Glucose transport activity and photolabelling with 3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl (IAPS)-forskolin of two mutants at tryptophan-388 and -412 of the glucose transporter GLUT1: dissociation of the binding domains of forskolin and glucose

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The tryptophan residues 388 and 412 in the glucose transporter GLUT1 were altered to leucine (L) by site-directed mutagenesis and were transiently expressed in COS-7 cells. As assessed by immunoblotting, comparable numbers of glucose transporters were present in plasma membranes from cells transfected with wild-type GLUT1, GLUT1-L388 or GLUT1-L412. Transfection of the wild-type GLUT1 gave rise to a 3-fold increase in the reconstituted glucose transport activity recovered from plasma membranes. In contrast, transfection of GLUT1-L412 failed to increase the reconstituted transport activity, whereas transfection of GLUT1-L388 produced only a 70% increase. Photolabelling of GLUT1-L412 with 3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl (^{125}IAPS)-forskolin was not different from that of the wild-type GLUT1, whereas the GLUT1-L388 incorporated 70% less photolabel than did the wild-type GLUT1. These data suggest a dissociation of the binding sites of forskolin and glucose in GLUT1. Whereas both tryptophan-388 and tryptophan-412 appear indispensable for the function of the transporter, only tryptophan-388 is involved in the binding of the inhibitory ligand forskolin.

INTRODUCTION

The diterpene forskolin inhibits the facilitated diffusion of glucose in adipocytes, erythrocytes and platelets by specific binding to a site at the glucose transporter protein (Kashiwagi et al., 1983; Sergeant and Kim, 1985; Kim et al., 1986; Joost and Steinfelder, 1987; Joost et al., 1988; Shanahan et al., 1987; Wadzinski et al., 1987, 1988). This site is believed to be located in close proximity to that of the transport inhibitor cytochalasin B, since forskolin inhibits the specific binding of cytochalasin B to the glucose transporter in erythrocytes and adipocytes (Sergeant and Kim, 1985; Kim et al., 1986; Joost and Steinfelder, 1987; Joost et al., 1988). Interestingly, the different transporter isoforms bind the diterpene with different affinities, in the order GLUT4 > GLUT1 > GLUT3 ≫ GLUT2 (Hellwig and Joost, 1991). For GLUT1, GLUT2 and GLUT4, this ranking order of affinities corresponds strikingly to the differences in the K_m values for glucose (reviewed by Mueckler, 1990). Thus forskolin appears to label a domain of the transporter involved in the recognition of the hexose or in its processing by the transport protein.

All previous attempts to identify the binding sites of cytochalasin B and forskolin by covalent labelling and identification of proteolytic fragments agree in that these sites are located in the C-terminal third of the transport protein (Cairns et al., 1984, 1987; Holman et al., 1986; Holman and Rees, 1987; Karim et al., 1987). A recent study indicated that a mutant of GLUT1 in which tryptophan-388 was exchanged for leucine or glycine exhibited a decreased sensitivity of the transporter activity to cytochalasin B, suggesting that this amino acid participates in the binding of cytochalasin B (Garcia et al., 1992). Furthermore, a photoreactive forskolin derivative, IAPS-forskolin, appears to bind to GLUT1 at a site near or within the membrane-spanning helix 10, possibly at tryptophan-388 (Wadzinski et al., 1990). Data derived from binding of this photolabel to the GLUT4 suggested a covalent binding site near helix 9 (Hellwig et al., 1992).

In the present study we investigated the binding of IAPS-forskolin to two mutants (tryptophan to leucine) of GLUT1 (GLUT1-L388 and GLUT1-L412) in order to identify further the binding region of forskolin. These mutants were expressed in COS-7 cells (Schürmann et al., 1992), and membranes of the transfected cells were photolysed in the presence of the label. Furthermore, the activities of the mutant glucose transporters were assessed by reconstitution in phosphatidylcholine liposomes. The data indicate that forskolin binding is decreased only in the GLUT1-L388 mutant, whereas glucose transport activity is decreased in GLUT1-L388 and abolished in GLUT1-L412.

