Structural and Functional Analysis of Glucose Absorption Mechanisms in the Rat Small Intestine *in vivo*

A. M. UGOLEV¹, Ya. Yu. KOMISSARCHIK², L. V. GROMOVA¹, A. A. GRUZDKOV¹, E. S. SNIGIREVSKAYA² and M. S. BRUDNAYA²

 Pavlov Institute of Physiology, Russian Academy of Sciences, Nab. Makarova 6, 199034, St.-Petersburg, Russia
 Institute of Cutology, Russian Academy of Sciences.

Tikhoretsky pr. 4, 194064, St.-Petersburg, Russia

Abstract. The absorption of glucose (free, and released from membrane hydrolysis of maltose) and water in the isolated loop of the rat small intestine was studied in chronic experiments. Even at maximum glucose (75 mmol/l) or maltose (37.5 mmol/l) concentrations the rate of glucose transfer by solvent drag and by diffusion did not exceed 13% and 25%, respectively, of the total rate of glucose absorption. Electron microscopic and immunocytochemical analysis revealed a significant widening of intercellular gaps in the basal epithelium region and an increase of actin density in the vicinity of the tight junctions and between the apical root filaments in enterocytes after glucose or glycine load in chronic and acute experiments. However, very rarely (in 1% of all cases), and only in chronic experiments, structural changes in the tight junctions such as "blisters" and dilatations were also recognised. It is concluded that under normal physiological conditions the absorption of glucose (free, and released from maltose hydrolysis) mainly uses active transport across the apical membrane of the enterocytes.

Key words: Small intestine — Membrane digestion — Glucose absorption — Immunocytochemical analysis

Introduction

At present there is no generally accepted opinion concerning the relative roles of the different mechanisms of glucose absorption in the mammalian small intestine. The concept of predominantly active transmembrane transport of this solute mediated

Correspondence to: L. V. Gromova, I. P. Pavlov Institute of Physiology, Academy of Sciences of Russia, Nab. Makarova, 6, 199034, St. Petersburg, Russia

by specific transporters seems to be the most common (Hopfer 1987; Diamond 1991; Ugolev and Iesuitova 1991; Koepsell and Spangenberg 1994). At the same time, some authors believe that at relatively high glucose concentrations, transfer of glucose through the epithelial barrier by diffusion (Kotler et al. 1981) or its paracellular transfer by solvent drag (Pappenheimer and Reiss 1987; Pappenheimer 1990, 1993; Atisook and Madara 1991) prevail.

The latter concepts mentioned are mainly based on studies of glucose absorption during perfusion of the isolated intestinal loop in anaesthesized animals. In this case conditions close to physiological are believed to be provided (Levin 1982; Roing and Vinardell 1991). However, a special technique developed by Ugolev and Zaripov (1979) for chronic experiments has recently been applied in many studies (Punin et al. 1986; Ugolev et al. 1986). This technique provides for a normal physiological state of animals and, despite some mucosal atrophy in the isolated intestinal loop, for a rather high efficiency of membrane hydrolysis and transport of nutrients as compared to acute experiments *in situ* (Ugolev et al. 1986). So, chronic experiments may be considered the most adequate physiological model.

In this work an attempt was made to evaluate the contributions of the transfer by solvent drag and/or by diffusion to the total absorption of glucose (both free and derived from maltose hydrolysis) in the isolated loop of the rat small intestine in chronic experiments. The transfer by solvent drag was calculated from data on glucose and water absorption at different luminal concentrations of glucose and maltose. The transfer by diffusion was estimated from the data on absorption of free glucose in the presence of phlorizin. Electron microscopy and immunocytochemistry were used to examine structural changes in the intestinal epithelium (alterations in the occluding junctions, the intercellular gaps and the spatial distribution of apical actin filaments) after the load of the rat small intestine with glucose, glycine, Ringer, and triolein solutions in acute and chronic experiments.

