Functional characterization of the human facilitative glucose transporter 12 (GLUT12) by electrophysiological methods

Short title: Functional characterization of hGLUT12

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ABSTRACT
GLUT12 is a member of the facilitative family of glucose transporters. The goal of this study was to characterize the functional properties of GLUT12, expressed in Xenopus laevis oocytes, using radiotracer and electrophysiological methods. Our results showed that GLUT12 is a facilitative sugar transporter with substrate selectivity: D-glucose ≥ α-methyl-D-glucopyranoside (α-MG) > 2-deoxy-D-glucose (2-DOG) > D-fructose = D-galactose. α-MG is a characteristic substrate of the Na⁺/glucose (SGLT) family, and has not been shown to be a substrate of any of the GLUTs. In the absence of sugar, ²²Na⁺...
was transported through GLUT12 at a higher rate (40%) than non-injected oocytes, indicating that there is a Na$^+$- leak through GLUT12. Genistein, an inhibitor of GLUT1, also inhibited sugar uptake by GLUT12. Glucose uptake was increased by the PKA activator 8-Br-CAMP, but not by the PKC activator PMA. In high K$^+$ concentrations, glucose uptake was blocked. Addition of glucose to the external solution induced an inward current with a reversal potential of ~-15 mV, and was blocked by Cl$^-$ channel blockers, indicating the current was carried by Cl$^-$ ions. The sugar-activated Cl$^-$ currents were unaffected by genistein. In high external K$^+$ concentrations, sugar-activated Cl$^-$ currents were also blocked, indicating that GLUT12 activity is voltage-dependent. Furthermore, glucose induced current was increased by the PKA activator 8-Br-cAMP, but not by the PKC activator PMA. These new features of GLUT12 are very different from those described for other GLUTs, indicating that GLUT12 must have a specific physiological role within glucose homeostasis, still to be discovered.

**Key words**

GLUT; GLUT12; Sugar transport; Two-electrode voltage clamp; *Xenopus laevis* oocytes
INTRODUCTION

Glucose uptake into the cells takes place through specific transporter proteins located within the plasma membrane. These transporters belong to two families of integral membrane proteins, the facilitative glucose transporter family GLUT/SLC2A (20, 27), and the Na⁺/glucose co-transporter family SGLT/SLC5A (49). The GLUTs transport monosaccharides passively down the hexose concentration gradient. Until now, 14 different GLUT isoforms have been identified. The distinct GLUT isoforms are distributed in a specific manner within the different tissues and cell types to satisfy their metabolic needs. Each particular physiological environment determines the expression, cellular location and regulation of the GLUT transporters.

GLUT12 was isolated from the mammary cancer cell line MCF-7 (34) and its expression has been described in insulin-sensitive tissues such as muscle and adipose tissue (34), where it seems to act as a secondary insulin-sensitive transporter (37, 38). GLUT12 expression has also been found in some tumor tissues (11, 33, 39) and it has been proposed as one of the key proteins in the energy supply to malignant cells through glycolysis (50).

Previous functional studies have found that GLUT12 is a facilitative sugar transporter, but surprisingly, protons influence sugar transport. In GLUT12-transfected MCDK cells, uptake of 2-deoxy-D-glucose was increased by external acidic pH against its concentration gradient, implicating a role of protons in sugar transport (48). It was demonstrated that 2-deoxy-D-glucose (2-DOG) was transported by GLUT12, and that the uptake of [³H]-2-DOG was inhibited by D-glucose>2-DOG> D-galactose>D-fructose>L-glucose (31). Since these are competition studies, except for glucose (27, 29), it is not known if the sugars are substrates or inhibitors.
Initial studies from our laboratory on *Xenopus laevis* oocytes expressing GLUT12 indicated that glucose transport is enhanced in the presence of external Na$^+$ and that glucose induced an inward current (29). In the present work, we set out to characterize the functional properties of GLUT12 using a combination of radiolabeled tracer and electrophysiological methods. We measured the transport of radiolabeled glucose, 2-DOG, galactose, fructose and α-methyl-D-glucopyranoside (α-MG) directly. We determined the dependence of sugar transport on ions (Na$^+$ and H$^+$), and the effect of activators of PKA and PKC on sugar transport. Finally, we used the two-electrode voltage clamp technique to identify and characterize the currents associated with the activation of the transporter.

**MATERIALS AND METHODS**

**Expression of glucose transporters in *Xenopus laevis* oocytes**

Stage VI oocytes from *X. laevis* (*Xenopus* Express, France) were obtained as previously described (15). They were microinjected with 50 ng of cRNA coding for human GLUT12 or GLUT1 and maintained at 18 ºC in Barth’s medium (88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO$_3$)$_2$, 0.41 mM CaCl$_2$, 0.82 mM MgSO$_4$, 2.4 mM NaHCO$_3$ and 10 mM HEPES-Tris, pH 7.4) supplemented with gentamycin (50 mg/L), ciprofloxacin (50 mg/L) and amikacin (50 mg/L). Experiments were performed at room temperature (20-23°C) 2-5 days after cRNA microinjection. The experimental protocol to manipulate the animals was approved by the Animal Ethics Committee of the University of Navarra (nº 008-11).

**Uptake assays**

Sugars uptake was measured by the radiotracer method (24). Briefly, groups of 6-10 oocytes were incubated for 15 min in 400 µl of Na$^+$ buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 10 mM HEPES-Tris, pH 7.5) containing the indicated
concentration of the respective unlabeled sugar and traces of the corresponding radiolabeled sugar. Once the incubation period has elapsed, the $[^{14}\text{C}]$ content of each oocyte was determined by liquid scintillation counting and uptake was calculated as pmol/oocyte/15min. Uptake by non-injected oocytes was measured in all experiments. Figures show representative experiments. Additionally, in some figures, uptake is also expressed as percentage of the mean uptake of the indicated control condition. To this end, the mean uptake by the non-injected oocytes was subtracted from the uptake value of each GLUT12-expressing oocyte and results represent the mean of the indicated number of oocytes obtained from at least 3 independent experiments.

