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Biochimica et Biophysica Acta 1559 (2002) 179–185

BIOCHIMICA ET BIOPHYSICA ACTA
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# Regulation of jejunal glucose transporter expression by forskolin

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Received 20 June 2001; received in revised form 8 October 2001; accepted 22 November 2001

**Abstract**

We have investigated the effects of forskolin on enterocyte membrane expression of the glucose transporters, SGLT1 and GLUT2, which are thought to be the main entry and efflux pathways for glucose, respectively. Forskolin treatment increased SGLT1 but decreased GLUT2 expression in mid and lower villus enterocytes. No change in transporter expression was noted in upper villus cells. Likewise, cyclic AMP levels were raised in mid and lower but not upper villus cells. The implications of these data for glucose transport are discussed. 

*Keywords:* Sodium glucose cotransporter; GLUT2; Rat jejunum; Cyclic AMP

## 1. Introduction

The small intestine is subjected to a wide range of humoral and lumenal stimuli and can modify its physiology accordingly. Perhaps the most important influences are provided by the rapid fluctuations in lumenal nutrient levels during and post feeding. In particular, macronutrients such as glucose need to be assimilated rapidly in the jejunum to avoid osmotic effects in the lower regions of the intestine. To achieve this rapid absorption, nature has developed a cohort of co-ordinated cellular mechanisms responsible for controlling the rate of transfer of nutrients such as glucose from the intestinal lumen to the blood. These cellular mechanisms can be regulated rapidly by both acute [1] and chronic [2–4] changes in lumenal carbohydrate levels, as well as by changes in plasma hormones [5] and in diseases such as insulin-dependent diabetes mellitus [6,7]. In the latter two cases some of these adaptive mechanisms are thought to be mediated by elevated intracellular levels of the second messenger cyclic AMP [8,9]. Previous work has indicated that increased intracellular cyclic AMP can increase both the enterocyte membrane potential, and thus the driving force for $Na^+$-glucose co-transport, and the rate of glucose uptake across both the brush border and basolateral membranes [8]. Whether the transport effects are due to increased expression of SGLT1 and GLUT2 transporters is unclear, and our current work addresses this issue. Specifically, we have investigated regional changes in SGLT1 and GLUT2 protein expression along the jejunal villus axis, since in many situations enterocytes respond in a heterogeneous manner to lumenal (glucose) [2] or humoral (glucagon or diabetes) [5,10] stimuli depending on their villus location.

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0005-2736/02/$ – see front matter 
PII: S0005-2736(01)00449-7

## 2. Methods and materials

### 2.1. Animals

All experiments used male Wistar rats (300 g body weight ± 10%), housed in the School's animal facility and maintained with free access to standard laboratory diet and water until the time of experimentation.

### 2.2. In vitro exposure of jejunal mucosa to forskolin

Rats were killed by cervical dislocation and the jejunum was excised rapidly and rinsed free of its lumenal contents. One end of the jejunal segment was ligated and the lumen filled, without distending the mucosa, with oxygenated HEPES-buffered salt solution (HBSS) containing (mM): NaCl 140; KCl 5; $Na_2HPO_4$ 1; $CaCl_2$ 1; $MgCl_2$ 0.5; HEPES 10; L-glutamine 2; IBMX 0.5; bovine serum albumin 0.2%; pH 7.4, with or without 5 μM forskolin. The jejuna were incubated in oxygenated HBSS for 1 h at 37°C.

### 2.3. Isolation of enterocytes

Cell were isolated by a modified $Ca^{2+}$ chelation technique in which the intestine is exposed to intracellular-like solutions [11]. Briefly, jejuna were filled with $Ca^{2+}$-free isolation buffer containing (mM): $K_2SO_4$ 7; $K_2HPO_4$ 44; $NaHCO_3$ 9; HEPES 10; L-glutamine 2; dithiothreitol 0.5; $Na_2EDTA$ 0.2; pH 7.4. Cells were dislodged by gentle rubbing of the intestine following incubation in $Ca^{2+}$-free, EDTA-containing buffer for 6 min (upper villus cells). Jejuna were re-filled and incubated for a further 8 min (mid villus cells) and finally re-filled and incubated for a further 12 min (lower villus cells). The purity of the cell fractions was assessed by measuring alkaline phosphatase activity in each fraction after 15 min at 37°C using *p*-nitrophenol phosphate as the substrate [12]. Isolated cell viability was based on the ability to exclude trypan blue. Upon isolation, the cell fractions were washed twice in HBSS to remove dead cells and other debris.

