Overexpression of P-glycoprotein in L1210/VCR cells is associated with changes in several endoplasmic reticulum proteins that may be partially responsible for the lack of thapsigargin sensitivity

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Abstract. L1210/VCR cells, which express an abundant amount of P-glycoprotein (P-gp), were found to be resistant to thapsigargin – an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). In the current paper, we have studied the possible differences among L1210 and L1210/VCR cells in expression of endoplasmic reticulum proteins involved in the regulation of calcium homeostasis and calcium-dependent processes. Amounts of mRNA encoding both calcium release channels (ryanodine receptor channels – RyR and IP_3-receptor channels – IP_3R) were found to be at similar levels in sensitive and resistant cells. However, mRNAs encoding IP_3R1 or 2 were decreased in resistant cells cultivated in the presence of VCR (1.08 μmol/l), while mRNA encoding RyR remained unchanged. The amount of mRNA for SERCA2 was decreased in resistant cells when compared with sensitive cells. This decrease was more pronounced when resistant cells were cultivated in the presence of vincristine (VCR). Calnexin was found to be less expressed at the protein level in resistant as in sensitive cells. The level of mRNA encoding calnexin was decreased only when resistant cells were cultivated in the presence of VCR. Calnexin was found to be associated with immature P-gp in resistant cells.

Thus, differences exist between sensitive and resistant cells in the expression of endoplasmic reticulum proteins involved in the control of intracellular calcium homeostasis or calcium-dependent processes. These changes may be at least partially responsible for the lack of sensitivity of resistant cells to thapsigargin.

Key words: P-gp-mediated MDR — Calcium release channels — Calnexin — SERCA — Thapsigargin

Abbreviations: IP_3R, inositol 1,4,5-trisphosphate receptor channels; MDR, multidrug resistance; PBS, phosphate buffered saline; P-gp, P-glycoprotein (according to ABC protein nomenclature ABCB1 protein); RyR, ryanodine receptor channels; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; Th, thapsigargin; VCR, vincristine; Ver, verapamil.

Introduction

Overexpression of P-glycoprotein (P-gp), a drug efflux pump of the plasma membrane, represents the most often observed molecular feature that confers multidrug resistance (MDR) in cancer tissue (for review see Breier et al. 2005). The accurate understanding of all metabolic features involved in the development of the P-gp-mediated MDR phenotype has not yet been achieved, although P-gp was the first ABC transporter (ABCB1) discovered more than thirty years ago (Gottesman and Ling 2006). Modulation of P-gp-mediated MDR was assumed to be related to, besides others, protein kinase pathway activities (Grunicke et al.

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1994), calcium signaling (Witkowski and Miller 1999), transglycosylation reaction robustness (Fiala et al. 2003), cytochrome P450 3A4 activity and pregnane X nuclear receptor regulatory pathways (Christians et al. 2005) and nuclear receptors for retinoid pathways (Sulová et al., 2008). The importance of intracellular calcium homeostasis in the regulation of P-gp-mediated MDR could be deduced from the fact that P-gp positive MDR cells were often found to be resistant also to thapsigargin (Th), an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Gutheil et al. 1994; Wagner-Souza et al. 2003; O'Neill et al. 2006). While Th was assumed to be a P-gp substrate (Gutheil et al. 1994; Rishi et al. 1998), the nature of Th resistance observed in P-gp positive cells was not fully understood because any calcium mobilizing effects of Th were not independently observed in P-gp positive cells (selected by vincristine, VCR) due to the presence of P-gp inhibitors, like verapamil (Ver) or cyclosporine A (Rumjanek et al. 2001; Wagner-Souza et al. 2003). Moreover, authors of the latter papers have shown that something other than differences in direct interaction with SERCA is responsible for the insensitivity to Th observed in P-gp positive cells. Additionally, they have proposed that mechanisms and systems involved in the control of intracellular calcium homeostasis could be responsible for the lack of sensitivity to Th observed in cells selected by VCR and consequently, may also be involved in the regulation of P-gp-mediated MDR (Wagner-Souza et al. 2003). While evidence about direct connections between calcium homeostasis and P-gp-mediated MDR has not yet been attained, several differences in calcium intracellular localization and content were observed between P-gp positive L1210/VCR and P-gp negative L1210 cells, respectively (Sulová et al. 2005).

In the present paper, differences that may exist in expression of endoplasmic reticulum proteins involved in calcium homeostasis between L1210/VCR and L1210 cells were studied.

Materials and Methods

Cell cultivation conditions

In the present paper, the following L1210 cell variants were used: sensitive L1210 cells (S); drug resistant L1210/VCR cells overexpressing P-gp (Fiala et al. 2003) cultivated prior to experiments in the absence of VCR (R) and in the presence of 1.08 μmol/l VCR (V). VCR was supplied by Gedeon Richter Co. (Hungary). The resistant cell subline L1210/VCR was obtained by selection with increasing stepwise concentrations of VCR (Poleková et al. 1992). These cells represent a well characterized model of MDR mediated by the efflux activity of P-gp. Other markers of MDR like glutathione S-transferase, MRP1 and BCRP were not found to be changed (Boháčová et al. 2000, 2006). Several additional characteristics of this cell subline were described elsewhere (Breier et al. 2000; Barančík et al. 2001; Sulová et al. 2005, 2008; Barančík et al. 2006). Cells (S, R and V) were cultivated in RPMI 1640 medium with L-glutamine (1 mg/ml), 4% fetal bovine serum and 1 μg/ml gentamycin (all from Gibco, USA) in a humidified atmosphere with 5% CO_2 in air at 37°C for 24 or 48 h.

Effect of Th on cell viability

S, R and V cells were incubated in the presence of various Th concentrations (0.001–15.000 μmol/l) (Sigma, USA) in the presence or absence of 10 μmol/l Ver (Sigma, USA). The final concentration of Th was adjusted by addition of a proper volume of stock solution (1.5 mmol/l in DMSO) directly into the cultivation medium with inoculum (5×10^4 cells/200 μl) in a 96-well tissue culture plate. After 48 h of cultivation, the survival of cells was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Gerlier and Thomasset 1986).

