Differential sensitivity of plant and yeast MRP (ABCC)-mediated organic anion transport processes towards sulfonylureas

Cyrille Forestier[a], Nathalie Frangne[b,1], Thomas Eggmann[b], Markus Klein[c,*]

[a] *CEA Cadarache, Direction des Sciences du Vivant, Département d'Ecophysiologie Végétale et de Microbiologie, Laboratoire des Echanges Membranaires et Signalisation, UMR 163 CNRS-CEA, F-13108 St Paul-lez-Durance, France*
[b] *Université de Neuchâtel, Institut de Botanique, Laboratoire de Physiologie végétale, Rue Emile Argand 13, CH-2007 Neuchâtel, Switzerland*
[c] *Zurich Basel Plant Science Center, Plant Biology, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland*

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Abstract The role of ATP-binding cassette (ABC) proteins such as multidrug resistance-associated proteins (MRPs) is critical in drug resistance in cancer cells and in plant detoxification processes. Due to broad substrate spectra, specific modulators of these proteins are still lacking. Sulfonylureas such as glibenclamide are used to treat non-insulin-dependent diabetes since they bind to the sulfonylurea receptor. Glibenclamide also inhibits the cystic fibrosis transmembrane conductance regulator, p-glycoprotein in animals and guard cell ion channels in plants. To investigate whether this compound is a more general blocker of ABC transporters the sensitivity of ABC-type transport processes across the vacuolar membrane of plants and yeast towards glibenclamide was evaluated. Glibenclamide inhibits the ATP-dependent uptake of β-estradiol 17-(β-D-glucuronide), lucifer yellow CH, and (2′,7′-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein. Transport of glutathione conjugates into plant but not into yeast vacuoles was drastically reduced by glibenclamide. Thus, irrespective of the homologies between plant, yeast and animal MRP transporters, specific features of plant vacuolar MRPs with regard to sensitivity towards sulfonylureas exist. Glibenclamide could be a useful tool to trap anionic fluorescent indicator dyes in the cytosol.

Key words: ATP-binding cassette transporter; Multidrug resistance-associated protein; Organic anion transport; Vacuole transport; Sulfonylurea; Fluorescent dye

1. Introduction

The multidrug resistance-associated proteins (MRPs) belong to the large multigene family of directly energized membrane ATP-binding cassette (ABC) transporters. Human MRP1 (ABCC1) was identified in small cell lung cancer cells since its overexpression resulted in the multidrug resistance phenotype leading to a decreased sensitivity of cancer cells to chemotherapeutic drugs of variable structure [1]. Subsequent work showed that most of the ABCC/MRP-type ABC transporter from different organisms are involved in extrusion of structurally diverse amphipathic drugs and conjugated organic anions out of the cytosol into the extracellular space or the vacuole. Assumed physiological functions for MRP transporters in different organisms are catabolite elimination from the liver into bile, e.g. during heme degradation, heavy metal tolerance in yeast, and herbicide tolerance or leaf senescence in plants. Consequently, it can be concluded that these membrane proteins are implicated in many different cellular detoxification processes [2].

Plant vacuolar membranes contain directly energized transport systems which are able to catalyze the transport of potentially toxic compounds into the large central vacuole which occupies up to 90% of the cellular volume [3–5]. These transport systems are therefore supposed to be crucial for the accumulation of toxic compounds and protection of the cytosol. After the initial characterization of a glutathione conjugate (GS-X) pump [6], further vacuolar ATP-dependent transport systems for a herbicide glucoside, glucuronides (β-estradiol 17-(β-D-glucuronide) ($E_2$17G) and plant-specific flavone glucuronides from rye), for the sulfonated fluorescent dye lucifer yellow CH (LY-CH) and for chlorophyll catabolites have been identified [7–11]. Presently, four *MRP* homologues from *Arabidopsis thaliana* have been cloned and characterized via heterologous expression in yeast [12–14]. All these AtMRPs are capable of transporting GS-X. AtMRP2 and AtMRP3 mediate chlorophyll catabolite transport [12–15]. AtMRP2 and AtMRP5 also transport glucuronides [15,16] but only AtMRP2 exhibits reciprocal activation of GS-X and glucuronide transport via distinct but coupled binding sites [16] which confirms previous experiments performed with intact vacuoles [10,11]. AtMRP3 partially restores cadmium tolerance to the Δ*ycf1* (yeast cadmium factor 1) mutant in *Saccharomyces cerevisiae* that exhibits hypersensitivity towards this heavy metal [13].

Among the ABC transporters, p-glycoprotein (p-gp; ABCB1), cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7) and the sulfonylurea receptor (SUR; ABCC8) have received considerable attention as they can con-

*Corresponding author. Fax: (41)-1-634 82 04.
E-mail address: markus.klein@botinst.unizh.ch (M. Klein).

[1] Present address: Université Poitiers, Laboratoire de Biochimie et Physiologie Végétale, Av. Recteur Pineau, F-8600 Poitiers, France.

