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The differential role of Cys-421 and Cys-429 of the Glut1 glucose transporter in transport inhibition by *p*-chloromercuribenzenesulfonic acid (pCMBS) or cytochalasin B (CB)

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Cys-421 and Cys-429 of Glut1 were replaced by site-directed mutagenesis in order to investigate their involvement in basal glucose transport and transport inhibition. Neither of the two cysteine residues was essential for basal 2-deoxy-D-glucose uptake in *Xenopus* oocytes expressing the respective mutant M421 and M429. If applied from the external side, the poorly permeable sulfhydryl-reactive agent pCMBS inhibited 2-deoxy-D-glucose uptake of Glut1- and M421-expressing *Xenopus* oocytes but failed to affect uptake of the Cys-429 mutant. This is in agreement with the proposed two-dimensional model of Glut1 confirming that Cys-429 is the only residue exposed to the surface of the plasma membrane. The replacement of Cys-421 at the exofacial end of helix eleven caused a partial protection of 3-*O*-methylglucose transport inhibition by CB; this residue may thus be involved in stabilizing an adjacent local tertiary structure necessary for the full activity of this inhibitor.

Glut1 glucose transporter; *Xenopus* oocyte; Cysteine mutant

1. INTRODUCTION

Molecular cloning has led to the identification of 5 different glucose transporters of the facilitative type, named Glut1 through Glut5 (reviewed in [1–5]). Derived from the nucleotide sequences of the cDNA the Glut1 glucose transporter is known to contain 6 cysteine residues [6]. Apart from Cys-347, all cysteines are located towards the ends of an α-helix and are expected to be in an aqueous environment. According to the two-dimensional model of Glut1 based on hydropathy plots, Cys-421 is located within an 'amphipathic' segment of helix 11 whereas Cys-429 is exposed to the cell surface. During conformational changes of the glucose trasnporter the cysteines at position 421 and 347 might also be exposed then being accessible to reagents from the exofacial side of the plasma membrane (reviewed in [5,7]).

Structure-function studies of Glut1 by the use of site-directed mutagenesis have been restricted so far to the role of the tryptophan residues concerning their role in glucose transport and inhibition after CB treatment [8,9]. Here we investigated the function of the residues Cys-421 and Cys-429 of Glut1 for (1) the basal glucose uptake, (2) their involvement in sulfhydryl reagent-mediated glucose transport inhibition using pCMBS, and (3) the possible interaction of these residues with transport inhibition by CB. *Xenopus* oocytes were used as a suitable cell system [10–12] which after injection of the respective mRNAs functionally expressed the transporter proteins of the wild-type Glut1 and the cysteine mutants.

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2. EXPERIMENTAL

2.1. *Engineering of Glut1 mutations and in vitro synthesis of mRNA*

A 2.4 kbp *Bam*H1 fragment derived from pSPGT [13] was subcloned into pBluescript SK⁺ (Stratagene, Heidelberg, Germany). Site-directed mutagenesis was performed according to the procedure of Kunkel et al. [14] using a BioRad kit (Bio-Rad, München, Germany). The resulting changes of the mutagenic oligonucleotides and the amino acids are as follows:

Amino acid no.	Nucleotide change	Amino acid change
Cys-421	5'TGC3' → 5'CGC3'	Cys → Arg
Cys-429	5'TGT3' → 5'TCT3'	Cys → Ser

The mutagenesis was confirmed by DNA sequence analysis with appropriate primers. The mutant DNAs were subcloned into the oocyte expression vector pSP64T [15]. In general, mRNAs were prepared from *Xba*I-linearized pSP64T according to the method of Melton et al. [16] modified by adding 0.5 μM ^{7m}GpppG and 50 μM rGTP and 0.8 μCi/ml [^{35}S]UTPαS. Quantitation of newly synthesized mRNA was performed by counting the incorporated [^{35}S]UTP, by determination of the OD_{260}, and by estimation of the respective mRNAs on a denaturing agarose gel using ribosomal 28S and 18S rRNA (Pharmacia, Freiburg, Germany) as standards. The final concentration of newly synthesized mRNA was adjusted to 1 mg/ml.

2.2. Xenopus *oocytes as expression system and transport measurement*

Collection, defolliculation and culture of *Xenopus* oocytes were conducted as described previously [10]. Routinely, oocytes were injected

with 50 nl of a solution containing capped mRNA and collected after 3 days for transport measurements. ^{14}C-labeled 3-*O*-methyl-glucose or 2-deoxyglucose uptake assays were conducted by maintaining groups of up to 10 oocytes in modified Barths' solution (MBS) at a final concentration of 50 μM of the respective glucose analogs. After the indicated time, the oocytes were washed 3 times with 3 ml of ice-cold MBS solution containing 0.1 mM phloretin (i.e. stop solution) and each single oocyte was dissolved in 0.5 ml of 1% SDS before quantitation of radioactivity by a liquid scintillation spectrophotometer.