The radiolabeled sugars used were: $[^{14}\text{C}]$ D-glucose (specific activity 55 mCi/mmol), $[^{14}\text{C}]$ D-galactose (specific activity 53 mCi/mmol), $[^{14}\text{C}]$ methyl-α-D-glucopyranoside (α-MG, specific activity 55 mCi/mmol), $[^{14}\text{C}]$ D-fructose (specific activity 300 mCi/mmol) and $[^{14}\text{C}]$ 2-deoxy-D-glucose (2-DOG, specific activity 58 mCi/mmol), all purchased from American Radiolabeled (St Louis, MO, USA).

Sodium uptake was measured in Na$^+$ buffer containing $^{22}\text{Na}^+$ (specific activity 100-2000 Ci/g) purchased from Perkin Elmer. Uptake of $^{22}\text{Na}^+$ was calculated as cpm/oocyte/15min, and results were normalized as above indicated.

**Electrophysiology methods**

The two-electrode voltage clamp method was used to control the membrane potential and monitor the transporter activity as previously described (7, 28). Microelectrodes were filled with 3M KCl solution (resistance 0.5-1.5 MΩ). Oocytes were maintained in a chamber continuously perfused with Na$^+$ buffer solution in the presence or absence of substrate. Continuous current measurements were made at a holding potential ($V_h$) of -50 mV, low-pass filtered at 0.05 Hz and digitized at 1 Hz using Axoscope V1.1.1.14 (Molecular Devices, Sunnyvale, CA, USA).
Substrate-induced currents, under voltage clamp conditions, were determined as the difference between the steady-state current measured in the presence and absence of sugars. In some experiments, the oocyte membrane potential was not clamped and variations on the resting membrane potential were measured.

To determine the theoretical reversal potential for ions \( E_x \), the Nernst equation was used:

\[
E_x = \frac{RT}{zF} \ln \left( \frac{[x_{\text{out}}]}{[x_{\text{in}}]} \right)
\]

Where \( R \) is the gas constant, \( T \) is the absolute temperatures, \( z \) is the electric charge of the ion, \([x]_\text{in}\) ionic intracellular concentration and \([x]_\text{out}\) ionic extracellular concentration.

At room temperature \( (RT/F) \) is around 25.8 mV, so according to this datum, utilized Nernst equation was:

\[
E_x = 2.303 \left( \frac{25.8 \text{ mV}}{z} \right) \log \left( \frac{[x_{\text{out}}]}{[x_{\text{in}}]} \right)
\]

Some figures show a representative experiment. In other figures, substrate-induced currents are expressed as percentage of the indicated control current obtained in the same oocyte, and represented as the mean of the indicated number of oocytes. All the experiments were performed with oocytes from at least 3 different frog donors.

**Effect of Na⁺, K⁺, and H⁺**

The previously described Na⁺ buffer was modified in indicated experiments by replacing 100 mM NaCl with 100 mM choline chloride (choline buffer) or 100 mM KCl (K⁺ buffer). The pH was varied between 7.5 and 6 by using Tris-HCl.

**Inhibition by Cl⁻ channel blockers and genistein**

Inhibition of glucose uptake and glucose-induced currents by the GLUTs inhibitor genistein (Sigma-Aldrich) or the Cl⁻ channel blockers niflumic acid (NFA, Sigma-Aldrich), 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB, Santa Cruz Biotechnology) and diphenylanthranic acid (DPC, Santa Cruz Biotechnology), was
measured by adding these compounds to the Na\(^+\) buffer solution in the presence of glucose. The concentrations used were: 100-200 µM genistein, 100 µM NFA, 100 µM NPPB, 1 mM DPC.

**Effect of PKA and PKC activators**

To analyze the effect of protein kinases, oocytes were incubated with 0.1 mM of the PKA activator 8-bromo adenosine 3’,5’-cyclic monophosphate (8-br-cAMP) (Sigma-Aldrich) or 0.1 µM of the PKC activator Phorbol-12-myristate-13-acetate (PMA) (Santa Cruz Biotechnology) for 30 and 15 min respectively (12, 17). Then, uptake of glucose or glucose-induced currents were determined and compared with control conditions.

**Statistical Analysis**

The results are expressed as mean values ± SE. Differences between groups were analyzed by unpaired Student’s test or Mann-Whitney U-test, depending to the result of the test of normal distribution of the data; significance was set at p<0.05.

**RESULTS**

I. Facilitative sugar transport by GLUT12

**Sugar selectivity**

We first examined the sugar selectivity of GLUT12 by measuring the uptake of a series of radio-labeled hexoses (20 mM). As shown in Fig. 1A, \(^{14}\text{C}\)-glucose and \(^{14}\text{C}\)-α-methyl-glucopyranoside uptake was ~2-fold higher in oocytes injected with GLUT12-cRNA compared to non-injected oocytes, whereas uptake of radio-labeled galactose, fructose, and 2-DOG was slightly higher. In Fig. 1B, the mean GLUT12-sugar uptake rate (normalized to the glucose uptake rate) was determined from 3 independent experiments in which the background transport rate for non-injected oocytes was subtracted. Sugar selectivity of GLUT12 was: glucose ≥ α-MG > 2-DOG >fructose = galactose. These results demonstrated for the first time that α-MG, a characterizing substrate for the
SGLT transporter family (49), which has never been described as a GLUT family substrate, is transported by GLUT12.