Abbreviations: ABC, ATP-binding cassette; BCECF, (2′,7′-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein; BmCl, monochlorobimane; CFTR, cystic fibrosis transmembrane conductance regulator; DNB-GS, 2,4-dinitrobenzene glutathione; $E_2$17G, β-estradiol 17-(β-D-glucuronide); GSH, reduced glutathione; GSSG, oxidized glutathione; GS-X, glutathione conjugate; KCO, potassium channel opener; LY-CH, lucifer yellow CH; MRP, multidrug resistance-associated protein; SUR, sulfonylurea receptor

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trol other membrane proteins and more specifically ion channels [17]. The clinical importance of mutations affecting these proteins has contributed to the development of powerful pharmacological tools. Sulfonylureas, such as glibenclamide, have become the major therapeutic agents used in the treatment of non-insulin-dependent diabetes since they have a nanomolar affinity for the pancreatic SUR receptor. This SUR1 isoform is associated with an inward-rectifying potassium channel to form K-ATP channels [18]. Conversely, K^+ channel openers (KCOs) are powerful vasodilator agents that increase the K^+ permeability of K-ATP channels and can reverse the effect of glibenclamide [19]. The situation for chloride channels is slightly different since both glibenclamide and KCOs have been reported to inhibit CFTR [20]. More recently, it has been shown that glibenclamide also inhibits p-gp [21], suggesting the presence of a conserved binding motif exhibited by p-gp, SUR and CFTR. In plants it has been shown that K^+ and anion channels are inhibited by glibenclamide in the micromolar concentration range and are reactivated by KCOs [22,23]. Glibenclamide induces the opening of the stomatal pores which are functional units of the leaf epidermis regulating plant transpiration. Stomata from *Arabidopsis* plants lacking *AtMRP5* are insensitive to glibenclamide and phytohormones resulting in increased drought resistance under dry conditions [15,24].

With regard to the redundancy of 14 and 12 ABCC/MRP-like genes in the *Arabidopsis* and rice genomes [25,26], respectively, and in view of the lack of information concerning the substrate specificity and physiological functions of plant and yeast MRP transporters, it appears interesting to test pharmacological tools in order to differentiate between MRPs or their different transport activities. We investigated the effects of sulfonylureas and KCOs on organic anion transport using plant and yeast vacuoles. We propose that besides their effects on ion channels, sulfonylureas are potent inhibitors of some MRP-mediated transport processes and thus could be used to distinguish between GS-X and glucuronide transport activities. By investigating the effect of glibenclamide on the cellular distribution of anionic fluorescent dyes we demonstrate that inhibitors of ABC transporters could be interesting tools to exclude different dyes from the vacuole.

2. Materials and methods

2.1. Plant material, growth conditions and chemicals

Barley (*Hordeum vulgare* L. cv. Bakara) was grown as described [8]. For the isolation of *A. thaliana* vacuoles, a cell suspension culture (ecotype Columbia, cell line T87) grown in Gamborg B5 medium with 2.5 μM 2,4-dichlorophenoxyacetic acid was used [27]. Unless otherwise stated, all chemicals were from Sigma or Fluka (Buchs, Switzerland). Plant cell culture media were from Duchefa (The Netherlands) and yeast media were supplied by Gibco BRL (Basel, Switzerland). β-Estradiol 17-(β-D-glucuronide) [estradiol-6,7-^{3}H] ([^{3}H]$E_2$17G) was obtained from NEN (Boston, MA, USA). [^{14}C]*S*-(2,4-Dinitrobenzene)-glutathione ([^{14}C]DNB-GS) was synthesized as described [28] and purified using a C18 reversed phase column. Fluorescent dyes were from Molecular Probes (Leiden, The Netherlands). RP49356 was a generous gift from Rhône-Poulenc Rorer (Vitry-sur-Seine, France), SR47063 from Sanofi Recherche (Montpellier, France).

2.2. Isolation of mesophyll cellular fractions and vacuolar uptake experiments

Protoplasts and vacuoles from barley and the *A. thaliana* cell suspension culture were prepared following published procedures [29–31]. Contamination of barley vacuoles with other cell constituents was less than 3% as measured by marker enzyme activities [30]. Intactness and purity of barley and *Arabidopsis* vacuoles was microscopically analyzed using 0.025% Neutral red which is trapped in the acid vacuolar lumen and the exclusion dye Evans blue (0.02%). Staining with fluorescein diacetate (0.1%) indicated that less than 2 and 10% of the barley and *Arabidopsis* vacuoles were contaminated with cytosolic patches attached to the tonoplast. Transport studies with barley mesophyll and *Arabidopsis* cell culture vacuoles were performed as previously described using the silicone oil centrifugation technique [10]. The following substrates were used in separate transport experiments: 0.05 μCi of 44 Ci/mmol [^{3}H]$E_2$17G, 0.05 μCi of 10 mCi/mmol [^{14}C]DNB-GS, 50 μM LY-CH or 25 μM (2′,7′-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein (BCECF). The vacuolar volume was calculated by the addition of 0.05 μCi ^3H_2O. For uptake experiments with [^{3}H]$E_2$17G, the vacuolar volume was determined in separate tubes. Time course experiments exhibited no decrease of the vacuolar volume during the transport experiment indicating stability of the vacuolar fractions. In experiments using radioactive substrates, vacuolar fractions were suspended in 3 ml of scintillation cocktail (Readysafe, Beckman) and radioactivity was determined by liquid scintillation spectrophotometry after adding 3 ml of scintillation cocktail (Readysafe). For BCECF and LY-CH uptake studies, the supernatants of two tubes were pooled and analyzed by spectrofluorometry (FL-500, Bio-Tek Instruments, Winooski, VT, USA) using the following excitation and emission wavelengths: BCECF: λ_{exc} 439 nm, λ_{em} 504 nm; LY-CH: λ_{exc} 430 nm, λ_{em} 530 nm. Unless stated otherwise, uptake rates were calculated by subtracting the radioactivity or relative fluorescence intensity measured after 2 min of incubation from corresponding 20 min values. The transport of all substrates in the absence of MgATP was less than 5% of the MgATP-stimulated uptake (data not shown). Protoplast preparations were incapable of transporting glutathione conjugates or $E_2$17G.