Materials and Methods

Experimental technique

The chronic experiments were performed on Wistar male rats (body mass 150-200 g). The surgical and experimental procedures were similar to those described earlier (Ugolev and Zaripov 1979; Ugolev et al. 1986). The animals were taken for experiments 6–7 days after a loop of the small intestine (20-cm in length at a distance of 15 cm from the duodenum) was isolated. The loop was perfused with glucose (12.5-75.0 mmol/l) or maltose (6.25-37.5 mmol/l) solutions. In some experiments the intestinal loop was also perfused with glucose (75 mmol/l) in the presence of phlorizin (2 mmol/l). The perfusion rate was 0.55-0.6 ml/min. On days when no experiments were performed the isolated intestinal loop was perfused with glucose (25 mmol/l) for 1 hour to maintain the functional capability of the mucosa and to delay its atrophy.

All substrate solutions were prepared in Ringer solution with a reduced NaCl con-

centration (120 mmol/l), so that with the highest of the glucose concentrations used (75 mmol/l) the osmolality of the perfusion solution was close to that of the standard Ringer solution. At lower substrate concentrations (12.5–50.0 mmol/l) mannitol was added in amounts needed to keep isoosmolality of the infusate. After a preliminary 15 min perfusion with the substrate at a particular concentration, three probes (5 min each) were sequentially taken for biochemical analysis.

Glucose concentration was determined by the glucose oxidase method, and total glucose and maltose concentrations (in mmol/l glucose) were measured by the anthrone method (Ugolev et al. 1986). The absorption rates for free glucose and glucose released by maltose hydrolysis (J_g and J_m , respectively, in μ mol/min), as well as the rate of fluid absorption (J_w , in ml/min) were calculated using the following formulas:

$$J_g = C_{q,\text{in}} \cdot V_{\text{in}}/5 - C_{g \text{ out}} \cdot V_{\text{out}}/5,$$

$$J_m = C_{m,\text{in}} \cdot V_{\text{in}}/5 - C_{m+q,\text{out}} \cdot V = \text{out}/5$$

$$J_w = (V_{\text{in}} - V_{\text{out}})/5.$$

where $C_{g,\text{in}}$ and $C_{m,\text{in}}$ are glucose and maltose concentrations, respectively, in the infusate (mmol/l of glucose); $C_{q \text{ out}}$ is glucose concentration in the outflowing perfusate as evaluated by the glucose oxidase method (mmol/l); $C_{m+q_{p},\text{out}}$ is the total concentration of sugars in the outflowing perfusate determined by the anthrone method (mmol/l of glucose); V_{in} and V_{out} are the volumes of inflowing and outflowing perfusates, respectively, for 5 min intervals (ml).

Electron microscopy and immunocytochemistry

The proximal segment of the rat small intestine was studied in acute and chronic experiments. In acute experiments the small intestine of animals anaesthesized with nembutal was ligated so as to obtain 5 isolated 3 or 5 cm long segments. Each of them was injected with one of the following solutions (0.5 ml per cm): (1) Ringer solution (pH 7.4); (2) 10 mmol/l glucose in Ringer solution; (3) 10 mmol/l glycine in Ringer solution; (4) 0.5% triolein emulsion in 5% gum arabic. The fifth segment was void and served as a control. In some experiments the glucose and glycine concentrations in the injected solutions were increased up to 40 mmol/l. In all cases the solutions were isoosmotic, close to the osmolality of the standard Ringer solution. After 20 min exposure of the intestinal preparations in the abdominal cavity of the animal intestinal pieces, 5–7 mm in length, were excised from the middle of each segment for fixation. Throughout the experiment the body temperature of the animals was kept at approximately 37 °C using a heat lamp.

In chronic experiments performed by the above-mentioned technique, an isolated intestinal loop was first perfused for 30 min with Ringer or glucose (40 mmol/l) solutions and subsequently perfused with 5 ml of a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer (PSB) for 2 min for both morphological and immunocytochemical analysis. The rats were then sacrificed, 5–7 mm pieces of the tissue were excised from the middle of the isolated small intestinal loop and postfixed in the same solution. Pieces of the normally functioning small intestine, proximal and distal to the anastomosis, served as controls.

