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TRANSPORT AND METABOLISM OF GLUCOSE IN ISOLATED ENTEROCYTES OF THE BLACK BULLHEAD *ICTALURUS MELAS*: EFFECTS OF DIET AND HORMONES

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Summary

The uptake and metabolism of glucose were assessed in enterocytes isolated from black bullhead *Ictalurus melas*. The objective of this study was to examine the effects of diet and hormone treatment on glucose transport and metabolism, so the enterocyte was the most appropriate preparation. Glucose transport was estimated using specific inhibitors: glucose uptake measured in the presence of phlorizin presumably represents transport at the basolateral membrane, whereas glucose uptake in the presence of cytochalasin B presumably represents transport at the brush border. Feeding bullheads a standard diet resulted in maximum enterocyte rates of glucose uptake of 438.2±35.5 nmol mg^{-1} cells h^{-1} for transport in the presence of cytochalasin B and 427.0±49.7 nmol mg^{-1} cells h^{-1} (means ± S.E.M., N=12) for transport in the presence of phlorizin. These values represent 50 % of the total 3-*O*-methylglucose transported. The rate of transport in the presence of cytochalasin B was increased in bullheads fed a high-carbohydrate diet. Incubating bullhead enterocytes with glucagon or glucagon-like peptide-1 (GLP) at 10^{-8} mol l^{-1} and with dexamethasone or isoproterenol at 10^{-6} mol l^{-1} significantly increased the rate of brush-border transport, but not the apparent affinity constant (K_t). Activation was dependent on hormone concentration. In contrast, insulin was without effect on transport rates, nor did it counteract activation by glucagon-family peptides. CO_2 production rates from D-[^{14}C]glucose indicated that glucose metabolism was not limited by transport rates in the enterocytes. Glucagon and GLP decreased maximal oxidation rates, whereas dexamethasone, isoproterenol and insulin did not alter these rates. The activities of enterocyte hexokinase exceeded the rate of glucose oxidation but not the rate of transport of glucose, at least at maximum activities, implicating this enzyme as one component of the strategy to ensure that glucose is maximally available to the blood of this species.

Key words: black bullhead, *Ictalurus melas*, glucose transport, glucose oxidation, diet, glucagon, glucagon-like peptide-1, dexamethasone, isoproterenol, enterocyte.

Introduction

The fish intestine exhibits at least two primary functional roles, ion–water regulation and nutrient absorption (Ferraris and Ahearn, 1984), although additional functions are known (Buddington *et al.* 1997). This structure shares many fundamental nutrient transport properties with the mammalian intestine, although differences do exist (Collie and Ferraris, 1995).

Previous studies performed in several species of fish using intestinal membrane vesicles have demonstrated that absorption of D-glucose occurs by a coupled mechanism. A primary concentrative Na^+-dependent, phlorizin-sensitive, carrier-mediated uptake of the hexose across the brush-border (apical or luminal) membrane of the intestinal epithelium, followed by a Na^+-independent, phloretin- and cytochalasin-B-sensitive facilitated diffusion of the sugar from the cell across the basolateral membrane to the blood (Reshkin and Ahearn, 1987*a*,*b*; Ahearn *et al.* 1992; Collie and Ferraris, 1995; Houpe *et al.* 1997).

Amongst vertebrates, there is a general tendency for species that consume diets containing primarily carbohydrate (herbivores and omnivores) to have greater intestinal glucose absorption rates compared with carnivorous species, and the same seems to occur in fish (Titus *et al.* 1991; Buddington *et al.* 1997). Several studies have shown that dietary substrates specifically regulate their intestinal transporters, with rates of intestinal sugar uptake being adjusted to match the carbohydrate levels of the natural diet of a species (Ferraris and Diamond, 1989, 1997). Glucose, a non-essential, non-toxic nutrient used as a source of calories, up-regulates its transporter in species that consume diets rich in carbohydrates

(Titus *et al.* 1991), but not in strictly carnivorous species (Titus *et al.* 1991; Buddington *et al.* 1991, 1997).

Little is known regarding the hormonal regulation of glucose transport, even though transporters contain potential target sites for phosphorylation by protein kinases (Hirsch *et al.* 1996; Wright *et al.* 1997). The importance of the mammalian intestine as an endocrine organ releasing factors to the circulation to affect distant organs is well known (Schmidt, 1997). The impact of these hormones directly upon intestinal transport/metabolism is less well understood. Glucose transport in the mammalian intestine is affected by hormones, including glucagon (Thompson and Debnam, 1986; Debnam and Sharp, 1993; Ferrer *et al.* 1994), insulin (Ozols, 1993), glucagon-like peptide (GLP) (Hermann *et al.* 1995), glucocorticoids and thyroid hormone (Collie, 1995). The only reported effects of hormones on nutrient transport in fish intestine are those of thyroid (Collie and Ferraris, 1995) and growth (Collie and Stevens, 1985) hormones and anabolic steroids (Hazzard and Ahearn, 1992).

Transepithelial transport is an energy-consuming process (needed for the maintenance of the Na^+ gradient), so enterocytes require a source of energy to continue nutrient transport. Once inside cells, nutrients are metabolized to extract stored energy. There are, however, few studies assessing the metabolic processes of enterocytes. Glucose utilization by the dog gut accounted for 20–25 % of whole-animal basal glucose utilization (Abumrad *et al.* 1982). As far as we are aware, there are no similar studies for any fish species.

Most intestinal transport studies in fish have used brush-border (BBMVs) or basolateral (BLMVs) membrane vesicles (Ahearn and Storelli, 1994; Collie and Ferraris, 1995). These preparations, however, do not allow the simultaneous study of both metabolism and transport of nutrients, nor the assessment of the influence of hormones on transcellular movements (Hazzard and Ahearn, 1992). Isolated enterocytes are amenable to such studies and have been used successfully to estimate transport rates directly in mammals (rat, Debnam and Sharp, 1993), a bird (chicken, Ferrer *et al.* 1994) and a fish (the eel *Anguilla anguilla*, Vilella *et al.* 1995).

The black bullhead *Ictalurus melas* is an omnivorous (and therefore presumably susceptible to up-regulation of glucose transporters by high-carbohydrate diets) freshwater fish species found in the midwestern USA and eastern Canada (Wooding, 1994). Isolating viable enterocytes in reasonable numbers is relatively easy in this species. Thus, the use of isolated bullhead enterocytes provides a model in which to study both metabolism and the uptake of nutrients, processes that cannot be studied in membrane preparations. Since enterocytes are polarized, the two membrane components of transport were estimated using specific inhibitors, namely phlorizin and cytochalasin B, in a way similar to that described in other enterocyte studies in mammals and birds (Debnam and Sharp, 1993; Ferrer *et al.* 1994). Thus, Na^+-independent sugar transport across the basolateral membrane was estimated using phlorizin, whereas Na^+-dependent sugar transport across the brush-border membrane was estimated using cytochalasin B.