2.3. Fluorescent dye accumulation in barley and Arabidopsis protoplasts

Mesophyll cell protoplasts were incubated for 1 h at room temperature in the presence of 0.1 mM of the fluorescent dyes monochlorobimane (BmCl) or BCECF-acetoxymethyl ester (BCECF-AM) in medium A (0.5 M sorbitol, 1 mM $CaCl_2$, 10 mM MES/KOH pH 5.8). When needed, glibenclamide was preincubated for 30 min at the concentrations indicated before addition of the dye and kept during all following steps. Protoplasts were washed twice with medium A. In order to facilitate the fluorescent observation of the vacuolar compartment, a partial lysis of protoplasts was triggered by a 10% dilution of the medium. Swelling mesophyll protoplasts were immediately observed under a Nikon Optiphot microscope fitted with the U fluorescence filter set for BmCl fluorescence. Confocal images of protoplasts incubated with BCECF were recorded using the confocal laser microscope Leica DMR and the Leica TCS 4D operating system. BCECF and chlorophyll fluorescence were detected with the filter sets for FITC and TRITC, respectively. The stored images were colored green (BCECF and chlorophyll) or red (chlorophyll only) using Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA, USA).

2.4. Isolation of yeast vacuoles and microsomes

For the preparation of vacuoles and microsomes, the following two strains of *S. cerevisiae* were used: (i) DTY7 (*Matα ura3-52 leu2-3,112 his6*), (ii) W303-1A (*Mat***a** *ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100*). Yeasts were grown in YPD medium. Yeast vacuoles were isolated as previously described [32] but omitting the vesiculation step. Vacuoles were recovered after ultracentrifugation in the 8% (w/v) Ficoll phase by suction into a syringe with a 90°-bent needle from below and immediately used for uptake experiments. Isolation of yeast microsomal vesicles was performed as previously described [13].

2.5. Uptake into yeast vacuoles

Uptake of [^{14}C]DNB-GS and [^{3}H]$E_2$17G into yeast vacuoles was measured at 25°C using the rapid filtration technique. The vacuolar suspension (100 μl) was mixed at time zero with 250 μl of medium B (0.4 M glycerol, 0.1 M KCl, 20 mM Tris–MES pH 7.4) supplied with 1 mM dithiothreitol, 10 μM (0.3 μCi) [^{3}H]$E_2$17G or 15 μM (0.15 μCi) of [^{14}C]DNB-GS, respectively, 1 mM $MgSO_4$ (absence of ATP) or 5 mM Na-ATP and 6 mM $MgSO_4$ (presence of ATP), and glibenclamide added at the concentrations indicated. Transport was terminated after 8 min by transfer of 100 μl aliquots (three replicates) on pre-

wetted filters (0.45 μm nitrocellulose for [^{14}C]DNB-GS and 0.22 μm durapore for [^{3}H]$E_2$17G supplied by Millipore, Volketswil, Switzerland) followed by rapid washes of the filters with cold medium B, all performed under vacuum. Dried filters were suspended into 5 ml of scintillation cocktail. All values were corrected for radioactivity values determined in the absence of ATP where no uptake of both substrates was observed (data not shown).

3. Results

It has been demonstrated that isolated vacuoles or tonoplast vesicles from various plant sources take up GS-X as well as $E_2$17G using directly energized transport systems [6,10,33]. In order to identify substances that could be used to discriminate between different transport activities or MRPs, we investigated the effect of CFTR and SUR modulators upon transport activities of [^{14}C]DNB-GS and [^{3}H]$E_2$17G, two known substrates of MRP transporters.

3.1. Glibenclamide inhibits glucuronide but not glutathione transport activities in barley mesophyll vacuoles

The sulfonylurea glibenclamide strongly inhibited the ATP-dependent transport of [^{3}H]$E_2$17G into barley vacuoles (Table 1). Glucuronide uptake was reduced to 13% of the control value by 150 μM glibenclamide. The inhibitory effect of glibenclamide on [^{3}H]$E_2$17G transport into vacuoles was dose-dependent, with a half-maximum inhibition ($K_{1/2}$) observed at 70 μM (Fig. 1). When 0.2 mM unlabelled DNB-GS was added, the ATP-dependent uptake of [^{3}H]$E_2$17G was increased up to 800% of the control rate as previously demonstrated [10]. Glibenclamide drastically reduced this DNB-GS-stimulated [^{3}H]$E_2$17G uptake activity to 36% of the uptake activity in the absence of DNB-GS. In the presence of DNB-GS as stimulatory agent, the $K_{1/2}$ value for glucuronide uptake inhibition by glibenclamide was significantly lower (12 ± 5 μM; Fig. 1) suggesting that the rise in glucuronide uptake activity induced by glutathione conjugates may also modulate the sensitivity of this transport mechanism towards glibenclamide. In contrast, glibenclamide did not affect the transport of [^{14}C]DNB-GS into barley vacuoles (Table 2). Tolbutamide, another sulfonylurea that was applied at concentrations up to 1 mM, had only negligible effects on either glucuronide or DNB-GS uptake (Table 1). This weak effect of tolbutamide compared to glibenclamide, a second-generation sulfonylurea, is in accordance with results obtained in animal tissues [19].

