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Mechanisms and Interaction of Vinblastine and Reduced Glutathione Transport in Membrane Vesicles by the Rabbit Multidrug Resistance Protein Mrp2 Expressed in Insect Cells

RÉMON A. M. H. VAN AUBEL, JAN B. KOENDERINK, JANNY G. P. PETERS, CAREL H. VAN OS, and FRANS G. M. RUSSEL

Departments of Pharmacology and Toxicology (R.A.M.H. van A., J.G.P.P., F.G.M.R.), Biochemistry (J.B.K.), and Cell Physiology (C.H. van O.), University of Nijmegen, the Netherlands

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ABSTRACT

The present study examined how the multidrug resistance protein (MRP) 2, which is an ATP-dependent anionic conjugate transporter, also mediates transport of the chemotherapeutic cationic drug vinblastine (VBL). We show that ATP-dependent [^{3}H]VBL (0.2 μM) uptake into membrane vesicles from Sf9 cells infected with a baculovirus encoding rabbit Mrp2 (Sf9-Mrp2) was similar to vesicles from mock-infected Sf9 cells (Sf9-mock) but could be stimulated by reduced glutathione (GSH) with a half-maximum stimulation of 1.9 $\pm$ 0.1 mM. At 5 mM GSH, initial ATP-dependent [^{3}H]VBL uptake rates were saturable with an apparent K_m of 1.5 $\pm$ 0.3 μM. The inhibitory effect of VBL on Mrp2-mediated ATP-dependent transport of the anionic conjugate [^{3}H]leukotriene C_4 was potentiated by increasing GSH concentrations. Membrane vesicles from Sf9-Mrp2 cells exhibited a ~7-fold increase in initial GSH uptake rates compared with membrane vesicles from Sf9-mock cells. Uptake of [^{3}H]GSH was osmotically sensitive, independent of ATP, and was *trans*-inhibited by GSH. The anionic conjugates estradiol-17β-D-glucuronide and leukotriene C_4 *cis*-inhibited [^{3}H]GSH uptake but only in the presence of ATP. Whereas ATP-dependent [^{3}H]VBL uptake was stimulated by GSH, VBL did not affect [^{3}H]GSH uptake. Our results show that GSH is required for Mrp2-mediated ATP-dependent VBL transport and that Mrp2 transports GSH independent of VBL.

The multidrug resistance protein (MRP) 2 (or canalicular multispecific organic anion transporter) is an ATP-dependent anionic conjugate transporter, which is expressed in small intestine and apical (canalicular) membranes of hepatocytes and renal proximal tubules (for review, see Keppler and König, 1997). Substrates of MRP2 include glutathione *S*-conjugates, such as *S*-(dinitrophenyl)-glutathione (DNP-SG), *S*-(prostaglandin A_1)-glutathione, and leukotriene C_4 (LTC_4) and glucuronide conjugates of bilirubin and estradiol (Jedlitschky et al., 1997; Madon et al., 1997; Evers et al., 1998; Ito et al., 1998; van Aubel et al., 1998). MRP2 belongs to a branch of at least six MRPs (MRP1–MRP6) within the superfamily of ATP-binding cassette proteins (see for latest update http://www.med.rug.nl/mdl/tab3.htm). Involvement in the phenotype of multidrug resistance, however, has only been proven for MRP1 because it is frequently overexpressed in various drug-selected cancer cell lines, and transfection of drug-sensitive cells with an *MRP1* cDNA results in resistance to various drugs (Cole and Deeley, 1998). Recently, overexpression of *MRP2* mRNA and MRP2 protein levels has been found in a few cancer cell lines selected for *cis*-diamminedichloroplatin (Kool et al., 1997). Down-regulation of endogenous MRP2 protein levels in HepG2 cells via antisense transfection reduces resistance to various chemotherapeutic drugs, including vincristine (VCR) and *cis*-diamminedichloroplatin (Koike et al., 1997). MDCKII cells stably transfected with an *MRP2* cDNA show enhanced vinblastine (VBL) transport across the apical membrane (Evers et al., 1998).

