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Caffeine inhibits glucose transport by binding at the

GLUT1 nucleotide-binding site

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Running title: Caffeine and ATP compete for binding to GLUT1
ABSTRACT

GLUT1 is the primary glucose transport protein of the cardiovascular system and astroglia. A recent study proposes that caffeine uncompetitive inhibition of GLUT1 results from interactions at an exofacial GLUT1 site. Intracellular ATP is also an uncompetitive GLUT1 inhibitor and shares structural similarities with caffeine suggesting that caffeine acts at the previously characterized endofacial GLUT1 nucleotide-binding site. We tested this by confirming that caffeine uncompetitively inhibits GLUT1-mediated 3-O-methylglucose uptake in human erythrocytes ($V_{\text{max}}$ and $K_m$ for sugar uptake are reduced 4-fold; $K_i(app) = 3.5$ mM caffeine). ATP and AMP antagonize caffeine inhibition of 3-O-methylglucose uptake in erythrocyte ghosts by increasing $K_i(app)$ for caffeine inhibition of transport from $0.9 \pm 0.3$ mM in the absence of intracellular nucleotides to $2.6 \pm 0.6$ and $2.4 \pm 0.5$ mM in the presence of 5 mM intracellular ATP or AMP respectively. Extracellular ATP has no effect on sugar uptake or its inhibition by caffeine. Caffeine and ATP displace the fluorescent ATP derivative, trinitrophenyl-ATP from the GLUT1 nucleotide binding site but D-glucose and the transport inhibitor cytochalasin B do not.

Caffeine, but not ATP, inhibits cytochalasin B binding to GLUT1. Like ATP, caffeine renders the GLUT1 carboxy-terminus less accessible to peptide-directed antibodies but cytochalasin B and D-glucose do not. These results suggest that the caffeine binding site bridges two non-overlapping GLUT1 endofacial sites – the regulatory, nucleotide binding site and the cytochalasin B-binding site. Caffeine binding to GLUT1 mimics the action of ATP but not cytochalasin B on sugar transport. Molecular docking studies support this hypothesis.

Keywords: GLUT1, Erythrocyte, ATP, caffeine, Glucose Transport
Introduction

The human facilitative glucose transporter, GLUT1, is the prototypic member of the family of carrier proteins responsible for equilibrative, cellular glucose transport (24). GLUT1 is the primary glucose transporter in smooth muscle (47), astrocytes (55), endothelial cells of blood tissue barriers (47, 59) and in primate and cetacean erythrocytes (22, 24). In human red cells, GLUT1 constitutes 10% of membrane protein (32). The resulting erythrocyte glucose transport is so rapid that the glucose space of the blood available for exchange with metabolically active tissues includes both the serum and erythrocyte intracellular water (24). GLUT1 catalyzes transcellular glucose transport at the blood-brain barrier to provide the primary metabolic fuel for the CNS (24, 55). The central role of GLUT1 in cerebral function and development is evinced by GLUT1-deficiency syndrome in which GLUT1 gene mutations produce GLUT1-haploinsufficiency, which in turn gives rise to seizures, microcephaly, and severe developmental delay in infants (27).

GLUT1 activity, sites of expression and total expression levels are subject to acute and chronic physiologic control (24, 61). In cardiac muscle, fibroblasts, and adipocytes, acute exposure to insulin or hypoxia can increase GLUT1 cell surface expression (7, 19, 30). Acute AMP kinase activation resulting from reduced glucose availability, rapidly and reversibly increases cell surface GLUT1 levels and cellular sugar transport in blood-brain barrier endothelial cells (25). Chronic hypoxia, hypoglycemia and AMP-kinase activation increase GLUT1 gene and protein expression (10) thereby increasing blood brain barrier glucose transport (33, 48) and enhancing glucose transport in some cancers (3, 13, 31).

GLUT1 is allosterically inhibited by ATP, which binds at a single, ATPase-null, GLUT1 nucleotide binding site (17, 35). Mutagenesis and peptide mapping studies localize the adenosine nucleotide-binding site to the endofacial surface of GLUT1 involving cytoplasmic loop
8-9 and transmembrane helices 8 and 9 (43, 45). ATP binding leads to GLUT1 conformational change involving the cytoplasmic carboxy terminus and large intracellular loop 6-7 (9). These structural changes result in decreased $K_m$ and $V_{max}$ for zero-trans sugar uptake characteristic of uncompetitive inhibition (17, 43, 44). AMP binds to GLUT1 and acts as a competitive antagonist of ATP-inhibition of glucose uptake (17).