MATERIALS AND METHODS

Expression vectors

The mutant glucose transporters were generated by oligonucleotide-directed mutagenesis as described previously (Garcia et al., 1992) and were subcloned as *Bam*HI–*Hin*dIII fragments into the expression vector pCMV (Schürmann et al., 1992). This mammalian expression vector comprises the cytomegalovirus promoter, the human growth hormone terminator and a polyadenylation site.

Abbreviations used: GLUT1, erythrocyte/brain-type glucose transporter; IAPS-forskolin, 3-iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl-forskolin.

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DEAE-dextran-mediated transfection of COS-7 cells

Transfection of COS-7 cells with glucose transporter cDNA was performed essentially as described previously (Schürmann et al., 1992). COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and were grown in Dulbecco's modified Eagle's medium (pH 7.4) containing 10% fetal-calf serum, 20 mM Hepes, 2 mM glutamine and 0.1% glucose. The cells were harvested by trypsin treatment and were seeded on to 175 cm^2 culture flasks at a final density of 4×10^6 cells/flask. After 24 h, the cells were washed twice with pre-warmed PBS (138 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) once with TBS-D (25 mM Tris, 138 mM NaCl, 2.6 mM KCl, 0.1% dextrose, pH 7.4), and then 3 ml of TBS-D containing the DNA (15 μg) together with 3 mg of DEAE-dextran was added to each culture dish. Thereafter the cells were incubated at 37 °C and monitored for early signs of distress. Approx. 60 min after addition of the DNA/DEAE-dextran mixture, the medium was removed by aspiration and the monolayers were washed once with TBS-D and a second time with PBS. Thereafter the cells were incubated for 3 h in culture medium containing 100 μM chloroquine. The cells were then washed three times with serum-free medium and incubated with fresh culture medium in a CO_2 atmosphere at 37 °C for 48 h.

Homogenization and fractionation of transfected COS cells

The transfected cells were homogenized and fractionated by a modification of a protocol previously employed in 3T3-L1 cells (Weiland et al., 1990). For each treatment (pCMV, pCMV-GLUT1, pCMV-GLUT1-L388, pCMV-GLUT1-L412), 15 culture flasks (175 cm^2) were used. At 3 days after transfection, cells were washed twice with TES buffer (20 mM Tris, 1 mM EDTA, 255 mM sucrose, 200 μM phenylmethanesulphonyl fluoride). The cells were scraped off the culture flasks with a rubber policeman and were homogenized in a 50 ml Potter–Elvehjem homogenizer (A. H. Thomas Scientific; cat. no. 3431-E25; specific clearance 0.15 mm) with 15 hand strokes. The homogenate was centrifuged at 12000 *g* for 20 min at 4 °C in a JA 20 rotor, and the resulting pellet was layered on a sucrose cushion (38%, w/v, buffered with 20 mM Tris, pH 7.4) and centrifuged for 60 min at 100000 *g* (rotor SW28, 23000 rev./min). Plasma membranes were collected from the interface between buffer and sucrose and were washed once with 20 mM Tris, pH 7.4. The supernatant of the first centrifugation was centrifuged for 30 min at 20000 rev./min in the JA 20 rotor (16000 *g*) to bring down high-density microsomes, consisting mainly of endoplasmic reticulum and contaminating plasma membranes. The resulting supernatant was spun at 200000 *g* in a TI60 rotor for 75 min and yielded a pellet consisting of low-density microsomes. All membrane fractions were suspended in Tris buffer at a protein concentration of approx. 2 mg/ml, and then frozen in liquid N_2 and stored at −75 °C.

Immunoblotting of GLUT1

Antiserum against the GLUT1 was raised with a peptide corresponding to the C-terminus [sequence in one-letter code: (C)EELFHPLGADSQV (Haspel et al., 1988)] of GLUT1 coupled to keyhole-limpet haemocyanin. SDS/PAGE of membranes, transfer on to nitrocellulose membranes and immunoassay with specific serum were performed as described previously (Schürmann et al., 1992).