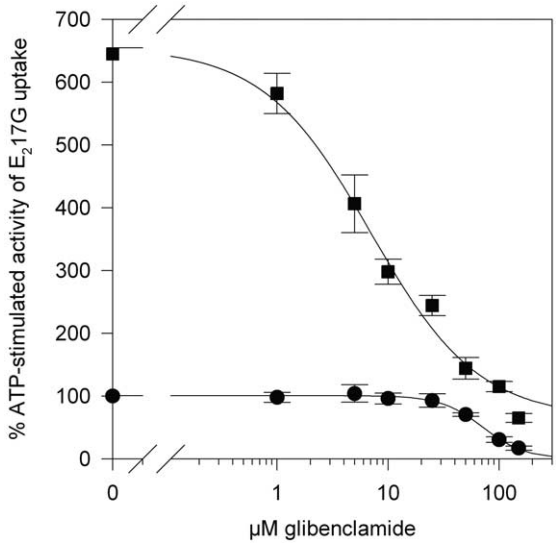

Fig. 1. Dose-dependent inhibition of vacuolar $E_2$17G uptake into barley vacuoles by glibenclamide in the absence (circles) or presence (squares) of 0.2 mM DNB-GS. Barley mesophyll vacuoles were incubated with 11.4 nM $E_2$17G in the presence of 4 mM $MgCl_2$, 3 mM ATP and glibenclamide at the concentrations indicated. A representative experiment with five replicates per condition is illustrated. For 100% rates and further details, see Table 1.

Table 1
Effects of sulfonylureas and KCOs on the ATP-dependent uptake of [^{3}H]$E_2$17G into barley vacuoles

Condition	Vacuolar transport of [^{3}H]$E_2$17G	
	without DNB-GS	+0.2 mM DNB-GS
	% of MgATP-stimulated value	
Control	100	832 ± 320
+150 μM glibenclamide (glib)	12.9 ± 12.7	36 ± 29
+0.1 mM tolbutamide	88.1 ± 7.4	638 ± 9
+1 mM tolbutamide	81.6 ± 5.2	599 ± 86
+10 μM cromakalim (crom)	90.2 ± 2.2	500 ± 15
+100 μM crom	63 ± 9.5	518 ± 13
+1 μM SR47063	87.9 ± 18.53	536 ± 17
+10 μM SR47063	35.6 ± 20.7	298 ± 61
+10 μM RP49356	79.2 ± 4.67	595 ± 11
+100 μM RP49356	63.1 ± 2.62	373 ± 29
+150 μM glib+100 μM crom	2.8 ± 3.9	49.4 ± 5
+15 μM glib+100 μM crom	64.1 ± 5.2	429 ± 56

Barley mesophyll vacuoles were incubated with 11.4 nM $E_2$17G in the presence of 4 mM $MgCl_2$, 3 mM ATP, in the absence or presence of DNB-GS as a stimulating agent and diverse pharmacological compounds interfering with CFTR and SUR. 100% corresponds to an uptake rate of 10.3 ± 1.5 pmol $E_2$17G×/l vacuolar volume/s. The vacuolar uptake rate was calculated by subtracting the radioactivity determined after 2 min from corresponding 20 min values. In the absence of ATP, no uptake was detectable (not shown, see [11]).

KCOs such as cromakalim, SR47063 or RP49356 are known to activate the SUR and to antagonize the inhibitory effects of glibenclamide. The application of 0.1 mM cromakalim had no effect on the vacuolar uptake of [^{14}C]DNB-GS (Table 2) and moderately inhibited the uptake of [^{3}H]$E_2$17G (60% of the control rate, Table 1). When glibenclamide and cromakalim were simultaneously applied, both basal and DNB-GS-stimulated glucuronide transport activities were inhibited to rates already observed in the presence of glibenclamide alone (Table 1) suggesting that these compounds do not compete for the same binding site. Furthermore, cromakalim does not antagonize glucuronide transport inhibition by gli-

Table 2
The ATP-dependent vacuolar uptake of [^{14}C]DNB-GS into barley vacuoles is not affected by glibenclamide

Condition	Transport of [^{14}C]DNB-GS
	% of MgATP-stimulated value
Control	100
+glibenclamide 150 μM	103 ± 9.86
+cromakalim 100 μM	110 ± 5.85
+SR47063 10 μM	76.7 ± 4.67
+RP49356 10 μM	102 ± 4.8
+RP49356 100 μM	67.7 ± 3.68

Barley mesophyll vacuoles were incubated with 50 μM DNB-GS in the presence of 4 mM $MgCl_2$, 3 mM ATP, glibenclamide and KCOs at the concentrations indicated. 100% corresponds to an uptake rate of 67 ± 9 nmol DNB-GS/l vacuolar volume/s. The vacuolar uptake rate was calculated by subtracting the radioactivity determined after 2 min from the corresponding 20 min values.

benclamide as reported for SUR and sulfonylurea-induced stomatal opening [22]. The KCOs SR47063 and RP49356 induced a partial inhibition of organic anion transport, SR47063 being slightly more efficient towards both substrates (Table 1). It is worth noting that the inhibition was similar in DNB-GS-stimulated and non-stimulated conditions.

