

# Proton/solute cotransport in rat kidney brush-border membrane vesicles: relative importance to both D-glucose and peptide transport

S. Vayro, N.L. Simmons \*

Department of Physiological Sciences, The Medical School, University of Newcastle-upon-Tyne, Framlington Place, Newcastle-upon-Tyne, NE2 4HH, UK

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## Abstract

We have determined the relative importance of the transmembrane proton electrochemical gradient to the transport of D-[<sup>14</sup>C]glucose and [<sup>14</sup>C]glycylsarcosine (gly-sar) in rat kidney brush-border membrane vesicles (BBMV) from superficial renal cortex. Electrogenic [<sup>14</sup>C]gly-sar transport was first optimised by imposing a pH gradient ( $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 8.4$ ) and an interior negative p.d. (using outwardly directed  $\text{K}^+$  gradient plus valinomycin). Under identical conditions ( $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 8.4$ ), an acceleration of initial D-[<sup>14</sup>C]glucose (at 100  $\mu\text{M}$ ) transport by  $2.0 \pm 0.7$ -fold was observed compared to no proton gradient ( $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$ ). This increase was due primarily to an effect of external protons, since acidic conditions ( $\text{pH}_o = \text{pH}_i = 5.7$ ) also resulted in an acceleration of D-glucose influx (2-fold). The increase in D-glucose transport in the presence of external acidity was reduced by the uncoupler FCCP, even in the absence of a proton gradient. Furthermore, the increased D-glucose transport with external acidity in the presence of a proton gradient was insensitive to a  $\text{K}^+$  gradient-driven diffusion potential in the presence of valinomycin. In no instance was an overshoot accumulation of D-[<sup>14</sup>C]glucose observed in  $\text{H}^+$  gradient conditions.  $\text{H}^+$ -stimulated D-[<sup>14</sup>C]glucose transport showed a linear dependence on D-glucose concentration up to 20 mM D-glucose, unlike electrogenic  $\text{Na}^+$ -dependent D-glucose transport, whose  $K_m$  was  $1.77 \pm 0.35$  mM. In contrast, the initial rate of [<sup>14</sup>C]gly-sar (100  $\mu\text{M}$ ) transport by the renal  $\text{H}^+$ /di-tripeptide transporter was accelerated  $15.7 \pm 3.3$ -fold and stimulated a marked overshoot of  $5.1 \pm 0.4$ -fold over equilibrium values. Conversely, the electrogenic,  $\text{Na}^+$ /glucose transporter could be readily demonstrated, whilst [<sup>14</sup>C]gly-sar transport could not be energised by an inward  $\text{Na}^+$  gradient. The absence of electrogenic D-glucose transport in  $\text{H}^+$  gradient conditions is clear evidence against  $\text{H}^+$ /glucose cotransport in  $\text{Na}^+$ -free conditions mediated by SGLT2 (sodium-glucose transporter, renal cortex). Furthermore, since a proton gradient does not increase brush-border membrane D-glucose uptake in  $\text{Na}^+$ -rich media, it is unlikely that in vivo renal D-glucose transport mediated via SGLT2 may be energised by the transmembrane proton gradient.

**Keywords:** Proton; D-Glucose transport; SGLT2; Brush-border membrane vesicle; (Rat kidney)

## 1. Introduction

The ability of a proton electrochemical gradient to energise the net uptake of certain solutes such as di- and tripeptides across the brush-border of renal proximal tubule has been recognised for several years [1,2]. The occurrence of other substrate transport linked to a proton rather than an  $\text{Na}^+$ -electrochemical gradient has received comparatively little attention. Recently, Hirayama et al. [3] have shown that the intestinal  $\text{Na}^+$ /glucose transporter (SGLT1) when expressed in *Xenopus laevis* oocytes generates sugar-dependent inward current in the presence of a trans-

membrane proton gradient in  $\text{Na}^+$ -free conditions [3]; they showed that the affinity of SGLT1 for protons was three orders of magnitude greater than for  $\text{Na}^+$  and that protons could support a higher maximal sugar-stimulated current than could  $\text{Na}^+$ .

Along the renal proximal tubule, it is now clear that  $\text{Na}^+$ /glucose transport is not homogeneous; a low-affinity, high-capacity  $\text{Na}^+$ /glucose transporter occurs in the early proximal tubule ( $S_1/S_2$ ), whilst a high-affinity, low-capacity  $\text{Na}^+$ /glucose transporter is expressed in the late proximal tubule ( $S_3$ ) [4]. This heterogeneity is due to the differential expression of two separate but related gene products, namely SGLT1 and SGLT2 [5]; SGLT1 is expressed in mammalian small intestine and  $S_3$  renal proximal tubule, whilst SGLT2 is expressed solely in the renal

\* Corresponding author. Fax: +44 191 2226706.

cortex ( $S_1/S_2$  portions) [5–7]. Studies of the properties of the cloned  $\text{Na}^+$ /glucose transporters expressed in oocytes show that the affinity of the transporters varies in accordance with previous physiological data, that both generate inward current in voltage-clamped oocytes but that the  $\text{Na}^+$ /glucose stoichiometry varies from 1:1 in SGLT2 to 2:1 in SGLT1 [5–7].

Since the ability to link D-glucose transport to dissipative proton flow is of considerable significance, not least in the co-expression with other  $\text{H}^+$ -driven transporters, we have examined the relative ability of a proton and  $\text{Na}^+$  gradient to energise both D-glucose transport and the hydrolysis-resistant dipeptide glycylsarcosine (gly-sar) in brush-border membrane vesicles from superficial ( $S_1/S_2$  proximal tubule) rat renal cortex.