Photolabelling with IAPS-forskolin

IAPS-forskolin (3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyldeacetyl-forskolin) was prepared as described by Wadzinski et al. (1987) and was stored in ethanol solution at −20 °C. Samples of membranes (100 μl) containing approx. 50 μg of membrane protein were incubated with [^{125}I]IAPS-forskolin (2 μl; final concn. 50 nM) for 30 min on ice. The samples were photolysed by six flashes (setting on 1000 W·s) with the high-pressure mercury lamp of the LIZZY photolysis flash (Raytest, Straubenhardt, Germany). The reaction was immediately quenched by addition of 2 μl of 10% β-mercaptoethanol, and the samples were diluted with 1 ml of ice-cold Tris buffer (10 mM). Membranes were separated by centrifugation in a refrigerated microfuge (15000 rev./min, 30 min), and the resulting pellets were separated by SDS/PAGE (10% gels). The gels were dried and autoradiographed for 2–7 days.

Reconstitution of glucose transport activity from membrane fractions

Glucose transport activity reconstituted into phosphatidylcholine liposomes was assayed as described by Robinson et al. (1982) with minor modifications (Schürmann et al., 1989) under equilibrium exchange conditions at a glucose concentration of 1 mM.

RESULTS

Figure 1 illustrates the immunochemical detection of glucose transporters in membrane fractions from COS-7 cells transfected with pCMV (Co), pCMV-GLUT1 (WT), pCMV-GLUT1-L412 and pCMV-GLUT1-L388. Antiserum against a peptide corresponding to the C-terminus of GLUT1 was used throughout. In the control samples, the serum reacted with a broad band migrating close to the 69 kDa standard. In cells exposed to DNA of wild-type or mutant GLUT1, the transfected glucose transporters were detected as a strong signal at an apparent molecular mass of 45 kDa. Thus the constitutive glucose transporter of the COS-7 cell can clearly be distinguished from the transfected GLUT1 by virtue of its lower electrophoretic mobility. We assume that a heterogeneous glycosylation of the constitutive and the transfected transporter accounts for the difference in their molecular masses (see Discussion).

The antiserum against the C-terminus of GLUT1 also reacted with a 27 kDa band in plasma membranes from transfected cells. This protein appears to be induced by transfection of the cells with transporter DNA, since its abundance is much higher in transfected cells than in the controls (Figure 1). Furthermore, it appeared to be specifically labelled by IAPS-forskolin (see Figure 2). Since its molecular mass corresponds to that of a known tryptic fragment of the GLUT1 (Haspel et al., 1988), it might represent a C-terminal fragment generated by limited proteolysis, during either homogenization of the cells or the electrophoresis of the membranes. In other recipient cells or with transfection of other transporter isoforms, we have so far never detected fragments of the transfected glucose transporters (A. Schürmann, S. Wandel and H. G. Joost, unpublished work).

The amount of immunoreactivity recovered per membrane protein was remarkably similar in all three transfections with glucose transporter cDNA (Figure 1). Furthermore, the different transfections showed no apparent difference in the subcellular distribution of the transfected transporters. Their highest abundance was observed in the plasma-membrane fraction (PM), whereas only a small fraction appeared to be recovered from the low-density microsomes (LD). In addition, the subcellular distribution of the transfected transporters was indistinguishable from that of the constitutive transporter in COS-7 cells (69 kDa). Thus transcription, translation and, with the possible exception

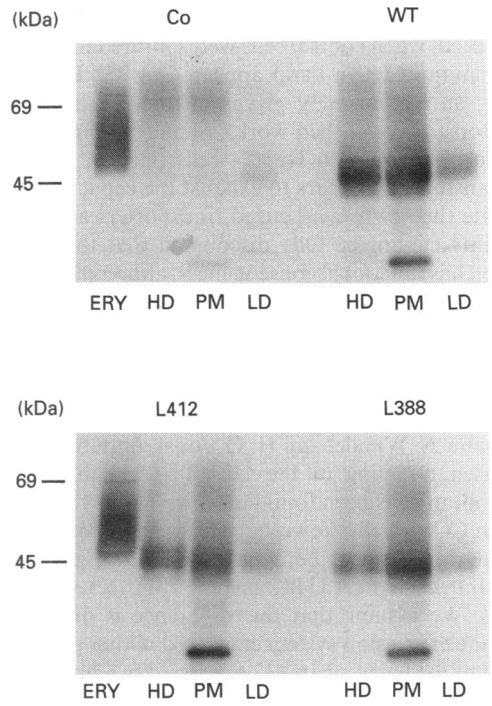

Figure 1 Immunochemical detection of glucose transporters in COS-7 cells transfected with GLUT1, GLUT1-L412 and GLUT1-L388