The purpose of the present study was fourfold: (1) to estimate glucose flux in black bullhead enterocytes; (2) to establish whether transport limits metabolism; (3) to test the possible effects of changes in carbohydrate and protein levels in the diet on glucose transport; and (4) to establish the effects of several hormones (glucagon, GLP, insulin, dexamethasone and isoproterenol) on both glucose transport and metabolism in isolated enterocytes.

Materials and methods

Fish

Cultured black bullheads (*Ictalurus melas* L.) were obtained from Dr Joe Buttner (Department of Biological Sciences, State University of New York, Brockport, New York, USA). Fish were maintained for 2 months in 1250 l circular tanks under laboratory conditions in dechlorinated Ottawa tap water at 13 °C. Mean fish mass was 180±15 g (N=180). The fish were fed daily to satiation with a standard Purina catfish cage chow (standard diet). The photoperiod used was 12 h:12 h (L:D). Experiments were undertaken from June to August. The effect of diet composition on glucose uptake by enterocytes was assessed in two groups of fish fed for 1 month with two experimental diets prepared by Dr C. Y. Cho (Department of Zoology, University of Guelph, Ontario, Canada), one formulated to be low-carbohydrate/high-protein (LC/HP), and the other formulated to be high-carbohydrate/low-protein (HC/LP). Diets were isocaloric and their composition is presented in Table 1.

Cell collection and preparation

Fish were dipnetted, immediately killed by a sharp blow to the head and then weighed. The whole intestine was excised and divided into an anterior (approximately two-thirds of the length) and a posterior (the remaining third) portion. The intestinal contents were flushed with modified Hanks' medium (in mmol l^{-1}: 136.9 NaCl, 5.4 KCl, 0.8 $MgSO_4 \cdot 7H_2O$, 0.33 $Na_2HPO_4 \cdot 7H_2O$, 0.44 KH_2PO_4, 5 Hepes, 5 Hepes–Na, 5

Table 1. *Formulation of the two experimental diets fed to the black bullheads for 1 month*

	Percentage of dry mass	
Ingredient	LC/HP	HC/LP
Fish meal	41	25
Corn gluten	24	15
Soybean meal	15	10
Whey	10	10
Starch	0	28
Vitamins premix-G	1	1
Minerals premix-G	1	1
Fish oil	8	10

Diets were either low-carbohydrate/high-protein (LC/HP) or high-carbohydrate/low-protein (HC/LP).

$NaHCO_3$ and 1.5 $CaCl_2$, pH 7.6) by attaching a syringe to the anterior end of the gut. The gut was then weighed, slit longitudinally, spread on an ice-cooled plate and rinsed twice with Hanks' medium. The mucosa was gently scraped using the edge of a glass slide, and the mucosa thus obtained was transferred immediately to a Petri dish containing modified Hanks' medium plus 1 mmol l^{-1} EGTA and 0.05 mg ml^{-1} collagenase (from *Clostridium histolyticum*, type IV, Sigma). This suspension was gently aspirated with a glass Pasteur pipette for 15 min to disperse the cells into the medium. The suspension was then filtered through 250 and 72 μm filters. The filtered cell suspension was centrifuged at 80 ***g*** for 3 min at 4 °C (Sorvall RC 5B Plus, SS-34 rotor). The cells were washed four times with 10 vols of modified Hanks' medium plus 1 % defatted bovine serum albumin, pH 7.6 (Hanks' complete). The final pellet was resuspended in Hanks' complete to a concentration of 17.5 mg ml^{-1}. Cell viability was assessed by Trypan Blue exclusion (Mommsen *et al.* 1994), and only preparations that yielded cell viabilities greater than 90 % were used. The pH of all media was adjusted to 7.6, and no significant changes in pH were observed during any experiment. The cells began to deteriorate 8 h after isolation, as judged by their reduced viability (Trypan Blue exclusion) and loss of shape. All experiments reported here were completed within 5 h of isolation. There was no indication of decreased viability during any of the studies. Cells maintained their distinctive microvillar structure on a portion of the membrane throughout this period.

Reagents

All labelled substrates were purchased from Amersham Canada Ltd (Oakville, Ontario, Canada). The specific activities were 3.81 GBq mmol l^{-1} 3-*O*-methyl-D-[U-^{14}C]glucose, 11.2 GBq mmol l^{-1} D-[U-^{14}C]glucose and 2.15 GBq mmol l^{-1} L-[1-^{14}C]glucose. Glucagon was a kind gift from Lilly Research Laboratories (Indianapolis, IN, USA), and glucagon-like peptide-1 (GLP) was obtained from American Peptide Company (Sunnyvale, CA, USA). The remaining hormones (insulin), hormone agonists (isoproterenol, dexamethasone), substrates and inhibitors were obtained from Sigma Chemical Co. (St Louis, MO, USA) or Boehringer Mannheim (Montréal, Québec, Canada).

Uptake studies

Uptake experiments were carried out in duplicate at 20 °C according to Soengas and Moon (1995) with slight modifications. Passive D-glucose uptake was calculated from the measured accumulation of the non-transported stereoisomer L-glucose (probably identical in its permeability to D-glucose). Uptake of 3-*O*-methyl-D-[U-^{14}C]glucose and L-[1-^{14}C]glucose were initiated by mixing 1 vol of the cell suspension containing 17.5 mg cells ml^{-1} with 0.5 vol of incubation medium (Hanks' complete) containing both radioactive and non-radioactive 3-*O*-methyl-D-glucose (3-OMG) or L-glucose. The final concentration of labelled and unlabelled substrates was 0.0185 MBq ml^{-1} and 0.01–60 mmol l^{-1}, respectively, in a final volume of 0.2 ml in a conical plastic centrifuge tube (1.5 ml). The mixture was shaken (70 strokes min^{-1}) during the pre-incubation and incubation periods to prevent settling of the cells. For incubations in the presence of inhibitors (1 mmol l^{-1} phlorizin, 50 μmol l^{-1} cytochalasin B), cells were pre-incubated with the inhibitor for 15 min at room temperature (20 °C) in the absence of substrate. Fresh stock solutions of inhibitors were prepared daily in 0.5 % dimethylsulphoxide. No effects were seen due to the vehicle alone on any variable studied (data not shown). The concentrations of inhibitors chosen were those reported to produce complete inhibition of brush-border (phlorizin) and basolateral (cytochalasin B) membrane glucose transport in other fish species (Naftalin and Kleinzeller, 1981; Reshkin and Ahearn, 1987*a*,*b*; Hazzard and Ahearn, 1992; Houpe *et al.* 1996; Maffia *et al.* 1996). Sugar uptake measured in the presence of phlorizin is assumed to represent facilitated transport at the basolateral membrane, while initial unidirectional entry across the brush border was estimated by measuring uptake in the presence of cytochalasin B. The Na^+-dependence of uptake rates was assessed in cells isolated and incubated in Na^+-free Hanks' medium (osmolarity was adjusted using *N*-methyl-D-glucamine instead of NaCl, with all other Na^+ salts substituted by their K^+ equivalent).