### 2.4. Cell membrane preparation

Enterocyte fractions (3–5 ml of packed cells) were resuspended in homogenisation buffer (mM): mannitol 50; HEPES 2; PMSF 0.5; pH 7.2 (10 ml buffer: 1 ml cells) and homogenised using a Polytron homogeniser (full speed 2×30 s bursts). The homogenate was subjected to centrifugation at 1500×*g* for 15 min to remove cell debris and nuclei, and the supernatant subjected to further centrifugation at 15 000×*g* for 30 min to prepare a crude plasma membrane fraction. All steps were performed at 4°C.

### 2.5. Immunoblotting

For Western blotting, membranes (10–50 μg protein) were solubilised in Laemmli sample buffer containing 5% sodium dodecyl sulphate (SDS) and electrophoresed on a 10% SDS–polyacrylamide gel. The proteins were transferred from the gel to nitrocellulose membranes by electrophoretic blotting for 1 h at a constant current of 1 mA/cm$^2$. Non-specific protein-binding sites were blocked with PBS-T (phosphate buffered saline containing 0.1% Tween 20) and 5% fat-free milk overnight at 4°C. The filters were incubated with commercially available antibodies to SGLT1 and GLUT2 (1:1000 dilution, Chemicon, UK) for 2 h at room temperature. The filters were washed (2×15 min) with PBS-T containing 1% fat-free milk and incubated with a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:2000, Dako, UK) for 2 h at room temperature and finally washed again with PBS-T. Bound antibodies were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK) and exposure to Hyperfilm ECL (Amersham Pharmacia Biotech).

In subsequent experiments for quantification of the relative amounts of transporter protein in enterocyte fractions, 20 μg of protein was dotted directly onto nitrocellulose (using a BioRad Dot Blot SF chamber). Nitrocellulose membranes were probed as described above and X-ray films analysed using a scanning densitometer (Hoefer Scientific Instruments, USA). Preliminary experiments revealed a linear relationship between protein loading and band density between 10 and 50 μg protein (data not shown).

### 2.6. Measurement of intracellular cyclic AMP

Aliquots (100 μl) from each enterocyte fraction were mixed with 3 M perchloric acid to precipitate cell proteins. The acid fractions were neutralised with $K_3PO_4$ (3.16 M) and the cell precipitates removed by centrifugation (13 000 × *g*, 5 min). Cyclic AMP in the subsequent aqueous fractions was determined using a commercially available cyclic AMP radioimmunoassay system (Amersham Pharmacia Biotech) and values expressed as pmol cyclic AMP/mg cell protein.

### 2.7. Statistics

All results are shown as mean ± S.E.M. Differences between means of the control and forskolin groups were evaluated by Student's unpaired *t*-test and considered significant at $P < 0.05$. Within-groups comparisons were carried out using one-way analysis of variance (ANOVA) and differences between groups determined by Scheffe's post hoc analysis.

### 2.8. Materials

Radiochemicals were purchased from Amersham Pharmacia Biotech. All other chemicals were of the best grade available and were obtained from either Sigma (Poole, Dorset, UK) or Merck (Poole, Dorset, UK).

## 3. Results

### 3.1. Villus enterocyte preparations

Over 90% of all cells isolated from upper, mid and lower villus regions excluded trypan blue, demonstrating that the cells maintained their membrane integrity. Furthermore, there was an increasing gradient for the activity of the differentiation marker alkaline phosphatase from the lower to upper villus fractions. Alkaline phosphatase activity was not affected by short-term forskolin treatment (data not shown). Approximately 50% of the total number of cells recovered was in the first fraction (designated upper villus). This recovery was not affected by forskolin treatment.