COS-7 cells were transfected with glucose transporter cDNA as described in the Materials and methods section. Co, cells transfected with pCMV; WT, wild-type cells transfected with pCMV-GLUT1; L412, cells transfected with pCMV-GLUT1-L412; L388, cells transfected with pGLUT1-L388. At 3 days after transfection, cells were homogenized and fractionated as described in the text. Membrane fractions were separated by SDS/PAGE, transferred on to nitrocellulose membranes and probed with specific antiserum against GLUT1. For comparison, samples of membranes from human erythrocytes (ERY) were run in parallel. Abbreviations: HD, high-density microsomes; PM, plasma membranes; LD, low-density microsomes. Apparent molecular masses (kDa) of standard proteins are shown.

Table 1 Reconstituted glucose transport activity in membrane fractions from COS-7 cells transfected with GLUT1, GLUT1-L412 and GLUT1-L388

Plasma membranes from COS-7 cells transfected with glucose transporter cDNA were prepared as described in the Materials and methods section. Glucose transporters were solubilized and reconstituted into phosphatidylcholine liposomes, and stereospecific uptake of D-glucose was assayed. Control, cells transfected with pCMV; wild-type GLUT1, wild-type cells transfected with pCMV-GLUT1; L412, cells transfected with pCMV-GLUT1-L412; L388, cells transfected with pGLUT1-L388. The data represent means ± S.E.M. of quadruplicate samples.

	Reconstituted glucose transport activity (nmol/s per mg of protein)
Control	0.71 ± 0.01
Wild-type GLUT1	2.39 ± 0.11
L412	0.54 ± 0.08
L388	1.22 ± 0.03

of the glycosylation, also the cellular processing of the two mutant transporters appeared to be similar to that of GLUT1.

Table 1 illustrates the glucose transport activities reconstituted from plasma membranes of transfected COS-7 cells. Transfection with the wild-type GLUT1 (WT) increased the reconstituted transport activity approx. 3-fold. In contrast, transfection with

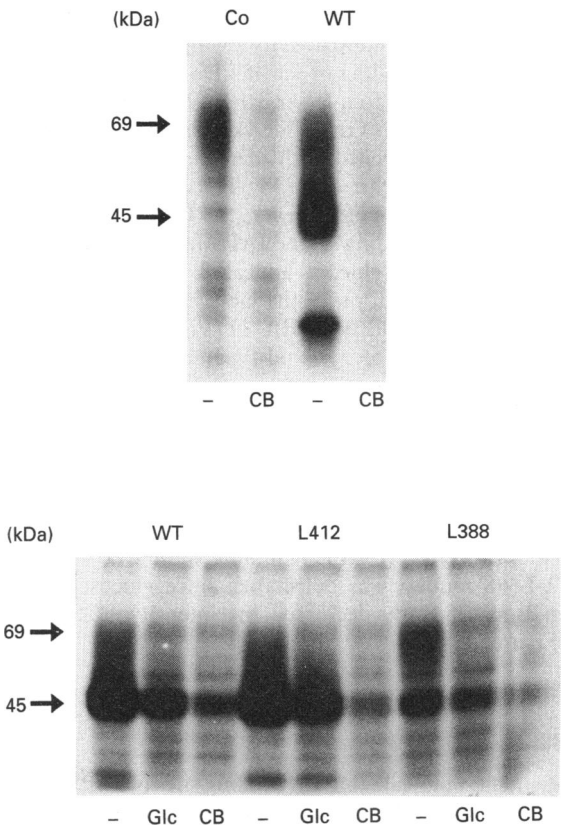

Figure 2 Photolabelling with IAPS-forskolin of plasma membranes from COS-7 cells transfected with GLUT1, GLUT1-L412 and GLUT1-L388

Plasma membranes from COS-7 cells transfected with glucose transporter cDNA were photolabelled with ^{125}I-IAPS-forskolin as described in the Materials and methods section. Co, cells transfected with pCMV vector only; WT, cells transfected with pCMV-GLUT1; L412, cells transfected with pCMV-GLUT1-L412; L388, cells transfected with pGLUT1-L388. The membranes were irradiated with u.v. light in the presence of photolabel only (–), or in the presence of 500 mM D-glucose (Glc) or 10 μM cytochalasin B (CB). The samples were separated by SDS/PAGE and autoradiographed for 7 days. Apparent molecular masses (kDa) of standard proteins are shown.