## 2. Materials and methods

### 2.1. Preparation of brush-border membrane vesicles

Renal cortical brush border-membrane vesicles were prepared by a double cation precipitation procedure as described by Biber et al. [8]. Kidneys from three male Wistar rats (220–250 g body weight) were removed and decapsulated before cutting thin slices (1–2 mm thick) of superficial cortex. The tissue was suspended 10% (w/v) in buffer containing: 100 mM mannitol, 5 mM EGTA, 0.2 mM PMSF, 10 mM Hepes/Tris (pH 7.4) and homogenised (setting 5 for 2 min) with a Kinematica homogeniser. The homogenate volume was adjusted to 40 ml with water, and 2.0 M  $\text{MgCl}_2$  added to give a final concentration of 12 mM. This suspension was centrifuged at  $2400 \times g$  for 15 min to remove large cellular debris and cation-aggregated material. Brush-border membrane vesicles remaining in the supernatant were collected by centrifuging at  $30900 \times g$  for 30 min. The pellet was resuspended in 20 ml buffer solution containing 50 mM mannitol, 2.5 mM EGTA, 0.1 mM PMSF, 5.0 mM Hepes/Tris (pH 7.4), and the cation precipitation step was repeated. The final brush-border membrane vesicle fraction was resuspended in 400–500  $\mu\text{l}$  of buffer containing (except where stated): 300 mM mannitol, 10 mM Hepes/Tris (pH 7.4). Vesicles (containing 8–10 mg/ml protein) were stored in liquid nitrogen until use. All steps of the procedure were carried out at  $4^\circ\text{C}$ .

### 2.2. Protein and enzyme assays

Protein determination was carried out by the method of Bradford, [9] using bovine serum albumin as the standard. Alkaline phosphatase and leucine aminopeptidase were used as marker enzymes for brush-border membrane vesicles. Alkaline phosphatase activity was enriched  $18.0 \pm 1.0$ -fold (mean  $\pm$  S.E.,  $n = 5$ ) over tissue homogenates, whilst leucine aminopeptidase activity was enriched  $14.1 \pm 1.6$ -fold (mean  $\pm$  S.E.,  $n = 5$ ).

### 2.3. Transport measurements

The uptake of D- $[^{14}\text{C}]$ glucose (specific activity 270 mCi/mmol) and  $[^{14}\text{C}]$ glycylsarcosine (specific activity 14 mCi/mmol) into rat renal brush-border membrane vesicles was measured at room temperature ( $20^\circ\text{C}$ ) using a rapid filtration technique as described by Berner et al. [10]. Brush-border membrane vesicles (containing 40–50  $\mu\text{g}$  protein/time point) were pipetted into a 1 ml tube. Reactions were started by the addition of 750  $\mu\text{l}$  incubation medium which contained (except where stated): 100 mM NaCl, 100 mM mannitol, 10 mM Hepes/Tris (pH 7.4) and 0.1 mM D- $[^{14}\text{C}]$ glucose or 0.1 mM  $[^{14}\text{C}]$ glycylsarcosine (about 40000 cpm). The reaction mix was taken up into the tip of a 1 ml electronic pipette (Biohit Proline, Helsinki, Finland). At appropriate time points, 60  $\mu\text{l}$  volumes of reaction mix were dispensed directly onto nitrocellulose filters (0.45  $\mu\text{m}$  pore size) under vacuum. Filters were washed,  $3 \times 3$  ml ice-cold stop solution containing: 150 mM KCl, 10 mM Hepes/Tris (pH 7.4). For proton-driven experiments, the stop buffer contained: 300 mM mannitol and 50 mM Hepes/Tris (pH 8.4) or 50 mM Mes/Tris (pH 5.7). The filters were then removed and processed for liquid scintillation counting. Zero time points were determined by dispensing 60  $\mu\text{l}$  volumes of incubation medium onto the filters and washing with  $3 \times 3$  ml stop solution. Where indicated, measurements of initial transport rates (15 s) were performed. Uptake was directly proportional to time over a period to 20 s.

### 2.4. Statistics

Data are expressed as mean  $\pm$  S.D. of  $n$  individual experiments where mean values were determined in triplicate. Tests of significance of difference between mean values was made using ANOVA (analysis of variance) using a BonFerroni method for multiple comparison  $t$ -tests between data pairs.

### 2.5. Materials

D- $[^{14}\text{C}]$ Glucose and  $[^{14}\text{C}]$ glycylsarcosine were obtained from Amersham International (UK). Valinomycin and FCCP (carbonyl cyanide  $p$ -trifluoromethoxyphenylhydrazone) and phloridzin were from Sigma and prepared as ethanolic concentrated stock solutions at 9 mM, 50 mM and 0.1 M, respectively. This gave final ethanol concentrations of 0.1%, 0.2% and 0.1% (v/v), respectively, in transport experiments. All other chemicals were of the highest quality available from Sigma (Poole, UK).

## 3. Results

Fig. 1A shows that uptake of the hydrolysis-resistant dipeptide gly-sar [1], in renal vesicles (BBMV) isolated

