Stimulation of fructose transport across the intestinal brush-border membrane by PMA is mediated by GLUT2 and dynamically regulated by protein kinase C

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Perfusion of rat jejunum *in vitro* with PMA increased fructose transport by 70 % compared with control values and was blocked by the protein kinase C (PKC) inhibitor chelerythrine. The brush-border membrane contained both the fructose transporters GLUT5 and GLUT2; the presence of the latter was confirmed by luminal biotinylation. PMA increased the GLUT2 level 4-fold within minutes, so that the level was comparable with that of the basolateral membrane, but had no effect on GLUT5 level. GLUT2 was functional, accessible to luminal fructose and could be inhibited selectively by phloretin to permit determination of GLUT2- and GLUT5-mediated transport components. The 4-fold increase in GLUT2 level induced by PMA was matched by a 4-fold increase in GLUT2-mediated transport: there was a compensatory fall in the GLUT5-mediated rate. The pattern of dynamic trafficking was seen only for GLUT2, not GLUT5 or SGLT1, implying that GLUT2 trafficks to the brush-border membrane by a different pathway. Trafficking of GLUT2 to the brush-border membrane correlated with activation of PKC βII, implying that this isoenzyme is likely to control trafficking. Since PKC is activated by endogenous hormones, GLUT2 levels *in vivo* are 3–4-fold those *in vitro*; moreover, because PKC is inactivated as soon as intestine is excised, GLUT2 is lost from the brush-border within minutes *in vitro*. It is therefore difficult to detect GLUT2 in most *in vitro* preparations and its role in intestinal sugar absorption across the brush-border membrane has accordingly been overlooked.

Key words: GLUT5, intestine, sugar, trafficking.

INTRODUCTION

Fructose is transported across the small intestine by two members of the facilitative glucose transporter family, namely GLUT5 at the brush-border and GLUT2 at the basolateral membrane in human [1] and rat [2–4]. GLUT5 in rat is specific for fructose and is not inhibited by glucose even at ratios as high as 100:1 or by phloretin. GLUT2 on the other hand transports glucose as well as fructose and is inhibited by phloretin [5,6].

Both GLUT5 and GLUT2 are involved in the adaptation of fructose transport to streptozotocin-diabetes; moreover, GLUT2 then also plays a role in transport across the brush-border membrane. Thus we have observed that GLUT5 levels in the brush-border membrane were enhanced almost 3-fold and that GLUT2 was readily detectable at the brush-border membrane in the jejunum of diabetic rats [5]. Crucially, GLUT2 was accessible to fructose in the luminal perfusate and was fully functional. We were able to demonstrate that the 1.6-fold overall enhancement in fructose transport observed in diabetes comprised a phloretin-sensitive component mediated by GLUT2 and a phloretin-insensitive component mediated by GLUT5. Diabetes diminished the intrinsic activity (the transport activity per molecule) of GLUT5 to 20 % of its value in normal rat jejunum.

The absorptive epithelial cells of intestine undergo continuous and rapid renewal. When cells leave the proliferative compartment of the crypts, they differentiate and mature into functional absorptive cells as they migrate up the villus to be extruded from the tip after a few days; at any one time the entire sequence of developmental events is displayed. Protein kinase C (PKC) is a serine/threonine kinase that plays an important role in the regulation of cell growth and differentiation. PKC comprises a family of up to 11 isoenzymes, which show different sensitivities to activators, including diacylglycerol, phospholipid and Ca^{2+} [7]. Several of the isoenzymes are present in intestine [8], where they play an important role in development. In many cell lines, PKC isoenzymes can regulate the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAP kinase) pathway, while in others the phosphatidylinositol 3-kinase pathway can regulate PKC isoenzymes, notably PKCξ. Both these pathways are regulated by insulin, which we have shown is involved in the regulation of both intestinal glucose and fructose transport [5,9–11]. Moreover, in pancreatic islets, perfusion with glucose causes rapid translocation and activation of PKCα to the plasma membrane [12]. We therefore decided to investigate the role of PKC in the regulation of rat jejunal fructose transport.

EXPERIMENTAL

Animals

Male Wistar rats (240–260 g) were fed *ad libitum* on standard Bantin and Kingman rat and mouse diet with free access to water.