Effect of Na\(^+\) and H\(^+\)

We have found in preliminary studies that GLUT12 glucose transport rate is increased in the presence of Na\(^+\) (29). In MDCK cells over-expressing GLUT12, proton-coupled active glucose transport has been reported (48). Furthermore, in some Na\(^+\)-coupled transporters, such as the SGLT family, protons (H\(^+\)) can substitute for Na\(^+\) in driving sugar transport (49). We therefore investigated the role of Na\(^+\) and H\(^+\) in sugar transport by GLUT12. Fig. 2A shows a representative experiment, in the presence of external Na\(^+\) at pH 7.5, glucose uptake was 2-fold higher for GLUT12-expressing oocytes than non-injected controls. The mean of 5 independent experiments (normalized to glucose uptake at pH 7.5) is shown in Fig. 2B. At pH 7.5, glucose uptake in the absence of Na\(^+\) decreased by \(-45\%\), but acidification of the medium (pH 6) in the absence of Na\(^+\) did not further modify uptake (\(-55\%\) inhibition), indicating that H\(^+\) were not involved in glucose transport in the absence of Na\(^+\). Interestingly, in Na\(^+\) buffer, decrease of pH diminished the uptake by \(-60\%\), indicating that protons, under certain conditions, also interact with GLUT12. Higher glucose uptake in the presence of Na\(^+\) suggested that GLUT12 activity could be coupled to the Na\(^+\) entry. To test this hypothesis, we studied the uptake of \(^{22}\)Na\(^+\). As shown in Fig. 2C, in the absence of glucose, Na\(^+\) uptake in GLUT12-expressing oocytes was higher (40\%) than in non-injected oocytes. This indicated that there was a Na\(^+\) leak (or Na\(^+\) uniport) mediated by GLUT12 (see also Fig. 5 below). When glucose was present in the external solution, Na\(^+\) uptake was \(-30\%\) higher than in the absence of glucose, although the difference between both conditions was not statistically significant (Fig. 2D). As controls, and to rule out a possible unspecific effect of Na\(^+\) in GLUT12-mediated glucose transport, we measured the
uptake of glucose in the presence and the absence of Na\(^+\) by GLUT1. Uptake of 20 mM glucose, saturating concentration for GLUT1 (3), was the same in the presence and in the absence of Na\(^+\) (data not shown), confirming that co-transport of Na\(^+\) and glucose was a specific property of GLUT12.

**Inhibition by genistein**

Genistein is a competitive inhibitor of glucose transport by GLUT1, with a \(K_i\) of 12 \(\mu\)M (41), and acts as a direct inhibitor of insulin-induced glucose uptake by GLUT4 in 3T3-L1 adipocytes, with a \(K_i\) of 20 \(\mu\)M (5). We examined the effect of genistein on glucose uptake by GLUT12. As shown in Fig. 3A, 100 \(\mu\)M genistein inhibited glucose uptake almost completely.

**Voltage dependence**

We examined the dependence of GLUT12 on membrane potential by bathing the oocytes in high K\(^+\) buffer (100 mM K\(^+\)), where the membrane potential is depolarized to a low value (eg. -4 mV, Fig. 10B). Fig. 3B shows that in K\(^+\) buffer, glucose uptake by GLUT12 was almost completely inhibited, indicating glucose-uptake by GLUT12 is voltage dependent. To rule out the possibility of an unspecific effect of K\(^+\), we measured the uptake of glucose by GLUT1 in the presence of K\(^+\) buffer, and found that it was not significantly modified (Fig. 3C).

**Effect of PKA and PKC activators**

It has been described that GLUT12 possesses internalization di-leucine motifs at the N- and C-terminal ends of the protein, which retains this transporter into intracellular compartments in the absence of stimulus (32, 34, 47). In agreement with this, GLUT12 has been described to localize in the plasma membrane and in the perinuclear region of MCF-7 cells (34). On the other hand, it has been demonstrated in *X. laevis* oocytes, that the expression at the plasma membrane of several membrane transporters is increased
by PKA or PKC activation (17, 23). Therefore, we decided to study the effect of the protein kinase activators 8-Br-cAMP and PMA in the regulation of GLUT12 functional expression. Fig. 3D shows glucose uptake in control (untreated) oocytes and in oocytes pre-incubated with 8-Br-cAMP (PKA activator) or PMA (PKC activator). Treatment with 8-Br-cAMP (30 min) increased glucose-uptake by ~50%; in contrast, treatment with PMA (15 min) did not modify glucose uptake. These results indicated that GLUT12 could be regulated by PKA.

II. Electrophysiological properties of GLUT12

Sugar-activated currents

To compare GLUT12 with the electrogenic SGLT family, which couples glucose and Na\(^+\) transport, electrophysiological measurements were performed to detect currents that might be associated with sugar transport through GLUT12 (29). Figure 4A shows a continuous current record from a GLUT12-expressing oocyte bathed in Na\(^+\) buffer. The oocyte membrane potential was clamped at -50 mV. Addition of glucose to the external solution induced an inward current. When glucose was washed out from the external solution in choline buffer, and subsequently in Na\(^+\) buffer, the holding current returned to the original baseline. The amplitude of the inward current increased with increasing glucose concentrations (1-100 mM) (~ 5, 10, 15, 25, 40 and 90 nA), but did not saturate at the highest concentration tested. In contrast, GLUT12-mediated glucose uptake saturated at a glucose concentration below 100 mM (data not shown). Glucose-induced currents were not observed in non-injected oocytes (data not shown) (29), indicating they were associated with GLUT12.

The sugar specificity of the currents induced by various sugars was studied. In contrast to sugar uptake by GLUT12, where the rate of uptake depended on the sugar (Fig. 1B), the currents induced by glucose, galactose, fructose, α-MG and 2-DOG (100 mM) were
of similar magnitude (80-110 nA) (Fig. 4B). These results suggested that the currents
induced by various sugars were uncoupled from their uptake by GLUT12.

**Dependence on Na\(^+\), H\(^+\) and Cl\(^-\).**

Experiments were performed to determine the effect of Na\(^+\) on the currents evoked by
glucose through GLUT12. We found there was a Na\(^+\) leak current mediated by
GLUT12, which is shown in the experiment of Fig. 5. A GLUT12-expressing oocyte
was held at -50 mV, and bathed initially in choline buffer. Replacing choline with Na\(^+\)
in the external solution induced an inward current of 35 nA, and addition of glucose
(100 mM) to the Na\(^+\) buffer induced an inward current of 40 nA (Fig. 5A). In non-
injected control oocytes, the increase in current in switching from choline to Na\(^+\) buffers
was much smaller, and addition of glucose to Na\(^+\) buffer did not induce an inward
current (Fig. 5B). The current difference for non-injected oocytes was 20% of that of
the GLUT12-expressing oocytes (Fig. 5C). The current difference (switching from
choline to Na\(^+\) buffers) between GLUT12- and non-injected oocytes is the Na\(^+\) leak
through GLUT12. This is consistent with the increase in \(^{22}\)Na\(^+\) uptake in GLUT12
compared to non-injected oocytes (Fig. 2C).