RNA isolation and relative quantification of mRNA levels by RT-PCR

The population of total RNA was isolated by the TRI Reagent (MRC Inc.). Briefly, cells were lysed in sterile distilled water and afterwards an equal amount of TRI Reagent was added. After 5 min, the homogenate was extracted with chloroform. RNAs in the aqueous phase were precipitated with isopropanol. The RNA pellet was washed with 75% ethanol and stored in 96% ethanol at –70°C. The purity and integrity of the isolated RNAs was checked on a GeneQuant Pro spectrophotometer (GE Healthcare Life Sciences). Reverse transcription was performed using 1.5 μg of total RNA and Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Life Sciences) with pd(N_6) primer. All primer sequences used, together with corresponding position and fragment size for each amplified gene, are listed in Table 1. Annealing temperatures for primers used were as follows: SERCA2, $IP_3R1$and IP_3R2 – 60°C, RYR1 and RYR2 – 56°C, calnexin – 55°C. Calibration curve for several numbers of cycles were done for each pair of primers in order to have an exponential phase of PCR. PCR specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GI 56187; Table 1) expression was used as a housekeeper gene control for semi-quantitative evaluation of PCR. PCR specific for GAPDH was started by an initial denaturation at 94°C and was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min. PCRs were terminated by a final polymerization at

72°C for 7 min. All PCR products were analyzed on 2% agarose gels.

Estimation of calnexin and P-gp levels by Western and dot blots

Expression of calnexin and P-gp was determined by Western blot using monoclonal anti-P-gp antibody C219 and rabbit polyclonal anti-calnexin antibody (both from Calbiochem, UK). S, R and V cells after 24 and 48 h cultivations were disrupted, and cytosolic and crude membrane fractions were prepared as described by Hamada and Tsuruo (1988). Cytosolic and membrane proteins (6 μg per line) were separated by SDS PAGE using the Laemmli system (Laemmli 1970). Signals of GAPDH and β-actin as housekeepers in crude membrane fractions were very weak (Sulová et al. 2005), and therefore we tested the accuracy of protein loading by Coomassie blue staining of protein bands in a separate gel. Proteins were transferred by electroblotting to nitrocellulose membrane (Amersham, USA), and both proteins were detected by corresponding primary antibodies and secondary anti-mouse as well as anti-rabbit antibodies linked to horseradish peroxidase (both from Calbiochem, UK) using the ECL detection system (Amersham, USA). Crude membrane fraction and soluble fraction proteins in Laemmli sample buffer were applied to a nitrocellulose membrane (2 μg protein per dot) for dot blot analysis. Calnexin was detected using the same primary and secondary antibodies as for the Western blot procedure. Samples were analyzed and quantified with the Kodak Image Station system.

Detection of calnexin – P-gp association by imunoprecipitation

The fractions of cytosolic proteins (80–100 μg) from S, R and V cells were adjusted to a final volume of 300 μl with 50 mmol/l Tris-HCl (pH 7.0) and anti-calnexin antibody (final dilution 1 : 500) was added. After 2 h of incubation at 4°C, 20 μl of protein A/G PLUS-agarose (Santa Cruz) was added, and the mixture was incubated overnight at 4°C. Afterwards, precipitates were pelleted by centrifugation (10 min; 10,000 rpm; 4°C) and washed two times with 50 mmol/l Tris-HCl buffer. Precipitates were applied on 8% SDS PAGE and then proteins were transferred by electroblotting to nitrocellulose membrane and the presence of P-gp or calnexin in the immunoprecipitate was detected by the same antibodies as were described for the Western blot procedure.

Detection of endoplasmic reticulum protein by imunofluorescence

S, R and V cells were resuspended in cultivation medium (1×10^6 cells/ml) and transferred to a 24-well cultivation plate with poly-L-lysine cover glasses and cultivated 24 h in a humidified atmosphere with 5% CO_2 in air at 37°C. Cover glasses with bound cells were washed two times with PBS and then cells were fixed by incubation in pre-cooled 100% methanol at –20°C for 10 min. Additionally, cells were washed with PBS (3 times for 5 min), blocked against nonspecific binding with 1% BSA in PBS for 30 min and incubated with primary antibody against calnexin (same as described for Western blot), inositol 1,4,5-trisphosphate receptor channels (IP_3R; Affinity Bioreagents) and ryanodine receptor channels (RyR; Chemicon International) in 1% BSA in PBS for 60 min at 37°C. Cells were washed three times with PBS and incubated with anti-rabbit or anti-mouse antibody labeled with fluorescein isothiocyanate (Calbiochem) for 60 min at 37°C. Cells incubated with secondary antibody only were used as control experiment. Finally, cells were washed three times with PBS and specific labels were evaluated as green fluorescence in a confocal microscope, Leica TCS SP-2 AOBS, using excitation at 488 nm.

Statistical analysis

Data are expressed as mean ± S.E.M. Statistical significance was assessed using an unpaired Student's *t*-test.