Perfusion of jejunal loops

Rats were anaesthetized with an intraperitoneal injection of a mixture of 1.0 ml of Hypnorm (Janssen Animal Health, High Wycombe, Bucks., U.K.) and 0.4 ml of Hypnovel (Roche Diagnostics, Welwyn Garden City, Herts., U.K.) per kg of body

Abbreviations used: PKC, protein kinase C; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; sulpho-NHS-LC-biotin, sulphosuccinimidyl-6-(biotinamido) hexanoate.

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weight. The systems for the luminal perfusion of jejunal loops *in vitro* and *in vivo* have been described in detail previously [5,11,13]. Because PKC activators and inhibitors take some time to become effective in whole tissue and because there is a limit to the time of viability for isolated loops *in vitro*, the following procedure for the treatment of jejunum with drugs was adopted. Jejunum was first perfused luminally *in vivo* with 5 mM D-fructose (plus 1 mM β-hydroxybutyrate as an energy source) in the presence or absence of drugs: the perfusion system was a gas-segmented, single-pass system with perfusate and gas flow rates of 0.75 and 0.38 ml·min^{-1} respectively. Jejunum was perfused with 200 nM PMA for 30 min or with chelerythrine for 45 min: when they were perfused in combination, PMA was added to the perfusate 15 min after the inhibitor. At the end of the *in vivo* treatment period, the cannulated loop was then excised and perfused *in vitro* using the gas-segmented, recirculated flow system: 5 mM D-fructose and the combination of drugs at the end of the *in vivo* treatment period were perfused luminally through the jejunum for 60 min; the flow rates of perfusate and gas were 7.0 and 3.4 ml·min^{-1} respectively. Control perfusions were performed in which no drugs were present; these showed that there was no falling off in the D-fructose uptake rate after the initial approach to the steady state, confirming that this approach to drug treatment and perfusion ensured that the jejunum was viable for the whole experimental period from the start of the *in vivo* to the finish of the *in vitro* perfusion. Samples (0.05 ml) were taken from the perfusate at 5-min intervals throughout the *in vitro* perfusion. After measurement of fructose concentration with a COBAS automatic analyser (Roche Diagnostics) using a test kit from Boehringer (supplied by Digen, Oxford, Oxfordshire, U.K.), the amount of fructose remaining in the perfusate was calculated, with correction for losses in perfusate volume caused by water transport. Because the perfusate is recirculated, the concentration of fructose decreases with time; it was therefore necessary to limit the period over which the rate of transport was measured to one in which the concentration decreased by no more than 20%. Over such periods, plots of luminal perfusate content versus time are effectively linear. The rate of fructose transport, expressed as μmol·min^{-1}·(g of dry weight)$^{-1}$, was therefore determined by linear regression analysis as the average rate of disappearance over this period from the luminal perfusate *in vitro*.

Membrane-vesicle preparation

Brush-border membrane vesicles were made as described previously [5]: every stage of the preparation was performed at 0–4 °C to prevent changes in trafficking after the intestine had been excised. Briefly, the jejuna of two rats were perfused with drugs as described above. The timing of the final *in vitro* perfusion was such as to get as direct a correspondence as possible between rates of transport and extent of trafficking. Perfusions for vesicle preparations were therefore terminated at a point corresponding to half the time period over which the average rate of transport was determined, as described above. Immediately after perfusion, each jejunum was rinsed with ice-cold buffered mannitol (20 mM imidazole buffer, pH 7.5, containing 250 mM mannitol and 0.1 mM PMSF), placed on an ice-cold glass plate and slit longitudinally so that the muscle of the jejunum flattened out on to the cold plate. Mucosal scrapings were taken with an ice-cold glass slide and homogenized immediately at 4 °C in buffered mannitol using a Kinematica Polytron homogenizer (four 30 s bursts using the large probe at setting 7). The rest of the preparation and its detailed characterization for purity were as described by Corpe et al. [5]. Enrichment of sucrase activity in these highly purified preparations ranged from 16- to 20-fold; there was no significant enrichment of Na^+/K^+-ATPase activity.

Basolateral membrane vesicles were also prepared as described in [5] and had an enrichment of 11–23-fold in ouabain-sensitive Na^+/K^+-ATPase activity; there was no significant enrichment of sucrase activity.