GLUT1-mediated sugar transport is also inhibited by families of structurally diverse small molecules, which affect the kinetics of transport in different ways (1, 51). Cytochalasin B (CB) is a micotoxin that binds at the endofacial surface of GLUT1 and functions as a competitive inhibitor of exchange and net sugar efflux and as a noncompetitive inhibitor of net uptake (6). Barbiturates such as phenobarbital, in contrast, appear to act as noncompetitive inhibitors of net sugar uptake and exit but as competitive inhibitors of exchange transport (30). The methylxanthines comprise an additional class of GLUT1 inhibitors (18). Among these, caffeine (1,3,7-trimethylxanthine) is most commonly encountered in a normal diet. Indeed 80% of the U.S. population consumes caffeine daily, making it the most widely used psychoactive drug in the world (34). Given the widespread use of caffeine and the central role of GLUT1 in cerebral metabolism, understanding how caffeine inhibits GLUT1 could be useful in the management of organismal carbohydrate homeostasis in health and disease.

In the present study, we ask whether the uncompetitive inhibition of GLUT1 produced by caffeine (38, 52) and ATP (17) and the structural similarities between caffeine and adenosine reflect a common mechanism of action on GLUT1.
Materials and Methods

Materials

$^3$H 3-O-methylglucose ($^3$H-3-OMG) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled 3-OMG, Cytochalasin B (CB), phloretin, ATP, AMP, and D-glucose were purchased from Sigma Aldrich (St. Louis, MO). Trinitrophenyl-ATP (TNP-ATP) was purchased from Life Technologies (Carlsbad, CA). Custom synthesized rabbit anti-GLUT1 C-terminal antibody (C-Ab) was produced by New England Peptide and was raised against GLUT1 residues 480-492.

Erythrocyte and ghost preparation

De-identified whole human blood was purchased from Biological Specialty Corporation (Colmar, PA). Erythrocytes were washed three times with cold wash buffer 150 mM KCl, 5 mM HEPES (pH 7.4) and 0.5 mM EDTA and prepared for transport experiments as previously described (53). Ghosts were prepared from washed erythrocytes as previously described (53) and resealed with erythrocyte wash buffer containing indicated concentrations of ATP or AMP.

Transport measurements

All transport reactions were performed in triplicate at ice temperature using 20 µL of a 50% suspension of either erythrocytes or ghosts as previously described (53). Uptake was initiated by the addition of 100 µL of uptake solution containing 2.5 µCi/mL $^3$H-3-OMG and the indicated concentrations of unlabeled 3-OMG and caffeine. After 30 s, reactions were stopped by addition of 1 mL ice-cold stop solution containing 10 µM CB and 100 µM phloretin in PBS. Cells were pelleted and washed once with 1 mL stop solution, then lysed with 0.5 mL of 3% perchloric acid. Clarified lysates were assayed in duplicate for the radiolabel using liquid scintillation counting.
**Kinetic analysis**

Non-linear regression analysis of transport data was performed using GraphPad Prism version 6. The concentration dependence of 3-OMG uptake was fit to Eq. 1

\[
v = \frac{V_{\max} [S]}{K_m + [S]}
\]  

(1)

where \( v \) is the rate of 3-OMG uptake, \([S]\) is the 3-OMG concentration and \( V_{\max} \) and \( K_m \) are the velocity and Michaelis constants respectively for transport. Caffeine dose-response data were fit to Eq. 2

\[
uptake = v_0 - \frac{v_i [I]}{K_{i(app)} + [I]}
\]  

(2)

where \( v_0 \) is the amount of sugar uptake in the absence of caffeine, \([I]\) is the caffeine concentration, \( v_0 - v_i \) is the sugar uptake remaining at saturating [caffeine], and \( K_{i(app)} \) is that concentration of caffeine reducing transport by \( v_i/2 \).

**TNP-ATP binding competition assay**

GLUT1 protein was purified to ≥ 90% purity from washed erythrocyte membranes as previously described (36). Binding reactions were assayed at room temperature in 50 mM Tris-HCl pH 7.4, 5 mM MgCl2 by adding 100 nM purified GLUT1 to 5 μM TNP-ATP. After a 2 min incubation to permit equilibrium binding (21), interfering ligands were added and the suspension assayed for fluorescence at 500 – 600 nm with excitation at 408 nm (Photon Technologies International, Edison, NJ). Data were plotted using GraphPad Prism version 6 (La Jolla, CA) and a locally weighted scatter plot-smoothing (LOWESS) curve fit.
**3H-Cytochalasin B binding**

3H-CB (100 nM) binding to purified human GLUT1 (1 µg GLUT1 in 40 µL reaction medium) was measured as described previously (36).