Results and Discussion

Th sensitivity of S, R and V cells

R and V cells were found to be less sensitive to Th than S cells (Fig. 1; the IC_{50}s of about 0.01, 0.3 and 0.1 μmol/l were obtained for S, R and V cells, respectively). In agreement with the data of Wagner-Souza et al. (2003), cells selected for MDR by VCR are also resistant to Th. This may be caused by the possibility that P-gp effectively reduced the intracellular Th concentration since it was assumed to be a P-gp substrate (Gutheil et al. 1994; Rishi et al. 1998). On the other hand, any intracellular calcium mobilization effect of Th was not observed in cells with P-gp-mediated resistance induced by VCR independently of the presence or absence of P-gp inhibitors like Ver and cyclosporine A (Wagner-Souza et al. 2003). Consistent with this, we observed only partial restoration of R cell sensitivity to Th in the presence of Ver at a concentration of 10 μmol/l (Fig. 2). Th in the presence of Ver at this concentration effectively depressed the survival of R cells solely at the greatest concentration, i.e. 0.1 μmol/l, but not at a concentration of 0.01 μmol/l that was found to be effective on S cells. Moreover, Ver at the applied concentration was found to completely antagonize P-gp activity in R cells measured with calcein/AM and fluo-3/AM as fluorescent substrates of P-gp (Orlický et al. 2004). These facts indicated that the lack of R cell sensitivity

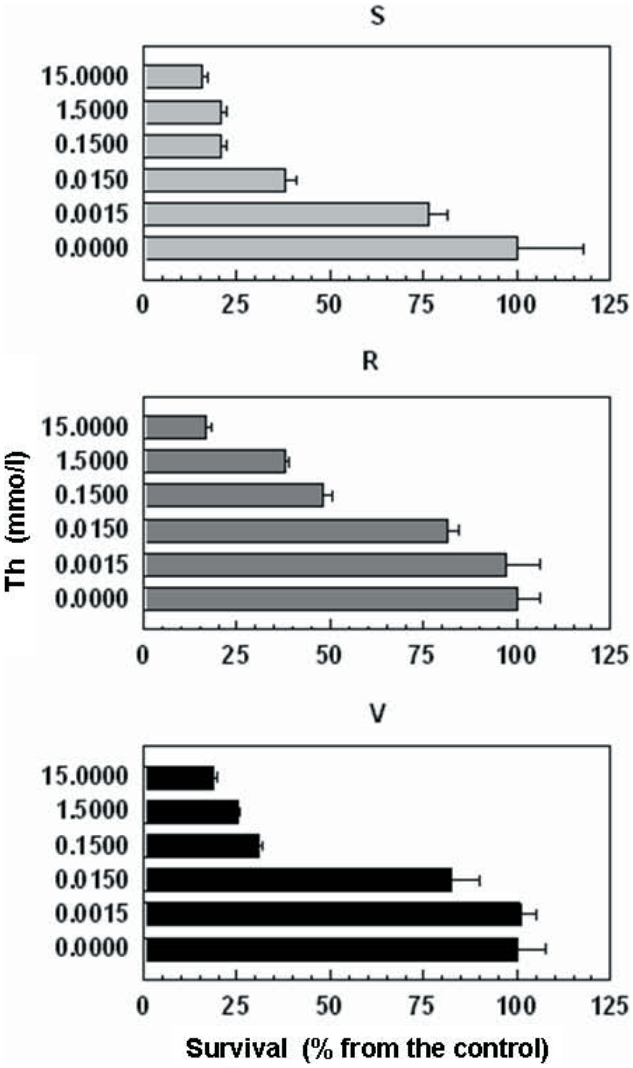

Figure 1. Sensitivity of S, R and V cells to thapsigargin (Th). Cells were cultivated for two days in the presence of Th at the indicated concentrations. In V cells, 1.08 μmol/l VCR was present during cultivation. Cell survival was measured by the MTT test. For all of the details about methods of measurement, see Materials and Methods. Data represent mean ± S.E.M. from five independent cultivations. * significantly different from the value obtained after cultivation in the absence of Th ($p < 0.02$).

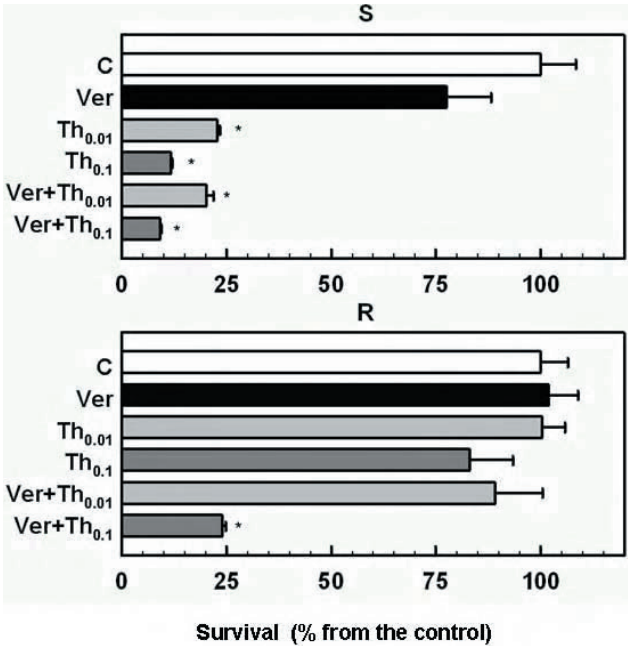

Figure 2. Effect of verapamil (Ver) on S and R cell survival depression induced by thapsigargin (Th). Cells were cultivated for two days in the presence of Th and Ver. Cell survival was measured by the MTT test. For all of the details about methods of measurement, see Materials and Methods. Data represent mean ± S.E.M. from five independent cultivations. C, control (without drugs); Ver, 10 μmol/l Ver; $Th_{0.1}$, 0.1 μmol/l Th; $Th_{0.01}$, 0.01 μmol/l Th; $Ver+Th_{0.1}$, 10 μmol/l Ver and 0.1 μmol/l Th; $Ver+Th_{0.01}$, 10 μmol/l Ver and 0.01 μmol/l Th; * significantly different from the value obtained after cultivation in the absence of Th ($p < 0.02$).

to Th is caused only partially by P-gp-mediated efflux of Th, so there must exist another mechanism that is involved in resistance of R cells to Th.