Vacuoles isolated from an *Arabidopsis* cell culture exhibited a directly energized transport systems for DNB-GS and $E_2$17G. In the presence of 150 μM glibenclamide, the uptake of [^{3}H]$E_2$17G in *Arabidopsis* vacuoles was inhibited to 6% of the control rate while the transport of [^{14}C]DNB-GS was not affected by this sulfonylurea (data not shown). Thus, the glibenclamide sensitivity of directly energized transport systems for DNB-GS and $E_2$17G is not species-specific in plants.

3.2. *Glibenclamide selectively inhibits the vacuolar deposition of anionic fluorescent dyes in barley vacuoles*

Since glibenclamide was a very efficient inhibitor of $E_2$17G but not DNB-GS transport, we investigated its action on the vacuolar accumulation of different negatively charged fluorescent probes used as substrates for ABC-type transport processes. The disulfonated fluorescent dye LY-CH accumulates in barley vacuoles via a directly energized transport system which can be distinguished from GS-X or glucuronide transport systems [9]. The ATP-dependent transport of LY-CH into barley vacuoles could be strongly inhibited by 150 μM glibenclamide (2% of the control; Fig. 2). Vacuolar LY-CH uptake was stimulated by 0.2 mM DNB-GS up to about 920% of the control suggesting a similar interaction with GS-Xs as shown for $E_2$17G. Again, glibenclamide strongly inhibited the DNB-GS-stimulated LY-CH uptake into barley vacuoles (from about 920 to about 5%; Fig. 2) suggesting a comparable pharmacology as shown for $E_2$17G.

BCECF is a widely applied polyanionic pH-sensitive fluorescent probe with four or five negative charges at cytosolic pH values. When barley mesophyll vacuoles were exposed to 25 μM BCECF, uptake could only be observed in the presence of ATP (Table 3). The ATP-dependent vacuolar fluorescence was strongly reduced by 1 mM vanadate but insensitive to bafilomycin A1, a specific inhibitor of the vacuolar H^+-ATPase, and NH_4Cl which produces a dissipation of the pH gradient across the vacuolar membrane (Table 3). This argues strongly for the presence of an MRP-like ABC transporter for BCECF on barley mesophyll vacuoles and confirms results obtained using barley protein storage vacuoles where it was shown to accumulate in the lumen of storage and lytic vacuoles in protoplasts treated with gibberellic and abscisic acid [34]. In contrast to LY-CH and $E_2$17G, the vacuolar uptake of BCECF was not stimulated by different GS-X and was inhibited by oxidized glutathione (GSSG) (Table

Table 3
BCECF uptake into barley vacuoles is mediated by an ABC-type transporter

Treatment		Uptake of BCECF
		% of MgATP-stimulated value
−ATP		0.1 ± 0.1
+MgATP	(control)	100
+MgATP	+1 mM vanadate	5.1 ± 7.3
+MgATP	+0.1 μM bafilomycin A1	103.9 ± 26.0
+MgATP	+5 mM NH_4Cl	129.4 ± 24.7
+MgATP	+150 μM glibenclamide	0.0 ± 2.2
+MgATP	+3 mM GSSG	55.1 ± 13.4
+MgATP	+3 mM GSH	89.8 ± 15.4
+MgATP	+0.2 mM DNB-GS	115.5 ± 6.2
+MgATP	+0.2 mM Decyl-GS	93.8 ± 19.8

Isolated barley vacuoles were incubated with 1 mM $MgSO_4$ (−ATP) or 3 mM ATP and 4 mM $MgSO_4$ (+MgATP), 25 μM BCECF and further compounds as indicated. The relative fluorescence measured in vacuolar supernatants after 20 min is corrected for the fluorescence observed after 2 min reflecting unspecific binding of the dye to vacuolar membranes. The results represent three independent experiments, each with five replicates per condition.

