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Na-dependent D-Glucose Transport by Intestinal Brush Border Membrane Vesicles from Gilthead Sea Bream (*Sparus aurata*)

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Abstract. Brush border membrane vesicles (BBMV) enriched in sucrase, maltase and alkaline phosphatase, and impoverished in Na^+-K^+-ATPase, were isolated from proximal and distal intestine of the gilthead sea bream (*Sparus aurata*) by a $MgCl_2$ precipitation method. Vesicles were suitable for the study of the characteristics of D-glucose apical transport. Only one D-glucose carrier was found in vesicles from each intestinal segment. In both cases, the D-glucose transport system was sodium-dependent, phlorizin-sensitive, significantly inhibited by D-glucose, D-galactose, α-methyl-D-glucose, 3-O-methyl-D-glucose and 2-deoxy-D-glucose, and showed stereospecificity. Apparent affinity constants of D-glucose transport (K_t) were 0.24 ± 0.03 mM in proximal and 0.18 ± 0.03 mM in distal intestine. Maximal rate of influx (*J*max) was 47.3 ± 2.2 pmols. mg^{-1} protein for proximal and 27.3 ± 3.6 pmols. mg^{-1} protein for distal intestine. Specific phlorizin binding and relative abundance of an anti-SGLT1 reactive protein were significantly higher in proximal than in distal BBMV. These results suggest the presence of the same D-glucose transporter along the intestine, with a higher density in the proximal portion. This transporter is compatible with the sodium-dependent D-glucose carrier described for other fish and with the SGLT1 of higher vertebrates.

Key words: SGLT1 — Proximal intestine — Distal intestine — Brush border membrane vesicles — Freezing — Fish

Introduction

The gilthead sea bream (*Sparus aurata*) is predominantly located in the Mediterranean Sea. In the last 15 years, the commercial farming of this fish has become a common practice along the Mediterranean coastline because of the increase in its consumption. In spite of its importance for the optimization of the farming procedures, there is still limited information available about the physiology in general (Montero et al., 1999) and the digestive process in particular (Lorenzo et al., 1989; Moyano et al., 1996) of this species.

The intestine of carnivorous fish like gilthead sea bream presents a well-developed ability to digest protein and a low capacity to digest carbohydrate when compared to omnivorous and herbivorous fish (Buddington, Krogdahl & Bakke-Mckellep, 1997). Thus, the commercial nutrient dense diets for gilthead sea bream usually contain high protein and low carbohydrate percentages (Buddington et al., 1997). Therefore, studies focused on hexose uptake by carnivorous fish are of interest to ascertain if absorption is a limiting step.

In the intestine of teleost fish, D-glucose absorption is mediated by a hexose carrier present in the brush border membranes of intestinal cells, which is followed by a facilitated diffusion across the basolateral membrane to the blood (Reshkin & Ahearn, 1987b; Drai et al., 1990). In a number of studies, the glucose transporter of the brush border membrane of fish intestinal epithelium has been characterized as a sodium-dependent system that is sensitive to phlorizin and strongly inhibited by D-glucose, α-methyl-D-glucose and D-galactose (Ferraris & Ahearn, 1984; Storelli, Vilella & Cassano, 1986; Reshkin & Ahearn, 1987a; Ahearn & Storelli, 1994). The different sodium-dependence relationships and responses to

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inhibitors reported (Ahearn et al., 1992; Ahearn & Storelli, 1994) suggest that heterogeneity in D-glucose transporters may exist in fish. At present, intestinal hexose uptake in gilthead sea bream has only been studied indirectly by means of electrophysiological measurements (Lorenzo et al., 1987).

Nutrient transport systems change quantitatively and qualitatively along the length of fish intestine. Thus, proximal regions show a higher substrate uptake capacity and a lower affinity than distal locations (Ferraris & Ahearn, 1983, 1984; Collie, 1985; Buddington & Diamond, 1987; Reshkin & Ahearn, 1987a). Some differences have also been described between glucose transporters at the upper intestine and pyloric ceca (Ahearn et al., 1992).

In the present study, brush border membrane vesicles have been isolated from proximal and distal intestine of the gilthead sea bream. A D-glucose transport system has been characterized along the intestine as the SGLT1 of higher vertebrates. The density of transporters is higher in the proximal segment, as indicated by specific phlorizin binding and Western blot analysis, performed using a polyclonal antibody against rabbit intestinal SGLT1. In addition, a peptide that is recognized by the same anti-rabbit SGLT1 antibody has been localized along the brush border of both intestinal regions of this fish, by means of immunohistochemical detection.

Materials and Methods

Animals

Cultured gilthead sea bream (*Sparus aurata*) averaging 300 to 350 g live body weight were obtained from a commercial fish farm (Cultivos Marinos S.A., Arenys de Mar, Barcelona, Spain). The fish had been grown in floating sea cages and fed daily a standard diet (Ecoplus Marino 4.5 mm, 43% protein, 21% crude fat, 15% carbohydrate, 16,2 MJ. kg^{-1} metabolizable energy; Dibaq-Diproteg, Fuentepelayo, Segovia, Spain) at an average of 1% of total biomass. At the time of sampling (late September to early October), the mean water temperature at the farm of origin was 21°C.