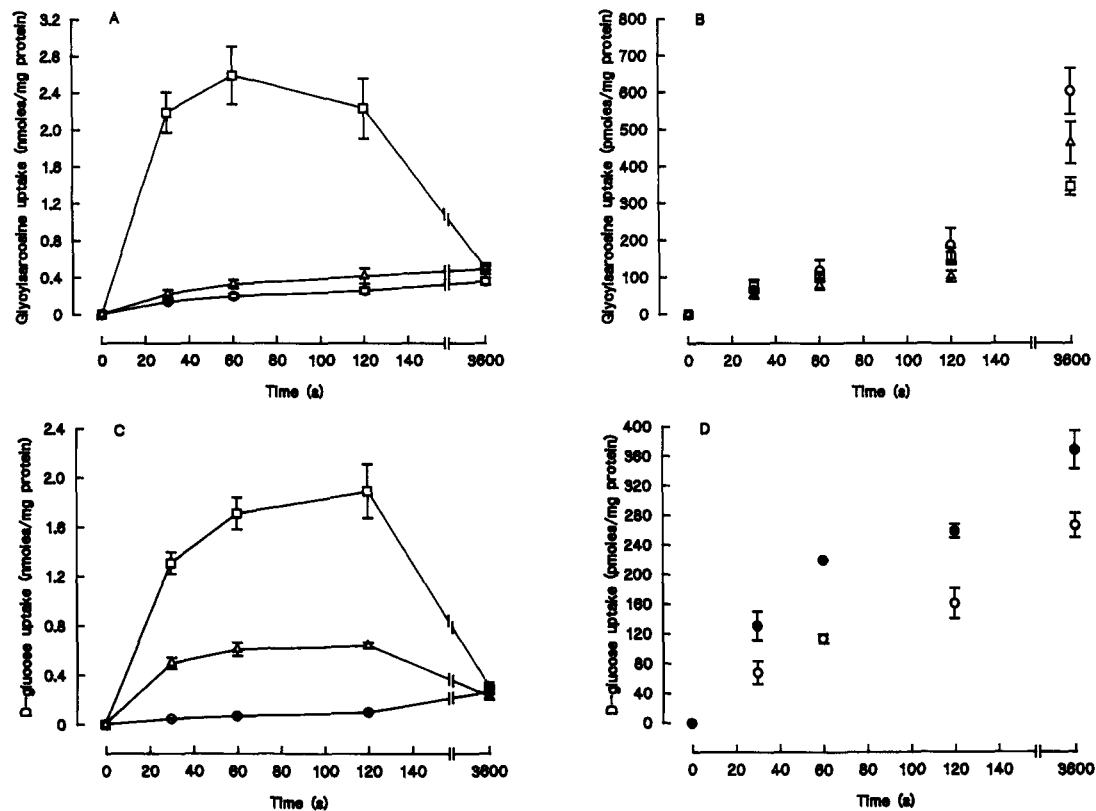


Fig. 1. Comparison of intravesicular  $[^{14}\text{C}]$ glycylsarcosine and  $\text{D}-[^{14}\text{C}]$ glucose accumulation under  $\text{H}^+$ -gradient and  $\text{Na}^+$ -gradient conditions. BBMV were prepared and gly-sar and D-glucose transport activity measured as described in the Methods section. (A) Gly-sar transport driven by a proton gradient. Intravesicular composition: 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 50 mM Hepes/Tris (pH 8.4). Extravesicular medium: 300 mM mannitol, 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.1 mM  $[^{14}\text{C}]$ gly-sar, 10  $\mu\text{M}$  valinomycin, either 50 mM Hepes/Tris (pH 8.4) (○) or 50 mM Mes/Tris (pH 5.7) (□) or 50 mM Mes/Tris (pH 5.7) plus 10  $\mu\text{M}$  FCCP (Δ). (B) Gly-sar transport under  $\text{Na}^+$ -gradient conditions. Intravesicular composition: 300 mM mannitol, 10 mM Hepes/Tris (pH 7.4). Extravesicular medium: 100 mM mannitol, 10 mM Hepes/Tris (pH 7.4), 0.1 mM  $[^{14}\text{C}]$ gly-sar, either 100 mM NaCl (□), 100 mM KCl (○) or 100 mM Na gluconate (Δ). (C) D-Glucose transport under  $\text{Na}^+$ -gradient conditions. Intravesicular composition: 300 mM mannitol, 10 mM Hepes/Tris (pH 7.4). Extravesicular medium: 100 mM mannitol, 10 mM Hepes/Tris (pH 7.4), 0.1 mM  $\text{D}-[^{14}\text{C}]$ glucose, either 100 mM NaCl (□), 100 mM KCl (○) or 100 mM sodium gluconate (Δ). (D) D-Glucose transport driven by a proton gradient. Intravesicular composition: 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 50 mM Hepes/Tris (pH 8.4). Extravesicular medium: 300 mM mannitol, 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.1 mM  $\text{D}-[^{14}\text{C}]$ glucose, 10  $\mu\text{M}$  valinomycin, either 50 mM Hepes/Tris (pH 8.4) (○) or 50 mM Mes/Tris (pH 5.7) (●). Note the similar equilibrium volume using  $\text{D}-[^{14}\text{C}]$ glucose to that measured using  $[^{14}\text{C}]$ gly-sar. Data are the mean  $\pm$  S.D. for three separate vesicle preparations, each assay performed in triplicate.

from the rat superficial cortex, is dependent upon a trans-membrane proton gradient. In the presence of an inward proton gradient ( $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 8.4$ ) with membrane potential clamped at an interior negative value in the presence of an outward  $\text{K}^+$  gradient and the presence of the  $\text{K}^+$  ionophore valinomycin, gly-sar initial uptake ( $4.38 \pm 0.43$  nmol/mg per min) is accelerated  $15.7 \pm 3.3$ -fold (with respect to no gradient, mean  $\pm$  S.D.,  $n = 3$ ) and displays an accumulative overshoot at 60 s ( $5.1 \pm 0.4$ -fold above equilibrium, mean  $\pm$  S.D.,  $n = 3$ ). In contrast, gly-sar uptake shows only a slow uptake to equilibrium in the absence of a pH gradient ( $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$  initial rate  $0.28 \pm 0.03$  nmol/mg per min) or in gradient conditions in the presence of the proton ionophore FCCP (initial rate  $0.45 \pm 0.08$  nmol/mg per min) (Fig. 1A). Table 1 shows that an outward  $\text{K}^+$  gradient in the presence of valinomycin significantly increases the initial rate of gly-sar uptake and this is dependent upon the presence of an