We described higher sensitivity of R and V cells to an increase in the external calcium concentration that corresponds with more pronounced calcium entry through the plasma membrane in R and V cells when compared with S cells (Sulová et al. 2005). Moreover, different localization of intracellular calcium in V or R cells and S cells was observed by cytochemical visualization of Ca^{2+} ions. These changes may indicate differences in calcium homeostasis between sensitive and resistant cells that may be partially responsible for the lack of Th sensitivity observed in P-gp overexpressing cells.

Expression of RyR, SERCA and IP_3R in S, V and R cells

The endoplasmic reticulum may be recognized as the dominant intracellular calcium store and therefore plays a crucial role in the regulation of intracellular calcium homeostasis (Gorlach et al. 2006). Thus, we studied if any differences exist between S, R and V cells in the expression of protein systems responsible for calcium handling in the endoplasmic reticulum.

While levels of mRNA encoding RyR subtypes 1 or 2 (RyR1 or RyR2) were practically similar in S, R and V cells, levels of the SERCA subtype 2 (SERCA2) were decreased in P-gp overexpressing R cells (Fig. 3). The presence of

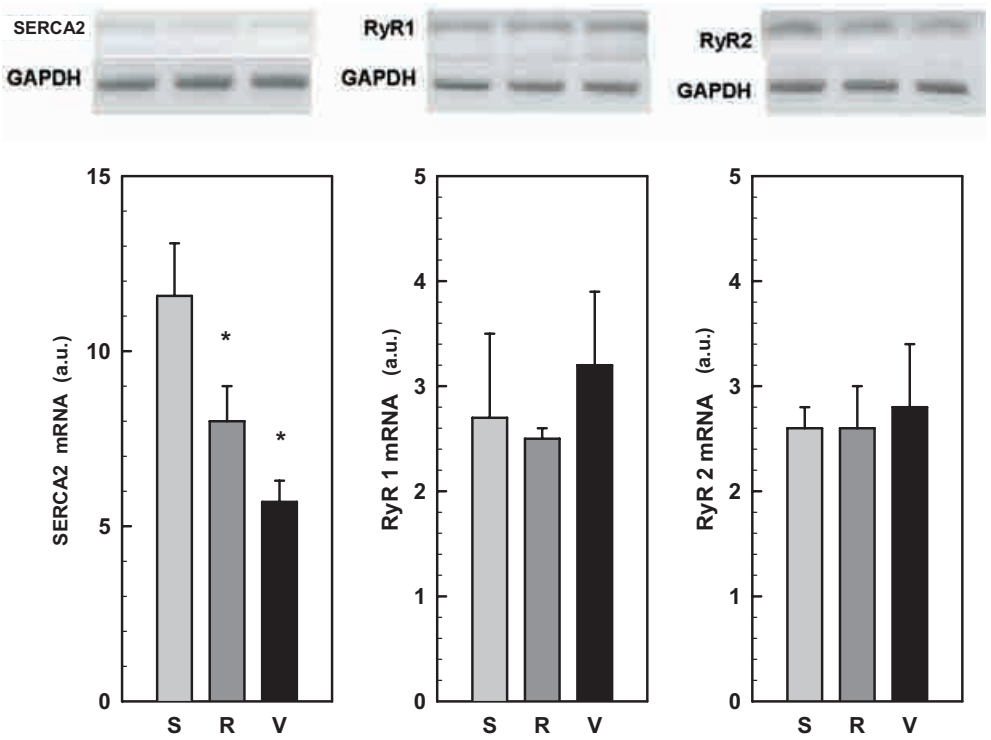

Figure 3. Levels of mRNA encoding SERCA2, RyR1 and RyR2 in the S, R and V cells measured by RT-PCR. Cells were cultivated two days prior measurements. For all of the details about methods of measurement, see Materials and Methods. Quantified density of PCR product bands were normalized to the density of the GAPDH signal as a housekeeper. Data represent mean ± S.E.M. from five independent cultivations. * significantly different from the value obtained after cultivation in the absence of Th ($p < 0.02$).

VCR in the last cultivation of L1210/VCR cells prior to estimation of mRNA levels in V cells induced an additional decrease in mRNA encoding SERCA2 (Fig. 3). Similar levels of RyR expression in S, R and V cells were also visible using immunofluorescence confocal microscopy with anti-RyR antibody documented in Fig. 4. However, fluorescence was found to be localized only in a region near the cell plasma membrane in R and V cells, while in S cells, fluorescence was also localized in intracellular structures.

Levels of mRNA encoding IP_3R subtypes 1 and 2 (IP_3R1 and IP_3R2) were found to be practically unchanged in S and R cells (Fig. 5). However, significant decreases in mRNA levels for IP_3R1 and IP_3R2 were found when L1210/VCR cells were cultivated prior to mRNA content estimation in the presence of VCR (V cells). Similar levels of IP_3R protein in S and R cells and decreased levels of IP_3R in V cells were also observable using immunofluorescence confocal microscopy with anti-IP_3R antibody (Fig. 6). Differences in fluorescence localization in S cells on one hand and R and V cells on the other hand were less visible but seemed to be similar as was described for immunofluorescence using anti-RyR antibody in Fig. 4.