Fig. 6A shows the addition of 100 mM glucose to Na\(^+\) buffer induced a current of ~120
nA. In choline buffer, 100 mM glucose induced a current of ~80 nA. Similar to the
uptake experiments (Fig. 2A and B), the presence of H\(^+\) in choline buffer did not modify
the magnitude of the current (Fig. 6B), indicating that H\(^+\) were not involved in the
generation of the glucose-induced currents under this condition. Currents induced by
glucose in the presence of Na\(^+\) were 25% higher than in its absence, demonstrating a
contribution of Na\(^+\) to the glucose-evoked currents. On the other hand, sugar-activated
currents observed in the absence of Na\(^+\) indicated that Na\(^-\) is not essential for induction
of the inward current by GLUT12.
To identify the current evoked by glucose through GLUT12, we recorded the changes in membrane potential ($V_m$) in response to sugar. In a GLUT12-expressing oocyte bathed in Na$^+$ buffer, addition of 100 mM glucose to the bath solution depolarized $V_m$ from -32 to -21 mV (Fig. 7A). After sugar exposure, the bath solution was replaced with choline buffer, resulting in a hyperpolarization of $V_m$ from -21 to -48 mV. When Na$^+$ was restored in the bath solution, $V_m$ returned to -32 mV. To confirm the depolarization induced by glucose was mediated by GLUT12, we repeated the experiment on control non-injected oocytes from the same batch. There was no specific effect of glucose on the $V_m$ on these oocytes (Fig. 7B). The experiment also confirmed that there was no endogenous expression of SGLT in the non-injected oocytes.

Glucose-induced depolarization in GLUT12-expressing oocytes was variable, but never reached values more positive than -15 mV. According to the $X. laevis$ oocytes intracellular ionic concentrations (45), and the extracellular concentration of ions in the Na$^+$ buffer, the theoretical reversal potentials calculated for Na$^+$, K$^+$ and Cl$^-$ were +57 mV, -100 mV and -16 mV respectively. Since the depolarization induced by glucose was near -15 mV, we hypothesized the currents were carried by Cl$^-$ ions.

**Effect of chloride channel inhibitors**

To confirm our hypothesis of a Cl$^-$ conductance through GLUT12, we tested some Cl$^-$ channel inhibitors, widely described as blockers of Cl$^-$ channels in $X. laevis$ oocytes (45, 46), on glucose-induced currents through GLUT12. Fig. 8A shows that in a GLUT12-expressing oocyte the addition of 100 mM glucose induced a current of ~80 nA, which was completely inhibited by the addition of 100 μM NFA (Fig. 8A and D). Similar results were obtained with NPPB (100 μM) (Fig. 8B and D), while DPC (at 1 mM) did not significantly inhibit the glucose-induced currents (Fig. 8C and D). Thus
the pharmacology supports the view that the glucose-induced currents mediated by GLUT12 were carried by Cl⁻ ions.

To probe the link between the Cl⁻ channel activated by glucose and sugar transport by GLUT12, we investigated the effect of NFA on GLUT12 glucose uptake. Fig. 8E shows that NFA had no effect on glucose uptake, but the compound completely blocked the Cl⁻ current induced by glucose (Fig. 8A).

**Effect of genistein**

We have shown that genistein is an inhibitor of GLUT12 sugar uptake (Fig. 3A). In contrast, the current induced by 100 mM glucose (~60 nA) was unaffected by genistein (Fig. 9).

**Voltage dependence**

Fig. 10A shows that, under voltage-clamp conditions, 100 mM glucose induced currents of ~80 nA in a GLUT12-expressing oocyte in K⁺ buffer, and ~100 nA in Na⁺ buffer, indicating that K⁺ did not affect the glucose-induced current. When Vₘ was not clamped, in Na⁺ buffer 100 mM glucose depolarized Vₘ from -31 to -20 mV, but in K⁺ buffer, glucose did not affect Vₘ (Fig. 10B). In K⁺ buffer, Vₘ is depolarized to -4 mV, beyond (more positive than) the reversal potential for Cl⁻, we anticipate that activation of a Cl⁻ current by glucose would result in an outward current or Cl⁻ influx into the cell, and hyperpolarize the membrane potential to the reversal potential for Cl⁻ (~ -16 mV).

The absence of glucose-induced hyperpolarization (in K⁺ buffer) suggested the GLUT12 glucose-induced Cl⁻ currents were voltage-dependent, and appeared to be inactivated at low membrane potential.

**Mechanism of Cl⁻ channel activation**

As described above, the Cl⁻ currents activated by different sugars depended more on the concentration than on the nature of the sugar. We examined the effect of sorbitol and
mannitol, sugars that are not transported by GLUTs. Sorbitol (Fig. 11) and mannitol (data not shown) induced currents through GLUT12 with similar magnitudes and time course to those of glucose. The effects of sorbitol and mannitol were not observed in non-injected oocytes. These results indicated that the GLUT-mediated currents might be activated by an osmotic mechanism associated with the transporter.

**Effect of PKA and PKC activators**

We examined the effect of PKA and PKA activators on the glucose-induced currents. Treatment with 8-Br-cAMP increased the glucose-induced currents by ~50% (Fig. 12A and C); however, treatment with PMA did not modify the currents (Fig. 12B and C). These results are similar to the effect of PKA and PKC on glucose-uptake by GLUT12 (Fig. 3D).

**DISCUSSION**

**Substrate selectivity.**

From the direct uptake of radiolabeled sugars, we have determined the selectivity of GLUT12: D-glucose ≥ α-MG > 2-DOG > D-fructose = D-galactose. This confirms the previous finding (D-glucose ≥ 2DOG > D-galactose) based on competitive inhibition studies of 2-DOG uptake (31). Furthermore, we show that α-MG, a substrate characteristic of the SGLT family, is also transported by GLUT12, and genistein, a common inhibitor of sugar transport by the GLUTs, also inhibits GLUT12 glucose transport.