The effects of hormones (insulin, glucagon and GLP) and hormone agonists (dexamethasone, isoproterenol) on 3-OMG uptake were studied as a function of time and dose. Cells obtained as described above were pre-incubated for 15 min with insulin, glucagon or GLP and for 2 h with isoproterenol or dexamethasone. Standard concentrations used were 10^{-8} mol l^{-1} for insulin, glucagon and GLP, and 10^{-6} mol l^{-1} for isoproterenol and dexamethasone.

Incubations were terminated after 5 min by layering 0.15 ml of the cell suspension over 0.5 ml of a mixture consisting of 2.1 vols of di-*n*-butylphthalate (Sigma Chemical Co.) and 1 vol of di-iso-octylphthalate (BDH Chemicals) at a final density of 1.022 g ml^{-1} in a 1.5 ml centrifuge tube and immediately centrifugation (30 s, 7000 ***g***; Fisher microcentrifuge 235B). The tube was stored on ice to minimize any endogenous metabolism. The medium layer was removed by aspiration, leaving the oil layer and the cell pellet. The tube was rinsed three times with ultrapure water, the oil layer was aspirated, and the radioactivity of the pellet was assessed by liquid scintillation counting after adding 1.2 ml of scintillation cocktail directly to the tube (ACS II, Amersham) and using an LKB Wallac 1215 Rackbeta scintillation counter with internal standard quench correction. Uptake values were corrected for extracellular trapped radioactivity, which was estimated using ^{14}C-labelled polyethyleneglycol (mean 4.6 %, *N*=8). Substrate uptake is presented as nmol substrate mg^{-1} cells h^{-1} (where substrate is 3-OMG or L-glucose) at 20 °C. The time course of uptake was determined at an extracellular substrate concentration of 1 mmol l^{-1} 3-OMG or L-glucose. Initial rates of uptake were linear up to 20 min, so 5 min was chosen as the standard time interval. Kinetic constants (K_t, apparent affinity constant; J_{max}, apparent maximal transport velocity) were

calculated after subtraction of the diffusion component of transport (obtained from L-glucose uptake rates) followed by linear regression analysis after transforming the data using the Eadie–Hofstee method.

CO_2 production studies

Carbon dioxide production by the cell suspension from D-[^{14}C]glucose was measured according to the method of Soengas and Moon (1995). Glass vials (20 ml) contained 0.1 ml of cell suspension (17.5 mg cells ml^{-1}) and 0.875 ml of Hanks' complete with D-glucose at a final concentration of 0.01–60 mmol l^{-1}. The vials were gassed with 99.5 % O_2/0.5 % CO_2 for 2 min and then sealed with a rubber septum through which was suspended a centre well containing a glass microfibre filter (GF/A, Whatman). After a 15 min pre-incubation period, the experiment was initiated by the addition of 0.025 ml of Hanks' complete containing D-[U-^{14}C]glucose (0.0185 MBq per vial). The vials were shaken during the 1 h incubation period at 20 °C. At 1 h, 0.1 ml of 1 mol l^{-1} hyamine hydroxide was injected through the rubber septum onto the filter in the centre well, and the cells were then lysed with 0.1 ml of 35 % (v/v) perchloric acid to release the CO_2 and terminate the incubation. The sealed vials were shaken for a further 2 h at 20 °C to ensure the collection of CO_2 onto the filter.

The effects of hormones (insulin, glucagon and GLP) and hormone agonists (isoproterenol and dexamethasone) on D-glucose metabolism were studied as for the uptake experiments (see above). Cells were pre-incubated 15 min with insulin, glucagon or GLP, and for 2 h with isoproterenol or dexamethasone as described above. Radioactivity trapped on the filter was determined by liquid scintillation counting (OCS, Amersham) as above. The rate of CO_2 production was calculated from the specific activity of the added labelled substrate, the mass of cells used and the length of the incubation period after correction for the CO_2 released from control vials (in the absence of cell suspension).

Hexokinase (E.C. 2.7.1.1) activity in enterocytes

An enterocyte suspension (17.5 mg ml^{-1}) was obtained as described above, and 1.2 ml was centrifuged (Fisher microfuge, 7000 ***g*** for 30 s). The medium was then removed, and the packed cells were sonicated (Kontes micro ultrasonic cell disrupter) in 10 vols of ice-cold 15 mmol l^{-1} β-mercaptoethanol, 5 mmol l^{-1} EDTA, 0.1 mmol l^{-1} phenylmethylsulphonyl fluoride (added as dry crystals immediately before homogenization), 10 % glycerol and 50 mmol l^{-1} imidazole–HCl, pH 7.4. The homogenate was centrifuged (Fisher microfuge, 7000 ***g*** for 30 s) and the supernatant was used as a source of enzyme. Enzyme activity was assessed using an ATP regenerating system consisting of creatine phosphate and creatine phosphokinase. The final incubation volume (1 ml) contained 50 mmol l^{-1} imidazole–HCl (pH 7.4), 0.5 mmol l^{-1} $NADP^+$, 2.5 mmol l^{-1} ATP, 2.5 mmol l^{-1} creatine phosphate, 0.9 units ml^{-1} creatine phosphokinase and different concentrations of D-glucose (from 0.0125 to 5 mmol l^{-1}), which was omitted in the controls. Hexokinase activity was monitored at 20 °C by following the appearance of NADPH at 340 nm (Beckman DU-65 spectrophotometer), and activities are reported as nmol mg^{-1} cells h^{-1} using the extinction coefficient of 6.22.

Statistics

Comparisons between kinetic variables were performed using a one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls multiple range test. The comparisons were performed using the SPSS statistical package. The differences were considered significant at $P<0.05$. Values are presented as means ± S.E.M.

Results

The number of cells obtained from the distal gut region was inadequate for a detailed study. Therefore, only cells isolated from the anterior two-thirds of the gut were studied in detail. However, preliminary data obtained from the posterior region clearly suggested the existence of a gradient in transport activity along the gut (high activity anteriorly, low activity posteriorly).