Biotinylation of brush-border membrane proteins

A 20-cm loop of normal jejunum was perfused with 5 mM fructose as described above: the loop was then flushed with ice-cold perfusate to stop trafficking and all subsequent operations were performed at 0–4 °C. The loop was tied off at the aboral end, gently filled with 1 mM sulphosuccinimidyl-6-(biotinamido) hexanoate [sulpho-NHS-LC-biotin; a membrane-impermeant reagent from Pierce & Warriner (UK), Chester, Cheshire, U.K.] and tied at the oral end. Following incubation for 30 min, the contents of the loop were flushed away with ice-cold 50 mM NH_4Cl to quench the reaction. After flushing with PBS, a mucosal scrape was homogenized in PBS containing 1% Triton X-100, 0.2% SDS and 1 mM PMSF: 0.2 ml of avidin-agarose (Sigma, Poole, Dorset, U.K.) was added to the homogenate and incubated on a rotating table for 1 h, when the agarose was isolated and washed three times with PBS by using a microfuge. Biotinylated proteins were solubilized by incubation in 0.1 ml of SDS sample buffer at 70 °C for 3 min.

Western blotting

SDS/PAGE and Western blotting were also performed as described previously using ECL® (enhanced chemiluminescence) detection [5]. Immunoblotting was performed using polyclonal antibodies raised in rabbit to the C-terminal sequences of GLUT5 and GLUT2, provided by Professor G. W. Gould (University of Glasgow, Glasgow, U.K.). SGLT1 antibody raised in rabbit was a gift from Professor H. Koepsell (Anatomisches Institut, Universität Würzburg, Würzburg, Germany). Antibody to PKC βII was raised in rabbit by Professor N. Groome (Oxford Brookes University, Oxford, U.K.) and provided by Dr M. G. Rumsby (University of York, York, U.K.). Quantification of Western blots was performed using a Flowgen AlphaImager 1200 analysis system (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). The level of GLUT5 determined in vesicle preparations from jejunum treated with different drugs was expressed relative to that in control preparations. All measurements were made within the linear range for the intensity response in ECL photographs; this was established carefully using a standard curve with different amounts of actin. The relative intrinsic activity of the transporter was defined as the transport activity per molecule of transporter, given by the transport rate divided by the transporter density and expressed relative to that in control preparations.

Statistical analysis

Values are presented as means ± S.E.M. and were tested for significance using a Student's t test.

RESULTS

In order to establish whether a PKC-dependent pathway might be involved in the regulation of fructose transport across the brush-border membrane, isolated loops of rat jejunum were

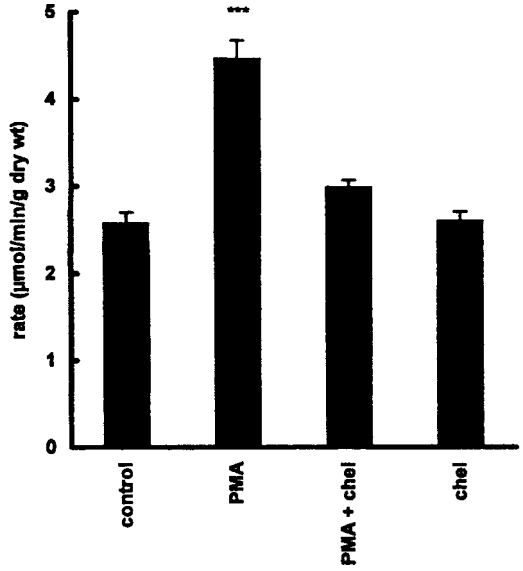

Figure 1 Regulation of brush-border fructose transport by PMA

The jejunum of an anaesthetized rat was perfused for 30 min *in vivo* and then *in vitro* for 60 min with 5 mM D-fructose, either alone (control) or in the presence of the PKC activator, PMA (200 nM); when present, the PKC inhibitor, chelerythrine (chel, 2 μM) was added to the perfusate 15 min before the PMA (for further details see the Experimental section). Values are given as means $\pm$ S.E.M. ($n = 12$ for control perfusions and 3–5 for all other perfusions). $^{***}P < 0.001$, by unpaired t test for comparison with control perfusions.