the GLUT1-L412 mutant failed to increase the reconstituted transport activity, although the plasma membranes contained amounts of glucose transporters comparable with those in the wild-type membranes (see Figure 1). Thus the GLUT1-L412 mutant appeared to be inactive in the reconstitution assay. Furthermore, the activity of the GLUT1-L388 mutant appeared to be considerably lower than that of the wild type, since transfection with its cDNA increased the reconstituted transport activity by only 70% (Table 1).

The transfection of the wild-type GLUT1 increased the glucose transport activity assayed in the intact cells by only 60% (results not shown; see also Schürmann et al., 1992), although it had increased the reconstituted transport activity more than 3-fold (Table 1), in parallel with a large increase in the transporter immunoreactivity (Figure 1). Thus the activities of different transporters transiently expressed in COS-7 cells must be compared by reconstitution, since small differences would not be detectable by assay of the glucose transport activity in intact cells.

Figure 2 illustrates the photolabelling of the glucose transporters with IAPS-forskolin. In control cells transfected with pCMV, only the constitutive glucose transporter (69 kDa) was labelled. In cells transfected with the wild-type GLUT1, photo-

labelling generated a strong additional signal at 45 kDa corresponding to the immunoreactivity of the transfected GLUT1 (see Figure 1). The labelling of the 45 kDa protein was inhibited by cytochalasin B and, to a lesser extent, by glucose (Figure 2). In cells transfected with GLUT1-L412, the glucose transporter (45 kDa) was photolabelled with identical intensity as in cells transfected with wild-type GLUT1. Thus, in contrast with its decreased glucose transport activity, the GLUT1-L412 mutant exhibited a normal binding of the IAPS-forskolin (Figure 2). It should be noted that glucose appeared to be less potent in inhibiting the incorporation of the label into the GLUT1-L412 mutant than into the wild-type GLUT1 (Figure 2); the inhibitory effect of glucose was 55% (wild-type) compared with 45% (GLUT1-L412), as determined by scintillation counting of the radioactivity incorporated into the 45 kDa band. In contrast with that of GLUT1-L412, photolabelling of the GLUT1-L388 mutant was considerably less than that of the wild-type GLUT1; determination of the incorporated label revealed a 70% lower photolabelling of the GLUT1-L388 mutant as compared with that of the wild-type GLUT1. A minute specific labelling of the GLUT1-L388 mutant was detectable, since glucose and cytochalasin B significantly decreased the intensity of the 45 kDa band (Figure 2).

DISCUSSION

The present data indicate that the binding sites of IAPS-forskolin and the domains of GLUT1 involved in the transport of glucose are related, but not identical. The mutation of tryptophan-388 in helix 10 decreased both photoaffinity labelling and glucose transport activity of GLUT1. In contrast, mutation of tryptophan-412 in helix 11 failed to affect its labelling with IAPS-forskolin, but abolished the transport activity of GLUT1, and appeared to decrease the potency of glucose to inhibit its photolabelling with IAPS-forskolin. Thus tryptophan-412 appears to be involved in the binding of glucose only, whereas tryptophan-388 appears to participate in binding of both forskolin and glucose. These data are in good agreement with a recent study in which the transport activity and the inhibitory potency of cytochalasin B in these mutants were compared after expression in *Xenopus* oocytes (Garcia et al., 1992). In that study, the inhibitory potency of cytochalasin B was decreased by one order of magnitude in the tryptophan-388 mutant, whereas it was unaffected in the tryptophan-412 mutant. The activity of the glucose transporter mutated at tryptophan-412 was dramatically decreased, whereas that of the tryptophan-388 mutant was only moderately decreased. Thus the present data not only dissociate the binding domains of glucose and forskolin, but also emphasize the close relationship of the binding sites for cytochalasin B and forskolin in the glucose transporters.