Characterization of 3-OMG uptake

The time course of uptake of a non-metabolizable analogue of D-glucose, 3-OMG (1 mmol l^{-1}), achieved a steady state after a 20 min incubation, with the rate of uptake being linear from 0.5 to 20 min (data not shown). Thus, a 5 min period was used in all further studies of 3-OMG uptake. 3-OMG uptake was concentration-dependent and was saturated at 3-OMG concentrations exceeding 40 mmol l^{-1} (Fig. 1). Uptake of 3-OMG in the presence of 50 µmol l^{-1} cytochalasin B or 1 mmol l^{-1} phlorizin to block basolateral and brush-border transport, respectively, was reduced, with saturation achieved at 40 mmol l^{-1} 3-OMG with phlorizin and at 30 mmol l^{-1} 3-OMG with cytochalasin B (Fig. 1). L-Glucose uptake was linear from 0.01 to 60 mmol l^{-1}, suggesting that L-glucose uptake represents the diffusion component of transport (Fig. 1). After subtraction of this diffusion component and using the Eadie–Hofstee regression analysis, K_t and J_{max} values were calculated (Table 2). Cytochalasin B significantly decreased both K_t and J_{max} values compared with total transport, but phlorizin only decreased J_{max}. 3-OMG transport in the absence of Na^+ decreased J_{max} compared with total transport (Fig. 1) to rates similar to those in the presence of Na^+ and 50 µmol l^{-1} cytochalasin B. In the presence of both a Na^+-free medium and 50 µmol l^{-1} cytochalasin B, uptake rates were linear and identical to those for L-glucose (Fig. 1), suggesting that diffusion is operating under these conditions.

Effect of different diets on 3-OMG uptake

Enterocytes isolated from fish fed for 1 month with the LC/HP diet showed no significant modification of 3-OMG uptake rates (J_{max}) or K_t values compared with those fed the standard diet (Table 2). However, 3-OMG transport (J_{max}) and

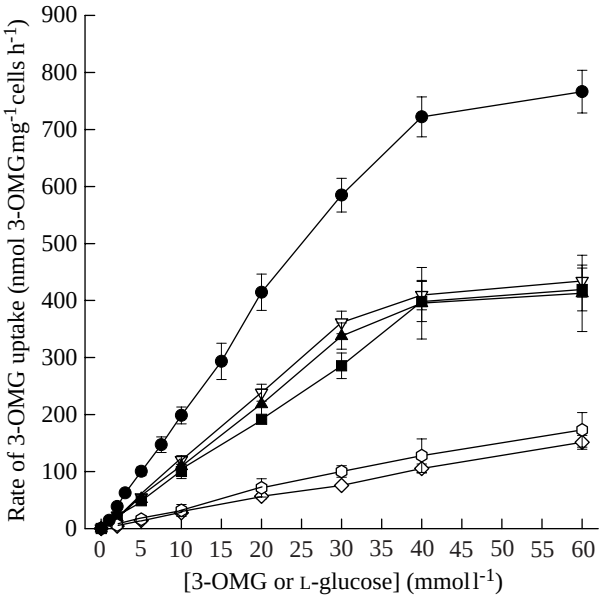

Fig. 1. Concentration-dependence of 3-*O*-methyl-D-glucose (3-OMG) or L-glucose uptake by bullhead enterocytes. Uptake was measured at room temperature after 5 min in the presence of 0.0185 MBq of 3-*O*-methyl-D-[U-^{14}C]glucose or L-[1-^{14}C]glucose and a range of concentrations of unlabelled substrate. Incubations were terminated by centrifuging samples through an oil layer as noted in Materials and methods. Radioactivity was assessed by scintillation counting (ACS, Amersham, UK) and uptake was corrected for trapped extracellular space. Initial uptake rates of 3-OMG (filled circles), 3-OMG in the presence of 1 mmol l^{-1} phlorizin (filled squares), 3-OMG in the presence of 50 μmol l^{-1} cytochalasin B (open triangles), 3-OMG in the absence of Na^+ (filled triangles), 3-OMG in the absence of Na^+ and in the presence of 50 μmol l^{-1} cytochalasin B (open circles) and L-glucose (open diamonds). Each value is the mean ± S.E.M. of N=12 independent experiments, except for 3-OMG uptake in the absence of Na^+ and 3-OMG uptake in the absence of Na^+ with 50 μmol l^{-1} cytochalasin B, where N=4.

transport in the presence of cytochalasin B were increased significantly in fish fed the HC/LP diet compared with those fed the commercial (standard) diet. The changes in kinetic constants clearly reflect a HC/LP-diet-induced increase in J_{max} in the absence of an alteration in K_t (Table 2). The absence of any significant impact of the HC/LP diet on 3-OMG uptake in the presence of phlorizin suggests that this diet preferentially affected the brush-border transporter. There were no significant effects of diet on L-glucose (diffusion) transport.

Effect of hormone treatments on 3-OMG uptake

Incubating enterocytes with 10^{-8} mol l^{-1} glucagon or GLP increased both total 3-OMG transport rate and the rate of transport in the presence of cytochalasin B without affecting K_t (Table 3). Again, there was no change in the diffusion component (L-glucose uptake) in the presence of these hormones. The dose–response effects of glucagon and GLP on uptake rates at 20 mmol l^{-1} 3-OMG plus cytochalasin B are shown in Fig. 2. A significant increase in uptake rate is observed

Table 2. *Kinetic constants for 3-OMG uptake into bullhead enterocytes derived from Eadie–Hofstee plots of data as in Fig. 1*

	Standard diet	LC/HP	HC/LP
K_t (mmol l^{-1})			
Total	43.5±5.3	46.4± 9.7	45.9±7.7
+ CytoB	23.3±4.0[c]	29.8±4.3	22.3±4.3[c]
+ Phlorizin	44.1±5.8[d]	43.0±7.6	35.5±8.2
J_{max} (nmol mg^{-1} cells h^{-1})			
Total	881.6±43.3	907.1±75.1	1233.6±77.2[a,b]
+ CytoB	438.2±35.5[c]	455.1±47.5[c]	754.3±44.8[a,b,c]
+ Phlorizin	427.0±49.7[c]	415.8±33.4[c]	458.3±44.7[c,d]

Diets were either the standard Purina catfish cage chow (standard diet) or a specially formulated diet consisting of low-carbohydrate/high-protein (LC/HP) or high-carbohydrate/low-protein (HC/LP) (see Table 1).

Total represents the standard assay, while +CytoB is in the presence of 50 μmol l^{-1} cytochalasin B and +Phlorizin is in the presence of 1 mmol l^{-1} phlorizin to inhibit basolateral and brush-border glucose transport, respectively.

Values are means ± S.E.M. of N=12 independent experiments each plotted individually and K_t and J_{max} values determined individually (Eadie–Hofstee method).