The mechanism by which MRP2 mediates transport of cationic chemotherapeutic drugs is unknown. Because MRP1 requires reduced glutathione (GSH) for ATP-dependent VCR uptake into membrane vesicles (Loe et al., 1996), MRP2-mediated drug transport might also depend on GSH. Furthermore, it has been suggested that GSH itself is an MRP2 substrate, but reports are contradictory (Oude Elferink et al., 1989; Fernandez-Checa et al., 1992). The Eisai hyperbilirubinemic (EHBR) and transport-deficient TR^- rat strains, respectively, which lack functional Mrp2, are characterized

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ABBREVIATIONS: MRP, multidrug resistance protein; LTC_4, leukotriene C_4; VBL, vinblastine; VCR, vincristine; GSH, reduced glutathione; $E_2$17βG, estradiol-17β-D-glucuronide; DNP-SG, *S*-dinitrophenyl-glutathione; EHBR, Eisai hyperbilirubinemic rat strain; DTT, dithiothreitol; MK571, 3-([{3-(2-[7-chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethyl-amino-3-oxopropyl)-thio}-methyl]thio)propanoic acid; YCF1, yeast cadmium factor-1.

by hyperbilirubinemia as well as low biliary GSH contents compared with wild-type rats (Oude Elferink et al., 1989; Takikawa et al., 1991; Keppler and König, 1997). However, GSH uptake into EHBR liver canalicular membrane vesicles has been reported to be similar to wild-type liver canalicular membrane vesicles (Fernandez-Checa et al., 1992). Furthermore, both wild-type and EHBR canalicular membrane vesicles only harbor ATP-independent GSH transport systems, suggesting that Mrp2 is not involved in GSH transport (Fernandez-Checa et al., 1992; Ballatori and Dutczak, 1994).

The purpose of this study was to investigate the mechanism by which MRP2 mediates ATP-dependent transport of the cationic drug VBL. Using membrane vesicles from Sf9 cells overexpressing rabbit Mrp2 as described previously (van Aubel et al., 1998), we determined uptake of [^{3}H]VBL with emphasis on a putative role for GSH. In addition, we investigated whether GSH itself is an Mrp2 substrate and how VBL and GSH affect Mrp2-mediated ATP-dependent uptake of [^{3}H]LTC$_4$.

Experimental Procedures

Materials. [G-^{3}H]VBL sulfate (15.5 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). [14, 15, 19, 20-^{3}H]LTC$_4$ (165 Ci/mmol) and [glycine-2-^{3}H]GSH (44.8 Ci/mmol) were purchased from NEN Life Science Products (Hoofddorp, the Netherlands). Estradiol-17β-D-[^{3}H]glucuronide ($E_2$17βG), LTC$_4$, glucuronate, *S*-methyl-GSH, GSH, ATP, dithiothreitol (DTT), and VBL were purchased from Sigma (Zwijndrecht, the Netherlands). Acivicin was purchased from ICN Biochemicals (Cleveland, OH). Creatine phosphate and creatine kinase were purchased from Boehringer Mannheim (Almere, the Netherlands). Glass fiber GF/F filters were purchased from Whatman (Omnilabo International, Breda, the Netherlands). ME-25 membrane filters were purchased from Schleicher & Schuell (Dassel, Germany). 3-([{3-(2-[7-Chloro-2-quinolinyl]ethenyl)phenyl}-{(3-dimethyl-amino-3-oxopropyl)-thio}-methyl]-thio)propanoic acid (MK571) was a generous gift of Dr. A. W. Ford-Hutchinson (Merck Frosst, Center for Therapeutic Research, Quebec, Canada).

Expression of Mrp2 in Sf9 Cells and Isolation of Membrane Vesicles. Sf9 cells (10^6/ml) were grown as 100-ml suspension cultures and infected for 3 days at a multiplicity of infection of 1 to 5 with a recombinant baculovirus encoding rabbit Mrp2 (Sf9-Mrp2) as described recently (van Aubel et al., 1998). For control experiments, Sf9 cells were infected with a baculovirus encoding the β-subunit of rat H^+/K^+ ATPase (Sf9-mock). Membrane vesicles were isolated as described (van Aubel et al., 1998). Briefly, cells were collected, resuspended in hypotonic buffer (0.5 mM sodium phosphate and 0.1 mM EDTA, pH 7.0) and centrifuged at 100,000*g* for 30 min. The pellet of crude membranes was resuspended in TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) and centrifuged at 12,000*g* for 10 min. The postnuclear supernatant was centrifuged at 100,000*g* for 40 min, and the pellet obtained was resuspended in TS buffer, layered over 38% sucrose in 5 mM HEPES/KOH (pH 7.4), and centrifuged at 100,000*g* for 120 min. The interphase was collected, homogenized, and centrifuged at 100,000*g* for 40 min. The resulting pellet was resuspended in TS buffer and passed through a 27-gauge needle 30 times. Membrane vesicles were frozen and stored at −80°C until use.