The transient transfection of COS-7 cells with glucose transporter cDNA represents a valid method for studying the structure–function relationship of the transporter proteins. However, two limitations of this system have to be considered. Firstly, transfection usually results in a 3–5-fold increase in glucose transporter immunoreactivity in plasma membranes and in transport activity reconstituted from these membranes. When glucose transport activity was assayed in intact cells, however, the effect of the transfection was only 1.6–2-fold, possibly reflecting a counter-regulatory phenomenon of the intact cell (Schürmann et al., 1992). Thus differences in the activity of transporters and mutants can so far only be studied by reconstitution of transport activity from isolated membranes. Secondly, the differences in the molecular masses of the constitutive and transfected GLUT1 suggest a different glycosylation of the proteins. Indeed, when control cells were cultured in low glucose, a second immunoreactive band appeared at 45 kDa, probably representing an incompletely glycosylated isoform (S. Wandel and H. G. Joost, unpublished work). Similar findings have previously been obtained with HepG2 cells (Haspel et al., 1986). Thus the transfection appears to exhaust the capacity of the cell to glycosylate the newly synthetized transporters appropriately. The possibility cannot be fully discounted that the incomplete glycosylation has functional consequences, although our previous data suggest a comparable (reconstitutable) activity of the constitutive and the transfected GLUT1 (Schürmann et al., 1992). We also observed variations in the electrophoretic mobilities of other transporters (GLUT3 and GLUT4) in transient as well as in stable transfection, depending on the recipient cell (A. Schürmann, S. Wandel and H. G. Joost, unpublished work).

A decreased targeting of the GLUT1-L388 mutant to the plasma membrane has been found in *Xenopus* oocytes (Garcia et al., 1992). In COS-7 cells, however, we observed no difference of the subcellular distribution between the wild-type glucose transporter, the transfected GLUT1, and the transfected mutants in COS-7 cells. We assume that this difference is due to factors specific for the expression system employed. Other previous data on a mutant of the tryptophan-412 expressed in Chinese-hamster ovary cells indicated decreased labelling by cytochalasin B (Katagiri et al., 1991). In contrast, no difference in the transport-inhibiting potency of cytochalasin B was observed when the GLUT1-L412 mutant was expressed in *Xenopus* oocytes (Garcia et al., 1992), and no difference in the binding of IAPS-forskolin was observed here. Thus the present data and those of Garcia et al. (1992) suggest that tryptophan-412 is not involved in binding of inhibitory ligands of glucose transporters, e.g. cytochalasin B and forskolin. Considering the data of Asano and co-workers (Katagiri et al., 1991), we cannot fully exclude a weak interaction of cytochalasin B with tryptophan-412, detectable only by photolabelling with cytochalasin B. It appears reasonable to conclude, however, that the affinity of this interaction would be considerably lower than that of the interaction with tryptophan-388.

Several investigators have used covalent labelling techniques in order to locate the sugar recognition site and the cytochalasin B binding site in GLUT1 (Cairns et al., 1984, 1987; Holman et al., 1986; Holman and Rees, 1987; Karim et al., 1987). These sites appear to be related to the binding site of forskolin, since binding of the diterpene is inhibited by D-glucose as well as by cytochalasin B (Sergeant and Kim, 1985; Joost and Steinfelder, 1987). From the fragmentation pattern of GLUT1 photolabelled with cytochalasin B or an impermeant photoreactive mannose derivative, it was suggested that the cytochalasin B binding site is located in the internal region of the transmembrane helix 10, and that the exofacial sugar-recognition site is located in the outer domain of helix 9 (Holman and Rees, 1987). Furthermore, the binding site of IAPS-forskolin in the erythrocyte-type glucose transporter (GLUT1) has previously been identified by full tryptic digestion and microsequencing of a small labelled fragment (Wadzinski et al., 1990). These data suggested that the label was covalently bound to a site within helix 10. In GLUT4, however, covalent binding of IAPS-forskolin to a site in helix 9 or in its proximity has been described (Hellwig et al., 1992). It was therefore suggested that forskolin binds to more than one contact sites in a region formed by the membrane-spanning helices 9 and 10. The present data support this hypothesis, because the mutation of tryptophan-388 in GLUT1 only decreased, but failed to eliminate, the photolabelling of the glucose transporter. If tryptophan-388 was the only contact site of

forskolin, its mutation would have fully abolished the binding of the label. Thus it is reasonable to conclude that there are additional contact sites of the inhibitory ligand.

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