K_t, apparent affinity constant (giving $J_{max}/2$); J_{max}, apparent maximum rate of transport of 3-OMG.

[a]Significant (Student–Newman–Keuls multiple range test, P<0.05) diet effects compared with bullheads fed standard diet; [b]significant (Student–Newman–Keuls multiple range test, P<0.05) diet effects compared with bullheads fed LC/HP diet; [c]significantly different (Student–Newman–Keuls multiple range test, P<0.05) from total transport; [d]significantly different (Student–Newman–Keuls multiple range test, P<0.05) from total + cytochalasin B transport.

at hormone concentrations exceeding 10^{-10} mol l^{-1}, although maximum changes do not exceed 1.4 times control values.

Dexamethasone and isoproterenol at 10^{-6} mol l^{-1} also increased both total and cytochalasin-B-sensitive 3-OMG transport rates without affecting K_t values (Table 3). The effect is dose-dependent for both hormones (Fig. 3). Significant increases in uptake rate occurred at hormone concentrations exceeding 10^{-9} mol l^{-1}, and both responses appear to plateau at hormone concentrations greater than 10^{-5} mol l^{-1}. The maximum responsiveness and sensitivity of uptake to dexamethasone are significantly greater than those to isoproterenol.

No effects on uptake rates were observed when bullhead enterocytes were incubated with 10^{-8} mol l^{-1} insulin (data not shown). In addition, the combined effect of incubation of enterocytes with 10^{-8} mol l^{-1} insulin and 10^{-8} mol l^{-1} glucagon or GLP produced results similar to those obtained with glucagon or GLP alone (data not shown).

CO_2 production studies

The time course of CO_2 production from D-[^{14}C]glucose by bullhead enterocytes (using 1 mmol l^{-1} D-glucose) achieved a

Table 3. *The effects of hormones on kinetic constants for 3-OMG uptake into bullhead enterocytes*

	Control	Glucagon	GLP	Dexamethasone	Isoproterenol
K_t (mmol l^{-1})					
Total	43.5±5.3	42.7±5.4	43.4±9.2	47.1±7.2	45.3±8.4
+CytoB	23.3±4.0c	28.9±4.5	28.8±6.8	22.0±3.9[c]	24.2±6.1[c]
J_{max} (nmol mg^{-1} cells h^{-1})					
Total	881.6±43.3	1050.0±47.3[b]	1162.6±86.3[a]	1305.4±48.1[a]	1190.7±74.5[a]
+CytoB	438.2±35.5[c]	613.8±19.9[a,b,c]	579.9±36.6[a,b,c]	740.3±32.1[a,c]	592.1±41.3[a,b,c]

Hormone concentrations were 10^{-8} mol l^{-1} for glucagon and glucagon-like peptide-1 (GLP) and 10^{-6} mol l^{-1} for dexamethasone and isoproterenol.

Cells were pre-incubated for 15 min (glucagon, GLP) or 2 h (dexamethasone, isoproterenol) in the presence of hormones before 3-OMG was added.

Total represents the standard assay, while +CytoB is in the presence of 50 μmol l^{-1} cytochalasin B as in Table 2.

Fish were fed the standard catfish cage chow.

Values are means ± S.E.M. of N=4 independent experiments determined as noted in Table 2.

K_t, apparent affinity constant (giving $J_{max}/2$); J_{max}, apparent maximum rate of transport of 3-OMG.

[a]Significant (Student–Newman–Keuls multiple range test, P<0.05) hormone effects compared with controls; [b]significantly different (Student–Newman–Keuls multiple range test, P<0.05) from dexamethasone effects; [c]significantly different (Student–Newman–Keuls multiple range test, P<0.05) from total transport.

steady state by 120 min, with a linear rate of oxidation observed between 5 and 120 min (data not shown). Thus, a 60 min period was used in all further studies of D-glucose oxidation. Oxidation rates saturated at approximately 20 mmol l^{-1} D-glucose (Fig. 4). Estimates of the Michaelis constant (K_m) and apparent maximum velocity (V_{max}) for glucose oxidation rates are reported in Table 4. Glucagon and GLP at 10^{-8} mol l^{-1} significantly decreased glucose oxidation rates compared with controls (Table 4). This decrease affected exclusively the apparent V_{max} values, not K_m (Table 4). In contrast, neither dexamethasone nor isoproterenol at 10^{-6} mol l^{-1} modified the oxidation rates (Fig. 4) or kinetic variables compared with control values (Table 4). Insulin at 10^{-8} mol l^{-1} did not modify D-glucose oxidation rates at a number of D-glucose concentrations (data not shown). As noted for 3-OMG

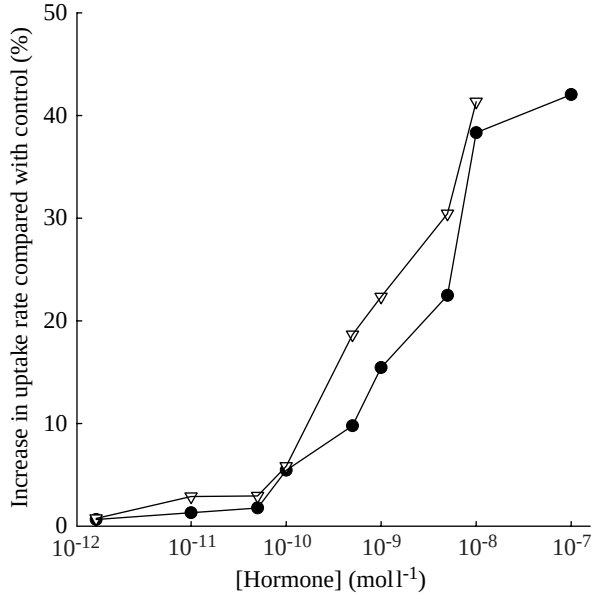

Fig. 2. Dose–response curves for the effect of glucagon (filled circles) and glucagon-like peptide-1 (open triangles) on the uptake of 20 mmol l^{-1} 3-*O*-methyl-D-glucose (3-OMG) in the presence of 50 μmol l^{-1} cytochalasin B by bullhead enterocytes. Uptake was measured as described in Fig. 1. Each value is the mean of two independent experiments run in duplicate and expressed as a percentage of the uptake in the absence of hormone. Variation between experiments was less than 7 %.

