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GTP analogs suppress uptake but not transport of D-glucose analogs in Glut1 glucose transporter-expressing *Xenopus* oocytes

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A *Xenopus* oocyte expression–co-injection system was used to study the influence of guanine nucleotides on D-glucose uptake. GTP analogs like GTPγS and GppNHp had no effect on 3-*O*-methylglucose transport determined by zero-*trans* uptake or equilibrium exchange, but suppressed 2-deoxyglucose uptake into Glut1 glucose transporter-expressing oocytes by up to 86%. Both GTP analogs showed concentration dependence of their effectiveness, with GTPγS being more potent than GppNHp. No statistically significant differences were observed between groups of oocytes co-injected with water or GDPβS (250 and 500 μM intracellular concentration). Glut1 transporter expression in plasma membrane was not different between water or GTPγS-co-injected oocytes. Thus, inhibition of hexokinase catalytic activity is the most likely causative factor for down-regulation of 2-deoxyglucose uptake.

Guanine nucleotide; Glut1 glucose transporter; *Xenopus* oocyte

1. INTRODUCTION

Molecular cloning has led to the identification of six different glucose transporters of the facilitative diffusion type named GLUT1–GLUT5, and Glut7 (reviewed in [1,2]). The glucose transporters have been expressed in *Xenopus laevis* oocytes by injection of mRNA made from the cDNAs under the control of the bacteriophage SP6 or T7 promoter, as reviewed in [3,4]. This expression system has proved to be extremely useful in addressing the question of transport kinetics of individual glucose transporter isoforms [5–8] and in testing their activity after substitution of single amino acid residues [9,10]. In addition, the cytoplasm of oocytes can be altered by microinjection of substrates or proteins in order to study the regulatory response to such perturbation.

Results from different experimental approaches indicate that guanine nucleotide-binding proteins regulate glucose uptake in adipocytes and muscle cells [11–18]. Baldini et al. [18], confirmed by Robinson et al. [19], reported that non-hydrolyzable GTP analogs exert an insulin-like effect on translocation of glucose transporter to the plasma membrane in permeabilized adipocytes. These data, however, left open the question of whether the intrinsic activity of the glucose transporter is altered. Since *Xenopus* oocytes are ideally suited to study the function of the heterologously expressed glucose transporter, Glut1 (i.e. the HepG2/erythrocyte type, [5–7,9,10]), we used this system to evaluate a putative involvement of GTP nucleotides in glucose uptake regulation. Since permeabilization of cells excludes transport measurements for this purpose, GTP analogs were applied by microinjection into *Xenopus* oocytes after prior injection and translation of the in vitro synthesized mRNA of Glut1.

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Footnote: When this manuscript was finished, data by Thomas et al. [(1993) Biochem. J. 290, 707–715] were published indicating that injection of 50 nl of 10 mM GTPγS into Glut4-expressing *Xenopus* oocytes has no effect on 3-*O*-methylglucose uptake.

2. EXPERIMENTAL

2.1. *Preparation of and injection into oocytes*

Collection, defolliculation and culture of *Xenopus* oocytes were conducted as described previously [4,6,9,10]. The *Xba*I-linearized oocyte expression vector, pSP64T, containing the Glut1 cDNA under the control of the bacteriophage promoter, SP6, was used for transcription. Routinely, oocytes were injected with 50 nl of a solution containing capped (^{7m}GpppG) mRNA at a concentration of 1 mg/ml. Healthy looking oocytes were collected after 3 days for co-injection of the indicated nucleotides. pH-adjusted aqueous solutions of GTPγS, GDPβS, GppNHp, and water as control, were microinjected through glass capillaries 60 min before 2-deoxyglucose or 3-*O*-methylglucose uptake measurements, and plasma membrane isolation. From previous equilibration experiments with labeled 3-*O*-methylglucose, the water space per oocyte was calculated to be about 0.5 μl [6]. The respective nucleotide-containing solutions were adjusted such that a microinjection of 25 nl of the respective nucleotide resulted in intracellular levels of 500, 250, 125, 62.5, and 31.25 μM.