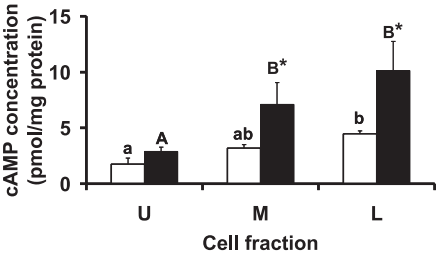


Fig. 1. Effects of 1 h incubation of isolated jejunum with buffer containing 5 μM forskolin (filled bars) or buffer alone (open bars) on cyclic AMP levels in upper, mid and lower villus enterocytes. Data are mean ± S.E.M. of 4–6 experiments in each group. Statistical analysis between control and forskolin-treated groups was carried out using Student's unpaired *t*-test and differences considered significant at $*P < 0.05$. Within-groups analysis utilised one-way ANOVA followed by Scheffe's post hoc analysis, and significant differences between cell fractions are indicated by the presence of different letters above the data bars ($P < 0.05$).

### 3.2. Intracellular cyclic AMP levels

Cyclic AMP levels decreased towards the villus tip (Fig. 1) and were significantly higher (one-way ANOVA and Scheffe's post hoc analysis) in lower villus fractions compared with upper ($P < 0.005$). There was no significant difference in cyclic AMP levels in upper vs. mid ($P = 0.11$) or mid vs. lower enterocytes ($P = 0.22$). Forskolin (5 μM) elevated intracellular cyclic AMP in all three villus fractions (Fig. 1) though this did not reach statistical significance in upper villus cells ($P = 0.12$). In forskolin-treated cells, intracellular cyclic AMP was increased in mid and lower villus populations compared with upper villus enterocytes ($P < 0.05$, one-way ANOVA).

### 3.3. Immunoblotting of villus enterocyte membranes for SGLT1 and GLUT2

Initial experiments utilised total villus membrane protein (prepared from mucosal scrapes) from rat jejunum exposed to forskolin (5 μM) or vehicle (0.01% DMSO) as a control. Western blotting (Fig. 2A) using the SGLT1 antibody demonstrated a single cross reacting band at 73 kDa, which was greater in the forskolin-treated samples. Similarly, GLUT2 was present as a 56 kDa band (Fig. 2B), but levels lower in forskolin-treated intestine.

To avoid variation arising from quantifying Western blots from several experiments performed on sep-

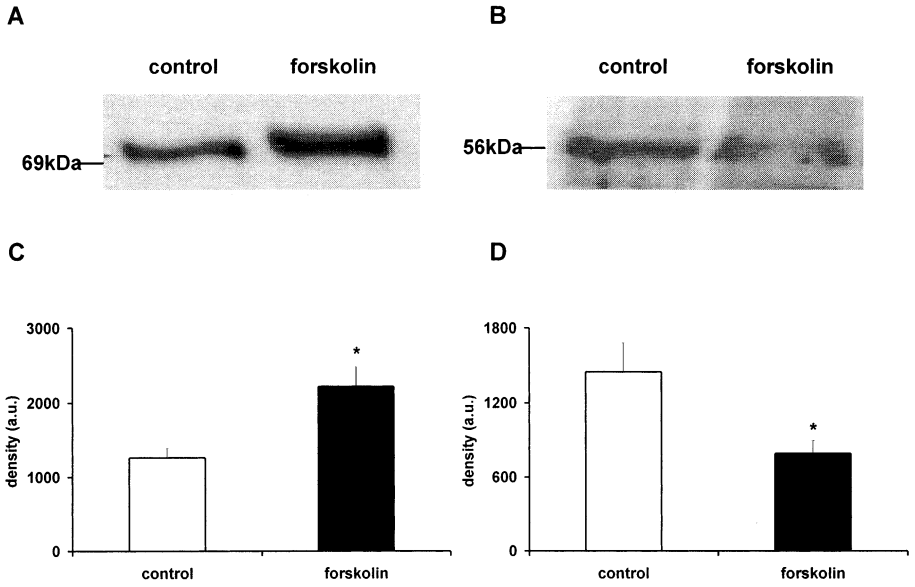


Fig. 2. Representative Western blot for SGLT1 (A) and GLUT2 (B) in enterocyte membranes from jejunal scrapes (50 μg protein/lane) following incubation of intact jejunum in vitro for 1 h with or without 5 μM forskolin. Semi-quantification of blots for SGLT1 (C) and GLUT2 (D) following forskolin treatment (filled bars) or incubation with buffer alone (open bars) was achieved by scanning densitometry. Data are shown as mean ± S.E.M. of four experiments. $*P < 0.01$, statistical differences between forskolin and control groups.