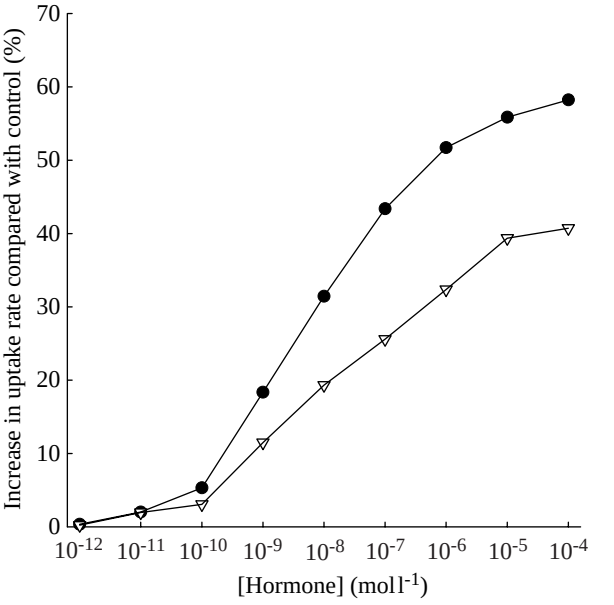

Fig. 3. Dose–response curves for the effect of dexamethasone (filled circles) and isoproterenol (open triangles) on the uptake of 20 mmol l^{-1} 3-*O*-methyl-D-glucose (3-OMG) in the presence of 50 μmol l^{-1} cytochalasin B by bullhead enterocytes. Uptake and conditions as in Fig. 2. Variation between experiments was less than 6 %.

Table 4. *Kinetic constants for the oxidation of D-[^{14}C]glucose by bullhead enterocytes in the presence and absence of hormones*

Condition	K_m (mmol D-glucose l^{-1})	V_{max} (nmol mg^{-1} cells h^{-1})
No addition	12.6±4.2	54.5±4.2
Glucagon	15.4±5.0	25.2±5.4[a,b]
GLP	14.5±3.9	17.3±3.8[a,b,c]
Dexamethasone	11.3±3.9	52.3±5.5
Isoproterenol	12.1±4.0	49.9±7.9

Enterocytes were pre-incubated for 15 min in the presence of glucagon or GLP, and for 2 h in the presence of dexamethasone or isoproterenol, before the addition of 3-OMG. Incubations were terminated as noted in Materials and methods after 60 min.

Hormone concentrations were 10^{-8} mol l^{-1} for glucagon and glucagon-like peptide (GLP) and 10^{-6} mol l^{-1} for dexamethasone and isoproterenol.

K_m, Michaelis constant; V_{max}, apparent maximum rate of oxidation.

Values are means ± S.E.M. of N=9, 5 and 4 for control, glucagon and the remaining hormones, respectively; each value represents an independent experiment as noted in Table 2.

[a]Significantly different (Student–Newman–Keuls multiple range test, P<0.05) from no addition; [b]significantly different (Student–Newman–Keuls multiple range test, P<0.05) from dexamethasone effects; [c]significantly different (Student–Newman–Keuls multiple range test, P<0.05) from isoproterenol effects.

transport, the combined effects of insulin and glucagon or GLP did not modify D-glucose oxidation rate (at 20 mmol l^{-1}) compared with incubation with glucagon or GLP alone.

Hexokinase activity in enterocytes

The activity of hexokinase was measured to test whether the rate of glucose transport or glucose phosphorylation limited glucose metabolism in bullhead enterocytes. Fig. 5 shows that hexokinase activity saturated above 1 mmol l^{-1} D-glucose. Using the Eadie–Hofstee regression analysis, estimated kinetic constants were 252.7±18.8 nmol D-glucose mg^{-1} cells h^{-1} for V_{max} and 47±3.4 μmol l^{-1} for K_m. This estimated V_{max} is lower than the J_{max} for 3-OMG uptake (Table 2) but higher than the V_{max} for D-glucose oxidation (Table 4). It is also apparent that enzyme activity saturated at concentrations lower than those observed for both oxidation rate and the rate of uptake so that, at 1 mmol l^{-1}, D-glucose uptake rate may in fact be lower than hexokinase activity.

Discussion

Fish intestinal glucose transport has been the subject of many studies (see Introduction), but our interest was to examine the role of hormones in glucose homeostasis in these cells. Intact enterocytes were prepared from bullhead intestines and, with the use of appropriate inhibitors, we were able to distinguish apical from basolateral glucose transport. Basolateral glucose transport (transport inhibited by cytochalasin B) represented

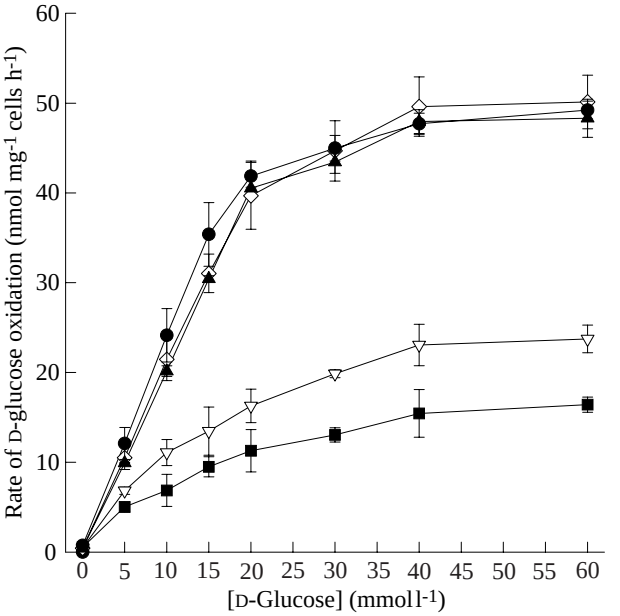

Fig. 4. CO_2 production from D-[^{14}C]glucose by bullhead enterocytes and the effects of glucagon, glucagon-like peptide, dexamethasone and isoproterenol. CO_2 production was measured at room temperature for 1 h in the presence of 0.0185 MBq of D-[U-^{14}C]glucose and different D-glucose concentrations. Radioactivity was estimated as described in Materials and methods. The rate of CO_2 production in the absence of hormone (filled circles) and in the presence of glucagon (open triangles) and glucagon-like peptide-1 (filled squares) at 10^{-8} mol l^{-1} and dexamethasone (open diamonds) and isoproterenol (filled triangles) at 10^{-6} mol l^{-1}. Each value is the mean ± S.E.M. of N=9 experiments for the control, N=5 for glucagon, and N=4 for the remaining hormones.

40–50 % of total 3-OMG transport in all experiments, and the sum of the two components (cytochalasin-B-sensitive and phlorizin-sensitive) always equalled total transport. These data validate the use of the enterocyte preparation for these studies, as has been shown previously for other systems (mammals, Debnam and Sharp, 1993; a bird, Ferrer *et al.* 1994; a fish, Vilella *et al.* 1995). The characterization of glucose transport demonstrated that the rate of Na^+-dependent transport was approximately equivalent to that of 3-OMG in the presence of cytochalasin B, as demonstrated by incubations of enterocytes in medium lacking Na^+. In contrast, the use of both cytochalasin B and medium lacking Na^+ gave transport rates equivalent to those observed for L-glucose, or equivalent to rates of diffusion. These findings all support the use of this preparation to assess enterocyte physiology, although considering the major differences from brush-border kinetic constants, the affinities reported here may not necessarily reflect *in vivo* values.