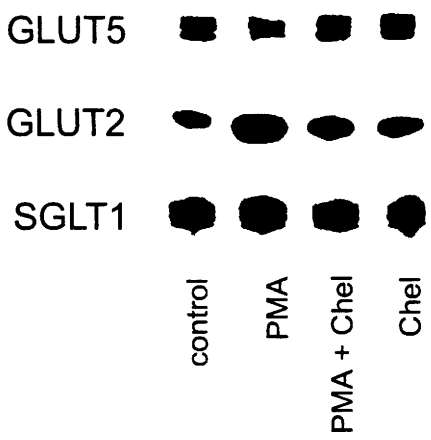

Figure 2 Control of GLUT5, GLUT2 and SGLT1 levels at the brush-border membrane of rat jejunum by a PKC-dependent pathway

Brush-border membrane vesicles were prepared from normal rat jejunum perfused with 5 mM D-fructose in the presence and absence of PMA and/or chelerythrine (Chel) as described in Figure 1. For full details, see the Experimental section. Vesicle protein (20 μg) was then separated on 10% SDS/PAGE gels, transblotted on to nitrocellulose and Western blotted for GLUT5, GLUT2 and SGLT1.

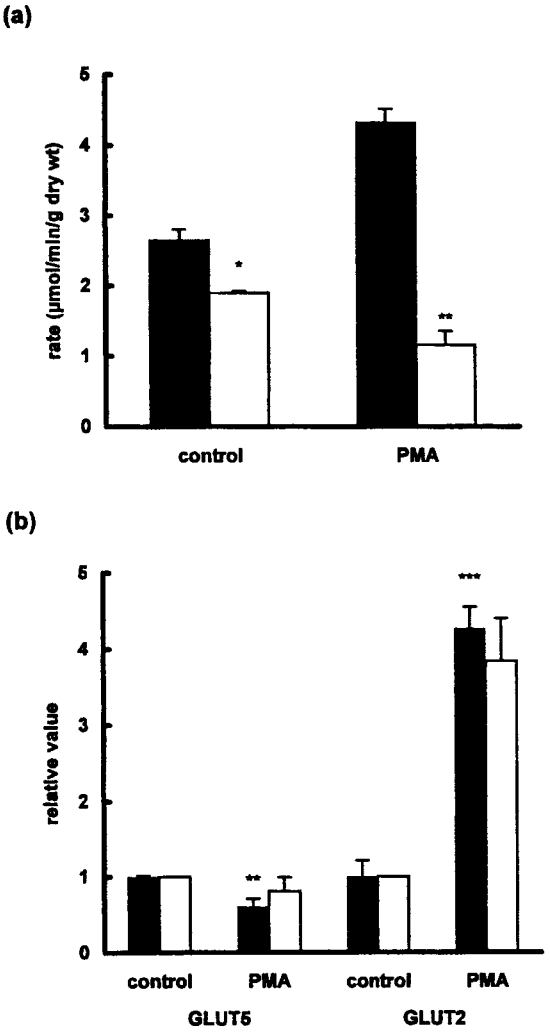

Figure 3 Relative contributions of GLUT5 and GLUT2 to fructose transport

(**a**) Selective inhibition of GLUT2 with phloretin permits determination of the contributions of GLUT5 and GLUT2 to the overall rate of fructose transport. Black bars, overall rate; white bars, GLUT5-mediated component determined in the presence of 0.2 mM phloretin. The difference between black and white bars corresponds to the GLUT2 component. (**b**) Comparison of how the GLUT5- and GLUT2-mediated components of fructose transport (black bars) and how the levels of GLUT5 and GLUT2 (white bars) change in response to perfusion of jejunum by PMA. Control values are taken as 1.0 and other values are expressed relative to the corresponding control. Note that since phloretin takes 15 min to achieve complete inhibition, the rates used in these calculations are taken over the time period 15–50 min for both the control and experimental periods; the values therefore differ slightly from those given elsewhere in this paper which are based on the full perfusion period of 5–50 min. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired t test for comparison with control perfusions.

perfused with either 200 nM PMA and/or 2 μM chelerythrine, concentrations at which their abilities to activate or inhibit PKC, respectively, are thought to be relatively specific. Since the drugs take some time to work and isolated loops of jejunum *in vitro* have limited viability, jejunum was pre-perfused *in vivo* with PMA for 30 min and/or chelerythrine for 45 min, as appropriate, before *in vitro* perfusion was commenced (see the Experimental section for further details). Figure 1 shows that PMA increased the rate of fructose transport to 1.7-fold ($P < 0.001$) compared with untreated control jejunum: stimulation was blocked by chelerythrine, which alone had no effect on fructose transport. Thus fructose transport appears to be regulated by a PKC-dependent pathway.