The obtained data indicated that S and R cells did not differ from the point of view of expression of calcium-induced calcium release channels. Thus, release of calcium ions from the endoplasmic reticulum should be of similar extent in S and R cells. On the other hand, a decrease in SERCA2 expression in R cells as compared with S cells indicated slower calcium ion uptake to the luminal space of the endoplasmic reticulum. Thus, the predominance of calcium release over calcium uptake should take place in R cells when compared with S cells. This indicated that in R cells, the calcium intracellular concentration has to be higher than in S cells. A similar conclusion was made recently (Sulová et al. 2005). A higher intracellular calcium concentration in P-gp positive MCF-7 cells as compared with its P-gp negative counterparts was also described (Mestdagh et al. 1994). Unfortunately, intracellular calcium concentrations could not be measured simply in P-gp positive cells, because calcium indicators like fura-2/AM, fluo-3/AM and calcein/AM, substrates of P-gp, are effectively eliminated from the intracellular space and thus could not be deesterified to the form that is able to bind Ca^{2+} (Orlický et al. 2004). In lymphoid leukemia cells, expression of the SERCA3 isoform may also take place (Papp et al. 2004). Whether depression of SERCA2 content in R cells is

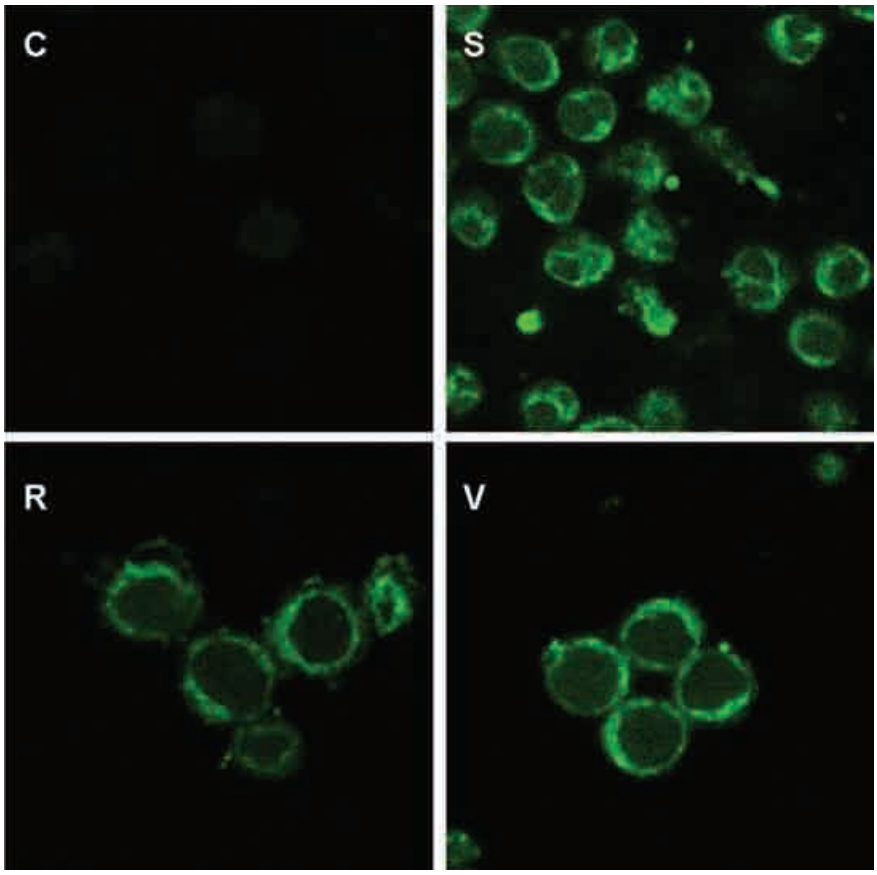

Figure 4. Immunofluorescence visualization of RyR in **S**, **R** and **V** cells. Cells were cultivated for two days prior to measurements and processed according to procedure described in Materials and Methods. RyR molecules were specifically visualized using anti-RyR (primary) antibody and anti-mouse (secondary) antibody labeled with fluorescein isothiocyanate. **C**, cells were processed similarly but incubation with primary antibody was omitted. Lack of staining was also observed when R and V cells were processed without primary antibody (not shown).

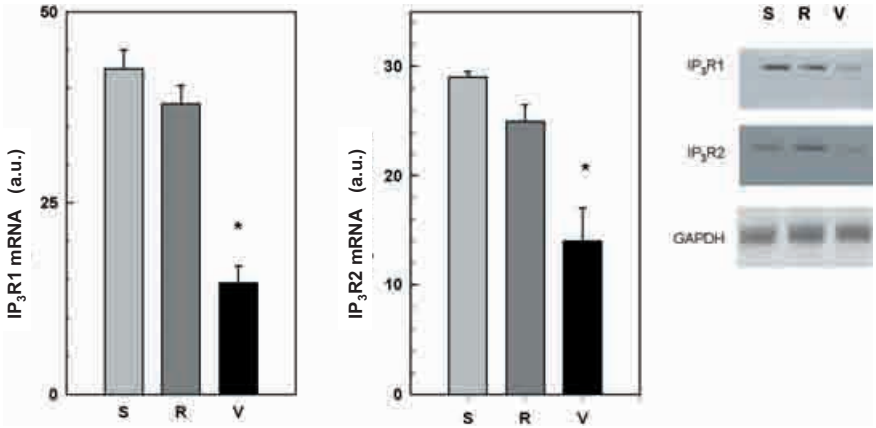

Figure 5. Levels of mRNA encoding IP_3R1 and IP_3R2 in the S, R and V cells measured by RT-PCR. Quantified density of PCR products bands were normalized to the density of the GAPDH signal as a housekeeper. Cells were cultivated for two days prior to measurements. For all details about methods of measurement, see Materials and Methods. Data represent mean ± S.E.M. from five independent cultivations. A sample of electrophoresis gel with visible PCR products (upper two panels) that represents one characteristic documentation from five measurements. * significantly different from the value obtained after cultivation in the absence of Th ($p < 0.02$).