The gradient of glucose uptake along the intestine parallels the normal gradient of luminal glucose concentrations (Thorens, 1996; Ferraris and Diamond, 1997), supporting the observation for a gradient of glucose transporter density in the same direction (Ferraris and Diamond, 1997). In the present study, preliminary experiments provided some evidence to

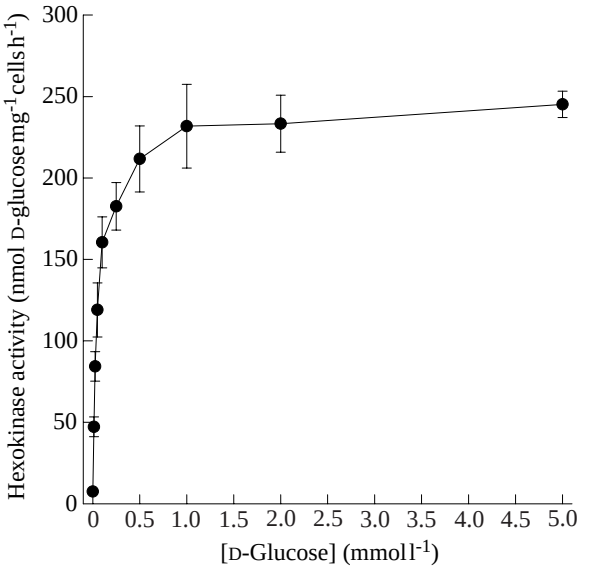

Fig. 5. Hexokinase activity in bullhead enterocytes. Enzyme activity was measured as noted in Materials and methods. Each value is the mean ± S.E.M. of N=5 experiments.

support the existence of such a gradient but, unfortunately, very few cells could be obtained from the distal third of the gut, so these studies were not continued. Only cells isolated from the proximal two-thirds of the gut were used.

Glucose absorption across the cells lining the gut is the net result of active transport, facilitated diffusion and metabolic utilization. A wide range of values for glucose absorption have been reported in the literature, but in many cases this may simply be due to differences in techniques, unstirred layer artefacts, the use of glucose analogues or variations in K_t across brush-border membrane (Ahearn *et al.* 1992; Hirayama *et al.* 1996). For instance, K_t of glucose absorption measured in intact humans was 6 mmol l^{-1}, but it was 0.5 mmol l^{-1} using BBMVs (Hirayama *et al.* 1996). Also, K_t for 3-OMG has been reported to be 40 times higher than that for α-methyl glucose or D-glucose (Hirayama *et al.* 1996). Solberg and Diamond (1987), using everted sleeves, did not achieve saturation even at 50 mmol l^{-1} 3-OMG.

Thus, differences in the kinetics and selectivity of intestinal sugar absorption between species are expected, but such differences cannot justify the higher estimates we obtained for K_t compared with those of most studies. The estimate of K_t obtained for brush-border transport of 3-OMG (transport in the presence of cytochalasin B) of approximately 20 mmol l^{-1} is considerably higher than those previously reported in fish using membrane vesicles (0.1–0.7 mmol l^{-1}) (Ahearn and Storelli, 1994; Collie and Ferraris, 1995). In contrast, the estimate obtained for basolateral transport (transport in the presence of phlorizin) of approximately 40 mmol l^{-1} is in accordance with many values reported for mammals and fish species (Ferraris and Diamond, 1997). The use of enterocytes instead of the more common methods of everted sleeves or membrane vesicles prevents an accurate estimate of kinetic constants. However, the effects of hormones on transport and metabolism cannot be measured using these conventional systems.

Bullhead enterocyte glucose transport capacity was altered by diet, as previously demonstrated in other fish species (Titus *et al.* 1991; Thamotharam *et al.* 1996). Fish fed with the HC/LP diet showed a significant increase in the apparent J_{max} of total glucose transport. This increase in 3-OMG transport did not occur in the presence of phlorizin, suggesting an increase in Na^+/glucose cotransport, presumably through an SGLT transporter (Na^+-dependent glucose transporter, inhibited by phlorizin). This result is comparable with that previously obtained in other fish species either using everted sleeves or BBMVs (Titus *et al.* 1991; Collie and Ferraris, 1995), again validating the technique used in the present study. SGLT1 mRNA has been detected in the mucosa of trout intestine (Wright, 1993), so this transporter presumably also exists in the bullhead enterocyte. The glucose transport capacity of fish fed the LC/HP diet did not differ significantly from that of fish fed the standard chow, probably because of its low carbohydrate content.

The main goal of our study was to examine the effects of different hormone treatments on glucose uptake and metabolism. Enterocytes have been used for this purpose in other animals (Debnam and Sharp, 1993; Ferrer *et al.* 1994). The hormones tested include some previously shown to have effects in mammalian intestines (glucagon-family peptides, Schmidt, 1997; Ferraris and Diamond, 1997; insulin, Kellet *et al.* 1984; Ozols, 1993; dexamethasone, Collie and Stevens, 1985; Collie, 1995) and isoproterenol because the autonomic nervous system may affect the regulation of nutrient transport (Ferraris and Diamond, 1997).

Glucagon and GLP pre-incubation for only 15 min increased the apparent J_{max} of bullhead enterocyte total transport and transport in the presence of cytochalasin B. This acute response to both hormones implies that these hormones may be important in the normal regulation of absorption. These results are comparable to those reported by Debnam and Sharp (1993) using mammalian enterocytes and glucagon at 10^{-8} mol l^{-1}; as in our study, they found a 40 % increase in transport capacity. The mammalian intestine does express glucagon receptors, and glucagon has been demonstrated to affect smooth muscle contraction and mucosal Na^+/K^+-ATPase activity (Christophe, 1996) as well as sugar transport (Debnam and Sharp, 1993; Ferraris and Diamond, 1997). Also, cyclic AMP levels are reported to increase in mammalian enterocytes after glucagon treatment (Sharp and Debnam, 1994). Glucagon receptors have been identified only in fish hepatic tissue, not in intestine (Navarro and Moon, 1994), although glucagon is produced by the endocrine tissues of the fish intestine (see Plisetskaya and Mommsen, 1996). Glucagon signalling does involve cyclic AMP in fish hepatic tissue (Plisetskaya and Mommsen, 1996) and, assuming that there are intestinal receptors, cyclic AMP may be the mediator of glucose transport by glucagon in fish enterocytes. However, the precise link between increases in levels of cyclic AMP and sugar transport are unknown,

although the studies of Sharp and Debnam (1994) implicating changes in Na^+ permeability in affecting membrane potential may be important. Specific fluorescent dyes could be used to test this hypothesis. The sensitivity of the bullhead enterocyte to glucagon is within the range of plasma glucagon concentrations (Navarro and Moon, 1994), again suggesting that this effect is of physiological importance in promoting glucose uptake at low plasma glucose concentrations.