**C-terminal antibody accessibility assay**

ELISA determination of GLUT1 C-terminal antibody binding was performed as previously described (9). Briefly, 200 ng of purified GLUT1 in PBS was bound to 96-well microtiter dishes at 37°C for 2 h. Plates were blocked with 3% bovine serum albumin (BSA) in PBS with the indicated concentrations of ligand at 37°C for 2 h. Bound protein was probed with C-terminal antibody solutions (0.3 µg/mL) containing indicated concentrations of ligand at 37°C for 2 h followed by five washes with PBS. Each well was then incubated with HRP-conjugated goat anti-rabbit secondary antibody (0.5 µg/mL) and ligand for a further 2 h. After washing 5 times with PBS, wells were developed with ABTS (Pierce). Absorbance was measured at 415 nm using an iMark microplate reader (Bio-Rad).

**Docking studies**

The GLUT1 crystal structure was obtained from the protein databank (http://www.rcsb.org/pdb/home/home.do) using the pdb code 4PYP (28). The β-nonylglucoside molecule was removed from the pdb structure. Structures for ATP, caffeine, and CB were obtained from ZINC (http://zinc.docking.org) (40). Computational docking was performed with AutoDock Vina (58). The ligands and protein were prepared for docking using Autodock Tools Version 1.5.6 (50, 54). Docking was constrained to a cube with dimensions of 44 x 28 x 28 Å volume encompassing loop 6 and the endofacial transport cavity of 4PYP.
Results

Caffeine is an uncompetitive inhibitor of 3-OMG uptake by human erythrocytes

The reported uncompetitive inhibition of erythrocyte sugar uptake by caffeine (38, 52) represents a relatively unusual form of transport inhibition. To confirm the inhibitory nature of caffeine on GLUT1, the uptake of 100 µM 3-OMG into sugar-free erythrocytes was measured over an interval of 30 s at ice temperature. Under these conditions, transport allows approximately 33% equilibration of cell water space with substrate, ensuring the measurement of close to initial rates of transport (53).

Exposure to caffeine produces a robust and dose-dependent inhibition of 3-OMG transport with an apparent $K_i$ of $3.5 \pm 0.2$ mM (Figure 1A). Analysis of the concentration dependence of 3-OMG uptake in the absence and presence of 5 mM caffeine indicates that caffeine reduces both $V_{\text{max}}$ and $K_m$ for sugar uptake (Figure 1B). $V_{\text{max}}$ for transport fall from $151 \pm 12$ µmol/L/min to $32 \pm 1$ µmol/L/min. $K_m$ falls from $1.4 \pm 0.3$ mM to $0.4 \pm 0.1$ mM (Figure 1B). This result is characteristic of uncompetitive inhibition and is consistent with previous studies of caffeine-mediated GLUT1 inhibition (38, 52). The decreases in $V_{\text{max}}$ and $K_m$ induced by caffeine are of the same magnitude ($4.7 \pm 0.6$ and $3.5 \pm 2.2$-fold respectively).

ATP antagonizes caffeine-mediated transport inhibition

Uncompetitive inhibition of GLUT1 by ATP has previously been described by our laboratory (17, 43, 44). Cytoplasmic (but not extracellular) ATP causes a dose-dependent decrease in both $V_{\text{max}}$ and $K_m$ for zero-trans sugar uptake (8- and 18-fold respectively). This unbalanced effect of ATP on $V_{\text{max}}$ and $K_m$ contrasts with the more balanced reduction in both parameters induced by caffeine and results in an apparent stimulation of net sugar transport under subsaturating [3-OMG] conditions. Reduction of $V_{\text{max}}$ and $K_m$ by ATP and caffeine suggests a common
mechanism of action - a hypothesis further supported by the structural similarities between
adenosine and caffeine.

To explore this possibility, we investigated the effects of ATP on caffeine-mediated GLUT1
inhibition. As with erythrocytes, resealed erythrocyte ghosts are inhibited by caffeine in a dose-
dependent manner with an apparent $K_i$ of $0.91 \pm 0.34$ mM (Figure 2, no ATP). When ghosts are
resealed with either 5 mM ATP or AMP, $K_i$ increases to $2.6 \pm 0.6$ mM and $2.4 \pm 0.5$ mM
respectively (Figure 2, ATP or AMP inside). The effect is specific to the cytoplasmic side of the
membrane, as including ATP in the uptake solutions and not inside the ghosts resulted in an
apparent $K_i$ of $0.96 \pm 0.63$ mM (Figure 2, ATP outside). The antagonism of caffeine-mediated
GLUT1 inhibition by only intracellular adenosine nucleotide (Figure 2) is consistent with existing
evidence indicating that ATP binds to GLUT1 at a cytoplasmic nucleotide binding site (44), and
suggests that caffeine also functions at the cytosolic surface of the protein. $K_{d_{app}}$ for ATP- and
AMP-antagonism of caffeine-inhibition of 3-OMG transport (assuming simple, competitive
antagonism) are $2.7 \pm 0.8$ and $3.1 \pm 0.7$ mM respectively. Recent studies suggest that $K_{d_{app}}$ for
ATP- and AMP-binding to GLUT1 are 0.6-2 mM and 2.2 mM respectively (9).