To correlate changes in fructose transport with changes in transporter density, the levels of GLUT5 and GLUT2 were determined by Western blotting of highly purified preparations of brush-border membrane vesicles prepared from jejunum treated with different combinations of drugs (Figure 2); the levels of SGLT1 were also monitored. As noted in the Experimental section, perfusions for vesicle preparation were terminated half-

Figure 4 Activation of PKC βII by PMA

(**a**) Jejunum was perfused with 5 mM D-fructose with or without PMA/chelerythrine (chel) as described in Figure 1 for 30 min *in vivo* and then 25 min *in vitro*. (**b**) Jejunum was perfused with 5 mM D-fructose only, either for 30 min *in vivo* and then 25 min *in vitro* or for 30 min *in vivo* only. After perfusion, trafficking was stopped by flushing with ice-cold perfusate. Brush-border membrane vesicles were prepared and blotted for PKC βII as described in Figure 2.

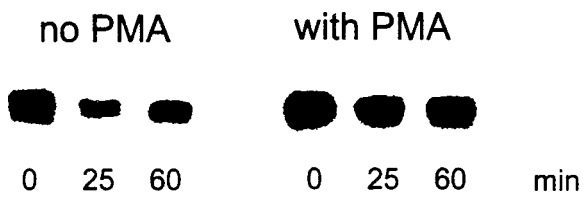

Figure 5 Excision of jejunum results in rapid loss of GLUT2 from the brush-border membrane during perfusion *in vitro*

Three pairs of normal jejuna were each perfused with 5 mM fructose *in vivo* for 30 min and then for different times *in vitro*: pair one was not perfused further (0 min *in vitro*), pair two was perfused for a further 25 min *in vitro* (standard perfusion) and pair three was perfused for a further 60 min *in vitro*. Three pairs were perfused in the absence of PMA and three pairs in the presence of PMA as indicated. After perfusion, trafficking was stopped by flushing with ice-cold perfusate. Brush-border membrane vesicles were prepared and blotted for GLUT2 as described for Figure 2.

way through the period over which transport measurements were made in order to provide the most direct correlation between transport and trafficking measurements. GLUT5 in these blots, which were developed using ECL detection, presented as a triplet from 62 to 57 kDa, consisting of a very closely spaced doublet and a lower-molecular-mass singlet; GLUT2 presented as a single band at 62 kDa or occasionally as a doublet (Figure 2; see also Figure 5) depending on the antiserum and exposure combination being used. Although there was a tendency for PMA to diminish GLUT5 levels (to 0.81 ± 0.18-fold of control levels), the effect was not significant (Figure 2): chelerythrine and PMA plus chelerythrine had no effect. SGLT1 presented as a closely-spaced triplet at 75 kDa; there was no effect on SGLT1 levels after any of the drug treatments (Figure 2). In contrast, PMA increased the levels of GLUT2 to 3.84 ± 0.56-fold the control values ($n = 6$, $P < 0.001$, Figure 2). Some 75% of this increase was blocked by 2 μM chelerythrine, which alone had no effect on GLUT2 levels. The pattern of trafficking of GLUT2 to the brush-border membrane induced *in vitro* is quite distinct therefore from that seen for both GLUT5 and SGLT1.

In order to determine how the different transport contributions made by GLUT5 and GLUT2 change in response to PMA, perfusions were also performed in the presence of 0.5 mM phloretin to selectively inhibit GLUT2. In control jejunum with 5 mM fructose alone, the phloretin-insensitive contribution mediated by GLUT5 (Figure 3a, white bar) was 72% of the overall rate (black bar); the 28% difference between the two bars represents the phloretin-sensitive GLUT2 component. In the presence of PMA, the relative contributions of GLUT5 and GLUT2 were reversed, being 27% and 73% respectively. We then compared how the GLUT5- and GLUT2-mediated rates were changed by PMA relative to their control values, and correlated those relative values with the relative changes in the amounts of the two transporters. Figure 3(b) shows that the GLUT5-mediated rate (black bar) diminished to about 60% of its control value ($P < 0.01$) with no significant change in GLUT5 amount (white bar). In sharp contrast, the GLUT2-mediated rate increased in the presence of PMA to 4.3-fold its control value, a value comparable with a 3.8-fold increase in GLUT2 levels. It is therefore clear that a large increase in GLUT2 level at the brush-border membrane, with no significant change in intrinsic activity, is responsible for the increase in transport induced by PMA.