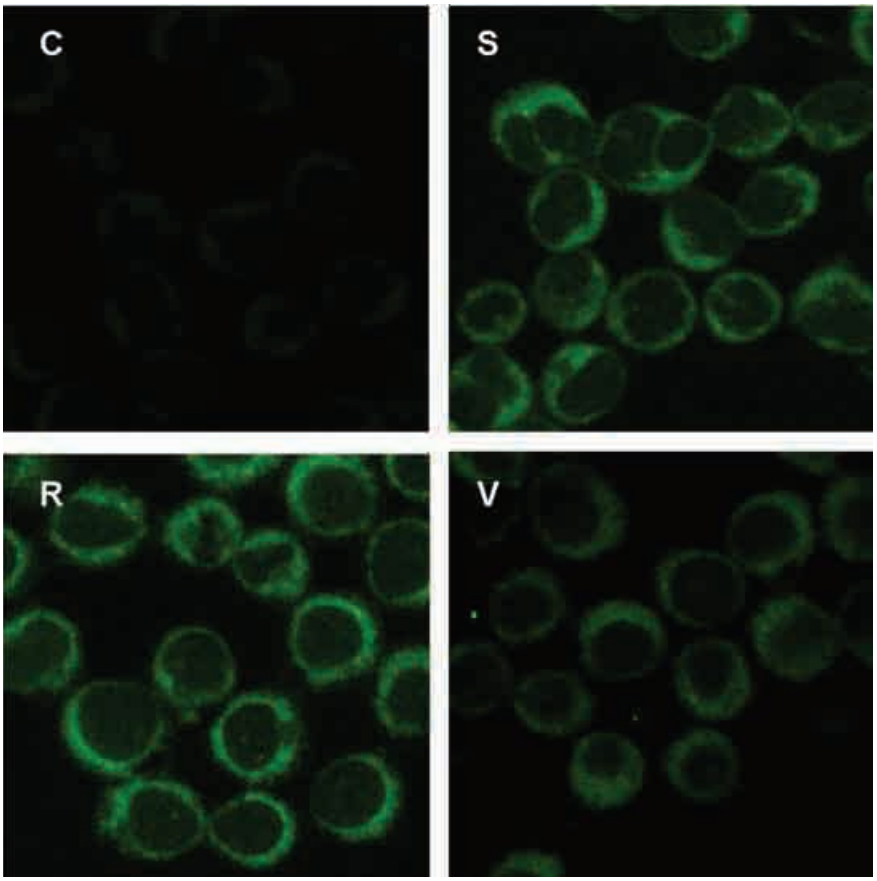

Figure 6. Immunofluorescence visualization of IP_3R in **S**, **R** and **V** cells. Cells were cultivated for two days prior to measurements and processed according to procedure described in Materials and Methods. IP_3R molecules were specifically visualized using anti-IP_3R (primary) antibody and anti-mouse (secondary) antibody labeled with fluorescein isothiocyanate. **C**, cells were processed similarly but incubation with primary antibody was omitted. Lack of staining was also observed when R and V cells were processed without primary antibody (not shown).

partially compensated by an increase in SERCA3 expression remains to be elucidated. However, SERCA3 is known to have a lower affinity for calcium than other isoforms (Wuytack et al. 1995). Thus, the prevalence of SERCA3 over SERCA2 expression has to induce an increase in intracellular calcium concentration (Papp et al. 2004).

Expression of calnexin in S, R and V cells

Calnexin is an endoplasmic reticulum lectin-chaperone with calcium binding ability that, together with another calcium-binding protein/lectin-chaperone calreticulin, plays a role in protein quality control in the lumen of the endoplasmic reticulum (Williams 2006). Calnexin was also described to control the quality and retention of the misfolded form of P-gp in the endoplasmic reticulum (Loo and Clarke 1994, 1995). Calcium ions are important for interaction of these chaperones with the controlled protein, and Th-induced calcium depletion of the endoplasmic reticulum has been found to secure cell surface expression of ΔF508-CFTR (cystic fibrosis transmembrane conductance regulator) that otherwise would be retained in the endoplasmic reticulum (Egan et al. 2002). Interestingly, while this effect was observed, CFTR and also P-gp after Th and curcumin (another SERCA inhibitor) treatments remain in the immature core-glycosylated forms (Loo et al. 2004).

The above facts inspired us to study whether any differences exist in calnexin expression in S, R or V cells. We found similar levels of mRNA encoding calnexin in S and R cells (Fig. 7). However, when VCR was present during the last cultivation prior to mRNA estimation, the level of calnexin mRNA decreased. Higher levels of calnexin were found in sensitive cells (S cells) than in R and V cells by both Western blot and dot blot techniques (Fig. 7). Results on the latter figure document that the time of cultivation (24 or 48 h) did not influence the calnexin content in S, V and