All substrates used were of highest grade quality. The mutant oligonucleotides were purchased from Molbiol (Berlin, Germany), the radiolabeled hexoses from DuPont (Bad Homburg, Germany); for sequencing the Sequenase version 2 DNA sequencing kit (USB, Cleveland, USA) was used. Cytochalasin B, phloretin and p-chloromercuribenzene sulfonic acid (pCMBS) are from Sigma Chemicals (St. Louis, USA). *Xenopus laevis* frogs were obtained from H. Kähler, Xenopus Laborzucht, Hamburg, Germany. Statistical evaluation was made using the Student's *t* test for paired values conducted with StatView for the Macintosh (Abacus Concepts, Inc.).

3. RESULTS

Table I shows that mRNAs which had been in vitro transcribed from wild-type Glut1-cDNA or the mutant Cys-421- and Cys-429-cDNAs were efficiently translated to functionally active heterologous glucose transporter proteins in *Xenopus* oocytes. No statistically significant differences (P>0.1) of 2-deoxy-D-glucose uptake were observed between the mutants missing the cysteine residues either at the external loop between transmembrane segment (TM) 11 and 12 (M429) or at the exofacial end of the 11th TM (M421), and the wild-type Glut1. Thus, neither cysteine residue in position 421 or 429 appeared to be essential for basal D-glucose uptake by the Glut1 glucose transporter.

Fig. 1 documents that the highly specific thiol-reagent pCMBS [17,18], which very poorly penetrates the plasma membrane in intact cells, inhibited glucose uptake exclusively by reacting with Cys-429. The 2-deoxy-D-glucose uptake was reduced to 28% in Glut1-expressing oocytes or to 24% in the M421 group (averaged value of Exp. 1 and 2). From theses data, Cys-421 is not accessible to inhibition by sulfhydryl reagents at least in a conformation of the glucose transporter that favors glucose binding from outside the plasma membrane (i.e. influx). As oocytes from different experiments do not necessarily express the same amount of glucose transporter proteins, for each group a paired comparison was conducted between untreated- and pCMBS-treated oocytes, thus setting the non-inhibited 2-deoxy-D-glucose uptake to 100%.

Table I

2-Deoxy-D-glucose uptake of the Cys-421 and Cys-429 mutants

2-Deoxy-D-glucose uptake (pmol/oocyte/30 min)			
Exp. 1			
	Sham	*Glut1*	*M 421*
Mean	2.7	74.3	76.2
± S.E.M.	0.4	6.5	6.5
Exp. 2			
	Sham	*Glut1*	*M 429*
Mean	2.5	148.0	125.8
± S.E.M.	0.3	11.0	6.9

In two independent experiments, *Xenopus* oocytes were injected with water (sham) or mRNA encoding the Cys-421 (C→R) and the Cys-429 (C→S) mutants. Three days after injection 2-deoxy-D-[U-^{14}C]glucose (50 μM) uptake was performed in 10–20 single *Xenopus* oocytes. Values represent mean ± S.E.M.

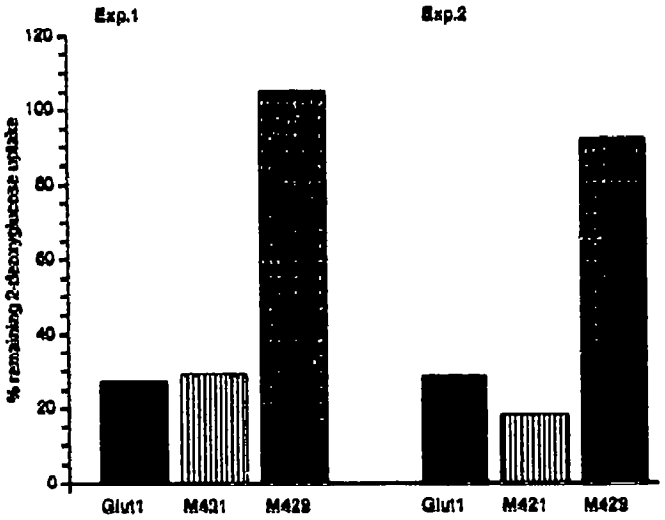

Fig. 1. Effect of pCMBS on 2-deoxy-D-glucose uptake. *Xenopus* oocytes expressing the wild-type Glut1, the M421 or the M429 glucose transporter were exposed to the relatively impermeant sulfhydryl reagent pCMBS (0.5 mM [17]) for 30 min at room temperature. For each group, the percentage of remaining activity was calculated from the differences of the averaged 2-deoxy-D-[U-^{14}C]glucose (50 μM) uptake of untreated and pCMBS-treated oocytes. For the two independent experiments 8–20 oocytes were included in each group.