**Effects of Caffeine on nucleotide and cytochalasin B binding to GLUT1**

ATP-antagonism of caffeine-inhibition of glucose transport suggests that ATP and caffeine
compete for binding to GLUT1. Competition for binding could result if ATP and caffeine bind at a
common site or if ATP and caffeine binding sites are physically distinct but mutually exclusive.
To test for competitive binding, we evaluated the ability of caffeine to interfere with the binding
of the fluorescent ATP analog, TNP-ATP to GLUT1 protein purified from human erythrocytes.
TNP-ATP mimics the effect of ATP on GLUT1-mediated 3-OMG transport kinetics (21). When
bound to purified GLUT1 in unsealed proteoliposomes, the probe exhibits an enhanced and
blue-shifted fluorescence (Figure 3A “control”). This bound fluorescence is unaffected by either
the presence of 5 mM D-glucose or the well-characterized GLUT1 inhibitor, CB (10 µM) (Figure 3A). The addition of 5 mM ATP, however, displaces pre-bound TNP-ATP, reducing fluorescence and restoring red-shifted fluorescence. This effect is mimicked by the addition of 5 mM caffeine (Figure 3A). Control reactions lacking GLUT1 reveal that TNP-ATP autofluorescence in the absence of protein is modulated by some ligands (Figure 3A, dashed lines). Following correction of fluorescence maxima for these changes, we observe that ATP and caffeine produce 28% and 27% reductions in TNP-ATP fluorescence respectively, while D-glucose and CB are without effect (Figure 3B). These results demonstrate that ATP and caffeine compete with TNP-ATP for binding to GLUT1. Caffeine inhibits ³H-CB binding to human GLUT1 but ATP does not (Table 1). D-Glucose competitively displaces CB from GLUT1 (62).

Caffeine and ATP induce similar structural changes in GLUT1

ATP binding to GLUT1 promotes GLUT1 conformational changes involving the cytosolic C-terminus and the large intracellular loop connecting trans-membrane helices 6 and 7 (9). One consequence of these changes is reduced GLUT1 C-terminal peptide-directed antibody (C-Ab) binding to GLUT1 in an ELISA-based assay (9, 17). We explored the possibility that caffeine may induce comparable structural changes in GLUT1. Purified GLUT1 was adsorbed to microtiter ELISA wells and probed with C-Ab in media containing increasing concentrations of either ATP or caffeine. Similar to previous findings, we observe that ATP induces a dose-dependent decrease in antibody binding to the C-terminus (9, 17). Interestingly, this effect is also seen with caffeine suggesting that caffeine binding to GLUT1 promotes similar GLUT1 conformational changes in (Figure 4). Neither the inclusion of 5 – 20 mM D-glucose nor 5 – 20 µM CB results in significant perturbation of C-Ab binding (Figure 4). Caffeine and ATP have no effect on C-terminal antibody recognition by HRP-conjugated secondary antibody over the concentrations of ligand used (data not shown).
Molecular docking analysis

We undertook a docking analysis of caffeine, ATP and CB binding to the recently published structure of human GLUT1 (28). Several putative binding sites are obtained for all 3 ligands. Figure 5 summarizes ATP, caffeine and CB binding at their highest affinity sites in GLUT1. While these studies are in silico and require biochemical verification, a number of points are worthy of comment: 1) The ATP and caffeine binding sites persist in both the e1 conformation of GLUT1 and in an e2 conformation obtained by homology modeling GLUT1 on the XylE e2 conformation but the CB binding site is lost in the e2 conformation (not shown). This is consistent with the observation that caffeine and ATP are uncompetitive with respect to sugar uptake but CB is a noncompetitive inhibitor of sugar uptake and a competitive inhibitor of sugar exit (6, 16). 2) ATP has previously been demonstrated to interact with GLUT1 transmembrane helices (TMs) 8 and 9 (44). 3) These highest affinity docking sites suggest that ATP and caffeine binding sites overlap, that caffeine and CB binding sites overlap but that ATP and CB binding sites do not overlap. These predictions are consistent with the ligand binding studies reported here (Figure 3 and Table 1).
Discussion

GLUT1 caffeine binding site

This study demonstrates that caffeine and TNP-ATP compete for binding to isolated, human GLUT1. Several mechanisms could explain this. 1) Caffeine and ATP share identical or overlapping binding sites. 2) Caffeine binds at an endo- or exofacial site that is physically distinct from the ATP binding site but where ATP or caffeine binding promotes a conformational change in the competing ligand’s binding site resulting in an indirect competition. Caffeine is also a competitive inhibitor of cytochalasin B binding to GLUT1 (52). We too observe caffeine inhibition of GLUT1 CB binding but find that cytochalasin B and ATP binding are not mutually exclusive. If caffeine inhibition of ATP and cytochalasin B binding results from steric overlap, this suggests that the caffeine binding site must bridge the otherwise independent endofacial ATP- and cytochalasin B-binding sites.