Transport Studies. Uptake of [^{3}H]VBL into membrane vesicles was performed as described (Horio et al., 1988; van Aubel et al., 1998), with modifications. Briefly, membrane vesicles (20 μg of protein equivalent) were thawed for 1 min at 37°C and added to prewarmed TS buffer supplemented with an ATP-regeneration mix (4

TABLE 1

ATP-dependent uptake of [^{3}H]VBL into membrane vesicles of Sf9-Mrp2 and Sf9-mock cells

Membrane vesicles were incubated in TS buffer with 0.2 μM [^{3}H]VBL and an ATP-regenerating system at 37°C for 2 min in the absence or presence of the compounds as listed below. All compounds were used at a concentration of 5 mM, except for MK571 (0.1 mM). ATP-dependent [^{3}H]VBL uptake was calculated by subtracting values in the absence of ATP from those in the presence of 4 mM ATP. Data are mean ± S.E. from two experiments performed in triplicate.

Compound	ATP-dependent [^{3}H]VBL Uptake	
	Sf9-Mrp2	Sf9-mock
	pmol/mg/2 min	
Control	5.0 ± 1.0	5.1 ± 1.2
GSH	23.1 ± 0.9[a]	4.9 ± 0.5
GSH + MK571	12.0 ± 1.1[a,b]	N.D.
S-Methyl-GSH	15.2 ± 0.9[a]	4.6 ± 0.7
DTT	4.8 ± 1.2	5.3 ± 0.9
Glucuronate	4.9 ± 0.8	5.0 ± 1.2

[a] $p < .01$ compared with control.
[b] $p < .01$ compared with uptake in the presence of GSH but absence of MK571 (ANOVA with Bonferroni's correction).
N.D., not determined.

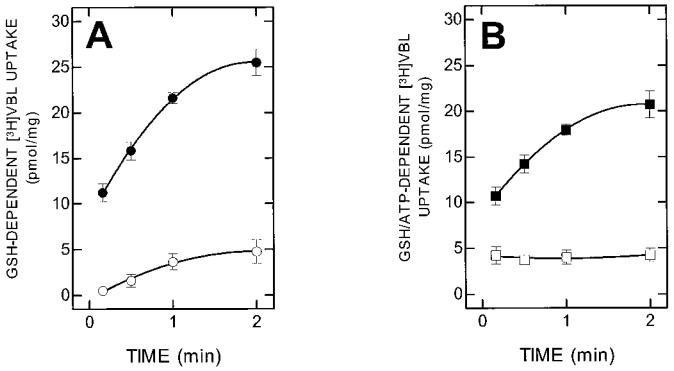

Fig. 1. Time course of GSH-stimulated ATP-dependent [^{3}H]VBL transport by Mrp2. Membrane vesicles prepared from Sf9-Mrp2 and Sf9-mock cells were incubated in TS buffer with 0.2 μM [^{3}H]VBL and 5 mM GSH at 37°C. Samples were taken from the reaction mixture for the times indicated. A, GSH-stimulated [^{3}H]VBL uptake into membrane vesicles from Sf9-Mrp2 in the absence (○) or presence (●) of ATP. B, net GSH/ATP-dependent [^{3}H]VBL uptake into membrane vesicles of Sf9-mock (□) and Sf9-MRP2 (■) cells. Data are mean ± S.E. from two experiments performed in triplicate.

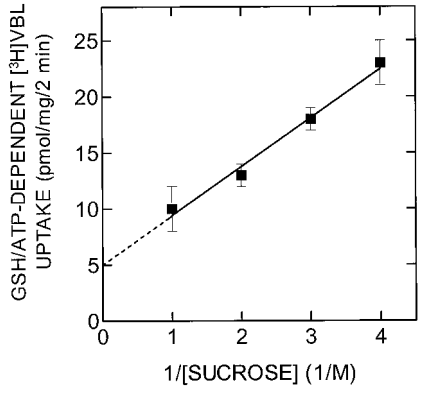

Fig. 2. Osmolarity dependence of GSH-stimulated ATP-dependent [^{3}H]VBL transport by Mrp2. GSH/ATP-dependent [^{3}H]VBL uptake into Sf9-Mrp2 membrane vesicles was determined for 2 min in the presence of sucrose concentrations ranging from 250 mM (isotonic condition) to 1000 mM. Net GSH/ATP-dependent [^{3}H]VBL transport was plotted against the inverse sucrose concentration in the reaction mixture. Linear fitting of the obtained data was performed with GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Data are mean ± S.D. from one experiment performed in triplicate.