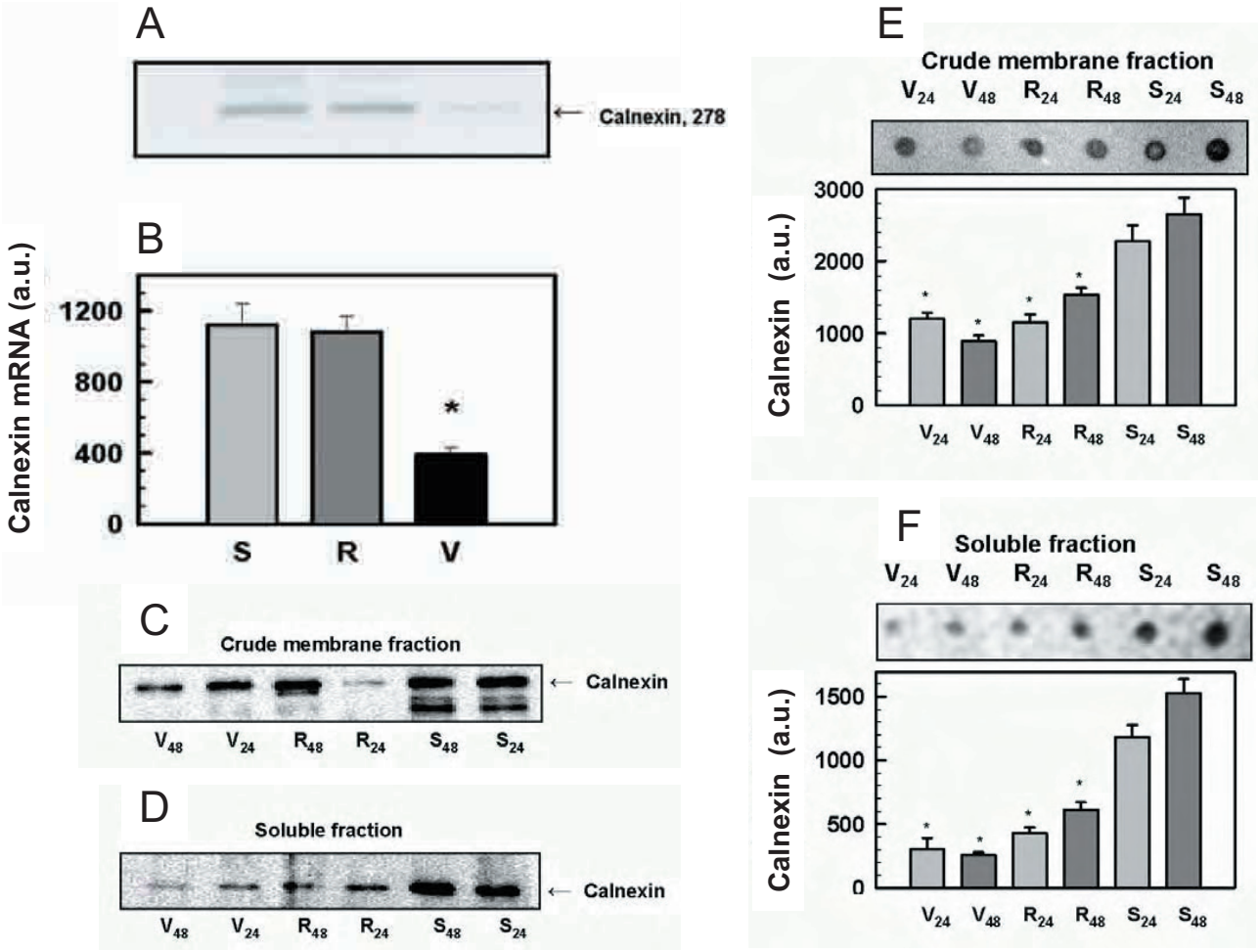

Figure 7. Expression of calnexin in S, R and V cells. **A.** Visualization of PCR products for calnexin in gel (typical example from five independent measurements). **B.** Levels of mRNA encoding calnexin. Quantified density of PCR product bands were normalized to the density of the GAPDH signal as a housekeeper. For all of the details about the methods of measurement, see Materials and Methods. Data represent mean ± S.E.M. from five independent cultivations. * significantly different from S or V cells ($p < 0.02$). **C.** Western blot of calnexin content in the crude membrane fraction isolated from the respective cells. Documentation is a typical example characteristic of five independent measurements. **D.** Western blot evaluation of calnexin content in the soluble fraction isolated from the respective cells. Documentation is a typical example characteristic of five independent measurements. **E.** Estimation of calnexin content in the crude membrane fraction using dot blot. Sample of dot blot represents a typical documentation characteristic of five independent cultivations. Data represent mean ± S.E.M. from five independent cultivations. * significantly different from S or V cells ($p < 0.02$). **F.** Estimation of calnexin content in the soluble fraction using dot blot. Sample of dot blot represents a typical documentation characteristic of five independent cultivations. Data represent mean ± S.E.M. from five independent cultivations. * significantly different from calnexin content obtained for S cells ($p < 0.02$). Signals of GAPDH and β-actin as housekeepers in the crude membrane fractions were very weak (Sulová et al. 2005) and therefore we tested the accuracy of protein loading for Western blots by Coomassie blue staining of protein bands in a separate gels (not shown). For Western blot and dot blot, S, R and V cells were cultivated prior to the experiment 24 or 48 hours, which are distinguished by indexes 24 and 48.

R cells. The lower level of calnexin expression in V cells was also detectable by immunofluorescence visualization by a confocal microscope (Fig. 8). Moreover, we also observed differences in fluorescence localization in S cells on the one hand and R and V cells. This seems to be similar to that described by immunofluorescence using anti-RyR or anti-IP_3R antibody (Fig. 4 and Fig. 6). Differences in cell localization of endoplasmic reticulum proteins (RyR, IP_3R and calnexin) visualized by immunofluorescence indicated an alteration of the architecture of this cell organelle in R and V cells compared with S cells. This is probably another change of the cell architecture of P-gp overexpressing R and V cells (in