The immunocytochemical analysis was performed according to the technique of Roth (1982) in Lowicryl sections. Pieces of the intestinal tissue were fixed in a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in PBS, pH 7.2, at room temperature for 20 min. Then, they were dehydrated in ethanol at low temperatures $(-4^{\circ}C \text{ to } -35^{\circ}C)$ and

embedded in hydrophilic resin (Lowicryl K4M) at -35 °C. The objects were infiltrated in a mixture of Lowicryl and absolute alcohol in the following ratios: 1:3 for 1 hour; 1:1 for 2 hours; 3:1 for 1 hour; pure Lowicryl (component A-2, 7 ml; component B-12, 3 ml; initiator, 0.1 mg) overnight. Then, the objects were placed into a fresh mixture of Lowicryl components in gelatinous or polyethylene capsules with lids and polymerized under ultraviolet light (L = 360 nm) at -35 °C for two days. The final polymerisation was carried out at room temperature for 12 hours. Ultrathin sections were cut on an LKB-8800 ultramicrotome with glass knives.

For the immunocytochemical analysis ultrathin sections were placed on nickel grids with Formwar films. The grids with the sections were incubated in 0.5% bovine serum albumine (Serva) in PBS at room temperature for 15 min. Subsequently, they were transferred to a drop of monoclonal antibodies against actin (Amersham, Boehringer Mannheim) and left for 1 hour at room temperature, washed repeatedly in 0.05% Twin-20 in PBS. RAM or GAM (Sigma) conjugated with 10 nm colloidal gold were used as secondary antibodies. Adjacent sections of the same intestinal pieces, unstained with primary antibodies, served as controls. In some cases non-immune mice serum was used instead of primary antibodies. Actin of smooth muscle cells detected within the same intestinal segments served as a positive control. Freeze-fracture was performed according to the technique described earlier (Komissarchik et al. 1985).

For a standard electron microscopic analysis the small intestinal pieces following initial fixation in a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in PBS were postfixed in 1% osmium tetroxide in the same buffer and embedded in a mixture of Epon-Araldyte using the common technique. The ultrathin sections were stained with uranyl acetate and lead citrate. Aqueous solution of uranyl acetate was used for Lowicryl sections and alcoholic solution for Epon-Araldyte sections. The sections were examined under a JEM-100C and a JEM-100U electron microscope.

The data obtained from physiological and immunocytochemical studies were treated statistically using the Student's *t*-test.



Figure 1. Glucose (A) and water (B) absorption in the isolated loop of the rat small intestine perfused with glucose and maltose in chronic experiments. Ordinate: (A) the rate of glucose absorption (μ mol/min in the isolated intestinal loop); (B) the rate of water absorption (μ l/min in the isolated intestinal loop). Abscissa: substrate concentration in the infusate (mmol/l of glucose). Solid lines, perfusion with glucose; dashed lines, perfusion with maltose. (Mean \pm S.E.M, n = 4).

Results

Physiological studies

The data on glucose and water absorption in the isolated loop of the rat small intestine are presented in Fig. 1. The rates of glucose absorption at different glucose concentrations in the infusate were similar to those obtained earlier in chronic experiments (Ugolev et al. 1986) and much higher than those in acute experiments *in situ* (Ugolev et al. 1986; Pappenheimer 1990, 1993). The water absorption rate increased from 0.40 to 1.75 μ l/min per cm of the intestinal loop when the glucose concentration in the initial infusate remained within the low to intermediate range (12 5 50 mmol/l) but did not change when the glucose concentration was markedly elevated (50–75 mmol/l). The rates of water absorption were close to those observed by other authors in acute *in vivo* experiments in rats (Kotler et al 1981; Westergaard et al. 1986; Meddings and Westergaard 1989).

The absorption rates of glucose derived from maltose hydrolysis did not actually differ from those of free glucose at equivalent substrate concentrations (Fig. 1). Water absorption in the isolated intestinal loop did not change statistically (P > 0.05) at maltose concentrations within a range of 6.25–37.5 mmol/l, and was approximately equal to the maximal water absorption observed during perfusion with free glucose.