arate days, an immunoblotting protocol was adopted in which membrane protein from several experiments was spotted directly onto the same piece of nitrocellulose using a slot blot chamber. Scanning densitometry of immunoblots showed that SGLT1 expression was greater in the forskolin treated groups (Fig. 2C), whereas GLUT2 levels were significantly reduced (Fig. 2D). To determine the villus locus of the changes in SGLT1 and GLUT2 expression, enterocytes were isolated sequentially to form discrete populations of cells.

SGLT1 expression along the villus axis was greatest in upper villus enterocytes and decreased in a graded fashion towards the lower villus cells (Fig. 3). Differences in SGLT1 protein between enterocyte populations were not statistically different. Following exposure to forskolin, SGLT1 levels were increased significantly in the mid and lower villus enterocytes (Fig. 3). Interestingly, there was no significant change in SGLT1 expression in the upper villus region.

GLUT2 expression was not significantly different in untreated upper, mid or lower villus cells (Fig. 4). Following forskolin treatment, in contrast to SGLT1, GLUT2 levels were significantly reduced in both mid and lower villus enterocytes. However, GLUT2 expression was unaltered by forskolin in upper villus cells.

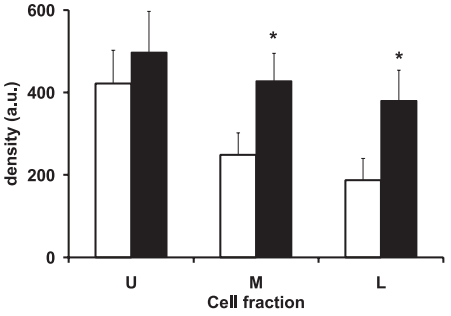


Fig. 3. Semi-quantitative scanning densitometry of slot blots (20 μg protein) for SGLT1. Following forskolin treatment (filled bars) or incubation with buffer alone (open bars) cells from upper, mid and lower villus regions we isolated by a well-characterised $Ca^{2+}$ chelation technique [11] and cell membranes prepared from each villus fraction. Data are shown as mean ± S.E.M. of four experiments. $*P < 0.01$, statistical differences between forskolin and control groups. There was no significant difference in SGLT1 expression in different cell fractions within treatment groups (one-way ANOVA, $P > 0.05$).

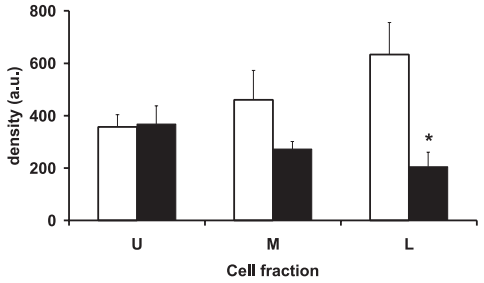


Fig. 4. Semi-quantitative scanning densitometry of slot blots (20 μg protein) for GLUT2. Membranes were prepared and used as described in Fig. 3. Data are shown as mean ± S.E.M. of four experiments. $*P < 0.01$, statistical differences between forskolin and control groups. There was no significant difference in GLUT2 expression in different cell fractions within treatment groups (one-way ANOVA, $P > 0.05$).

## 4. Discussion

Previous studies have shown that activating the cyclic AMP signalling cascade increases the movement of glucose across both the brush border [8,13–16] and basolateral [8] membranes of intestinal enterocytes. At the brush border, the cellular mechanisms include changes in membrane potential, providing a greater driving force for $Na^+$-glucose cotransport and an increased rate of SGLT1-mediated uptake. Basolateral changes were due to an increase in the Vmax for transport across this membrane. The purpose of our present work was to determine whether the cyclic AMP-mediated increase in glucose uptake occurred via SGLT1 at the brush border membrane and GLUT2 at the basolateral membrane, respectively, as a consequence of increased transporter expression along the villus axis.