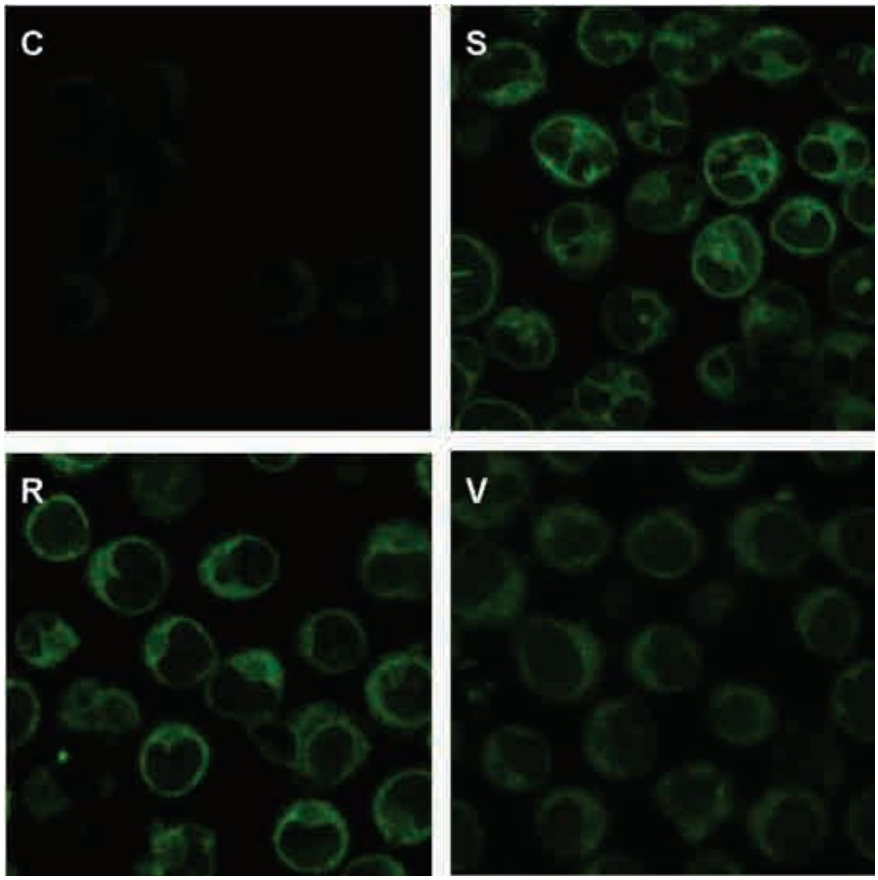

Figure 8. Immunofluorescence visualization of calnexin in **S**, **R** and **V** cells. Cells were cultivated for two days prior to measurements and processed according to procedure described in Materials and Methods. Calnexin molecules were specifically visualized using anti-calnexin (primary) antibody and anti-rabbit (secondary) antibody labeled with fluorescein isothiocyanate. **C**, cells were processed similarly but incubation with primary antibody was omitted. Lack of staining was also observed when R and V cells were processed without primary antibody (not shown).

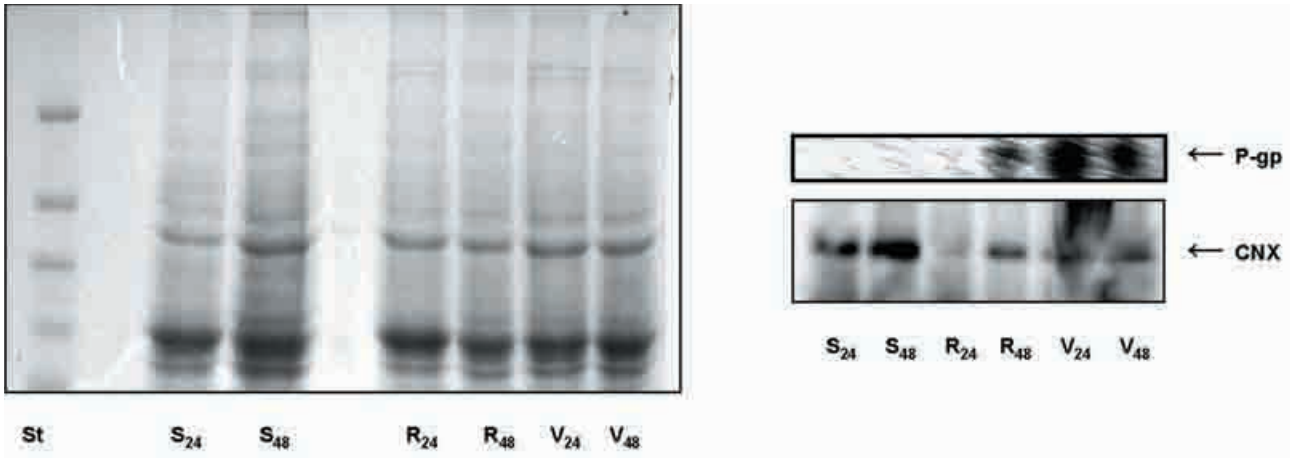

Figure 9. Detection of calnexin (CNX) – P-glycoprotein (P-gp) association by immunoprecipitation with anti-CNX antibody. Left panel – SDS-PAGE gel of immunoprecipitates. Right panel – specific visualization of proteins by anti-CNX and anti-P-gp antibodies using Western blot. P-gp M_r could be calculated at about 155 kDa, which indicated the immature core-glycosylated form. Documentation is characteristic of five independent measurements that always gave similar results. S, R and V cells were cultivated prior to the experiment 24 or 48 hours that are distinguished by indexes 24 and 48.

comparison to S cells), which is complementary to those we described elsewhere (Uhrík et al. 1994, 2006).

The fact that levels of proteins important for P-gp maturation, i.e. calnexin, were found to be decreased in V and probably R cells, in which massive expression of P-gp takes place, was astonishing. Therefore, we tested if P-gp in R and V cells is associated with calnexin, which is a crucial condition for its function in quality control, by immunoprecipitation with anti-calnexin antibody. Immunoprecipitates isolated from R cells (after 48 h cultivation) and V cells (after 24 and 48 h cultivations) contained protein (about 155 kDa) that could be stained by c219 antibody against P-gp (Fig. 9). This indicates that a detectable portion of immature P-gp also exists in complex with calnexin in R and V cells. Thus, calnexin is active in P-gp maturation control in R and V cells. However, robustness of calnexin active in the quality control processing of different proteins should be depressed in R and V cells as compared with S cells.

In conclusion, it could be stressed that the resistance against Th observed in R and V cells is only partially caused by P-gp-dependent efflux of this drug. Other reasons for this feature may be associated with an alteration in calcium homeostasis in R and V cells that should reflect changes in the expression of RyR, IP_3R and SERCA, i.e. proteins that are responsible for calcium-induced calcium release from the endoplasmic reticulum and calcium store formation in the endoplasmic reticulum. These changes are consistent with the higher level of intracellular calcium concentration in P-gp overexpressing cells in comparison with their P-gp negative counterparts, which was experimentally proven previously (Mestdagh et al. 1994). Levels of calnexin were also found to be decreased in V and probably R cells in comparison to S cells. This indicated that another function of the endoplasmic reticulum, i.e. protein quality control, should be altered in MDR cells. However, additional intensive study of this topic will be necessary for its detailed understanding.

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