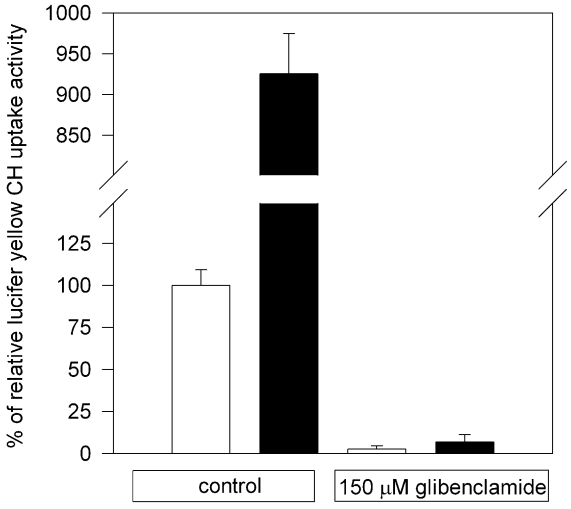

Fig. 2. The ATP-dependent transport of LY-CH into barley vacuoles is strongly inhibited by 150 μM glibenclamide in the absence (open bars) and presence (solid bars) of 0.2 mM DNB-GS. Barley vacuoles were incubated with 50 μM of LY-CH in the presence of 4 mM $MgCl_2$, 3 mM ATP and DNB-GS or glibenclamide as indicated. For the analysis of the vacuolar concentration of LY-CH, the aqueous supernatants of two tubes were pooled and the fluorescence was measured spectrofluorometrically. Illustrated are the results of three independent experiments, each performed in triplicate. 100% corresponds to the relative fluorescence measured in the presence of MgATP after 18 min of uptake. In the absence of MgATP, no uptake was observed.

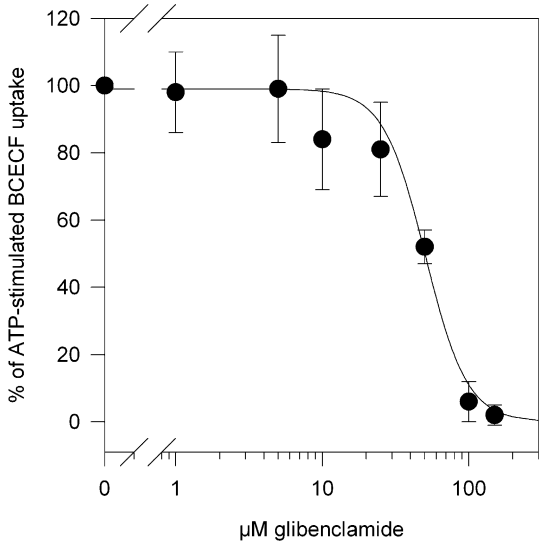

Fig. 3. Dose-dependent effect of glibenclamide on the MgATP-stimulated transport of BCECF into barley vacuoles. Barley mesophyll vacuoles were incubated with 25 μM BCECF, 4 mM $MgCl_2$, 3 mM ATP and glibenclamide at the concentrations indicated. For details, see Table 3.

3). Reduced glutathione (GSH) had no effect on the ATP-dependent transport of this dye into mesophyll vacuoles. Again, the transport of 25 μM BCECF into barley vacuoles was strongly inhibited by glibenclamide. The dose-dependent curve demonstrated a $K_{1/2}$ value of 40 μM for inhibition (Table 3 and Fig. 3).

The localization of BCECF in the central vacuole could be verified with *Arabidopsis* protoplasts incubated with 0.1 mM of BCECF-AM (Fig. 4A,B) using a confocal laser microscope. Furthermore, a strong green fluorescence was detected in smaller structures clearly distinguishable from chloroplasts (Fig. 4C) which may correspond to small vacuoles (Fig. 4B, arrow) [34]. Thus, as stated by Swanson et al. [34], BCECF may be capable of entering different vacuolar compartments. Confirming the vacuolar transport experiments, glibenclamide strongly inhibited the accumulation of BCECF in the central vacuole of *Arabidopsis* protoplasts resulting in a cytosolic localization of the dye (Fig. 4D,E).

Bimane dyes are well-known fluorescent tools to study the intracellular distribution of glutathione conjugates in cells [35]. In the cytosol, a covalently bound glutathione is transferred on the membrane-permeable, non-fluorescent halogenated bimane in a nucleophilic substitution reaction. The fluorescent bimane–GS conjugate is stored in the large central vacuole of mesophyll cells due to the action of a GS-X pump [36]. In *Saccharomyces*, YCF1 is responsible for the deposition of bimane–GS in the yeast vacuole [37]. Barley mesophyll protoplasts incubated in the presence of 100 μM BmCl exhibited the typical blue fluorescence present after enzymatic formation of the bimane–GS conjugate (Fig. 4G,H). In the absence of BmCl, a weak bright blue background fluorescence could be observed probably due to the presence of soluble hydroxycinnamic acids (not shown). The fluorescent bimane–GS mainly accumulated in the large central vacuoles of the mesophyll protoplasts (white arrow in Fig. 4H). When protoplasts were preincubated with 150 μM glibenclamide, no change in the vacuole-localized fluorescence of bimane–GS was observed (Fig. 4I). This result confirms our observation using direct vacuolar uptake experiments with [^{14}C]DNB-GS (Table 2). It can therefore be concluded that the transport not only of DNB-GS but of different glutathione conjugates is insensitive to glibenclamide.