Glucagon-like peptide-1 (GLP) is a metabolic hormone in fish, but not in mammals, and both GLP-1 and GLP-2 are produced in the fish intestine (see Plisetskaya and Mommsen, 1996). Recent evidence (S. Mojsov, personal communication) indicates that GLP receptors are expressed in the zebrafish intestine. The signalling pathway for GLP in fish is a subject of controversy (Mommsen and Moon, 1989), although T. P. Mommsen (personal communication) recently found changes in cyclic AMP concentrations in fish gut rings after addition of GLP. Again, any possible link between cyclic AMP or other messengers and changes in glucose uptake are unknown. Injection of growth hormone (a growth factor in fish) is known to enhance gut amino acid transport in coho salmon *Oncorhynchus kisutsch* undergoing the parr–smolt transformation (Collie and Ferraris, 1995), which involves changes in intestinal structure and function (Buddington *et al.* 1997), but its effects on glucose transport have not been studied. Similar to the effects of glucagon, GLP appears to act in an acute manner and at concentrations that are within the physiological range for fish (Plisetskaya and Mommsen, 1996). In mammals, GLP-1 inhibits gastric emptying (Holst, 1997), but there is no indication of a role in metabolism or transport. The role of both glucagon and GLP in enterocyte substrate transport needs to be further evaluated.

Insulin had no impact upon transport capacity or affinity in black bullhead enterocytes, in contrast to studies performed in mammals (Kellet *et al.* 1984; Ozols, 1993; Ferraris and Diamond, 1997). A few incubations were performed with insulin and one of the glucagon-family peptides, but the results did not differ from incubations of enterocytes with glucagon-family peptides alone. This suggests that, at least in these fish enterocytes, insulin is not involved in transport regulation either by itself or by counteracting the effects of glucagon. Whether insulin is involved in other components of fish intestinal physiology is unknown.

The effects of dexamethasone and isoproterenol on glucose transport support the involvement of the glucocorticoid and adrenoceptor pathways in the regulation of glucose transport. The effect of the hormones was to increase the apparent J_{max} to values higher than those observed for glucagon. A 2 h pre-incubation was used for both hormones, so it is not possible to distinguish between an acute or a more chronic effect of the hormone. Cortisol coordinates stress responses, leading to fuel mobilization, in fish and is known to stimulate gut Na^+/K^+-ATPase, an enzyme essential for maintaining Na^+-coupled nutrient absorption across the gut (Collie, 1995). In addition, an increased J_{max} of L-proline transport has been reported in the gut of coho salmon (*Oncorhynchus kisutch*) after cortisol treatment (Collie, 1995). Corticosteroid receptor activity has been identified in the intestine of the brook trout *Salvelinus fontinalis* (Chakraborti *et al.* 1987). Therefore, a role for cortisol in increasing glucose absorption in an omnivorous species would be a reasonable hypothesis.

Whether intestinal sugar transport is modified by neural input remains to be clearly demonstrated in mammals (Ferraris and Diamond, 1997). Noradrenergic nerves are generally inhibitory in mammals, although catecholamines produce variable responses in fish, but in all cases these are associated with gut motility (Jensen and Holmgren, 1994). Isoproterenol is a β-adrenoceptor agonist and it increases cyclic AMP levels in a mammalian intestinal cell line (Denning *et al.* 1994). The impact of isoproterenol on bullhead enterocytes may simply relate to its effects on cyclic AMP concentrations, as suggested above for glucagon and possibly GLP.

The up-regulation of transport observed either after feeding with an HC/LP diet or after hormone treatment changed only maximal transport rates (J_{max}) rather than altering the apparent affinity constant (K_t). This result is consistent with other regulatory pathways in fish enterocytes (Hazzard and Ahearn, 1992).

The second part of this study dealt with bullhead enterocyte glucose oxidation, to investigate whether glucose transport limited metabolism in the presence and absence of hormones. Changes in glucose uptake require additional energy that could be supplied by enhanced glucose utilization. Glucose oxidation has been studied in hepatocytes (Pereira *et al.* 1995) and red blood cells (Soengas and Moon, 1995) isolated from fish. No similar study has been performed using teleost enterocytes, and our results clearly showed that metabolic capacity is lower than transport capacity and, therefore, that metabolism is not limited by transport. As oxidation rates were much lower than uptake rates (55 *versus* 438 nmol mg^{-1} cells h^{-1}; Tables 4 and 2, respectively), these fish enterocytes metabolize only a fraction of the nutrient that is transported, with the largest amount left to enter the blood through the basolateral membrane. The hormones assayed demonstrated two different effects on glucose metabolism. Glucagon and GLP sharply decreased glucose oxidation, while no effects were observed for either dexamethasone or isoproterenol. Oxidation is generally unaffected by either glucagon or GLP when applied to fish hepatocytes *in vitro* (Plisetskaya and Mommsen, 1996). No similar studies have been reported using either dexamethasone or isoproterenol. These results are difficult to interpret mechanistically given the lack of similar studies or an understanding of metabolic regulation in these cells. Physiologically, however, it is interesting that both glucagon and GLP activate transport and inhibit metabolism, thus redirecting glucose towards transepithelial transport and increasing blood glucose levels.

Hexokinase activities are key to high glycolytic rates in pig enterocytes, even though enzyme activities far exceed glycolytic flux (Posho *et al.* 1994). Hexokinase activity in the bullhead enterocyte also exceeds that of metabolic flux, but activities are below those of glucose uptake rates, at least at

maximum values (253 *versus* 438 nmol glucose mg^{-1} cells h^{-1}; see Results and Table 2, respectively). However, the very low K_m (D-glucose) for hexokinase means that, irrespective of maximal activities, hexokinase will be poised to metabolize glucose entering the enterocyte. How this glucose is redirected to the basolateral membrane and to the blood is unknown.

In summary, the results obtained in the present study using enterocytes from bullhead demonstrate significant effects of diet and of hormones on *in vitro* glucose transport and oxidation. Further studies on the control of enterocyte metabolism are justified by the apparently rapid effects of hormones and by their differential effects on transport and metabolism. The role of hexokinase in the metabolism of glucose appears to be key to ensuring that transepithelial glucose transport predominates and that blood glucose level is not compromised.

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