Table 1

Effect of a  $\text{K}^+$  diffusion p.d. on initial rates of gly-sar transport into rat kidney BBMV

Condition	$\text{H}^+$ -driven gly-sar uptake (nmol/min per mg) $\pm$ valinomycin	
	(+)	(-)
(a) $\text{pH}_o = 8.4$ , $\text{pH}_i = 8.4$	$0.26 \pm 0.03$	$0.28 \pm 0.08$ <sup>ns</sup>
(b) $\text{pH}_o = 5.7$ , $\text{pH}_i = 8.4$	$3.13 \pm 0.22$ ***	$1.63 \pm 0.15$ **

Brush-border membrane vesicles were prepared and gly-sar transport activity measured as described in the Methods section. Intravesicular composition: 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 50 mM Hepes/Tris (pH 8.4). The initial rate (15 s) of gly-sar uptake was carried out in reaction media containing 300 mM mannitol, 0.1 mM  $[^{14}\text{C}]$ gly-sar, and either (a) 50 mM Hepes/Tris (pH 8.4) or (b) 50 mM Mes/Tris (pH 5.7). Reactions were carried out  $\pm$  10  $\mu\text{M}$  valinomycin. Values are the mean  $\pm$  S.D. for three separate vesicle preparations: \*\*\*, significantly different from control media ( $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$ ) ( $P < 0.001$ ); \*\* significantly different from equivalent media ( $P < 0.01$ ); <sup>ns</sup>, not significantly different from equivalent media ( $P > 0.05$ ).

inwardly directed proton gradient. These data are expected for the electrogenic  $H^+$ /dipeptide cotransport and are in complete agreement to those previously reported by Miyamoto et al. [11].

Under inward  $Na^+$ -gradient conditions (Fig. 1B), no accumulative gly-sar transport is observed. Comparing Fig. 1A and 1B, the initial rate of gly-sar uptake in NaCl media ( $0.15 \pm 0.03$  nmol/mg per min) is not accelerated compared to mannitol media where no pH gradient exists ( $pH_o = 8.4$ ) or in acidic media in the presence of FCCP ( $0.28 \pm 0.03$  nmol/mg per min and  $0.45 \pm 0.08$  nmol/mg per min, respectively, mean  $\pm$  S.D.,  $n = 3$ ). Thus, an  $Na^+$  gradient alone cannot energise gly-sar transport.

D-Glucose absorption in superficial cortical BBMV will be mediated primarily by SGLT2 [5–7]. Fig. 1C shows that the presence of an inward NaCl gradient accelerates the initial rate of D-glucose uptake 29-fold (compared to KCl; initial rates  $2.62 \pm 0.18$  and  $0.09 \pm 0.007$  nmol/mg per min, respectively) and drives accumulative transport (5.5-fold at 60 s) above equilibrium values at 60 min. In the presence of an inward 100 mM sodium gluconate gradient, initial D-glucose uptake ( $1.00 \pm 0.09$  nmol/mg per min) and accumulative transport are depressed compared to NaCl media ( $P < 0.01$ ) due to the presence of a  $Cl^-$  conductance in rat BBMV through which a  $Cl^-$  diffusion potential difference (inside negative) acts on  $Na^+$ /glucose transport [13].

When D-glucose transport is measured under identical conditions to those optimal for proton-driven gly-sar accumulative transport, no accumulative transport is observed (Fig. 1D). However, the rate of D-glucose transport observed is significantly stimulated by  $2.0 \pm 0.7$ -fold (mean  $\pm$  S.D.,  $n = 3$ ) with an imposed pH gradient ( $0.26 \pm 0.04$  nmol/mg per min for  $pH_o = 5.7$ ,  $pH_i = 8.4$  and  $0.14 \pm 0.03$  nmol/mg per min for  $pH_o = 8.4$ ,  $pH_i = 8.4$ ,  $P < 0.01$ , Fig. 1D). The ability of protons to stimulate D-glu-

cose transport [3] is minor compared to that proton gradient stimulation observed with gly-sar transport. The overall level of D-glucose transport observed in pH gradient conditions (after 2 min), is only 13% of that seen in NaCl media (compare Fig. 1C and 1D). The acceleration of the rate of D-glucose uptake in acidic conditions ( $pH_o = 5.7$ ,  $pH_i = 8.4$ ) is not modified when mannitol is replaced by *N*-methyl-D-glucamine gluconate ( $0.26 \pm 0.04$  nmol/mg per min versus  $0.11 \pm 0.04$  nmol/mg per min, respectively, mean  $\pm$  S.D.,  $n = 3$ ) showing that ionic strength does not modify the effect of an imposed pH gradient. Varying the external pH ( $pH_o = 5.5$ – $8.5$ ,  $pH_i = 7.5$ ) had no significant effect on the initial rate of  $Na^+$ -dependent D-glucose transport into the vesicles (Fig. 2A,  $P > 0.05$ , ANOVA,  $4.10 \pm 0.23$  nmol/mg per min versus  $4.60 \pm 0.54$  nmol/mg per min for  $pH_o = 5.5$ ,  $pH_i = 7.5$  and  $pH_o = 7.5$ ,  $pH_i = 7.5$ , respectively,  $P > 0.05$ , mean  $\pm$  S.D.,  $n = 3$ ). In contrast, a significant increase in D-glucose uptake under acidic conditions was observed when NaCl was replaced with KCl media (Fig. 2B),  $0.34 \pm 0.04$  nmol/mg per min versus  $0.12 \pm 0.04$  nmol/mg per min for  $pH_o = 5.5$ ,  $pH_i = 7.5$  and  $pH_o = 7.5$ ,  $pH_i = 7.5$ , respectively ( $P < 0.01$ , mean  $\pm$  S.D.,  $n = 3$ ). The lack of modification of D-glucose uptake in  $Na^+$ -media at different external pH suggests that vesicle integrity (leak) permeability is not altered in acidic media.