Until now, 14 different GLUT isoforms have been identified and divided into three different classes, based on sequence homology. Class I contains GLUT1, GLUT2, GLUT3, GLUT4 and GLUT14 (gene duplication of GLUT3); Class II comprises the fructose transporter GLUT5, GLUT7, the urate transporter GLUT9 and GLUT11; and class III includes GLUT6, GLUT8, GLUT10, GLUT12 and GLUT13 (3, 29). Fructose
is a common substrate for the Class II of GLUTs (3), and the transport of the sugar has been related to the lack of the QLS motif in the helix 7 of the structure of GLUT transporters. The lack of the QLS motif in GLUT12 is consistent with its ability to transport fructose. However, unlike the Class II transporters, GLUT12 transports 2-DOG and galactose, typical substrates of Class I (27). Overall, GLUT12 transports all the hexoses that are substrates of the GLUT family (29); it also transports α-MG, which until now had not been reported to be a GLUT substrate (8). Moreover, α-MG appears to be as good substrate as glucose. Thus GLUT12 seems to be less restrictive among the members of the GLUT family.

**Effect of Na⁺ and H⁺**

We have found that glucose uptake by GLUT12 is increased by ~40% in the presence of external Na⁺ compared to the absence, and there is a Na⁺-leak (uniport) through GLUT12 in the absence of sugar. Na⁺ uptake by GLUT12 does not significantly increase in the presence of glucose, and in the absence of external Na⁺, there is glucose uptake (~60 %). Taken all together, these results indicate the activity of GLUT12 is dependent on Na⁺, but Na⁺ is not essential for GLUT12 function. Further experiments are needed to determine if there is Na⁺-glucose coupling, or to establish the mechanism of interaction between Na⁺ and GLUT12.

Glucose uptake was not modified by acidic pH, indicating that GLUT12 is independent of H⁺ as well. In the presence of Na⁺, protons further decreases sugar uptake, indicating that H⁺ could also interact with GLUT12. In relation to this, Wilson-O’Brien et al. (2010) have demonstrated in MDCK cells that, in the presence of both Na⁺ and H⁺, GLUT12 was able to transport glucose against its concentration gradient, coupling the transport to the favorable proton gradient. However, under favorable glucose
concentration gradient, protons slightly inhibited glucose uptake (48) as we here demonstrate.

The use of Na\(^+\) or H\(^+\) gradients has been described for human transporters such as SGLT1 (16), CNT3 (15), and the EAATs (21). For GLUT12, the inhibition of glucose uptake observed in the presence of both Na\(^+\) and H\(^+\) could be the result of competition of both ions for a common binding site, which would alter the conformation and function of the transporter; nevertheless, further experiments are needed to address this question. In this regard, the nucleoside transporter hCNT3 presents different substrate affinity and maximal transports rate depending on whether Na\(^+\), H\(^+\) or both cations are coupled to the substrate during the transport (35). Likewise, glutamate uptake through EAAT4, which involves the transport of both Na\(^+\) and H\(^+\), is inhibited by low pH through the decrease of the apparent affinity for Na\(^+\) (6).

**Effect of K\(^+\)**

At high external K\(^+\) concentrations, the cell resting membrane potential is depolarized to a low value. Voltage-dependence has been described for different Na\(^+\)-driven co-transporters (15, 44, 49), but has not been reported for facilitative transporters. The blockade of glucose uptake in the absence of Na\(^+\) (in choline buffer) under high external K\(^+\), suggests that the facilitative transport of glucose by GLUT12 is voltage dependent. In Na\(^+\)-coupled co-transporters such as SGLT1, depolarizing membrane voltages reduce sugar transport due to the reduction in the electrochemical potential gradient for Na\(^+\) ions as well as the preference of the empty (Na\(^+\)- and sugar- free) transporter for the inward-facing conformation at positive membrane voltages (28).

It has been demonstrated that glutamate transport through the EAAT transporters is driven by the co-transport of Na\(^+\) and counter-transport a K\(^+\) ion, which relocates the transporter into the outward facing conformation (4, 22). However, as high
extracellular K$^+$ was not able to inhibit glucose-induced currents through GLUT12 under voltage-clamp conditions, it seems unlikely that K$^+$ counter-transport is involved in GLUT12 transport mechanism.

**Regulation of GLUT12 by PKA and PKC**

Activation of PKA but not PKC increases glucose uptake by GLUT12. The regulation of GLUT12 by protein kinases could occur directly via phosphorylation of the transporter, changing its transport kinetics, or indirectly, by regulating its insertion into the plasma membrane (17, 23). Interestingly, in L6E9 rat muscle cells, the activation of PKA by 8-br-cAMP increases expression of GLUT1, while decreases GLUT4 expression (42). By contrast, PMA up-regulates the translocation of GLUT4 to the plasma membrane in isolated rat adipocytes, without any effect on GLUT1 (43). GLUT12, as GLUT1, has been proposed as one of the key proteins involved in the glycolytic metabolism of cancer cells (50), which would fit with the fact that both are up-regulated by PKA. Activation of PKC is also involved in the insulin-stimulated translocation of GLUT4 to the plasma membrane (25). Comparison of the glucose uptake levels in response to PKC or insulin in isolated rat adipocytes showed that, even though PKC activation increased glucose transport, insulin-mediated glucose transport was still higher (43), indicating the activity of another glucose transporter, probably GLUT12, which would be activated by another pathway. As GLUT4 and GLUT12 are differently regulated by PKA and PKC, it would make sense that each transporter could counter balance the lack of the other, depending on the physiological situation, as it has been proposed (29).

