foregoing cell swelling is almost systematically described. This osmolyte efflux is mainly due to activation of chloride channels [11] and considered as a facilitation of RVD [10]. Hence, chloride channels participate in the regulation of a number of fundamental cellular processes. However, despite a series of investigations [5,6,12–16], the exact molecular identities of the volume-sensitive outward rectifying (VSOR) chloride channels involved in RVD and AVD remain uncertain. Historically, because of its sequence similarities with the CFTR, the P-glycoprotein has been proposed as a good candidate for being the chloride channel involved in cell volume regulation. In preliminary studies, the overexpression of the P-gp in certain cell types was found to increase the magnitude of cell volume activated chloride currents [17,18]. Conversely, other works with cell lines transfected with *mdr1* cDNA did not find any functional correlation between P-gp and VSOR chloride channels [19–21]. Since then, many contradictory results have been reported, the conclusions apparently depending on the cell type studied or on whether cells had been transfected with *mdr1* gene or selected for the MDR phenotype [10,22,23]. More relevant to cell physiology now is the question of the putative regulatory role of the P-gp in the mechanisms underlying volume regulation.

Besides the presence of the P-gp in resistant human tumor cells, it appears that the P-glycoprotein is also expressed in numerous normal tissues of many organisms, including marine species. In such organisms, the activity of the P-gp is responsible for the multi-xenobiotic resistance (MXR) phenotype [24–27]. In two recent studies, we have demonstrated that the activity of the P-gp was osmoregulated in primary culture of mussel blood cells[2,3]. These results were consistent with the idea that the P-gp might be functionally linked to the VSOR chloride channels. To address this question in tumoral P-gp overexpressing cells, herein we conducted a series of experiments based on electrophysiological recordings of swelling-activated chloride currents and measurements of the P-gp transport activity in wild-type human breast cancer MCF7 cells, and in doxorubicin-selected and reversed-selected MDR variants.

Experimental procedures

Reagents and test substances. All compounds were of the highest available grade of purity. They were obtained from Sigma (St. Louis, MO). C219 monoclonal anti-P-gp antibody was purchased from AbCys (Paris, France) and calcein-AM was from Molecular Probes (Eugene, OR). Stock solutions of verapamil (VRP), doxorubicin (Doxo), vincristine, and calcein-AM were dissolved in DMSO. Final concentration of DMSO was less than 0.1%. For patch-clamp experiments, final concentrations of 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS), verapamil, vincristine, and doxorubicin were prepared freshly every day and obtained by appropriate dilution of a stock solution in hypotonic bath medium.

Cell culture. MCF7 cell lines were kindly supplied by Professor J.-P Marie (Hôtel Dieu, Paris, France). Multi-drug resistant MCF7 (MDR^+) cells were obtained from the parental MCF7 cells (MDR^-) by a stepwise selection in a culture medium supplemented with increasing concentrations of doxorubicin. The cells were grown in RPMI 1640 medium completed with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic/antimycotic solution. Doxo (1 μM) was added to the culture medium for the maintenance of the multi-drug resistant phenotype of MDR^+ cells. Cultured cells were incubated at 37 °C under a water-saturated 95% air-5% CO_2 atmosphere. The culture medium was renewed every three days. At 80–90% confluence, cells were dissociated by treatment with trypsin/EDTA and seeded at an initial density of 5×10^5 cells mL^{-1} in 25 cm^2-culture flasks for subculture, 96-well microplates for proliferation/activity assays or in 35 mm dishes for electrophysiological recordings. All cultures were discarded after 3 months (15 passages) and new cells were thawed from frozen stocks.

Protein extraction and Western blot analysis. Cells were lysed in 10 mM Tris–HCl buffer (pH 8.0) containing 0.1% Triton X-100, 0.15 mM KCl, 5 mM β-mercaptoethanol, 1.3 mM EDTA, 3.2 μM leupeptin, and 2 μM aprotinin. Protein lysates were placed on ice for 30 min, vortexed every 5 min, and cleared by centrifugation at 12,000*g* for 15 min at 4 °C. The supernatants were collected and frozen at −80 °C until analysis. Protein concentration in extracts was determined using the Bradford method.

Aliquots (20 μg) of total proteins extracted from cultured MCF7 cells were subjected to electrophoresis through a 7.5% SDS–polyacrylamide gel at a constant voltage of 80 V (25 mM Tris–HCl (pH 8.3), 192 mM glycine, and 0.1% SDS running buffer). Proteins were then transferred to nitrocellulose membrane overnight at 100 mA current using a 25 mM Tris–HCl (pH 8.3), 150 mM glycine, and 5% v/v methanol transfer buffer. The nitrocellulose membranes were blocked with 5% BSA in 1% Tween-20/Tris-buffered saline (TBST) (20 mM Tris (pH 7.6), 137 mM NaCl) for 1,5 h. Immunostaining was carried out using a mammalian P-gp C219 monoclonal antibody (0.2 μg mL^{-1}) and a secondary polyclonal rabbit anti-mouse IgG peroxidase conjugated antibody (1/80,000, Sigma–Aldrich, St. Louis, MO). Mouse monoclonal anti-actin antibody (1/10,000, Calbiochem, UK) was used as internal standard. The enhanced chemiluminescence (ECL) detection system (Amersham, UK) was used for visualization of the P-gp protein and actin as described in the manufacturer's manual. The signals from the blots were quantified using the ImageMaster TotalLab v1.11 software (Amersham-Pharmacia Biotech, UK).

Proliferation assays. Cell proliferation was quantified using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay

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