2.2. *Uptake/transport measurement and detection of Glut1 expression*

Three days after injection of water (sham group), or mRNAs (Glut1

group), nucleotide solutions or water were co-injected into oocytes Usually, 8–10 single oocytes of each group were taken for glucose uptake/transport measurement. The uptake assay consisted of ^{14}C-labeled 2-deoxyglucose or 3-*O*-methylglucose (50 μM, 1 μCi/ml) in 0.5 ml of modified Barths' solution (MBS) at room temperature. ^{14}C-Labeled 3-*O*-methylglucose transport assay was also conducted under equilibrium exchange influx conditions with MBS containing 10 mM 3-*O*-methylglucose. To test for transport inhibition, uptake was also conducted in the presence of 20 μM cytochalasin B (CB). After the indicated periods of time the oocytes were washed 3 times with 3 ml of ice-cold MBS containing 0.1 mM phloretin (i.e. stop solution). Each single oocyte was dissolved in 0.5 ml of 1% SDS before quantification of radioactivity by a liquid scintillation spectrophotometer.

In order to isolate oocyte plasma membranes, single oocytes of the sham group (A), the Glut1 group after water injection (B), and the Glut1 group after GTPγS co-injection (500 μM intracellular concentration) (C) were triturated in Barths' modified solution (MBS) by use of an adjustable 10 μl Eppendorf pipette [6]. For Western dotblot analysis, these ghosts were further treated as described previously [6,9]. Nitrocellulose membranes (Hybond-C, Amersham Buchler, Braunschweig, Germany) were loaded with 5 and 40 μg, respectively, of protein from the sham group and the Glut1 groups. A purified transporter (a kind gift from Dr. Gustav Lienhard, Dartmouth Medical School, Hanover, NH, USA) was used as standard. The detection system consisted of an IgG fraction from a rabbit antiserum raised against a synthetic peptide (F350) and of ^{125}I-labeled protein A (1.5 mCi/ml).

2.3. *Hexokinase activity*

Maximum activity of hexokinase was determined spectrophotometrically according to Bergmeyer [20]. The reaction was started with the addition of 100 μl of the 100,000 × *g* supernatant of oocyte homogenate. Inhibition of the catalytic activity by GTPγS was tested after addition of GTPγS-containing solutions leading to the same final concentrations as used for oocyte co-injection (see above). Values were presented as the percentage of uninhibited catalytic activity. ATP was determined according to Adams [20].

2.4. *Materials*

All substrates used were of the highest grade quality. The nucleotides were purchased from Boehringer-Mannheim GmbH, Germany; cytochalasin B and phloretin from Sigma Chemicals, St. Louis, MO, USA; [^{125}I]protein A and Hybond-C from Amersham Buchler, Braunschweig, Germany. *Xenopus laevis* frogs were obtained from H. Kähler, Xenopus Laborzucht, Hamburg, Germany.

3. RESULTS

Fig. 1A shows that mRNA, which had been in vitro transcribed from Glut1 cDNA, translated a functionally active glucose transporter protein, as documented by the 2-deoxyglucose uptake data. Co-injection of the poorly hydrolyzable GTP analog GTPγS into Glut1-expressing oocytes suppressed the uptake by more than 85% at 500 μM intracellular concentration. The recorded uptake was further suppressed by cytochalasin B (CB). Periods of time longer than 1 h after co-injection were not used because of morphological changes of the oocytes, as judged by light microscopy. Since oocytes from different experiments do not necessarily express the same amount of glucose transporter proteins, comparison between various nucleotides was performed on a percentage basis, with Glut1 glucose transporter activity measured in the absence of nucleotides set to 100% (Fig. 1B). The non-hydrolyzable GTP ana-