**The glucose-activated current**

The reversal potential of the glucose-activated inward current approaches the theoretical Nernst potential for Cl$^-$, indicating the current is predominantly carried by Cl$^-$ ions.
exiting the cell. Blockade of the glucose-induced current by Cl⁻ channel blockers NFA and NPPB is consistent with this interpretation. However, Na⁺ may also contribute to the glucose-activated current since the current is increased in the presence of Na⁺ (Fig. 6A). Several findings indicate the sugar-activated Cl⁻ current and GLUT12 sugar transport are uncoupled: 1) glucose-uptake saturated, but the glucose-induced Cl⁻ current did not saturate with increasing sugar concentrations (Fig. 4A); 2) sugar-uptake showed a sugar specificity, while the currents induced by different sugars at the same concentration were similar (Fig. 1B and 4B); 3) genistein inhibited sugar uptake but not the sugar-induced current (Fig. 3 and 9); and 4) the chloride channel inhibitor NFA completely block the glucose-induced current, in contrast, NFA did not block glucose-uptake (Fig. 8D and E).

The magnitude of the sugar-activated current depended on the sugar concentration and not on the nature of the sugar. Mannitol and sorbitol, which are not transported by GLUT12, also activated the Cl⁻ current, indicating the sugar-activated current might be induced by osmotic pressure. Interestingly, GLUT1 has also shown to be influenced by osmotic pressure, since their mRNA and protein levels, and plasma membrane location are increased by high external mannitol concentrations (18). It has been found that on *X. laevis* oocytes expressing the amiloride-sensitive Na⁺ sensitive channel (rENaC), osmotic pressure activated Na⁺ currents through the protein (19).

Does GLUT12 possess dual transporter/channel activity? Alternatively, given that *X. laevis* oocytes express several endogenous Cl⁻ channels (36, 40), could the expression of GLUT12 or perhaps our experimental conditions activate endogenous channels? Four types of Cl⁻ channels have been described in *X. laevis* oocytes, differing in their mode of activation (36): hyperpolarization-activated, Ca²⁺-inactivated, volume-sensitive and Ca²⁺-activated channels. Our experiments were conducted at -50 mV, in the presence of
extracellular Ca\textsuperscript{2+} and using isosmotic solutions, discarding the activation of the first three endogenous channels. Hypertonic solutions have been shown to activate endogenous ionic conductance in *X. laevis* oocytes (1), but this activation was only observed with solutions with an osmolarity higher than 480 mosmol l\textsuperscript{-1} (52). Therefore, Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels would remain as the main candidates to be activated by GLUT12 expression in *X. laevis* oocytes, as they are blocked as well by NFA and NPPB and its activity can be modulated by foreign proteins (45). However, even though the sugar-induced currents associated with GLUT12 are inhibited by both NFA and NPPB, they do not show the characteristic biphasic currents (channel activation followed by inactivation) of the endogenous Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels (46). Therefore, the sugar-activated Cl\textsuperscript{-} current is likely due to the GLUT12 transporter.

Ligand-gated Cl\textsuperscript{-} conductance through co-transporters has been previously demonstrated in *X. laevis* oocytes for the human amino acid transporters EAAT1-5 (2, 13, 44), ASCT1-2 (9, 51) and NaPi-1 (10), and the trout band 3 anion exchanger (14). GLUT12 behavior resembles that of an hSGLT1 mutant in which neutralization of Asp204 (D204C and D204N) results in a H\textsuperscript{+} channel activated by glucose that allows proton independent glucose transport, which is increased in the presence of either Na\textsuperscript{+} or H\textsuperscript{+} (30).

**Physiological relevance**

In summary, GLUT12 is a versatile transporter: it transports a wide diversity of hexoses, it can work as a Na\textsuperscript{+} or H\textsuperscript{+}/glucose symporter (48), and shows electrogenic properties (29). In addition, it can be translocated to the plasma membrane by a wide variety of stimulus such as nutrients availability through the mTOR pathway (47), growth factors and estradiol (26), insulin under the activation of PI3K pathway (37) and by PKA. Therefore, it is clear that GLUT12 has unique functional features, indicating
that it must have a specific role in the whole-body glucose homeostasis, instead of being
an evolutionary GLUT4 ancestor maintained as a backup, as previously proposed (37).

ACKNOWLEDGMENTS

We thank A. Redín for technical assistance and Dr. E. Gorraitz for her important
contributions to the preliminary experiments.

GRANTS

This work has been supported by “Fundación Marcelino Botín” and PIUNA (University
of Navarra). J. Pujol-Giménez was a recipient of a fellowship from “Asociación de
Amigos” (University of Navarra).

DISCLOSURES

All authors disclose any actual or potential conflict of interest including any financial,
personal or other relationships with other people or organizations that could
inappropriately influence this work.

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**Legends to figures**

**Figure 1 - Substrate selectivity of GLUT12 - A)** A representative experiment showing the uptake by GLUT12-expressing oocytes of 20 mM radiolabeled glucose, α-MG, galactose, fructose and 2-DOG in Na+ buffer. Data represent the mean of 5-10 measurements and error bars indicate standard errors (SE). Sugar uptake by non-injected oocytes (NI) is also shown. *p< 0.05 vs. NI. B)** Mean GLUT12 sugar uptake-rate was pooled from 3 independent experiments (13-19 oocytes). Values were corrected for basal uptake in non-injected oocytes and normalized to the mean glucose uptake in non-injected oocytes.
uptake (568 ± 137 to 1610 ± 774 pmol/oocyte/15min) and are represented as the mean±SE. Not significant (NS); *p< 0.05; ***p< 0.001 vs. Glucose.