mM ATP, 20 mM $MgCl_2$, 10 mM creatine phosphate, and 100 μg/ml creatine kinase) and 0.2 μM [^{3}H]VBL in a final volume of 60 μl. The reaction mixture was incubated at 37°C, and at indicated times, samples were taken from the mixture, diluted in 1 ml of ice-cold TS buffer and filtered through Whatman GF/F filters [soaked overnight in 5% (w/v) BSA at 37°C] using a filtration device (Millipore Corp., Bedford, MA). Filters were washed once with 5 ml of ice-cold TS buffer and dissolved in liquid scintillation fluid to determine the bound radioactivity. Uptake of [^{3}H]LTC_4 at 2 nM was performed as described (van Aubel et al., 1998). Uptake of [^{3}H]GSH at 37°C was performed according to the same procedure as for [^{3}H]LTC_4, except that 5 mM DTT was added at every GSH concentration used and that samples were filtered through ME-25 membrane filters. Uptake of GSH was not affected by 250 μM acivicin, indicating negligible activity of γ-glutamyltransferase in membrane vesicles of Sf9 cells. In all uptake experiments, net ATP-dependent transport was calculated by subtracting values in the absence of ATP from those in the presence of ATP. Measurements were corrected for the amount of ligand bound to the filters (usually <2% of total radioactivity).

Results

We investigated uptake of [^{3}H]VBL into membrane vesicles prepared from Sf9 cells infected with a baculovirus containing a rabbit *mrp2* cDNA (Sf9-Mrp2). We have recently shown that these membrane vesicles contain functional Mrp2 as assessed by uptake studies with [^{3}H]LTC_4 and ([^{3}H]$E_2$17βG) (van Aubel et al., 1998). As shown in Table 1, ATP-dependent uptake of 0.2 μM [^{3}H]VBL into Sf9-Mrp2 membrane vesicles did not differ from uptake into membrane vesicles from Sf9 cells infected with a baculovirus encoding the β-subunit of rat H^+/K^+ ATPase (Sf9-mock). In the presence of 5 mM GSH, however, ATP-dependent uptake of [^{3}H]VBL into Sf9-Mrp2 membrane vesicles increased 5-fold. The leukotriene D_4-receptor antagonist MK571 (Jones et al., 1989), which is an inhibitor of Mrp2-mediated transport (Büchler et al., 1996; van Aubel et al., 1998), inhibited GSH-stimulated ATP-dependent [^{3}H]VBL uptake by ~60%. *S*-Methyl-GSH also increased ATP-dependent [^{3}H]VBL uptake, however, this stimulation was 55% of uptake with GSH. Glucuronate and DTT, which was used to maintain GSH in the reduced form (Ballatori and Dutczak, 1994), did not stimulate uptake.

In the presence of GSH and ATP, Sf9-Mrp2 membrane vesicles exhibited time-dependent uptake of [^{3}H]VBL (Fig. 1A). In the absence of ATP, GSH-stimulated [^{3}H]VBL uptake into Sf9-Mrp2 membrane vesicles was low. For Sf9-Mrp2 membrane vesicles, initial uptake rates of net GSH-stimulated ATP-dependent [^{3}H]VBL were 5.2 ± 0.7 pmol/mg/20 s (Fig. 1B). In contrast, transport into Sf9-mock membrane vesicles was independent of time (4.9 ± 0.5 pmol/mg) and probably reflects nonspecific membrane binding. To confirm that vesicle-associated increase of ligand reflects transport into a vesicular space, the effect of medium osmolarity on [^{3}H]VBL uptake was investigated (Fig. 2). GSH-stimulated ATP-dependent [^{3}H]VBL uptake into Sf9-Mrp2 membrane vesicles decreased linearly with increasing concentrations of sucrose. Because vesicle space decreases with increasing osmolarity, extrapolation of the data to zero vesicle space indicates that 5.0 ± 0.6 pmol VBL/mg protein is bound to the vesicle membrane.