Ojeda and colleagues have previously demonstrated that caffeine is an uncompetitive inhibitor of GLUT1-mediated sugar uptake, a noncompetitive inhibitor of sugar exit into sugar-free medium and a noncompetitive inhibitor of GLUT1-mediated equilibrium exchange sugar transport (52). The absence of competitive inhibition establishes that caffeine does not bind at the endo- or exofacial sugar binding sites of GLUT1 (16). One curious observation made by Ojeda and colleagues is that the methylxanthines caffeine and theophylline decrease $V_{\text{max}}$ for infinite-cis sugar exit (efflux of sugar from cells containing saturating [sugar] into media containing varying [sugar]) without affecting the affinity of the external sugar binding site for sugar. However, pentoxifylline (a methylxanthine containing a 5-oxohexyl group in place of a methyl group at position 1 of the purine) reduces $V_{\text{max}}$ for infinite-cis exit but increases $K_{\text{m(app)}}$ for sugar interaction with the exofacial sugar binding site (52). These results were interpreted to be consistent with an exofacial methylxanthine binding site independent from the substrate-binding
site. Kinetic experiments of this type, however, do not permit unambiguous interpretation of site or sidedness of action (16, 29, 56). While definitive characterization of the GLUT1 methylxanthine binding site(s) awaits either direct labeling studies or mutagenesis of putative interaction domains, molecular docking studies can provide some insights.

Molecular docking studies suggest that the so-called "e1" conformation of GLUT1 (28) binds cytochalasin B, ATP and caffeine in decreasing order of affinity. Although several permutations of binding sites were computed for each ligand, the highest affinity binding sites are consistent with the transport and ligand-binding studies presented here and with previous biochemical analyses of ATP and CB binding to GLUT1. ATP is predicted to interact with TMs 8 and 9 – a GLUT1 region previously demonstrated to become covalently labeled by azido-ATP (44). CB is predicted to interact with the cytoplasmic loops of GLUT1 and especially with those extending between TM10 and 11 – a region previously demonstrated to be essential for CB binding (39).

The predicted ATP and caffeine binding sites persist in a GLUT1 “e2” conformation obtained by homology modeling GLUT1 on the XylE e2 conformation (57) but the CB binding site is lost in the e2 conformation (not shown). These docking studies suggest that sites occupied by the purine groups of ATP and caffeine overlap, that the methyl group at position 1 of caffeine sterically hinders binding of the toluene ring of CB but that ATP and CB binding sites do not overlap - predictions consistent with the ligand binding studies reported here (Figure 3 and Table 2). Docking studies also suggest that the purine group of pentoxifylline overlays the space occupied by the ribose moiety of GLUT1-liganded ATP (not the purine binding site occupied by the adenine moiety of ATP nor the purine of caffeine) and extends its 5-oxohexyl group into space occupied by the triphosphate group of ATP or into a hydrophobic pocket between TMs 7, 8 and 10 (not shown). This supports the hypothesis that pentoxifylline binding to GLUT1 may not involve the GLUT1 purine binding site and thus provides a rationale for the different effects
of caffeine and pentoxyfilline on GLUT1-mediated sugar transport observed by Ojeda and colleagues (52).

**GLUT1 structural changes induced by caffeine binding**

ATP binding promotes GLUT1 conformational changes involving the cytoplasmic C-terminus and loop 6-7 (9, 17). The structural similarities between adenosine and caffeine, and the observation that caffeine binding also occludes the GLUT1 C-terminus in a dose-dependent manner suggests that caffeine and ATP binding to GLUT1 promote similar structural changes. ATP binding drives transport inhibition by bringing the C-terminus of GLUT1 in close proximity to the C-terminal half of intracellular loop 6-7 (9). Quench flow experiments in erythrocytes indicate that rapid translocation of sugar through the transport pore is unaffected by ATP, however, the release of sugar into the cytosol is significantly slowed (8). It has been suggested, therefore, that the GLUT1 C-terminus and loop 6-7 form a cytosolic “cage”, trapping the substrate in the transport channel thereby preventing its release. A similar, “ball and chain” mechanism mediates Na and K channel inactivation (2, 49). The trapped sugar has a greater probability of reentering the translocation pore thereby promoting its return to the extracellular environment and thus causing the apparent saturation of sugar uptake at lower extracellular sugar concentrations (15). The net effect (reduced $V_{\text{max}}$ and $K_m$) resembles uncompetitive inhibition in which the inhibitor stabilizes the enzyme-substrate complex preventing catalysis and product release (transport).