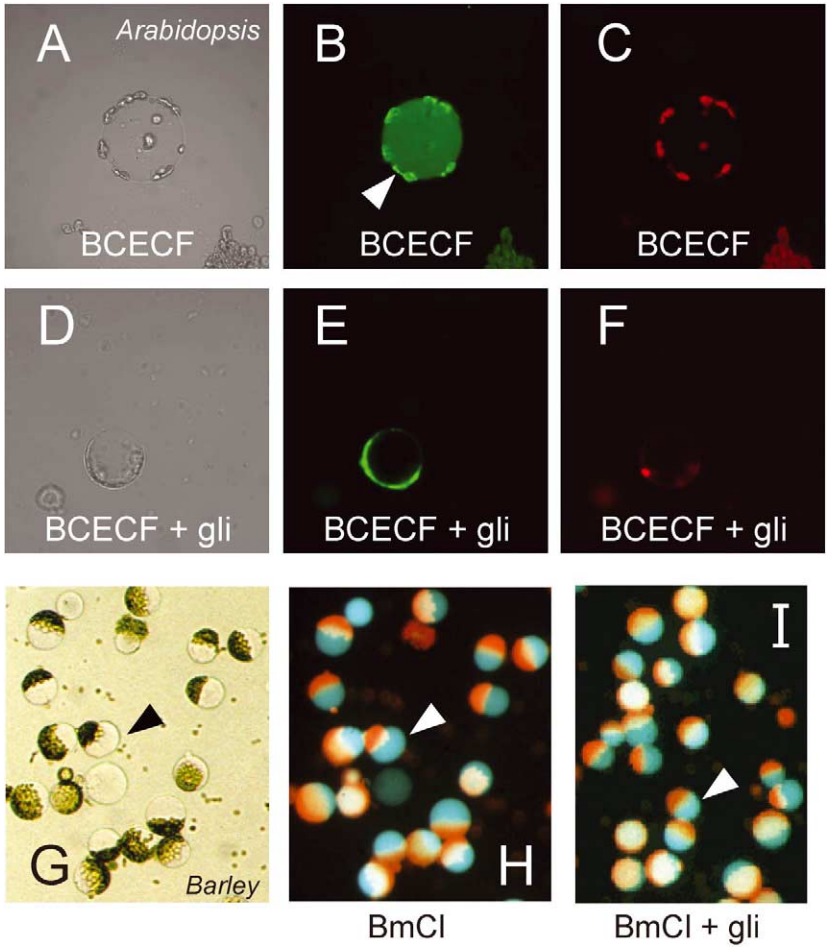

Fig. 4. Effects of glibenclamide on fluorescent probe distribution in protoplasts. A–F: *Arabidopsis* mesophyll protoplasts incubated with 0.1 mM BCECF-AM in the absence (A–C) or presence (D–F) of 0.2 mM glibenclamide were observed using a confocal laser microscope. A,D: Light microscopic images of the false color images B,C and E,F, respectively. B and E were detected with the FITC (BCECF and chlorophyll fluorescence), C and F with the TRITC (chlorophyll fluorescence only) filter set. The arrow in B highlights BCECF accumulation in small vacuoles. G–I: Light and epifluorescence images of barley mesophyll protoplasts incubated with 0.1 mM BmCl in the absence (G,H) or presence (I) of 0.15 mM glibenclamide. Fluorescence microscopy (H,I) using a standard UV filter set shows that bimane–GS is localized within the central vacuole regardless of the presence of glibenclamide (I). G: Light microscopic image of H. Arrows indicate central vacuoles extruding from protoplasts in a swelling buffer.

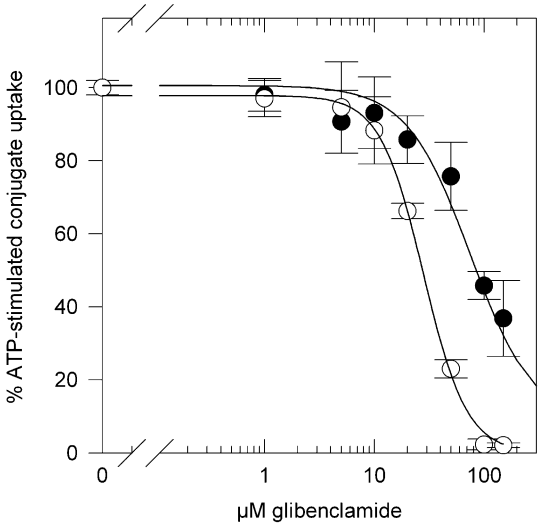

Fig. 5. Dose-dependent glibenclamide inhibition of the ATP-stimulated uptake of $E_2$17G (closed circles) and DNB-GS (open circles) in yeast vacuoles. Yeast vacuoles isolated from the W303-A1 strain were incubated with 10 μM $E_2$17G or 15 μM DNB-GS in the presence of 5 mM MgATP. Uptake was terminated after 8 min by rapid filtration as described. All rates were corrected by subtracting the values measured in the absence of MgATP which were less than 10% of the plus MgATP rates.

3.3. *Glibenclamide inhibition of MRP-mediated transport processes in* S. cerevisiae *vacuolar membranes*

In contrast to plants, [^{14}C]DNB-GS as well as [^{3}H]$E_2$17G transport activities were inhibited by glibenclamide in a dose-dependent manner when intact vacuoles isolated from *S. cerevisiae* strain W303 were used (Fig. 5). The $K_{1/2}$ values observed for glibenclamide were 30 and 70 μM for [^{14}C]DNB-GS and [^{3}H]$E_2$17G substrates, respectively. Glibenclamide inhibition of GS-X transport was also confirmed with a microsomal fraction isolated from the DTY7 yeast strain (data not shown), a strain used for the investigation of the tolerance of yeast to heavy metals and for the glutathione conjugate transport activities of MRPs [13].