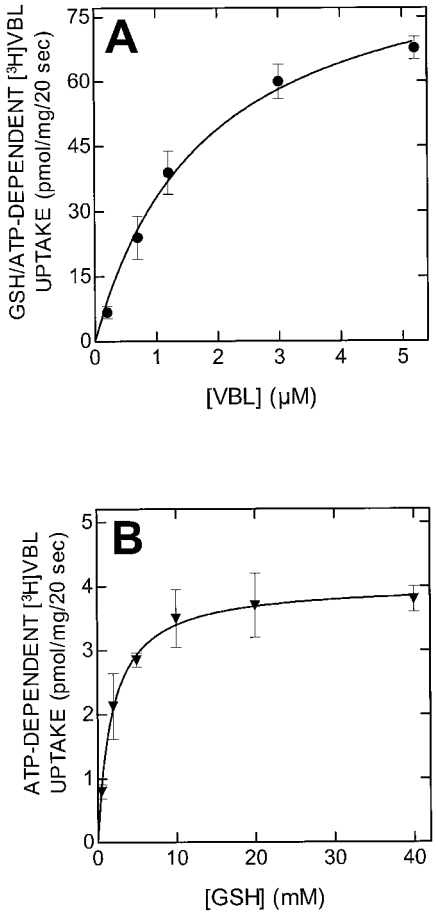

Fig. 3. VBL and GSH concentration dependence of GSH-stimulated ATP-dependent [^{3}H]VBL transport by Mrp2. A, membrane vesicles prepared from Sf9-Mrp2 cells were incubated at 37°C for 20 s in TS-buffer supplemented with 5 mM GSH and [^{3}H]VBL concentrations ranging from 0.2–5 μM. B, Sf9-Mrp2 membrane vesicles were incubated at 37°C for 20 s in TS buffer supplemented with 0.2 μM [^{3}H]VBL and GSH concentrations ranging from 0.5 to 40 mM. All data were corrected for binding of VBL to the vesicle membrane and were fitted according to the Michaelis-Menten equation with GraphPad Prism version 3.00. Data are mean from two experiments performed in triplicate (±S.E.).

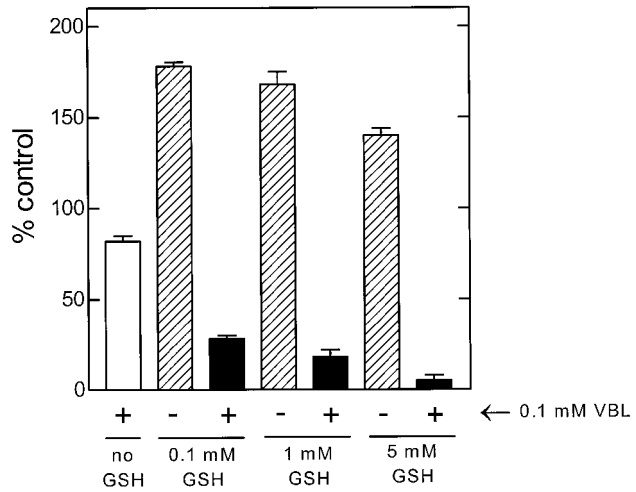

Fig. 4. Effect of GSH and VBL on Mrp2-mediated [^{3}H]LTC_4 transport. Sf9-Mrp2 membrane vesicles were incubated with 2 nM [^{3}H]LTC_4 at 23°C for 30 s in the absence (control) or presence of VBL (0.1 mM) and/or GSH at concentrations as indicated. Transport was expressed as percent inhibition of net ATP-dependent [^{3}H]LTC_4 uptake in the absence of GSH and VBL. Final concentrations of the solvent dimethyl sulfoxide were <0.1%. Data are mean ± S.E. from two experiments performed in triplicate.

To further investigate GSH-stimulated VBL transport by Mrp2, initial transport rates were determined at various concentrations of [^{3}H]VBL (Fig. 3A). In the presence of 5 mM GSH, initial ATP-dependent [^{3}H]VBL uptake rates increased with increasing VBL concentrations. Fitting of the obtained data according to the Michaelis-Menten equation revealed a K_m of 1.5 ± 0.3 μM and V_{max} of 89 ± 6 pmol/mg/20 s. We also investigated the GSH concentration dependence of Mrp2-mediated ATP-dependent VBL transport (Fig. 3B). Initial ATP-dependent [^{3}H]VBL uptake rates increased with increasing GSH concentrations to a maximum at 10 mM. According to Michaelis-Menten kinetics, half-maximum stimulation was determined at a GSH concentration of 1.9 ± 0.1 mM.

We investigated the effect of GSH and VBL on ATP-dependent uptake of the high-affinity substrate LTC_4 into Sf9-Mrp2 membrane vesicles. As shown in Fig. 4, initial rates of ATP-dependent [^{3}H]LTC_4 uptake were not inhibited but rather stimulated by GSH at concentrations of 0.1, 1, and 5 mM. VBL (0.1 mM) was only a poor inhibitor of initial [^{3}H]LTC_4 uptake rates. However, the inhibitory effect of VBL (20%) was greatly stimulated in the presence of 0.1, 1, and 5 mM GSH to 72, 82, and 95%, respectively.