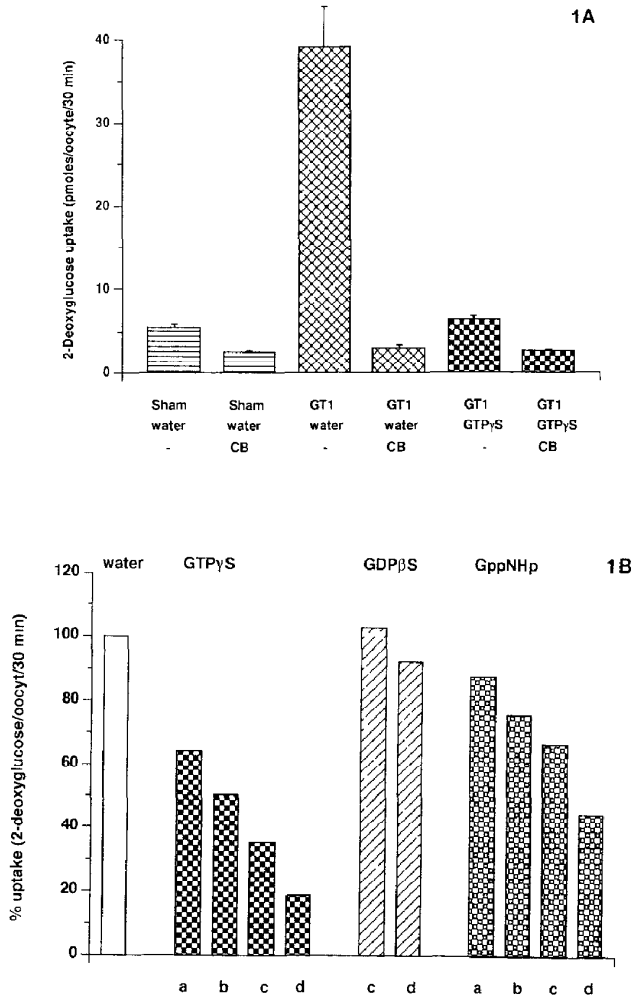

Fig. 1. 2-Deoxyglucose uptake into Glut1 mRNA-injected oocytes is inhibited by GTP analogs. (A) Oocytes were injected with 50 nl of water (sham) or mRNA-containing solution (Glut1). Three days after injection, oocytes were co-injected with 25 nl of water or GTPγS (intracellular concentration 500 μM) 60 min before uptake measurement. 2-Deoxyglucose uptake for 30 min was determined in the absence and presence of 20 μM cytochalasin B (CB), respectively. Values represent the mean ± S.E.M. of data from 7–8 oocytes, except for the CB groups that each include 4–5 oocytes. (B) Glut1-expressing oocytes were co-injected with various concentrations of the indicated nucleotide analogs, i.e. GTPγS, GDPβS, and GppNHp, 60 min before 2-deoxyglucose uptake measurement. Based on the assumption of an average oocyte volume of 0.5 μl, injection of 25 nl of nucleotide-containing solutions led to the indicated intracellular concentrations. Since these data were from two independent experiments 2-deoxyglucose uptake of the water-injected Glut1-expressing groups was set to 100% in order to mutually compare the effectiveness of the tested nucleotides. Intracellular concentrations after co-injection were: (a) 62.5, (b) 125, (c) 250, and (d) 500 μM

log, GppNHp, was a less effective inhibitor compared to GTPγS, whereas co-injection of GDPβS, a metabolically stable GDP analog leading to intracellular concentrations of 250 and 500 μM was without effect.

To determine whether the inhibition of glucose uptake in Glut1 glucose transporter-expressing oocytes by