4. Discussion

In animal cells, the effects of glibenclamide and KCOs on K-ATP channels have been extensively studied in numerous tissues. The nanomolar affinity of glibenclamide for the SUR1 receptor [38] has to be compared with the micromolar affinity for SUR2 or the CFTR chloride channel [39]. The fact that glibenclamide also inhibits p-gp [21] raises the question of a conserved binding motif exhibited by p-gp, SUR and CFTR. Moreover, in the plant kingdom, it has recently been reported that K^+ and anion channels are inhibited by glibenclamide ($K_{1/2} = 3$ μM) and refreshed by KCOs. These modulators strongly affect the regulation of stomatal movements at the basis of water loss control in plants [22,23].

The increasing number of MRP-like proteins found in animals and plants makes it difficult to differentiate between transporters if activities are measured in crude membrane preparations. We were interested to find pharmaceutical tools in order to distinguish in vitro or in vivo between different transport activities and we could demonstrate that the sulfonylurea glibenclamide differentially affects the transport of glutathione conjugates and other organic anions.

Glibenclamide selectively inhibited the uptake of glucuronide conjugates and other organic anions but not that of glutathione conjugates in barley mesophyll and *Arabidopsis* cell culture vacuoles. In contrast, in *Saccharomyces* both GS-X transport via the vacuolar YCF1 and BPT1 protein and glucuronide transport were drastically reduced by glibenclamide. Thus, the result obtained with vacuoles of the two plant species tested may be true for plants but cannot be generalized if plant and non-plant organisms are compared. The observation that some but not all transport processes mediated by MRPs are inhibited by glibenclamide may indicate that a certain class of MRP proteins contains a glibenclamide binding site while others do not. To our knowledge, little is known about the exact binding site for sulfonylureas, even in the intensively studied CFTR and SUR. It has been proposed that two separate regions in the third and eighth cytosolic loops of SUR1 could be involved in the binding of glibenclamide [40] but only the localization of the amino acids involved in the binding of glibenclamide to SUR will help to elucidate these questions.

To our knowledge, CFTR is the only ABC protein inhibited by KCOs [20]. In humans, properties of these compounds to increase the K^+ permeability have led to their development as vasodilatators, myorelaxants and antihypertensive agents [41]. In plants, KCOs are able to counteract the effect of glibenclamide and to reactivate K^+ or anion channels after a glibenclamide inhibition [22,23]. Thus, in a certain manner the sensitivity of CFTR to KCOs is unique. Herein, we show that cromakalim, SR47063 and RP49356 cannot be used to reverse the inhibitory effect of glibenclamide on transport activities (Tables 1 and 2). Therefore, one could propose that the plant vacuolar ABC transporters studied here are, from a pharmacological point of view, closer to CFTR than to SUR. This hypothesis is in accordance with the fact that the CFTR chloride channel is blocked by glibenclamide and taurocholate or $E_2$17G [42].

Fluorescent dyes are widely used tools to measure temporal and spatial concentration changes of pH, ions or other substances such as glutathione. The observation that glibenclamide inhibits the vacuolar distribution of BCECF in protoplasts could help to overcome some problems of pH measurements in plant cells. It has often been noticed that fluorescent dyes such as BCECF accumulate within the central vacuole [43]. Consequently, the fluorescence in the cytosol is either strongly reduced by a decrease in the cytosolic concentration of the dye or hard to detect due to the strong 'background' signals by vacuolar fluorescence. This results in an inaccurate measurement of cytosolic pH, e.g. by ratio imaging of BCECF. In general, inhibitors of dye transporters would help to target and fix fluorescence signals in the cytosol. A range of different dyes represent potential substrates of MRPs due to the presence of negative charges. Dyes such as chloromethylfluorescein diacetate [44,45], carboxy-2′7′-dichlorofluorescein [46] or the calcium indicator fluo-3 [47,48] are substrates of human MRPs. In contrast, other uncharged dyes, especially different acetoxymethyl ester derivatives of fura-2, fluo-3, indo-1, BCECF or calcein, are used to establish functional assays for p-gp since they are rapidly extruded in multidrug-resistant cell lines [49,50]. In plants, evidence for vacuolar ABC-type transporters for fluorescent dyes was presented for LY [9]. Swanson et al. [34] suggested the presence of a vacuolar ABC-type transport system for BCECF in bar-

ley aleurone cells. As shown in Table 3, BCECF is also transported into lytic mesophyll vacuoles of barley with the typical characteristics of an ABC transporter. Thus, the principle of inhibiting the vacuolar deposition of an indicator dye by glibenclamide forcing cytosolic accumulation could enable or improve measurements of various parameters in the cytosol using fluorescent probes. However, before performing such experiments it must be verified that glibenclamide itself has no effect on the cellular parameter(s) that are desired to be imaged.

Taken together, we propose that glibenclamide can be used as an efficient blocker of some but probably not all MRP proteins ranging from plants and yeast to humans [51–53] and as a tool to investigate vacuolar targeting of fluorescent dyes.

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