To investigate whether GSH itself is an Mrp2 substrate, we measured uptake of [^{3}H]GSH at concentrations of 0.1 and 5 mM. Sf9-Mrp2 membrane vesicles exhibited time-dependent uptake of [^{3}H]GSH at 0.1 mM, however, uptake was only modestly increased as compared with uptake into Sf9-mock membrane vesicles (Fig. 5A). At 5 mM, [^{3}H]GSH was taken up into Sf9-Mrp2 membrane vesicles with an initial rate of 1.4 ± 0.3 nmol/mg/min, which was ~7-fold higher compared with initial rates in Sf9-mock membrane vesicles (Fig. 5B). In the presence of ATP, time-dependent uptake of [^{3}H]GSH (0.1 or 5 mM) into Sf9-Mrp2 and Sf9-mock membrane vesicles was not significantly different from uptake in the absence of ATP (Fig. 5, A and B). Uptake of [^{3}H]GSH was sensitive to osmolarity, indicating that vesicle-associated [^{3}H]GSH levels represent true uptake into membrane vesicles rather than binding to the vesicle membrane (Fig. 5C). Preloading of Sf9-Mrp2 membrane vesicles with 5 mM GSH reduced initial [^{3}H]GSH uptake rates by ~85% either in the presence or absence of ATP (Table 2). Initial [^{3}H]GSH uptake rates were also inhibited by LTC_4, $E_2$17βG, and MK571 but only in the presence of ATP, whereas *S*-methyl-GSH had no effect (Table 2).

Recently, Loe et al. (1998) demonstrated that VCR stimulates GSH uptake into membrane vesicles from *MRP1*-transfected HeLa cells, providing evidence for a VCR/GSH cotransport mechanism. In view of a possible VBL/GSH cotransport mechanism by Mrp2, we investigated the effect of various VBL concentrations on uptake of [^{3}H]GSH into Sf9-Mrp2 membrane vesicles. However, the initial uptake rate of [^{3}H]GSH at 0.1 mM (95 ± 12 pmol/mg/min; n = 2) or 5 mM (1.3 ± 0.2 nmol/mg/min; n = 2) was not affected by either 0.2 μM, 10 μM, or 0.1 mM VBL (Table 2; data not shown). Furthermore, none of these VBL concentrations affected uptake of [^{3}H]GSH at 5 mM for 4 min (data not shown).

Discussion

The present study demonstrates that the ATP-dependent anionic conjugate transporter Mrp2 requires GSH for ATP-dependent transport of the cationic drug VBL. *S*-Methyl-GSH also stimulated ATP-dependent VBL transport but to a lesser extent than GSH, whereas glucuronate was not able to induce VBL transport. These results are in line with previous reports showing that MRP1-mediated VCR transport is stimulated by GSH and to a lesser extent by *S*-methyl-GSH but not by glucuronate (Loe et al., 1996, 1998). Furthermore, we show that stimulation of Mrp2-mediated ATP-dependent VBL transport by GSH is saturable, with a half-maximum stimulation at 1.9 mM. In the presence of 5 mM GSH, saturability of transport is observed at increased VBL concentrations, with a K_m of 1.5 μM. Moreover, the small inhibitory effect of VBL on Mrp2-mediated ATP-dependent [^{3}H]LTC_4 transport was potentiated by increasing the GSH concentration.

Second, we show that membrane vesicles from Sf9-Mrp2 cells exhibited a ~7-fold increase in initial GSH uptake rates compared with membrane vesicles from Sf9-mock cells. Uptake of GSH was sensitive to medium osmolarity independent of ATP and almost completely abolished when membrane vesicles were preloaded with GSH. Initial GSH uptake

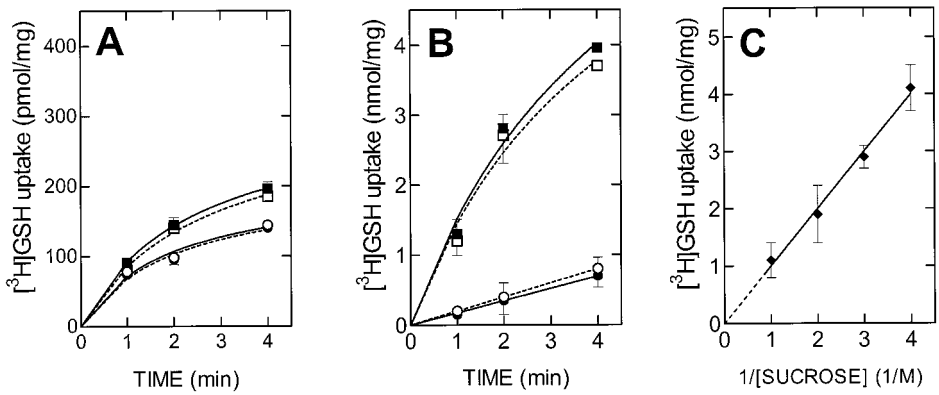

Fig. 5. Time course and osmolarity dependence of Mrp2-mediated [^{3}H]GSH transport. A and B, membrane vesicles prepared from Sf9-Mrp2 (■, □) and Sf9-mock (●, ○) cells were incubated at 37°C in TS-buffer with [^{3}H]GSH at concentrations of 0.1 mM (A) or 5 mM (B) in the presence (■, ●) or absence (□, ○) of ATP. Data are mean ± S.E. from two experiments performed in triplicate. C, [^{3}H]GSH uptake into Sf9-Mrp2 membrane vesicles was determined for 4 min in the presence of sucrose concentrations ranging from 250 mM (isotonic condition) to 1000 mM. [^{3}H]GSH transport was plotted against the inverse sucrose concentration in the reaction mixture. Linear fitting of the obtained data was performed with GraphPad Prism version 3.00. Data are mean ± S.D. from one experiment performed in triplicate.