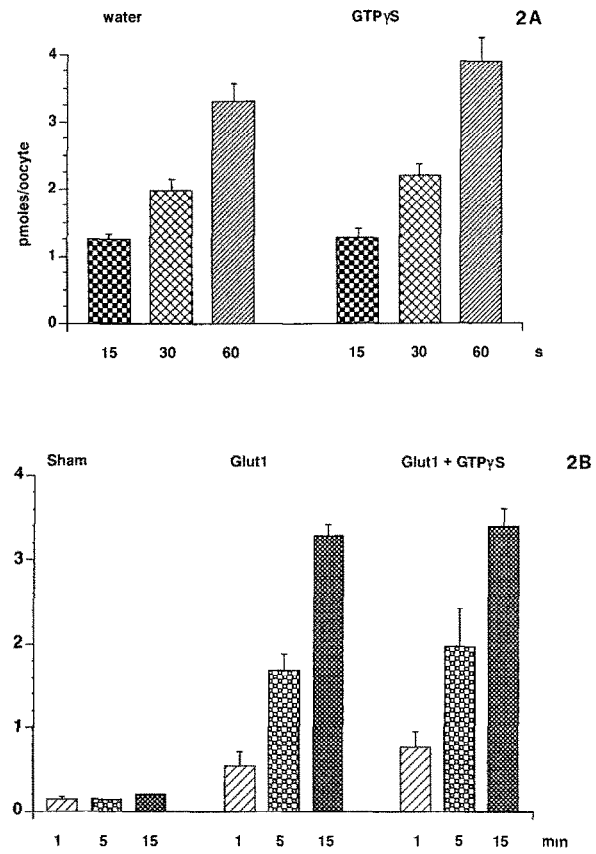

Fig. 2. GTPγS does not affect 3-*O*-methylglucose transport. Groups of 8–10 oocytes were used to measure ^{14}C-labeled 3-*O*-methylglucose transport into oocytes at the indicated periods of time under zero-*trans* (A) and equilibrium exchange influx conditions (B) The equilibrating concentration was 10 mM 3-*O*-methylglucose. Glut1-expressing oocytes were co-injected with either 25 nl of water or GTPγS-containing solution (intracellular concentration 500 μM) about 60 min before transport measurement. Stop of transport was achieved exactly as described for 2-deoxyglucose uptake Values represent the mean ± S.E.M.

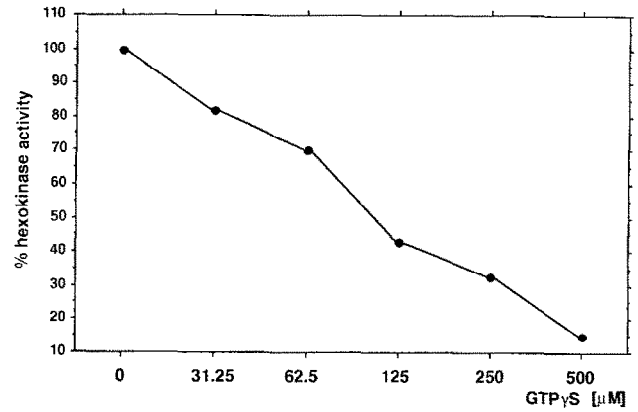

Fig. 3. Inhibition of hexokinase activity by GTPγS. Hexokinase activity of a 100,000 × *g* supernatant from oocyte homogenate was determined according to Bergmeyer [20] and tested for inhibition by GTPγS at the indicated concentrations. The maximum catalytic activity of non-injected oocytes amounted to 0.38 μmol converted D-glucose/oocyte/min and was set to 100%.

GTPγS was due to a direct effect on the catalytic activity of the transporter, we complemented the data with ^{14}C-labeled 3-*O*-methylglucose transport measurements. GTP analogs may down-regulate the glucose transporting system by influencing hexokinase activity, rendering it the rate-limiting step of glucose uptake, and/or could affect intracellular ATP necessary for 2-deoxyglucose phosphorylation. Fig. 2 compares the intracellular accumulation of ^{14}C-labeled 3-*O*-methylglucose under zero-*trans* (Fig. 2A, for time periods up to 1 min) and equilibrium exchange influx conditions (Fig. 2B, for 1–15 min) in Glut1-expressing oocytes between the water-injected group and GTPγS-treated cells. The data indicate that 3-*O*-methylglucose uptake/exchange at different periods of time was not affected by GTPγS, even at its highest concentration, thus definitely excluding a direct GTP or a G-protein-mediated effect on Glut1 transporter function. ATP levels tested in sham oocytes, Glut1 water-injected, and Glut1 GTPγS-co-injected oocytes did not differ significantly (2.8, 2.8, and 2.5 mM, respectively); however, GTPγS (Fig. 3), used at the same concentrations as for co-injection, strongly inhibited hexokinase activity from the 100,000 × *g* supernatant of oocyte homogenate. Since uptake of D-glucose includes transport and phosphorylation, the concentration-dependent inhibition of hexokinase activity was the most likely cause for down-regulation of 2-deoxyglucose uptake.