Figure 2 - Effect of Na⁺ and H⁺ on glucose uptake - A) A representative experiment showing the uptake of 5 mM glucose by GLUT12-expressing and non-injected oocytes (NI) at the indicated pH in Na⁺ and choline buffers. Data represent the mean (± SE) of 5-10 measurements. *p< 0.05 vs. NI. B) Mean GLUT12 glucose uptake-rate was obtained from 5 independent experiments (22-34 oocytes), normalized to the mean glucose uptake in Na⁺ at pH 7.5 (71 ± 9 to 340 ± 70 pmol/oocyte/15 min) and represented as the mean ± SE. ***p< 0.001 vs. Na⁺ pH 7.5. C) Uptake of 100 mM Na⁺ by GLUT12-expressing oocytes was determined in the absence and presence of 5 mM glucose. Data represent the mean (± SE) of 5-10 measurements. Sugar uptake by non-injected oocytes (NI) is also shown. *p< 0.05 vs. NI. D) Mean GLUT12 Na⁺ uptake-rate was obtained from 3 independent experiments (11-13 oocytes). Values were corrected for basal non-mediated uptake in non-injected oocytes, normalized to the mean Na⁺ uptake in the presence of glucose (399 ± 153 to 911 ± 239 cpm/oocyte/15min) and represented as the mean ± SE. Not significant (NS) vs. 22Na⁺ glucose.

Figure 3 - Effect of genistein, K⁺ and PKA and PKC activators on glucose uptake
A) Inhibition of GLUT12-mediated glucose transport by genistein. Uptake of 5 mM glucose by GLUT12-expressing and non-injected oocytes (NI) was determined in Na⁺ buffer in the absence and presence of 100 μM genistein. Data represent the mean (± SE) of 5-10 measurements. *p< 0.05; Not significant (NS) vs. NI. B) Effect of K⁺ on glucose transport. Uptake of 5 mM glucose by GLUT12-expressing oocytes was determined in choline and K⁺ buffers. Data represent the mean (± SE) of 5-10 measurements. *p< 0.05; Not significant (NS) vs. NI. C) Uptake of 20 mM glucose by GLUT1-expressing
oocytes was determined in Na\(^+\) and K\(^+\) buffers. In both figures, data represent the mean (± SE) of 5-10 measurements. Sugar uptake by non-injected oocytes (NI) is also shown. **p<0.01; *p< 0.05 vs. NI.  

**D** Effect of activators of PKA and PKC on GLUT12 mediated glucose induced-currents and transport. Uptake of 20 mM glucose was determined in Na\(^+\) buffer after pre-incubating the GLUT12-expressing oocytes with 8-br-cAMP (30 min) or PMA (15 min). Data represent the mean (± SE) of 5-10 measurements. Sugar uptake by non-injected oocytes (NI) is also shown. ***p<0.001; **p< 0.01; *p< 0.05 vs. NI.

**Figure 4 – Sugar-induced currents through GLUT12.**  

A) A GLUT12-expressing oocyte was held at -50 mV and perfused with Na\(^+\) buffer (black line) until a stable baseline was recorded. The addition of increasing glucose concentrations (1-100 mM) in Na\(^+\) buffer (dotted black line) induced inward currents of increasing magnitude (~ 5, 10, 15, 25, 40 and 90 nA). After exposure to glucose, the oocyte was washed out with choline buffer (grey line). Traces are representative of 4 experiments. B) The membrane potential of a GLUT12-expressing oocyte was clamped at -50 mV. The addition of the indicated sugars (100 mM) in Na\(^+\) buffer (dotted black line) induced currents of similar magnitude (80-110 nA). After exposure to each sugar, the oocyte was washed out with choline buffer (grey line). For clarity of presentation, a linear drift in the baseline in Na\(^+\) buffer has been subtracted from the current record. Traces are representative of 3 experiments.

**Figure 5 – Na\(^+\)- leak through GLUT12 –** A) A GLUT12-expressing oocyte was held at -50 mV and perfused with choline buffer (grey line) until a stable baseline (I\(_{Ch}\)) was reached. Replacement of the choline by a Na\(^+\) buffer (black line) induced an inward current of 35 nA, and subsequent addition of glucose to the Na\(^+\) buffer (dotted black line) increased the holding current by 40 nA. Traces are representative of >10 experiments.
experiments. B) A non-injected oocyte was held at -50 mV and perfused with Na⁺ buffer (black line), inducing a current of 5 nA. Addition of glucose (100 mM) in Na⁺ buffer (dotted black line) did not induce a response. Traces are representative of 6 experiments. C) Comparison of the difference in holding current (Iₜₐₜ, between Na⁺ and choline buffers) for non-injected and GLUT12-expressing oocytes. Results represent the mean ± SE (6-11 oocytes) normalized to the mean of the difference in holding current through GLUT12-expressing oocytes (25-90 nA). ***p< 0.001 vs. Na⁺-leak (GLUT12).

**Figure 6 - Effect of Na⁺ and H⁺ on glucose-induced currents through GLUT12 - A)**
A GLUT12-expressing oocyte was held at -50 mV and bathed in Na⁺ buffer (black line). The addition of 100 mM glucose in Na⁺ buffer (dotted black line) induced a current of 120 nA. After the oocyte was washed out with choline buffer (grey line), addition of 100 mM glucose in choline buffer (dotted grey line) induced a current of 80 nA. Traces are representative of >10 experiments. **B)** Glucose-induced current at the indicated pH in Na⁺ and choline buffers. Results are the mean ± SE (4-5 oocytes) normalized to the mean glucose-induced current in Na⁺ buffer (40-100 nA). *p< 0.05 vs. Na⁺ pH 7.5.

**Figure 7 - Effect of glucose on the membrane resting potential** A) In a GLUT12-expressing oocyte, addition of 100 mM glucose in Na⁺ buffer (dotted black line) depolarized the membrane potential (Vₘ) from -32 to -21 mV. Vₘ returned to baseline value in Na⁺ after glucose was washed out from the external solution in choline buffer (grey line). Traces are representative of 8 experiments. **B)** A non-injected (NI) oocyte was bathed in Na⁺ buffer (black line); addition of 100 mM glucose (dotted black line) did not modify membrane potential. Traces are representative of >10 experiments.
Figure 8 - Effect of chloride channel inhibitors on GLUT12-mediated glucose induced-currents and transport - A) A GLUT12-expressing oocyte was held at -50 mV and perfused with Na⁺ buffer (black line). The addition of 100 mM glucose in Na⁺ buffer (dotted black line) induced a current of 60 nA, which was totally inhibited by 100 μM NFA (dashed black line). The oocyte was washed out with choline buffer (grey line). Similar experiments were performed using 100 μM NPPB (B) or 1mM DPC (C). Traces are representative of 4 (A), 4 (B) and 3 (C) experiments D) Results are the mean ± SE (3-4 oocytes) of glucose-induced current in the presence of chloride channel inhibitors normalized to mean glucose-induced currents before their perfusions (25-90 nA). Not significant (NS); *p< 0.05; **p<0.01 vs. control. E) Uptake of 20 mM glucose by GLUT12-expressing and non-injected oocytes (NI), was determined in Na⁺ buffer in the absence and presence of 100 μM NFA. Data represent the mean (± SE) of 5-10 measurements. *p< 0.05 vs. NI.