Given these considerations, we propose that caffeine-inhibition of GLUT1-mediated sugar transport mirrors the mechanism of action of ATP on transport. In this model, caffeine binding at a cytoplasmic domain of the transporter leads to a stabilized interaction between the GLUT1 C-terminus and loop 6-7. This interaction results in a physical barrier preventing sugar release into the cell and, therefore, decreased net sugar uptake. It should be emphasized that this
explanation of transport inhibition by caffeine and ATP applies equally well to the simple
(alternating conformer) carrier model for sugar transport which presents only one sugar binding
site (exofacial or endofacial) at any instant (41, 60) and to the multi-site carrier model in which
the transporter presents exofacial, endofacial and cavity (occluded) sugar binding sites
simultaneously (4, 5, 15, 23). The multi-site carrier model is consistent with experimental
observations showing that glucose becomes physically occluded within GLUT1 only in the
presence of CCB (8) and that occupancy of an internal ligand binding site can exert a negative
or positive allosteric effect on ligand binding to an external site and vice versa depending on the
nature of the ligands that bind (16, 20, 37). In sum, the effects of caffeine on sugar transport
support previous demonstrations that sugar binding at exofacial domains may be subject to
modification by occupancy of the carrier by endofacial ligands.

**Implications for GLUT1 inhibition by caffeine**

Our studies and those of previous groups indicate that membrane resident GLUT1 is profoundly
inhibited by caffeine. This effect is observed at high concentrations with half-maximal inhibition
seen at 2.5 - 3.5 mM caffeine. Serum caffeine levels typically measured in humans are
approximately 6 µM (14) suggesting that the sugar transport inhibition resulting from normal
consumption of caffeine is less than 1%.

Glucose transport across the endothelial cells of the blood-brain barrier into the brain is also
catalyzed by GLUT1 (55). GLUT1 mutation can cause impaired glucose transport across the
blood brain barrier resulting in severe neurological disorders (38). Currently, the clinical
recommendation for such individuals is to avoid the consumption of caffeine due to a possible
exacerbation of glucose transport deficiency (11). In light of the results presented here and the
absence of clear clinical evidence, it seems unlikely that normal caffeine consumption (≤ 300
mg/day; (46)) could give rise to significant inhibition of GLUT1-mediated glucose transport
unless caffeine or its metabolites are concentrated in GLUT1-expressing membranes. Caffeine also enhances Ca release via the sarcoplasmic reticulum Ryanodine receptor (63) and inhibits cAMP-phosphodiesterase (12). Thus it is possible that blood brain barrier glucose transport could be impacted via elevated cytoplasmic Ca or cAMP levels. However, the IC$_{50}$ for caffeine modulation of these off-target effects is $> 1$ mM suggesting again that normal caffeine consumption is unlikely to inhibit sugar transport via these pathways. The arousal effects of caffeine appear to be mediated by high affinity antagonism at adenosine A$_2$A receptors present in specific areas of the CNS (26, 42). It is possible therefore, that increased neuronal activity resulting from CNS arousal could exacerbate demand for an already limited glucose supply in GLUT1-deficiency.

**Conclusions**

ATP and caffeine compete for binding to the endofacial, GLUT1 nucleotide-binding site and act as uncompetitive inhibitors of GLUT1. The action of caffeine on glucose transport is half-maximal at concentrations at least 2 orders of magnitude greater than those produced by normal consumption of caffeine.
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**Figure Legends**

**Figure 1**  
**A** Concentration dependence of caffeine inhibition of 100 µM 3-OMG uptake.  
Ordinate: rate of sugar uptake in µmol/L cell water/min; Abscissa: Caffeine concentration in the uptake medium, mM. Results are shown as mean ± SEM for at least 4 measurements made in duplicate. The curve drawn through the points was computed by nonlinear regression assuming that saturable inhibition of transport is described by eqn 2. The results are: $V_0 = 16.7 \pm 0.2$ µmol/L/min; $V_i = 18.1 \pm 0.3$ µmol/L/min; $K_{i(app)} = 3.5 \pm 0.2$ mM, $R^2 = 0.99$.  