To stimulate cyclic AMP, enterocytes were incubated for 1 h in the presence of the diterpene, forskolin, which is known to increase cyclic AMP in many tissues [17]. This manoeuvre increased intracellular cyclic AMP in all enterocyte fractions, though this did not reach statistical significance in the upper villus cells (Fig. 1). Furthermore, in untreated cells there is a gradient of basal cyclic AMP levels, which was highest in the lower villus cells and decreased towards the villus tip. This pattern agrees with a preliminary study of cyclic AMP levels in rat ileal enterocytes [18] and is likely to be linked to the essential role of cyclic AMP in regulating intestinal chloride secretion by the lower villus enterocytes and crypt cells [19].

Our current work provides direct evidence that, in jejunal enterocytes, forskolin induces increased expression of SGLT1 but decreases the expression of GLUT2. We have investigated the villus location of these changes in transporter expression using enterocytes isolated into three discrete cell populations, designated to be from the upper, mid or lower villus. Our experiments illustrated that increased SGLT1 expression and reduced GLUT2 levels were evident in the mid and lower villus cells, whilst there was no observable difference in either SGLT1 or GLUT2 levels in the upper villus enterocyte population. This is in keeping with the changes in enterocyte cyclic AMP levels, which were observed following forskolin treatment. Previous data from our laboratory, using the protein kinase A activating agent dibutyryl cyclic AMP, supports the hypothesis that the forskolin-induced changes in glucose transporter expression occur as a consequence of increased intracellular cyclic AMP rather than via indirect metabolic effects of forskolin on enterocytes [20].

Previous studies using the cyclic AMP raising agent, cholera toxin, have demonstrated a direct link between exposure to the toxin and induction of $Na^+$-glucose transport in the secretory intestinal cell line HRT-18 [21,22]. Furthermore, activation of protein kinase A has been shown to lead to increased insertion of SGLT1 into the plasma membrane [23] of *Xenopus* oocytes, suggesting that increased SGLT1 in the brush border membrane of intestinal enterocytes may occur as a consequence of trafficking from intracellular compartments. This latter finding is consistent with increased SGLT1-mediated transport across the apical membrane of rat jejunal enterocytes [8].

The regulation of GLUT2 by cyclic AMP in intestinal enterocytes is less well studied. Previous work in Caco-2 cells has shown that forskolin markedly and transiently decreased the expression of GLUT2 mRNA, whilst stimulating GLUT5 mRNA abundance [24]. Furthermore, in pancreatic β-cells, protein kinase A-dependent phosphorylation of GLUT2 leads to a significant decrease in glucose transport [25]. Taken together, these studies support our findings that cyclic AMP decreased plasma membrane expression of GLUT2 in mid and lower villus enterocyte (Fig. 4).

The changes in transporter expression observed

here are partly at odds with previous data on the effects of cyclic AMP on glucose transport by intestinal enterocytes. An increase in transport at the brush border membrane is consistent with our finding that SGLT1 levels are increased in the cell membrane. However, increased efflux across the basolateral pole cannot be accounted for by a corresponding increase in GLUT2, unless the intrinsic activity of the transporter is upregulated. This seems unlikely given the previous data in pancreatic β-cells [25]. It is possible, however, that other members of the GLUT superfamily, for example GLUT1, which is known to be present in normal jejunal enterocytes [26], might be involved in increased basolateral glucose efflux following elevation of intracellular cyclic AMP levels.

In conclusion, we have demonstrated that exposure of the jejunal mucosa to forskolin increases intracellular cyclic AMP levels. This increase is greatest in the mid and lower villus regions and is associated with an increase in SGLT1 but a decrease in GLUT2 protein expression. These data confirm that the mid and lower villus cells are important zones of adaptation along the villus axis [2,5,6,10]. Interestingly, we observed no change in transporter expression in the upper villus cells following forskolin treatment. We would suggest that because these cells are fully differentiated they are already working at maximal transport capacity. In contrast, mid and lower villus enterocytes are younger and less advanced along the differentiation pathway and can therefore exhibit the necessary degree of plasticity required to match modulations in physiological function with changes in the local environment.

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