Does the increased D-glucose uptake observed in  $Na^+$ -free acidic conditions result from a direct effect upon SGLT2? We have tested the phloridzin sensitivity of the increased D-glucose transport in acidic media. With  $pH_o = 8.4$ ,  $pH_i = 8.4$ , D-glucose transport was increased from  $0.14 \pm 0.02$ ,  $n = 3$ , to  $0.27 \pm 0.03$  nmol/min per mg,  $n = 3$  ( $P < 0.05$ ) with  $pH_o = 5.7$ ,  $pH_i = 8.4$ . In the presence of 200  $\mu$ M phloridzin, this increase was abolished ( $pH_o = 8.4$ ,  $pH_i = 8.4$ , transport was  $0.09 \pm 0.02$ ,  $n = 3$ , with  $pH_o = 5.7$ ,  $pH_i = 8.4$  transport was  $0.10 \pm 0.02$  nmol/min per mg protein  $n = 3$ ,  $P > 0.05$ ). In addition,

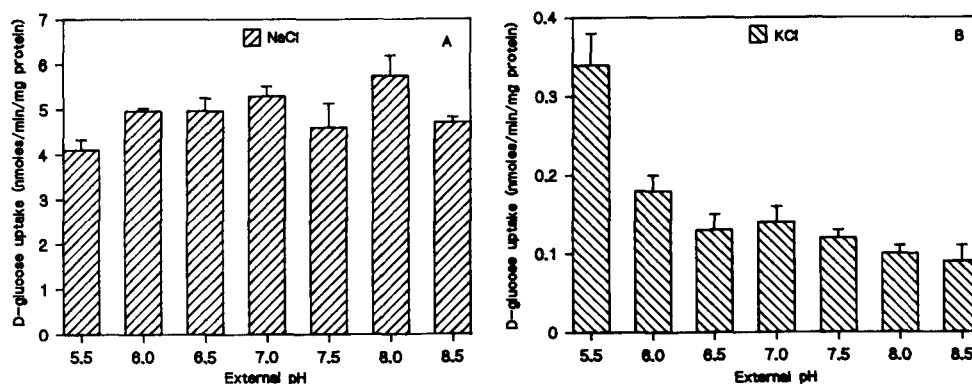


Fig. 2. Effect of external pH on D-glucose transport in  $Na^+$  media and  $K^+$  media. BBMV were prepared and D-glucose transport activity measured as described in the Methods section. The initial rate (15 s) of D-glucose transport was measured in either NaCl or KCl media over a range of external pH conditions (pH 5.5–8.5). Intravesicular composition: 300 mM mannitol, 50 mM Hepes/Tris (pH 7.5). (A) Extravesicular medium: 100 mM NaCl, 100 mM mannitol, 0.1 mM D-[ $^{14}C$ ]glucose, either 50 mM Mes/Tris for pH 5.5–6.5 or 50 mM Hepes/Tris for pH 7.0–8.5. (B) Extravesicular medium: 100 mM KCl, 100 mM mannitol, 0.1 mM D-[ $^{14}C$ ]glucose, either 50 mM Mes/Tris for pH 5.5–6.5 or 50 mM Hepes/Tris for pH 7.0–8.5. Data are the mean  $\pm$  S.D. for three separate vesicle preparations, each assay performed in triplicate.

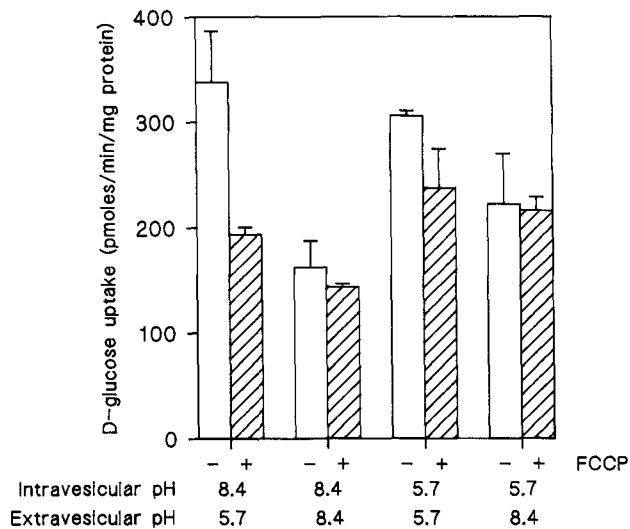


Fig. 3. Effect of acidic media and of 10  $\mu\text{M}$  FCCCP on initial D-glucose uptake. Initial rates of D-glucose uptake were measured with the intravesicular composition established with 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , either 50 mM Hepes/Tris (pH 8.4) or 50 mM Mes/Tris (pH 5.7). Extravesicular medium contained 300 mM mannitol, 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.1 mM D-[ $^{14}\text{C}$ ]glucose, 10  $\mu\text{M}$  valinomycin, either 50 mM Hepes/Tris (pH 8.4) or 50 mM Mes/Tris (pH 5.7), in the absence (open bars) or presence (hatched bars) of 10  $\mu\text{M}$  FCCCP. Data are the mean  $\pm$  S.D. for three separate vesicle preparations, each assay performed in triplicate.

the effect of external D-fructose or D-galactose on the initial rate of D-glucose transport was investigated. The expected increase in initial rate of D-glucose uptake in  $\text{Na}^+$ -free acidic conditions ( $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 8.4$  relative to  $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$ ) was observed. The initial rate of D-glucose uptake in acidic conditions ( $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 8.4$ ) was unaltered in the presence of either D-fructose or D-galactose (both 10 mM;  $0.31 \pm 0.04$  versus  $0.31 \pm 0.05$  and  $0.30 \pm 0.004$  nmol/min per mg for control vesicles and vesicles  $\pm$  D-fructose or D-galactose, respectively, mean  $\pm$  S.D. for two separate experiments, each assay performed in triplicate). This suggests that D-glucose uptake (in acidic conditions) is not mediated by the kidney cortex D-fructose transporter, GLUT5 [17].