**B** Caffeine is an uncompetitive inhibitor of 3-OMG uptake by human RBCs. Ordinate: rate of sugar uptake in µmol/L cell water/min; Abscissa: extracellular 3-OMG concentration in mM. Results are shown as mean ± SEM for at least 4 measurements made in duplicate with control cells (○) and for cells exposed to 5 mM Caffeine (●). Control experiments indicate that results obtained by preincubating cells with caffeine for 15 minutes are indistinguishable from the those obtained by simply adding caffeine during the 30 seconds of sugar uptake measurement. The curves drawn through the points were computed by nonlinear regression assuming that transport is described by Michaelis-Menten kinetics (eqn 1). The results are: Control, $V_{\text{max}} = 151 \pm 12$ µmol/L/min; $K_m = 1.3 \pm 0.3$ mM, $R^2 = 0.87$; Caffeine, $V_{\text{max}} = 32 \pm 2$ µmol/L/min; $K_m = 0.4 \pm 0.1$ mM, $R^2 = 0.80$.

**Figure 2**  
Dixon plot of caffeine inhibition of 3-OMG uptake in RBC ghosts. Ordinate: 1/sugar uptake (min/mol/L); Abscissa: caffeine concentration in mM. Results are shown as mean ± SEM for at least 3 measurements made in duplicate and are shown for control ghosts (○), ghosts containing 4 mM (●), ghosts containing 4 mM AMP (△) and nucleotide-free, resealed ghosts exposed to 4 mM extracellular ATP (▲). The lines drawn through the points were computed by linear regression and have the following constants: control slope = 337.0 ± 44.9 min, x-intercept = -0.91 ± 0.34 mM, y-intercept = 305,226 ± 111,768 min/M, $R^2 = 0.72$; 4 mM
intracellular ATP slope = 44.5 ± 4.6 min, x-intercept = -2.58 ± 0.55 mM, y-intercept = 111,949 ± 10,641 min/M, R² = 0.83; 4 mM intracellular AMP slope = 131.8 ± 11.1 min, x-intercept = -2.41 ± 0.45 mM, y-intercept = 318,946 ± 27,465 min/M, R² = 0.85; 4 mM extracellular ATP slope = 354.6 ± 56.7 min, x-intercept = -0.96 ± 0.63 mM, y-intercept = 340,677 ± 135,181 min/M, R² = 0.67. In all instances the slope is significantly different from zero (p < 0.0001).

Figure 3  Caffeine displaces ATP from GLUT1. A Emission spectra of 5 μM TNP-ATP in the absence (dashed lines) or presence (continuous lines) of 100 nM GLUT1. Suspensions were excited at 408 nm and the resulting emission (ordinate) measured over 500-600 nm (abscissa). Results are shown for TNP-ATP emission in the absence of added ligands (black lines) and for emission in the presence of 5 mM D-glucose (purple lines), 10 μM CB (blue lines), 5 mM ATP (red lines) or 5 mM caffeine (green lines). B GLUT1-specific TNP-ATP maximum fluorescence shown in the absence or presence of ligands. Ordinate: fluorescence; Abscissa: ligand additions. Results are shown as mean ± SEM for 3 separate measurements. * indicates that the measured emission is significantly less than that observed in the absence of added ligand (p ≤ 0.037, 1-tailed, paired T-test)

Figure 4  Caffeine and ATP promote conformational change in the GLUT1 C-terminus. Ordinate: C-Ab binding to GLUT1 measured by ELISA (relative antibody binding, %); abscissa: C-Ab binding conditions. C-Ab binding to unsealed GLUT1 proteoliposomes was measured in the presence and absence of ATP (5 - 20 mM, gray bars), caffeine (5 -20 mM, yellow bars), D-glucose (D-Glc, 5-20 mM, pink bars) or cytochalasin B (CB, 5 - 20 μM, black bars). The numbers below the chart indicate the concentration of ligand present during the binding assay. Results are shown as the mean ± SEM of 3 - 16 measurements made in duplicate. *Unpaired, 1-tailed T-test analysis indicates that the results are significantly less that control antibody binding (p ≤ 0.0027).
Docking analysis of caffeine, ATP and CB binding to GLUT1. A GLUT1 crystal structure (28) showing ATP, caffeine and CB located in their respective, highest affinity docking sites. The images were generated in PyMol and show GLUT1 in cartoon representation. ATP (red), Caffeine (blue) and CB (yellow) are shown as space-filling representations. GLUT1 transmembrane helix (TM) 5 is eliminated to reveal the putative ligand binding sites. TMs 8 and 9 which have previously been demonstrated to be the site of azidoATP photo-incorporation (44) are indicated in red. TMs 1, 3, 6, 8, 9 and 10 are numbered. The approximate margins of the lipid bilayer are indicated by the horizontal lines. Residues coordinating ATP binding (and their TM locations) are: F26 (TM1), Q161 (TM5), Q282 and Q283 (TM7), E380 and P385 (TM10), N411 and N415 (TM11). The computed ΔG for ATP binding is -8.5 kcal/mol. Residues coordinating caffeine binding (and their TM locations) are: Q161 (TM5) and P385 (TM10). The computed ΔG for ATP binding is -5.7 kcal/mol. Residues coordinating CB binding (and their TM and loop locations) are: V83 (TM2), M142 (TM4), W388, F389 and A392 (TM10 and loop 10-11), I404 and G408 (TM 11). The computed ΔG for CB binding is -10.3 kcal/mol. B Space filling models of ATP (red) and caffeine (blue) and their corresponding stick models docked in their respective GLUT1 binding sites. C Space filling models of Caffeine (blue) and CB (yellow) and their corresponding stick models docked in their respective GLUT1 binding sites. D Space filling models of ATP (red) and CB (yellow) and their corresponding stick models docked in their respective GLUT1 binding sites.
<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Caffeine $K_{i(app)}$ mM</th>
<th>Nucleotide $K_{i(app)}$ mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP$_i$, 0 ATP$_o$</td>
<td>0.9 ± 0.3</td>
<td>NA</td>
</tr>
<tr>
<td>4 mM ATP$_i$, 0 ATP$_o$</td>
<td>2.6 ± 0.6</td>
<td>2.2</td>
</tr>
<tr>
<td>0 ATP$_i$, 4 mM ATP$_o$</td>
<td>1.0 ± 0.6</td>
<td>NA</td>
</tr>
<tr>
<td>4 mM AMP$_i$, 0 ATP$_o$</td>
<td>2.4 ± 0.5</td>
<td>2.4</td>
</tr>
</tbody>
</table>