Saxon et al. [8] have reported that intestine contains α, βII, δ and ξ isoenzymes of PKC and we have identified all these in brush-border membrane vesicles in previous preliminary work. Activation of PKC isoenzymes is often associated with their movement to a specific membrane within or at the cell periphery. In order to establish which isoenzyme might control the trafficking of GLUT2 to the brush-border membrane, we therefore investigated how the membrane-associated levels of the α, βII, δ and ξ isoenzymes changed in response to PMA. Of these, only the PKC βII isoenzyme showed large changes (Figure 4a; other isoenzymes not shown). PKC βII presents as a 49-kDa band that is eliminated by pre-incubation of antibody with excess peptide, confirming that the band is specific (results not shown). PMA strongly activates PKC βII compared with control perfusions, and activation is blocked by chelerythrine, which alone has no effect. Significantly, PKC βII is activated in *in vivo* perfusions with 5 mM fructose alone, presumably because of the supply to the mucosa of stimulating hormones (Figure 4b).

An immediate question, then, is whether the levels of GLUT2 are increased *in vivo* compared with our normal perfusions *in vitro*; if so, the implication is that excision of jejunum for *in vitro* perfusions, or indeed any *in vitro* experiment, results in rapid loss of GLUT2 from the brush-border membrane. We therefore prepared brush-border membrane vesicles from three pairs of jejuna perfused for different times *in vitro*. Each pair was perfused with 5 mM fructose in the absence of PMA for 30 min *in vivo*; pair one was not perfused further (0 min *in vitro*), pair two was perfused for a further 25 min *in vitro* (standard perfusion) and pair three was perfused for a further 60 min *in vitro*. Figure 5 shows that it is indeed the case that GLUT2 is rapidly lost from the membrane when no PMA is present: *in vivo* levels of GLUT2 were high, but diminished to 25–30% within 25 min of perfusion *in vitro* and remained low thereafter. The fact that the whole process was complete within 25 min implies that the half-life for the trafficking away of GLUT2 from the brush-border membrane after excision is no more than a few minutes. Inclusion of PMA in the perfusions promotes trafficking of GLUT2 to the brush-border membrane *in vivo* (Figure 5, 0 min *in vitro*) and there is some trafficking away after perfusion *in vitro* for 25 and 60 min. However, this appears to be much slower than in the absence of PMA, so that, starting from a higher baseline, the level of GLUT2 in the presence of PMA for a standard perfusion (30 min *in vivo*, 25 min *in vitro*) is 3.8-fold that in the absence of PMA.

In order to provide additional confirmation that GLUT2 was indeed at the brush-border membrane, the levels of GLUT5 and

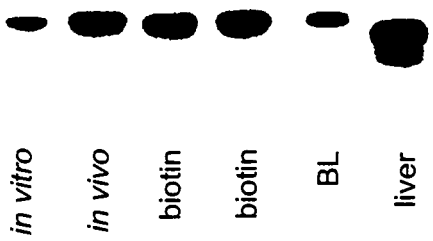

Figure 6 Luminal biotinylation of brush-border membrane proteins in intact jejunum confirms that GLUT2 is at the brush-border membrane

Lane 1 (numbered from the left; *in vitro*), a control brush-border membrane vesicle preparation made by standard methods from jejunum that that had been perfused with 5 mM fructose *in vivo* for 30 min and then *in vitro* for 25 min to allow GLUT2 to traffick away from the membrane. Lane 2 (*in vivo*), a standard brush-border membrane vesicle preparation made from jejunum that had been perfused *in vivo* for 30 min only; trafficking was stopped by flushing with ice-cold perfusate before vesicle preparation. Lanes 3 (biotin) and 4 (biotin), two preparations of biotinylated proteins, prepared by luminal biotinylation of intact jejunum after trafficking had been stopped; the membrane-impermeant reagent was 1 mM sulpho-NHS-LC-biotin and isolation of brush-border proteins was by avidin-agarose from the mucosal homogenate without vesicle preparation at any stage. Lanes 6 (BL) and 7 (liver) are basolateral and postnuclear liver membrane preparations, respectively, for comparison of levels. Samples were equivalent to 15 μg of membrane protein solubilized in SDS and blotted for GLUT2 as described for Figure 2.