Does the effect of external acidic conditions reflect a proton gradient per se? Fig. 3 shows how D-glucose transport is affected when either the external or internal environment of the vesicle preparation is altered. Extravesicular acidity stimulates D-glucose uptake as described above (Fig. 3, compare  $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$ , with  $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 8.4$ ,  $P < 0.05$ ). However, when both intravesicular and extravesicular pH are set to 5.7 (in the absence of a proton gradient) D-glucose uptake is also enhanced compared to  $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$ ,  $P < 0.05$ . When the intravesicular pH is set at 5.7 ( $\text{pH}_o = 8.4$ ), D-glucose uptake is increased, but not significantly compared to  $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$ . A test of the proton gradient dependence of the increased D-glucose transport rate is to use the protonophore FCCCP. Fig. 3 shows the increased rate of D-glucose uptake in acidic gradient conditions is abolished when the

protonophore FCCCP (10  $\mu\text{M}$ ) was present (compare data for  $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 8.4$ , with  $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$  in the presence or absence of FCCCP). However, confirmation of an apparent proton gradient dependence by this experiment is confounded by the reduction of D-glucose uptake by FCCCP with intra and extravesicular acidic media ( $P < 0.047$  paired data, Fig. 3). Taken together with the observation that D-glucose transport reached levels of uptake in acidic pH-clamped conditions similar to those in pH gradient conditions (above), this suggests that SGLT2 undergoes allosteric modification by protons.

A test of  $\text{H}^+$ /glucose cotransport would be that the increased D-glucose uptake observed in proton gradient  $\text{Na}^+$ -free conditions should be sensitive to the transmembrane electrical p.d. as is the case for  $\text{H}^+$ /gly-sar transport (above). Table 2 summarises experiments conducted to investigate the electrogenicity of D-glucose transport. It is evident that external acidic media stimulates D-glucose uptake (Table 2a vs. 2b). This effect of external acidity is also observed in the absence of a proton gradient ( $\text{pH}_o = 5.7$ ,  $\text{pH}_i = 5.7$ , Table 2a vs. 2e,f). In addition, it is apparent that this increased transport is insensitive to the imposition of an outward  $\text{K}^+$  diffusion p.d. (Table 2b  $\pm$  valinomycin). Valinomycin has no effect upon D-glucose transport under any of the conditions tested, i.e., in the absence of a  $\text{K}^+$  gradient, and in the presence or absence of a transmembrane pH gradient. The lack of sensitivity of

Table 2

Initial rates of D-glucose transport into rat kidney BBMV in the presence or absence of a pH gradient or membrane potential gradient

Condition		D-Glucose uptake (nmol/min per mg) $\pm$ valinomycin	
$\text{K}^+$ -gradient	pH gradient	(+)	(-)
0 $\text{K}_o$ / 100 $\text{K}_i$	(a) 8.4 $_o$ / 8.4 $_i$	0.15 $\pm$ 0.02	0.16 $\pm$ 0.03
	(b) 5.7 $_o$ / 8.4 $_i$	0.28 $\pm$ 0.04 <sup>s</sup>	0.28 $\pm$ 0.02 <sup>s,ns</sup>
100 $\text{K}_o$ = 100 $\text{K}_i$	(c) 8.4 $_o$ / 8.4 $_i$	0.14 $\pm$ 0.02	0.16 $\pm$ 0.02
	(d) 5.7 $_o$ / 8.4 $_i$	0.26 $\pm$ 0.04 <sup>s</sup>	0.23 $\pm$ 0.02 <sup>s,ns</sup>
0 $\text{K}_o$ / 100 $\text{K}_i$	(e) 5.7 $_o$ / 5.7 $_i$	0.29 $\pm$ 0.04 <sup>s</sup>	0.27 $\pm$ 0.009 <sup>s,ns</sup>
100 $\text{K}_o$ = 100 $\text{K}_i$	(f) 5.7 $_o$ / 5.7 $_i$	0.29 $\pm$ 0.06 <sup>s</sup>	0.27 $\pm$ 0.03 <sup>s,ns</sup>

Brush-border membrane vesicles were prepared and D-glucose transport activity measured as described in the Methods section. Intravesicular composition: (a–d) 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 50 mM Hepes/Tris (pH 8.4); (e–f) 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 50 mM Mes/Tris (pH 5.7). The initial rate (15 s) of D-glucose uptake was carried out in reaction media containing: (a,b) 300 mM mannitol, 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.1 mM D-[ $^{14}\text{C}$ ]glucose, and either 50 mM Hepes/Tris (pH 8.7) or 50 mM Mes/Tris (pH 5.7); (c,d) 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.1 mM D-[ $^{14}\text{C}$ ]glucose, 50 mM Hepes/Tris (pH 8.4) or 50 mM Mes/Tris (pH 5.7); (e) 300 mM mannitol, 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.1 mM D-[ $^{14}\text{C}$ ]glucose, 50 mM Mes/Tris (pH 5.7); (f) 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.1 mM D-[ $^{14}\text{C}$ ]glucose, 50 mM Mes/Tris (pH 5.7). All reactions were carried out  $\pm$  10  $\mu\text{M}$  valinomycin. Values are the mean  $\pm$  S.D. for four separate vesicle preparations: <sup>s</sup>, significantly different from control media ( $\text{pH}_o = 8.4$ ,  $\text{pH}_i = 8.4$ ) ( $P < 0.001$ ); <sup>ns</sup>, not significantly different from equivalent media ( $P > 0.05$ ).