Fig. 4 demonstrates that, at least during 60 min post-injection, effects on translocation of glucose transporter molecules were not involved in the GTP inhibition of Glut1-mediated uptake. Plasma membranes isolated from Glut1-expressing oocytes that had been co-injected with either water or GTPγS (intracellular concentration 500 μM) exhibited identical signals on the immunoblot. Aliquots of purified erythrocyte glucose transporter were used as standards and plasma membranes of sham-injected oocytes served as negative controls.

4. DISCUSSION

Xenopus oocytes have been reported to possess a G-protein system that appears to be involved in various cellular responses [21,22]. Here they are used as a convenient expression–co-injection system for the study of effector-mediated influence on glucose transporter activity. G-proteins, as transducers of receptor signals, have been proposed to be involved in the counter-regulatory actions on glucose transporter intrinsic activity by β-adrenergic receptor agonists like isoproterenol. For many systems like the G-protein-regulated adenylate cyclase system [23,24], non-hydrolyzable or poorly

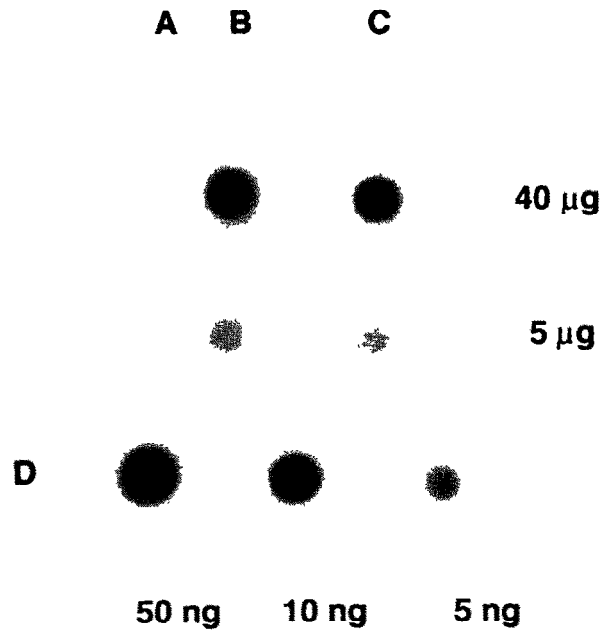

Fig. 4. GTPγS co-injection does not alter the amount of Glut1 glucose transporter expressed in plasma membrane. Western dotblot analysis was performed from plasma membranes of sham-injected (A, control) and mRNA-injected oocytes after co-injection of 25 nl of either water (B) or GTPγS-containing solution (C). For details of plasma membrane isolation and Western blot analysis see section 2. 5 and 40 µg of protein were loaded on the nitrocellulose membrane and compared with aliquots (i.e. 50 ng, 10 ng, and 5 ng) of purified glucose transporter (D).

hydrolyzable analogs appear to be more effective than GTP itself, which is readily metabolized. Data are presented that after injection of Glut1 mRNA into oocytes the intrinsic activity of the Glut1 glucose transporter was not affected by GTP analogs such as GTPγS and GppNHp, whereas the uptake of 2-deoxyglucose was decreased in a concentration-dependent manner. Consistent with reports from other systems [25], GTPγS was a more powerful tool than the imido analog, GppNHp. GDPβS co-injection, used at the two highest concentrations chosen (i.e. 250 and 500 µM), served as a standard indicator for an exclusive GTP involvement in glucose uptake regulation.

As reported recently [26,27], translocation might be involved in the increase of D-glucose uptake after treatment of oocytes with insulin. Although *Xenopus* oocytes possess functional insulin-like growth factor-1 receptors on their surface, we (unpublished observations) and others [7] were unable to detect an effect of insulin on glucose uptake. The results in Fig. 4 argue against an inhibitory effect of GTP nucleotides on translocation as a possible mechanism for 2-deoxyglucose transport down-regulation. Thus, by co-injection of GTP analogs into oocytes a putative signal transduction towards the catalytic activity of the transport protein should be detectable. Since nucleotide-co-injected oocytes exhibit normal ATP levels, the inhibition of hexokinase by GTP analogs most likely contributes to the observed effect. This excludes a direct GTP-mediated effect on the transport function of Glut1, at least after its expression in the plasma membrane of *Xenopus* oocytes.

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