rates in Sf9-Mrp2 membrane vesicles were inhibited by the anionic conjugates LTC_4 and $E_2 17\beta G$, but only in the presence of ATP. These results suggest that Mrp2 is permeable for GSH and that the site for GSH permeability interacts with the site for active transport of anionic conjugates. In this respect, MK571 inhibits both Mrp2-mediated ATP-dependent anionic conjugate transport and ATP-independent GSH transport (van Aubel et al., 1998; this study). Similar to our results, GSH permeability has recently been shown for the cystic fibrosis transmembrane conductance regulator, which belongs to the MRP branch of ATP-binding cassette transporters (Linsdell and Hanrahan, 1998). Alternatively, Mrp2 itself may not be involved in GSH transport but may regulate an endogenous ATP-independent GSH transporter. Such a regulatory mechanism for Mrp2 has been proposed based on results from uptake studies with liver canalicular membrane vesicles (Fernandez-Checa et al., 1992; Ballatori and Dutczak, 1994; Mittur et al., 1998). Radiation inactivation of canalicular GSH transport has indicated the complexity of low-affinity GSH transport, which involves multiple putative transporters, including presumably Mrp2 (Mittur et al., 1998). Furthermore, low-affinity GSH uptake into liver canalicular membrane vesicles is *cis*-inhibited by DNP-SG in the absence of ATP but *trans*-stimulated by DNP-SG in the presence of ATP (Fernandez-Checa et al., 1992; Ballatori and Dutczak, 1994). The characteristics of this Mrp2-regulated GSH transport, however, are different from our results, because anionic conjugates inhibited GSH uptake into Sf9-Mrp2 membrane vesicles in the presence of ATP, whereas they did not affect uptake in the absence of ATP.

A recent report by Paulusma et al. (1999) is at variance with the hypothesis of Mrp2 as both an ATP-dependent anionic conjugate transporter and an ATP-independent GSH transporter. These authors demonstrated that inhibition of ATP synthesis in *Mrp2*-transfected MDCKII cells greatly reduced efflux of both DNP-SG and GSH across the apical membrane (Evers et al., 1998; Paulusma et al., 1999). Nonetheless, a difference in uptake of GSH between membrane vesicles of transfected and parental cells either in the presence or absence of ATP was undetectable (Paulusma et al., 1999). Similar contradictory results obtained with intact cells and isolated membrane vesicles have been reported for MRP1. Although expression levels of MRP1 correlate with efflux of GSH from intact cells (Zaman et al., 1995; Rappa et al., 1997; Paulusma et al., 1999), MRP1-mediated uptake of GSH into membrane vesicles either in the presence or absence of ATP could not be demonstrated (Leier et al., 1996; Loe et al., 1998). It has recently been suggested that MRP1 and Mrp2 might mediate GSH efflux from intact cells in the form of a short-lived complex with an intracellular compound, which is lacking in preparations of membrane vesicles (Zaman et al., 1995; Paulusma et al., 1999). Such a mechanism would explain why Mrp2-deficient rats have very low biliary GSH levels, although GSH uptake into EHBR canalicular membrane vesicles is intact (Oude Elferink et al., 1989; Fernandez-Checa et al., 1992). On the other hand, membrane vesicle preparations from various cell lines and tissues may be unsuitable for detecting GSH uptake because of their mixed orientation and possible leakage. Recently, Rebbeor et al. (1998a) have demonstrated GSH uptake into yeast *sec*6-4 membrane vesicles, which are orientated almost exclusively inside out (Ambesi et al., 1997). Uptake of GSH into these vesicles was saturable (K_m = 15–20 mM), competitively inhibited by DNP-SG ($K_i \sim 0.2$ mM), and dependent on ATP. A further study identified the yeast cadmium factor-1 (YCF1), a yeast ortholog of mammalian MRP1 and MRP2 (Li et al., 1996), as the major contributor to ATP-dependent GSH transport (Rebbeor et al., 1998b). However, membrane vesicles from wild-type yeast also show low levels of ATP-independent GSH uptake, and at present it is unknown whether YCF1 or a different transporter is involved (Rebbeor et al., 1998a,b).