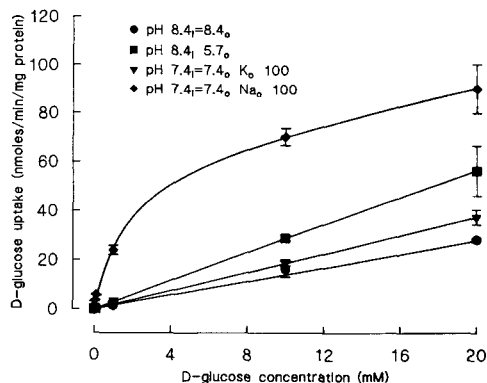


Fig. 4. D-Glucose concentration dependence of initial D-glucose transport into rat kidney BBMVs in  $\text{Na}^+$ -media and  $\text{Na}^+$ -free acidic media. BBMVs were prepared and the initial rate (15 s) of D-glucose transport activity measured as described in the Methods section. For  $\text{H}^+$ /glucose transport, BBMVs intravesicular composition was 100 mM  $\text{K}_2\text{SO}_4$ , 50  $\mu\text{M}$   $\text{MgSO}_4$ , 50 mM Hepes/Tris (pH 8.4). Extravesicular medium was 300 mM mannitol, 50  $\mu\text{M}$   $\text{MgSO}_4$ , 0.05–20 mM D-[ $^{14}\text{C}$ ]glucose, 10  $\mu\text{M}$  valinomycin, either 50 mM Hepes/Tris (pH 8.4) (●) or 50 mM Mes/Tris (pH 5.7) (■). For D-glucose transport under  $\text{Na}^+$ -gradient conditions the intravesicular composition was 300 mM mannitol, 10 mM Hepes/Tris (pH 7.4). Extravesicular medium was 100 mM mannitol, 0.05–20 mM D-[ $^{14}\text{C}$ ]glucose, 10 mM Hepes/Tris (pH 7.4), either 100 mM NaCl (◆) or 100 mM KCl (▼). Data mean  $\pm$  S.D. for three separate vesicle preparations, each assay performed in triplicate.

acid-stimulated D-glucose transport contrasts to that observed with gly-sar (Table 1, above) and strongly supports the idea that this effect does not represent  $\text{H}^+$ /transport.

Fig. 4 shows the kinetics of initial  $\text{Na}^+$ -dependent D-glucose uptake: the  $K_m$  was  $1.77 \pm 0.39$  mM, whilst the  $V_{\max}$  was  $63.33 \pm 7.35$  nmol/mg per min (NaCl minus KCl data). This relatively low affinity for D-glucose (compared to SGLT1) is typical for SGLT2 [4,5]. Confirmation of the SGLT2 identity of D-glucose transport in superficial cortical BBMVs is shown by adding excess D-galactose (10 mM) [5], which does not markedly depress  $\text{Na}^+$ -driven D-glucose uptake (initial rates  $3.26 \pm 0.39$  nmol/mg per min and  $2.77 \pm 0.19$  nmol/mg per min,  $P > 0.05$ , mean  $\pm$  S.D.,  $n = 3$ , respectively).

The  $\text{H}^+$ -gradient-driven D-glucose uptake shows a linear dependence upon D-glucose concentration (Fig. 4) up to 20 mM, indicating that the affinity of D-glucose for  $\text{H}^+$ /glucose transporter is very low compared to that seen in  $\text{Na}^+$  gradient conditions (Fig. 4). At physiological levels of D-glucose within the proximal tubule (5 mM decreasing progressively along the  $\text{S}_1/\text{S}_2$  portions), external acid media display only a limited ability to increase D-glucose uptake, whilst no accumulative D-glucose transport via SGLT2 is observed (above).

#### 4. Discussion

The ability of an  $\text{H}^+$ -gradient alone to energise D-glucose transport in oocytes expressing SGLT1 has been suggested by voltage-clamp experiments in which a D-glu-

cose and proton-dependent inward current in  $\text{Na}^+$ -free media was recorded. Furthermore, the  $\text{H}^+$  (D-glucose-dependent) inward current was partially reversed in the presence of 10  $\mu\text{M}$  inhibitor phloridzin. The kinetics of the D-glucose-dependent  $\text{H}^+$  current show a low affinity (20 mM with respect to  $\alpha$ -methyl-D-glucopyranoside ( $\alpha$ -MDG)), but higher capacity compared to  $\text{Na}^+$  inward current mediated by  $\text{Na}^+$ /glucose transport ( $K_m = 0.2$  mM for  $\alpha$ -MDG). In small intestine it has been suggested that the proton gradient may be physiologically relevant to D-glucose accumulation mediated by SGLT1 [3]. Indeed, in toad small intestine in  $\text{Na}^+$ -free media, D-glucose may stimulate an inward short-circuit current in the presence of  $\text{Na}^+$ -free apical (brush-border) acidic solutions [14].