GLUT2 in preparations of biotinylated brush-border proteins were compared with those in a standard preparation of brush-border membrane vesicles. Biotinylation was carried out from the luminal side in loops of intact jejunum that had been perfused previously in exactly the same way as when vesicle preparations were made, namely with 5 mM fructose for 30 min *in vivo*. The biotinylated proteins were then isolated from the mucosal homogenate (without preparing vesicles) by the use of avidin beads (see Experimental section for details). The biotinylation reagent, sulpho-NHS-LC-biotin, is membrane-impermeant and would in any event immediately block any potential transporters for it by reaction with amino groups. Lane 2 of Figure 6 (reading from the left) shows that the standard brush-border vesicle preparation from jejunum perfused *in vivo* had a GLUT2 level 3.8-fold that of the standard vesicle preparation from jejunum which has been perfused *in vivo* for 30 min and then *in vitro* for 25 min (standard control perfusion, lane 1). The two samples biotinylated *in vivo* (Figure 6, lanes 3 and 4, no vesicle preparation) had an average GLUT2 level of 3.3-fold that for the control preparation (lane 2). Figure 6, lane 5 shows the GLUT2 level in a standard basolateral membrane vesicle preparation for which several mucosal scrapes were collected from excised intestine [5]; this is therefore broadly comparable in procedure with the control brush-border preparation (lane 1). A liver postnuclear membrane preparation is shown for comparison (lane 6). These data confirm that GLUT2 is at the brush-border membrane and show that the levels are comparable with those from other sources.

DISCUSSION

A role for GLUT2 in the transport of fructose across the brush-border membrane of normal rats

GLUT2 and GLUT5 are thought to be the only members of the facilitative glucose transporter family that transport fructose; they are both known to be present in jejunum. Although GLUT2 has until now been considered to be solely a basolateral protein in the jejunum of normal rats, it was readily detected by Western blotting of standard brush-border membrane vesicle preparations. An obvious concern is that GLUT2 might be detected in brush-border membrane vesicles because it was present as a result of basolateral contamination. In the present work, vesicles were highly purified with sucrase specific activities in the range of 16–20-fold greater than the homogenate. In common with other workers using similar procedures, there was no significant enrichment of the Na^+/K^+-ATPase-specific activity [14] and we could not detect Na^+/K^+-ATPase by Western blotting [5]. Yet brush-border GLUT2 levels *in vivo* are comparable with those in highly purified basolateral membrane vesicle preparations (Figure 6). It is therefore clear that the very small level of basolateral contamination that occurs in any brush-border membrane vesicle preparation cannot begin to account for the levels of GLUT2 found in brush-border membrane vesicles.

Nevertheless, because of the importance of this question, we felt it necessary to confirm this conclusion by a route that did not involve the preparation of brush-border membrane vesicles at any stage. This we did by luminal biotinylation of brush-border membrane proteins under ice-cold conditions with a membrane-impermeant reagent, sulpho-NHS-LC-biotin, in loops of intact jejunum *in vivo*. After quenching the reaction with NH_4Cl, a mucosal scrape was homogenized and the biotinylated proteins retrieved by the use of avidin-agarose. Figure 6 shows clearly that the levels of GLUT2 are the same as those present in standard brush-border membrane vesicle preparations prepared after trafficking of GLUT2 was stopped *in vivo* by flushing with ice-cold perfusate. The fact that there is no difference between the results from the two completely different types of preparation confirms that GLUT2 is present at the brush-border membrane and is not an artifact of the standard brush-border membrane preparation.

Further strong support for this view is provided by the observation that GLUT2 is functional and so must be accessible to luminal fructose; two components of fructose transport were identified, one phloretin-sensitive and mediated by GLUT2, the other phloretin-insensitive and mediated by GLUT5. The ability to measure both the individual transport components and transporter levels permitted us to distinguish between regulation through control of transporter intrinsic activity and transporter trafficking. Thus the direct correlation between GLUT2-mediated transport and GLUT2 levels (Figure 3b) enabled us to show that PMA stimulated fructose transport across the jejunal brush-border membrane by increasing the level of GLUT2 almost 4-fold without any change in its intrinsic activity; in contrast there was no significant change in the level of GLUT5, although there was a moderate reduction in its intrinsic activity leading to a reduction in the GLUT5-mediated component of transport.