The physiological significance of MRP2-mediated GSH secretion into bile and urine is not clear. Biliary and urinary GSH may provide protection against oxidative damage and, in addition, may be a source for glutamate, glycine, and cysteine after degradation of GSH by extracellular membrane-bound peptidases (Ookhtens and Kaplowitz, 1998). Furthermore, urinary GSH may account for detoxification of agents that enter the urine directly via glomerular filtration. Besides MRP2, other transport proteins also are involved in biliary GSH secretion (Ballatori and Rebbeor, 1998). In liver canalicular membranes, the putative low- and high-affinity ATP-independent GSH transporters both have an estimated molecular mass of ~70 kDa (Mittur et al., 1998). Although their molecular identity is unknown, these transporters might be canalicular isoforms of the 74-kDa sinusoidal organic anion transporting polypeptide (Oatp1), which functions as a GSH/organic solute exchanger (Ballatori and Rebbeor, 1998).

In this study, we tried to address the mechanism by which GSH affects Mrp2-mediated ATP-dependent VBL transport. Possible explanations for the effect of GSH are spontaneous formation of a glutathione-*S*-conjugate, a cotransport mechanism, or an indirect interaction. So far, conjugates of GSH with *vinca* alkaloids, such as VCR and VBL, have not been demonstrated (Tew, 1994; Ban et al., 1996). Furthermore, HPLC analysis of the products excreted by *MRP1*-transfected SW-1573/S1 cells after preloading with VCR only revealed unmodified drugs (Zaman et al., 1995). Thus, either GSH is cotransported with VBL or GSH indirectly stimulates ATP-dependent VBL transport. GSH stimulates Mrp2-mediated ATP-dependent VBL transport with an apparent half-maximum stimulation at ~2 mM. A similar value (2.7 mM) was

TABLE 2

Effect of various compounds on [^{3}H]GSH uptake into membrane vesicles of Sf9-Mrp2 cells

Membrane vesicles were incubated in TS buffer with 5 mM [^{3}H]GSH at 37°C for 1 min in the absence or presence of ATP supplemented with or without (control) the compounds as listed below. The *trans* effect of GSH was studied by preloading Sf9-Mrp2 membrane vesicles with 5 mM GSH for 1 h at 37°C and resuspending them in TS buffer with [^{3}H]GSH. Transport is expressed as percentage of the control. Data are mean ± S.E. from two experiments performed in triplicate.

Compound	[^{3}H]GSH Uptake	
	+ATP	−ATP
Control	100	100
GSH (5 mM, *trans*)	12 ± 3[a]	15 ± 4[a]
S-Methyl-GSH (5 mM)	98 ± 5	100 ± 2
LTC_4 (5 μM)	23 ± 5[a]	97 ± 17
$E_2 17\beta G$ (0.1 mM)	50 ± 12[a]	89 ± 12
MK571 (0.1 mM)	32 ± 7[a]	99 ± 7
VBL (0.2 μM)	98 ± 5	98 ± 10
VBL (0.1 mM)	102 ± 3	107 ± 3

[a] $p < .01$ (ANOVA with Bonferroni's correction). Control uptake of [^{3}H]GSH into Sf9-Mrp2 membrane vesicles was 1.3 ± 0.2 nmol/mg/min in the presence or absence of ATP.

found for GSH-stimulated ATP-dependent daunorubicin transport by MRP1 (Renes et al., 1999). The transport affinity of GSH for MRP2/Mrp2 appears to be 10-fold lower (~20 mM), which is in the same range as reported for YCF1 (Rebbeor et al., 1998b; Paulusma et al., 1999). This suggests that VBL enhances the affinity of GSH for Mrp2, but we could not demonstrate that VBL induces Mrp2-mediated GSH transport, as one would expect for a cotransport mechanism. Loe et al. (1998) recently have shown both GSH-stimulated VCR and VCR-stimulated GSH uptake into membrane vesicles of HeLa cells overexpressing human MRP1, suggesting a VCR/GSH cotransport mechanism. VBL also stimulated GSH uptake into MRP1-enriched membrane vesicles, although to a lesser degree than VCR (Loe et al., 1998). With respect to our results, we cannot exclude that it is impossible to detect a VBL-dependent stimulation of GSH transport above the background of GSH uptake, because of the relatively low affinity and high V_{max} of the latter process. However, even if VBL does not stimulate GSH transport, we conclude from our data that Mrp2-mediated GSH transport does not require the cotransport of VBL.

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Send reprint requests to: Dr. F. G. M. Russel, University of Nijmegen, Department of Pharmacology and Toxicology 233, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands. E-mail: F.Russel@farm.kun.nl