In the presence of phlorizin (2 mmol/l) the rate of glucose (75 mmol/l) absorption was significantly lower as compared to that without phlorizin (0.172±0.051 vs. $0.703\pm0.043 \ \mu$ mol/min per cm intestinal loop, P < 0.01). The net water secretion markedly increased in the presence of phlorizin (from 0.108 ± 0.37 to $2.38\pm0.87 \ \mu$ l/min per cm intestinal loop, P < 0.05).

Structural and immunocytochemical studies

In general, the electron micrographs of control (without substrate load) sections of the small intestine from acute experiments were consistent with those published (Hirokawa et al. 1982; Drenckhahn and Dermietzel 1988).

The method of fixation and embedding allowed us to elucidate the relationships between the structural elements of the cytoskeleton and the junctional complex consisting of a tight junction, an intermediate junction and desmosomes. These specialized contacts showed an organization pattern typical for such structures. In the area of intermediate and tight junctions we managed to distinguish their associated microfilaments of 7–8 nm in diameter while in the region of desmosomes, thicker (intermediate) filaments, could be seen. Lateral membranes of the adjacent cells in the basal area formed invaginations, the size of the intercellular gap remaining invariable (15–17 nm).

The ultrastructural analysis of the same elements of the intestinal epithelium after glycine (10 mmol/l) or glucose (10 mmol/l) absorption in acute experiments



Figure 2. Intercellulai boundaries of the rat small intestinal enterocytes during glucose (40 mmol/l) absorption in chronic experiments. Freeze-fracture replica of three neighbor ing enterocytes in the tight junction zone without visible alterations. PF protoplasmic face EF extracellulai face MV microvilli. Arrows indicate the intercellular bound aries (a) Bar = 0.5 μ m. Ultrathin section of unchanged cell junction complex (b) tj tight junction. ij intermediate junction. d. desmosomes. Bar = 0.25 μ m. Very rate case of blisters observed in the the tight junction (c). Bar = 0.25 μ m.

revealed a significant widening of intercellular gaps in the basal epithelium region. In some cases the intercellular gaps in the area of invaginations of adjacent cells became narrower. No changes were observed in the structure of intercellular junctions.



Figure 3. Distribution of the actin label in apical region of the enterocyte during absorption of different substrates in acute experiments. In the control (without substrate load) the label is revealed within microvilli, on root filaments, and sometimes in tight junction region (a). After glycine load (10 mmol/l - b, 40 mmol/l - c) or glucose load (10 mmol/l - d) the label is seen between root filaments (arrows) and in tight junction region After triolein load (0.5%) the distribution of actin label (e) does not differ from that in the control. Bar = 0.25 μ m.

(tight, intermediate junctions, and desmosomes). When concentrations of glycine and glucose were raised to 40 mmol/l the organization of intercellular junctions did not differ from that at concentrations of 10 mmol/l. In case of triolein load

no changes in the region of both intercellular gaps and junctional complex were observed.

The electron microscopic analysis of the small intestinal loop after the glucose load (40 mmol/l) or Ringer solution in chronic experiments revealed some changes as compared to the control (normally functioning) small intestine. They mainly consisted in shortened microvilli and increased numbers of Goblet cells. This was in agreement with the light microscopic data reported by Punin et al. (1986). In most cases, the structural organization of intercellular junctions and space did not differ from that observed in acute experiments. An analysis of tight junctions both in freeze-fracture (Fig. 2a) and ultrathin sections (Fig. 2b) did not, as a rule, reveal any visible structural changes. Extremely seldom were observed blisters in tight junctions (Fig. 2c).

An analysis of the distribution of labeled anti-actin antibodies in the longitudinal and transverse sections of the control intestinal segments obtained from acute experiments showed that the label were traceable throughout the length of the bundle of filaments, from tops of the microvilli to the ends of the root filaments (Fig. 3a). The label was in fact absent between root filaments, it never appeared in the desmosome area, and was rather rare nearby tight and intermediate junctions. However, after the load with glucose or glycine at concentrations of 10 and 40 mmol/l the label was not only found in actin filament bundles of the microvilli and the root filaments but also in the area of the terminal web, between the root filaments, and its density in the vicinity of tight and intermediate junctions was



Figure 4. Distribution of actim label in the apical region of the enterocyte during glucose absorption in chronic experiments. The actim label is seen within microvilli region, in root filaments region, between root filaments, and close to tight junction after perfusion with glucose (*a*), and within microvilli and in root filaments region after perfusion with Ringer (*b*). Bar = $0.25 \ \mu$ m.

increased (Fig. 3b, c, d). The distribution of the immune label within the apical area of enterocytes and the junctional complex after the Ringer and triolein load did not actually differ from that seen in the control sections (Fig. 3e).