The dynamic regulation of GLUT2 trafficking: consequences for preparations *in vitro*

Trafficking of GLUT2 to the brush-border membrane is likely to be under the control of PKC βII, whose activation by PMA correlates with an approx. 4-fold increase in brush-border GLUT2 levels. Of particular note, trafficking of GLUT2 to the membrane is dynamic, occurring on a time scale of minutes under conditions where no significant changes in the levels of GLUT5 and SGLT1 were observed. We have recently seen the same pattern of trafficking during an investigation of the control of brush-border membrane fructose transport by ERK/MAP kinase, p38 MAP kinase and phosphatidylinositol 3-kinase intracellular signalling pathways [15]. In perfusions *in vitro*, around 25% of the GLUT2 remained at the membrane, representing a steady-state level. The implication of these observations

is that the majority of GLUT2 trafficks by a pathway that is distinct from that for GLUT5 and SGLT1.

The dynamic nature of brush-border GLUT2 trafficking and its likely control by PKC βII has the most important technical consequences for the investigation of nutrient transport *in vitro*. PKC βII is activated *in vivo* by the action of endogenous hormones; when jejunum is excised from an animal, therefore, PKC βII is inactivated (Figure 4). An immediate consequence is that GLUT2 trafficks away from the brush-border within a matter of minutes (Figure 5). In many *in vitro* preparations, then, GLUT2 or GLUT2-mediated transport is difficult to detect, especially when studying glucose absorption, which is dominated by SGLT1 at the low glucose concentrations often used for uptake studies. We believe this is one of the principal reasons why the role of GLUT2 in sugar absorption has been overlooked.

Different *in vitro* preparations present different problems. In some cases, it is possible to prevent the loss of GLUT2. For example, when preparing vesicles, GLUT2 trafficking can be arrested by flushing with ice-cold buffers before excision and performing mucosal scrapes under ice-cold conditions; however, failure to observe the very strictest procedures with respect to GLUT2 will result in considerable inconsistency or even failure of detection. In other cases, notably where whole tissue is used in perfusions of isolated loops *in vitro* or *in situ*, everted sacs or Ussing chambers – all extensively used intestinal preparations – there is no way of preventing loss of GLUT2, short of artifactually activating PKC βII or an appropriate intracellular signalling pathway by the use of hormones or drugs.

In our experiments with normal jejunum, GLUT2 diminishes *in vitro* to around 25% of its level *in vivo* and makes a proportionately small contribution to fructose transport. We have not established where the GLUT2 trafficks to, nor have we established whether GLUT2 at the basolateral membrane undergoes a similar phenomenon. However, just how much GLUT2 is at the membrane will depend to a significant extent on the composition of the diet on which animals have been maintained, or on their hormonal status, and the ability to detect GLUT2 *in vitro* will change accordingly. We now see why we readily detected GLUT2 at the brush-border membrane of diabetic, but not normal, jejunum in previous work [5]. In diabetic jejunum, the brush-border GLUT2 levels are almost 7-fold higher than normal jejunum and, even though GLUT2 probably still trafficks away to some extent *in vitro*, the fact that the rate is slower and starts from a higher baseline renders GLUT2 readily detectable in diabetic compared with normal jejunum. This difference, which is rather like that between untreated and PMA-treated jejunum *in vitro* (illustrated in Figure 5), was exacerbated by the fact that mucosal scrapes were performed at room temperature in the mistaken belief that speed was of the essence, rather than the control we now recognize. We also used ^{125}I-linked secondary antibodies for Western blotting rather than the much more sensitive ECL detection used in the present work. In many experiments, we pre-perfused jejunum *in vitro* for 60 min, giving GLUT2 more than enough time to traffick away in normal jejunum. All the above considerations and experience show why a role for GLUT2 in brush-border sugar absorption has taken so long to be established.

Conclusion

Given that the changes in fructose transport reported here take place within minutes, the PKC-dependent pathway has the potential to regulate fructose transport during the period of digestion and assimilation of a meal by controlling the brush-border levels of GLUT2. A potentially fruitful avenue for investigation of the molecular mechanisms underlying the assimilation of sugars after a meal is therefore to determine which are the nutrients and local or endocrine hormones whose actions are mediated by PKC-dependent pathways. One observation of particular interest is that glucose causes the activation and translocation of PKCα to the plasma membrane of pancreatic cells [12]. In the following paper [16], we report that the transport of glucose by SGLT1 correlates with the activation of PKC βII and rapid activation and recruitment of GLUT2 to the brush-border membrane. GLUT2 is the only transporter known to transport both fructose and glucose. Thus we show that GLUT2 mediates the diffusive component of glucose absorption, which is the major route by which glucose is absorbed across the small intestine.

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