Observed in three independent experiments, inhibition of 3-*O*-methylglucose transport by the specific transport inhibitor CB was always about half as effective in M421-expressing *Xenopus* oocytes when compared with cells expressing the wild-type or the Cys-429 mutant. In Fig. 2 we compared the remaining transport activity at two different concentrations of CB. Oocytes from the same frog were used in order to directly compare the inhibition between the two mutants. 3-*O*-methylglucose transport was inhibited by 62% (Glut1), 76% (M429), and 30% (M421) at the lower concentration or by 86% (Glut1), 81% (M429), and 57% (M421) at the higher concentration of CB. As shown for both concentrations, the effectiveness of CB-inhibition of glucose transport was dependent on the presence of Cys-421 as can be documented by comparing M421 vs. M429 (P<0.01 for 2 μM CB; P<0.01 for 20 μM CB) or M421 vs. wild-type Glut1 (P=0.01 for 2 μM CB; P<0.01 for 20 μM CB).

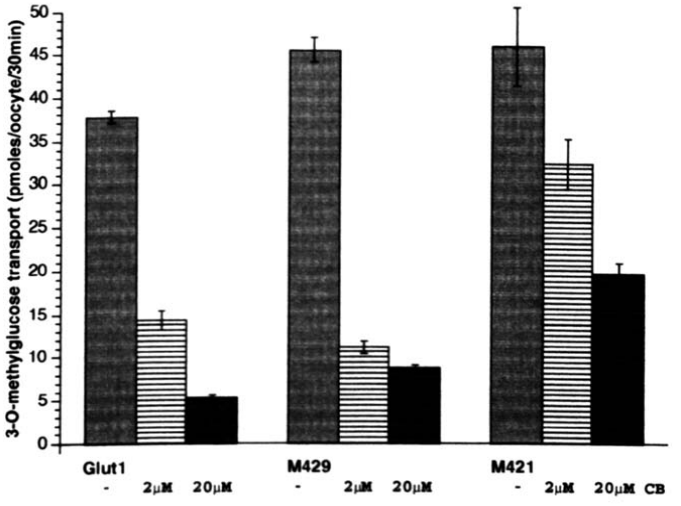

Fig. 2. 3-*O*-Methylglucose transport inhibition by cytochalasin B on oocytes expressing cysteine mutants. 3-*O*-[^{14}C]Methylglucose (50 μM) transport assay was performed in *Xenopus* oocytes expressing the wild-type Glut1 and the Cys-421 or Cys-429 mutants. Transport was inhibited by CB at two concentrations (2 μM and 20 μM). Values present mean ± S.E.M. of 10–20 oocytes from each group.

4. DISCUSSION

The importance of the cysteine residues for the function of glucose transporters has been addressed by many authors (reviewed in [5,7,19]). The role of Cys-421 at the exofacial end of helix 11 and Cys-429 which is located in the extracellular loop between TM 11 and 12 has been of primary interest for ligand binding and transport inhibition by sulfhydryl reagents. In contrast to Cys-207 and Cys-347 that is conserved in Glut1–Glut4, Cys-421 and Cys-429 are expressed exclusively in the Glut1 and Glut2 transporters. In accordance with their expression in only a limited number of tranporter isoforms, these cysteins are not essential for the basal glucose transport.

It has been concluded that among the three cysteins of a 19 kDa carboxy-terminal cleavage fragment Cys-429 is the most likely candidate for binding from the exofacial surface of the tranporter [20]. This assumption, however, has to be confirmed by amino acid sequence analysis. As pointed out by others [5,7] additional cysteins might be also exposed during the confirmational changes of the transporter. Therefore, it remained to be answered whether under defined conditions Cys- 421 is also accessible to ligands reacting with thiols from outside the cell and/or after covalent binding is involved in transport inhibition. PCMBS is known as an effective inhibitory reagent which very poorly penetrates plasma membranes of intact cells [17]. In a conformation of the transporter ready to bind glucose from outside the cell (influx), pCMBS at the used concentration (0.5 mM) was without effect on the Cys-429 mutant, whereas a comparable transport inhibition of both Glut1- and M421-expressing oocytes was observed. With regard to the alternating-conformation mechanism of transport, further studies will have to be conducted to answer the question of whether inhibition from outside the plasma membrane can affect the efflux of glucose from the *Xenopus* oocyte and vice versa after co-injecting the sulfhydryl reagent into the cells.

As crystallization of the glucose transporter has still failed to succeed, site-directed mutagenesis remains at present the only alternative strategy in order to study structure-function relationship. It is well established that CB competitively inhibits glucose transport from the cytoplasmic side by binding at or near the inward-facing substrate binding site of Glut1. We know from mutation of all six tryptophans that Trp-388 at the end of helix 10 is involved in the equilibrium binding of CB [9]. Substitution of Cys-421 by Arg caused a partial protection of the glucose transport inhibition by this specific transport inhibitor. As we failed to perform CB binding in membranes of glucose transporter-expressing oocytes, the putative effect of this point mutation on the K_d for CB remains an open question. However, it seems reasonable to assume that Cys-421 exerts a stabilizing effect on the adjacent helix 10 harboring Trp-388. Since the replacement of Cys-421 cannot completely abolish the inhibitory effectiveness of CB, one might speculate that either Trp-388 still remains a binding site for CB even with lower affinity and/or CB molecules that are bound to other domains are additionally involved.

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