In chronic experiments, like in acute ones, the density of labeled anti-actin antibodies close to tight and intermediate junctions was higher after the glucose load than after the Ringer load (Fig. 4a, b).

Discussion

One main feature of the active membrane transport is saturation of the transport system with the increasing substrate concentration at the membrane. The Michaelis constant for the active glucose transport (K_t) is about 1–3 mmol/l. Therefore, if glucose concentration in the intestinal lumen is within a range of 25–30 mmol/l one may expect a nearly complete (more than 90%) saturation of its active transport system (Thomson and Dietschy 1984; Pappenheimer and Reiss 1987; Pappenhiemer 1993). At the same time, it was found in perfusion experiments *in vivo* that at relatively high glucose concentrations (more than 50 mmol/l) no saturation occurred (Ugolev et al. 1986; Meddings and Westergaard 1989).

Some authors believe that at high glucose concentrations an unsaturated component of absorption provided by passive diffusion of glucose through the epithelial barrier may play a significant role (Kotler et al. 1981). However, other investigators (Madara and Pappenheimer 1987; Pappenheimer and Reiss 1987; Pappenheimer 1990, 1993; Atisook and Madara 1991) suggest that under physiological conditions at relatively high luminal concentrations of glucose its absorption predominantly occurs by solvent drag through the paracellular channels, while the active membrane transport of glucose plays a secondary role as a trigger mechanism. Glucose transport across the apical membrane activates contraction of the enterocyte cytoskeleton, thereby opening tight junctions, and creates a concentration gradient of the substrate across the junctions for an increased fluid absorption.

It remained unclear, however, to what extent conclusions made by the above authors on the basis of *in situ* perfusion experiments on isolated small intestinal loop in anaestesized rats may be applied to glucose absorption under the most adequate physiological conditions, such as in chronic experiments. Particularly as the narcosis and surgery-induced trauma significantly diminish, as demonstrated, the digestive-absorptive functions of the small intestine (Ugolev et al. 1986) and permeability of the pre-epithelial layer (Anderson et al. 1988).

To evaluate the relative role of the paracellular transfer of glucose by solvent drag we made calculations using the modified Kedem and Katchalsky equation (Pappenheimer and Reiss 1987):

$$J_{sd} = (1 - \sigma) \cdot J_w \cdot (C_1 + C_2) \cdot 0.5$$

where J_{sd} is the rate of the paracellular transfer of glucose by solvent drag $(\mu \text{mol/min/intestinal segment})$: C_1 and C_2 are glucose concentrations at the external and the internal ends of the intercellular tight junction (mmol/l): J_w is the rate of transepithelial fluid flux (ml/min/intestinal segment): $1 - \sigma$ is reflection coefficient of solvent drag $(0 \le \sigma \le 1)$.

We considered C_1 as an average glucose concentration in the lumen of the perfused intestinal loop ($C_1 = (C_{\rm in} + C_{\rm out})/2$, where $C_{\rm in}$ and $C_{\rm out}$ are glucose concentrations at the entry and the exit of the perfused intestinal loop). The value of C_2 was assumed to be $1/2 C_1$, taking into consideration the experimental data obtained by other authors (Parsons 1984).

Also, it has been accepted that $\sigma = 0$, and that water absorption occurs completely through the intercellular channels. This corresponds to the case of maximally possible paracellular transport of glucose by solvent drag.

The results obtained show a close correlation (r = 0.94) between the absorption rates of glucose and water in isolated intestinal loop in chronic experiments. This is consistent with the conception of paracellular transfer of glucose by solvent drag under these conditions.