Figure 9 - Effect of genistein on GLUT12 mediated glucose induced-currents - A GLUT12-expressing oocyte was held at -50 mV and perfused with Na⁺ buffer (black line). Addition of 100 mM glucose in Na⁺ buffer (dotted black line) induced a current of 50 nA. The current was unaffected by 200 μM genistein (dashed black line). Traces are representative of 4 experiments.

Figure 10 - Effect of K⁺ on GLUT12 mediated glucose induced-currents. Glucose-induced currents under voltage-clamp (-50 mV) or membrane resting potential (mV) in GLUT12-expressing oocytes. A) Under voltage clamp (Vₘ = -50 mV), in a GLUT12-expressing oocyte, perfusion of 100 mM glucose in K⁺ (thin dotted grey line) and Na⁺ (dotted black line) buffers induced currents of 80 and 100 nA respectively. Traces are representative of 3 experiments. B) In another GLUT12-expressing oocyte, 100 mM glucose depolarized the membrane in both Na⁺ (dotted black line) and choline (thin grey...
line) buffers by ~10 mV (from -31 to -20 mV) and ~29 mV (from -55 to -26 mV) respectively. Perfusion of K\textsuperscript{+} buffer (dotted grey line), depolarized the membrane by ~35 mV (from -39 to – 4 mV). In K\textsuperscript{+} buffer, addition of 100 mM glucose (thin dotted grey line) did not modify membrane potential. Traces are representative of 3 experiments.

Figure 11 – Sorbitol induced currents through GLUT12 – A) A control non-injected oocyte was held at -50 mV and initially bathed in Na\textsuperscript{+} buffer (black line). Addition of glucose or sorbitol (100 mM) to the Na\textsuperscript{+} buffer (dotted black line) did not induce any response. Traces are representative of 3 experiments. B) A GLUT12-expressing oocyte was held at -50 mV in Na\textsuperscript{+} buffer (black line). Addition of glucose or sorbitol (100 mM) to Na\textsuperscript{+} buffer (dotted black line) induced currents of similar magnitude (30-35 nA). After exposure to each sugar, the oocyte was washed out with choline buffer (grey line). Traces are representative of 3 experiments. C) In a GLUT12-expressing oocyte, addition of glucose or sorbitol (100mM) in Na\textsuperscript{+} buffer (dotted black line) depolarized the membrane potential from -20 to -15 mV, and from -19 to -15 mV, respectively. The sugar-induced depolarization disappeared when the oocyte was washed out with choline buffer (grey line). Traces are representative of 4 experiments.

Figure 12 - Effect of activators of PKA and PKC on GLUT12 mediated glucose induced-currents and transport - GLUT12-expressing oocytes were held at -50 mV and perfused with Na\textsuperscript{+} buffer (black line). The 100 mM glucose-induced current (thin dotted black line) was recorded before and after incubating the oocyte for 30 min with Na\textsuperscript{+} buffer containing the PKA activator 8-br-cAMP (dotted black line) (A) or for 15 min with Na\textsuperscript{+} buffer containing the PKC activator PMA (dotted black line) (B). Traces are representative of 6 (A) and 3 (B) experiments respectively. C) Results are the mean
± SE (3-6 oocytes) normalized to the mean glucose-induced current prior to incubation with the activators (25-50 nA). Not significant (NS); *p< 0.05 vs. control.
Figure 2

A

Glucose uptake (pmol/100g/15min)

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<th>Condition</th>
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<th>GLUT12</th>
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</tr>
<tr>
<td>Ch pH 7.5</td>
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<td>Ch pH 6</td>
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B

% Glucose uptake

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<tr>
<td>Ch pH 7.5</td>
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<td>Ch pH 6</td>
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C

Sodium uptake (cpm/100g/5min)

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<tbody>
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<td>GLUT12</td>
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D

% Sodium uptake

<table>
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<tbody>
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<tr>
<td>Na²⁺</td>
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</tr>
</tbody>
</table>

NS

***
Figure 3

A

Glucose uptake (pmol/cell/15min)

Control
Genistein

B

Glucose uptake (pmol/cell/15min)

100 mM Ch
100 mM K⁺

C

Glucose uptake (pmol/cell/15min)

100 mM Na⁺
100 mM K⁺

D

Glucose uptake (pmol/cell/15min)

Control
8-br-cAMP
PMA
Figure 4

A

B
Figure 6

A

![Graph showing current (I) over time (200s) with different conditions: Na⁺, Na⁺ + Glucose, Choline, and Choline + Glucose.]

B

![Bar chart showing percentage of glucose (% glucose) for Na⁺ pH 7.5, Ch pH 7.5, and Ch pH 6. The bars for Ch pH 7.5 and Ch pH 6 have asterisks (*), indicating statistical significance.]
Figure 9
Figure 11

A

Glucose

Sorbitol

\[I (20 \text{ nA})\]

50 s

\[\text{Na}^+\]

\[\text{Na}^+ + \text{Substrate}\]

B

Glucose

Sorbitol

\[I (20 \text{ nA})\]

200 s

\[\text{Na}^+\]

\[\text{Na}^+ + \text{Substrate}\]

\[\text{Choline}\]

C

Glucose

Sorbitol

\[I (20 \text{ nA})\]

200 s

\[\text{Na}^+\]

\[\text{Na}^+ + \text{Substrate}\]

\[\text{Choline}\]
Figure 12

A

B

C

![Graphs showing current measurements and comparisons.](image-url)