In the present study we have utilised brush-border membrane vesicles from rat to determine whether the protonmotive force at the apical membrane of renal proximal tubule ( $\text{S}_1/\text{S}_2$  segments) may also be important for D-glucose accumulation. In these experiments we have utilised the expression of an  $\text{H}^+$ /oligopeptide transport system as a useful intrinsic control of  $\text{H}^+$ /transport. The distribution of  $\text{H}^+$ /oligopeptide transport along the nephron is uncertain; saturable absorption of intact peptide in the early/mid proximal tubule is evident, but the bulk of absorption is likely to be present in the late proximal tubule ( $\text{S}_3$ ) [1]. Both a high-affinity low-capacity and low-affinity high-capacity transport of peptides in renal vesicles have been reported [12], but this was from mixed medullary/cortical tissue. The exact segmental distribution of these transporters is at present not defined. Recently, an  $\text{H}^+$ /oligopeptide transport system unique to renal epithelium (PEPT2) has been identified using homology cloning and functional expression using a vaccinia vector and HeLa cells [15]. This expression contrasts with the homologous intestinal  $\text{H}^+$ /oligopeptide transporter (PEPT1), which is expressed in both intestine and kidney [15]. In the present study, BBMVs isolated from the superficial cortex will consist of  $\text{S}_1/\text{S}_2$  proximal tubules [4]. Notwithstanding these facts, under the conditions established (external pH = 5.7, internal pH = 8.4, with an outward  $\text{K}^+$  gradient in the presence of valinomycin to generate an intravesicular negative p.d.), a marked pH-gradient-dependent and electrogenic transport of gly-sar is observed that results in a marked overshoot (accumulative transport). An  $\text{Na}^+$  gradient is incapable of energising gly-sar transport.

SGLT1 is expressed in mammalian small intestine and  $\text{S}_3$  renal proximal tubule, whilst SGLT2 is expressed solely in the renal cortex ( $\text{S}_1/\text{S}_2$  portions) [5–7]. With SGLT1, maximal rates of D-glucose stimulated  $\text{H}^+$  inward current (at saturating conditions measured using voltage clamp) in  $\text{Na}^+$ -free media are observed that exceed those for  $\text{Na}^+$ /glucose transport (at saturating conditions), though D-glucose affinity is lower than that observed in  $\text{Na}^+$  gradient conditions [3]. Measurement of D-glucose transport rates under  $\text{H}^+$  gradient conditions in oocytes in order to confirm that  $\text{H}^+$  gradients may generate stoichio-

metric D-glucose fluxes have yet to be published. In small intestine BBMV, the effects of  $H^+$  gradients on  $Na^+$ -independent D-glucose fluxes have been investigated [14]; a pH gradient ( $pH_o = 5.5$ ,  $pH_i = 7.5$ ) is able to increase (2-fold) the initial D-glucose transport in  $K^+$ -loaded rabbit small intestinal vesicles in the presence of valinomycin. The present study shows that, for SGLT2 in rat renal BBMV, D-glucose transport is increased by external acidic media and that this is sensitive to inhibition by phloridzin (200  $\mu M$ ).

The magnitude of the effect of external protons on D-glucose transport via SGLT2 is small relative to fluxes observed in  $Na^+$ -media. Initial D-glucose uptake is accelerated  $2.0 \pm 0.7$ -fold by external acidic media, whereas an inward  $Na^+$  gradient accelerates D-glucose uptake 29-fold (compared to KCl media) at identical substrate concentrations. This rate of  $H^+$ /D-glucose transport is also to be compared to that observed for gly-sar uptake under equivalent  $H^+$  gradient conditions in the same vesicle population. In comparison to  $Na^+$  gradients, no accumulative D-glucose transport is observed and the acceleration of D-glucose transport shows no evidence of saturation up to 20 mM D-glucose. The D-glucose affinity for transport in acidic conditions is low compared to that in  $Na^+$ -rich media (1.77 mM), in agreement with D-glucose-stimulated  $H^+$  currents mediated by SGLT1 [3]. However, the increased D-glucose transport with acidic external media is seen in both gradient and non-gradient (but acidic) media. A key test of  $H^+$ /glucose cotransport would be that such transport would be sensitive to the transmembrane p.d. For  $H^+$ /gly-sar transport in this vesicle preparation this is indeed the case. In contrast, D-glucose transport in these conditions is not sensitive to an imposed  $K^+$  diffusion p.d. in the presence of valinomycin. We have confirmed these data for both gly-sar and glucose transport in the same experiment using the same vesicle population (not shown). It must therefore be concluded that the low-affinity, acid-stimulated D-glucose transport in renal cortical BBMV does not represent  $H^+$ /cotransport. Alternative explanations are possible. External acidic media are likely to titrate key amino-acids of the SGLT2 protein involved in substrate recognition as is observed with the human erythrocyte D-glucose transporter in acidic media [16]. Thus, the increased D-glucose transport rates observed in  $Na^+$ -free acidic media may represent diffusive D-glucose flux via SGLT2. Since this transport is sensitive to phloridzin and unaffected by an excess of D-fructose, it is unlikely that this represents a contribution from the GLUT5 isoform of the facilitative D-fructose transporter [17]. Though the lack of potential dependence implies an absence of  $H^+$ /cotransport, we cannot, however, exclude the possibility that a D-glucose-induced conformational change might induce a proton conductance via SGLT2. It is possible that SGLT1 and SGLT2 differ markedly with respect to the cation (driving ion) specificity. It is apparent that further

experiments are required to measure both  $H^+$  and D-glucose fluxes and their interdependence in oocytes expressing both SGLT1 and SGLT2.

For the early proximal tubule, the data presented here show that, whereas  $H^+$ /dipeptide transport must be of functional importance ( $Na^+$  is ineffective as a driving cation), it is unlikely that  $H^+$ -stimulated D-glucose transport is of physiological significance relative to  $Na^+$ /glucose transport, given the prevailing conditions in which a brush-border  $Na^+$  gradient is maintained at a lumen pH of 7–7.5 with a lumen D-glucose concentration at maximum 5 mM. Additionally, it is likely that any  $H^+$  influx generated via SGLT2 will be minor compared to dissipative  $H^+$  influx generated across the brush-border membrane by  $H^+$ /oligopeptide transport. Confirmation as to the relative importance of these  $H^+$  fluxes to cytoplasmic  $pH_i$  homeostasis will need to be assessed in an intact system.

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