The results of calculations have shown that even at maximal glucose concentration (75 mmol/l) the rate of the solvent drag transfer of glucose does not exceed 10% of the total glucose absorption. According to similar calculations for glucose derived from maltose hydrolysis, this mechanism may account for only about 13% of the total rate of glucose absorption with the maximum maltose concentrations in the infusate used (37.5 mmol/l).

A relative contribution of the diffusion component to the total glucose absorption was estimated from the results of glucose absorption in the presence of phlorizin (2 mmol/l). Phlorizin inhibits both the active transport of glucose and its paracellular transfer by solvent drag. At the same time, this value of the diffusion component is overestimated since in the case of active glucose transport its concentration at the apical membrane of the enterocytes must be lower than in the absence of active glucose transport. According to our data, transfer of glucose by diffusion through the epithelial barrier at the highest of the glucose concentrations used (75 mmol/l) accounts for approximately 25% of the total glucose absorption, i.e. more than twice that of the paracellular glucose transfer by solvent drag.

The results of our electron microscopic and immunocytochemical studies somewhat disagree with the experimental data substantiating the hypothesis of predominant paracellular transport of glucose or amino acids by solvent drag in the small intestine under physiological conditions (Madara and Pappenheimer 1987; Madara 1990; Atisook and Madara 1991). Indeed, our electron microscopic data obtained in acute and chronic experiments suggest that glucose and glycine induce an expansion of the lateral intercellular spaces. This is in agreement with the data of the above authors. However, in contrast to their data, in acute experiments we have never observed structural changes in the tight junctions in response to glucose or glycine. In our previous publication (Komissarchik et al. 1992) we suggested that the reason for this disagreement might be lower concentrations of glucose or amino acid than those used by Pappenheimer's group. However, in the current study, higher concentrations of these substances (40 mmol/l) produced the same results as those reported earlier.

Another reason for this discrepancy might be different conditions of fixation. The authors of the solvent drag hypothesis claim that the "opening" of tight junctions was detected only after a replacement of perfusate by the fixing solution, and could not be discovered in the case of conventional fixation procedures (i.e. by excising tissue pieces and immersing them into the fixing solution) (Madara and Pappenheimer 1987).

Tissue fixation for the structural analysis by electron microscopy and immunocytochemistry in chronic experiment was more adequate and close to that used by Madara and Pappenheimer since the isolated intestinal loop was washed by the fixing solution immediately after its perfusion with glucose or Ringer solution. However, even in this case we also failed to clearly identify the "opening" of tight junctions after glucose absorption. Based on a few observations only, it is hardly possible to substantiate a dominant role of the paracellular mechanism for glucose absorption in normal physiological conditions. One may suggest that the natural range of alterations in tight junctions is much smaller and in fact undeterminable by direct structural methods. Nevertheless, some of our data obtained in acute and chronic experiments by the immunochemical technique showed that during absorption of glucose or glycine certain changes might have occured in the tight junctions of enterocytes. We observed alteration in the distribution of anti-actin label after the glucose or glycine load (the label appeared between root filaments, its density increasing in the area close to tight junction), but failed to detect any changes in the label distribution after the Ringer or triolein load.

The supporters of the solvent drag hypothesis believe that the paracellular transfer of glucose is a predominant mechanism for its absorption at high concentrations of this monosaccharide in the small intestinal lumen (higher than 200 mmol/l). However, normally, glucose concentration in the intestinal lumen never exceeds 50 mmol/l (Diamond 1991).

Thus, although not excluding the possibility of predominantly paracellular transfer of some substances by solvent drag, especially, in the presence of glucose (Madara and Pappenheimer 1987; Pappenheimer and Reiss 1987; Pappenheimer 1990, 1993; Atisook and Madara 1991), the results obtained allow us to conclude that under physiological conditions and at normal glucose concentrations in the small intestinal lumen the absorption of glucose itself occurs mainly by its active transport across the apical membrane of the enterocytes. At the same time, at extremely high luminal concentrations of glucose some other mechanisms of its

absorption may play a significant role, particularly glucose diffusion through the apical membrane of the enterocyte and its transfer through the intercellular tight junctions by solvent drag.

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