$^a$Effects of ATP and AMP on $K_{i(app)}$ for caffeine inhibition of 3MG uptake obtained by analysis of data in Figure 2. $^b$ATP and or AMP (4 mM) were either absent or present on the inside (e.g. ATP$_i$) or outside (e.g. AMP$_o$) of red cell ghosts. Nucleotides were added to the interior of ghosts during resealing of hypotonically lysed red cells. $^c$ $K_{i(app)}$ for caffeine inhibition of 3MG uptake was computed as -X-intercept of the Dixon plot of Figure 2. $^d$ $K_{d(app)}$ for nucleotide –dependent competition with caffeine for inhibition of uptake was computed assuming that caffeine and intracellular nucleotide compete for binding at the same site. $^e$Not applicable.
Table 2
Effects of Caffeine and ATP on $^3$H-Cytochalasin B binding to GLUT1 proteoliposomes

<table>
<thead>
<tr>
<th>Binding conditions</th>
<th>$^{b}\text{CB}<em>{\text{bound}}/\text{CB}</em>{\text{free}}$</th>
<th>$^{c}\text{P}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.71 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Caffeine 1 mM</td>
<td>0.60 ± 0.01</td>
<td>0.0488</td>
</tr>
<tr>
<td>Caffeine 5 mM</td>
<td>0.54 ± 0.01</td>
<td>0.0015</td>
</tr>
<tr>
<td>Caffeine 20 mM</td>
<td>0.39 ± 0.02</td>
<td>0.000035</td>
</tr>
<tr>
<td>ATP 5 mM</td>
<td>0.76 ± 0.03</td>
<td>0.1749</td>
</tr>
<tr>
<td>Cytochalasin B 20 µM</td>
<td>0.24 ± 0.04</td>
<td>0.000003</td>
</tr>
</tbody>
</table>

$^{a}$Equilibrium binding of 100 nM $^3$H-Cytochalasin B to unsealed GLUT1 proteoliposomes was measured in the absence (control) or presence of caffeine (1 to 20 mM), ATP (5 mM) or unlabeled cytochalasin B (20 µM). $^{b}$Binding was measured as the ratio of bound : free $^3$H-CB. $^{c}$The significance of the binding assay result was computed by a 1 tailed, unpaired t-test and is shown as the probability that binding in the presence of the ligand is identical to binding in the absence of ligand (control). Results are shown as the mean ± SEM for a minimum of 3 measurements made in duplicate.
Figure 1

A

3-OMG uptake µmol/L/min

[caffeine] (mM)

B

control

5 mM caffeine

3-OMG uptake µmol/L/min

[3-OMG] (mM)
Figure 2

Caffeine (mM) vs. $1/v$ (min/M) for different conditions:
- ATP outside
- No ATP
- ATP inside
- AMP inside

Graph shows a linear relationship between caffeine concentration and $1/v$, indicating a direct effect of caffeine on the enzyme activity.
Figure 3

A

B

* significance
Figure 4

% antibody binding

ATP mM 0 5 10 20 0
Caffeine mM 0 5 10 20 0
D-Glc mM 0 5 10 20 0
CB µM 0 5 10 20 0
Figure 5