Chemicals

All unlabelled chemicals, reagent grade, were obtained from Sigma (St. Louis, MO). D-[U-^{14}C]Glucose (specific activity 315 mCi. $mmol^{-1}$) was purchased from American Radiolabelled Chemicals (St. Louis, MO). L-[1–^{3}H(N)]Glucose (specific activity 20 Ci.mol^{-1}) and [^{3}H]phlorizin (specific activity 56 Ci.$mmol^{-1}$) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [4-^{3}H(N)]Cytochalasin B (specific activity 17 Ci.$mmol^{-1}$) was obtained from Amersham Biosciences Limited (Buckinghamshire, UK).

Tissue Sampling

Fish were netted and kept in a well-oxygenated tank until being killed by severing the spinal cord. The abdominal cavity was immediately opened and the entire intestine was carefully removed and cut in two segments, the proximal and the distal, according to abrupt visual dissimilarities in diameter and mucosal thickness. Segments were placed separately in ice-cold isosmotic saline in the presence of 0.2 mM phenylmethylsulfonyl fluoride (PMSF, protease inhibitor), flushed with 10 mL of saline plus PMSF, freed from adherent mesenteric tissue and cut open lengthwise. Pools of proximal or distal segments were frozen in liquid nitrogen and stored at −80°C until used in the isolation of intestinal brush border membrane vesicles (BBMV).

Preparation of Intestinal Brush Border Membrane Vesicles

BBMV were prepared from the proximal or distal segment pools by a $MgCl_2$ precipitation method (Vazquez et al., 1997). Samples of 8–12 g were thawed in 40 mL of a hyposmotic buffer containing (in mM) 100 mannitol and 2 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-Tris(hydroxymethyl)aminomethane (TRIS) (pH 7.4), cut into small portions, and placed in a Waring blender at low speed for 8 min, to separate the mucosa from the muscular layers. The result was filtered through a Büchner funnel, and the eluent was brought up to 100 mL with the same buffer and homogenized at high speed for 3 minutes. The homogenate was subsequently filtered through nylon stocking material and the eluent was brought up to 150 mL with the same buffer. $MgCl_2$ was added to reach a final concentration of 10 mM, for selective precipitation of most cellular membranes except for the brush border. After stirring gently for 15 minutes, the homogenate was centrifuged (3,000 × *g* for 15 min). The supernatant was reposed in ice for 10 min, during which lipids in excess formed a thin layer that was subsequently discarded. The remaining volume was then centrifuged (30,000 × *g* for 30 min) and the pellet was carefully resuspended in 100 mM mannitol, 2 mM HEPES-TRIS (pH 7.4) and 0.1 mM $MgSO_4$. The suspension was homogenized with a glass-Teflon homogenizer (40 strokes) and centrifuged at 30,000 × *g* for 25 min. The final pellet, containing purified brush border membrane segments, was resuspended in 300 mM mannitol, 20 mM HEPES-TRIS (pH 7.4), 0.1 mM $MgSO_4$ and 0.41 μM LiN_3 with a syringe fitted with a 25-gauge needle. The result was then homogenized with a 29-gauge needle to facilitate vesicle formation.

All isolation procedures were carried out at 0–4°C. Centrifugation steps were conducted with a Centrikon H-401 centrifuge (Kontron Instruments, Milano, Italy) with a 18.24 rotor.

Enzyme and Protein Determinations

Sucrase (EC 3.2.1.48) and maltase (EC 3.2.1.20) were assayed by the method of Messer and Dahlqvist (1966), whereas alkaline phosphatase (EC 3.1.3.1) was determined by the method of Weiser (1973). The activity of the ouabain-sensitive Na^+-K^+-ATPase (EC 3.6.1.3) was analyzed by the method of Colas and Maroux (1980). Protein was quantified using the Bio-Rad protein assay, with bovine serum albumin as standard. All determinations were carried out at 21°C.

Orientation of Vesicles

Membrane orientation was assessed from sucrase activity according to the method of Del Castillo & Robinson (1982). Sucrase activity was determined in intact vesicles and in vesicles incubated for 30 min with a mixture of 3 μM Na^+ deoxycholate and 15 mM EDTA. It was observed that more than 90 % of the vesicles were outside-out oriented (*data not shown*).

D-Glucose and L-Glucose Transport Assays

The uptake of D-[^{14}C]glucose or L-[^{3}H]glucose was measured at 21°C by a rapid filtration technique (Garriga, Moretó & Planas, 1999). Each uptake was started by mixing 10 μL of BBMV suspension (equivalent to 150–200 μg of protein) with 40 μL of the adequate incubation medium. At selected times, reaction was stopped by the addition of 1 mL of ice-cold isotonic stop solution. An aliquot (900 μL) of the resulting suspension was rapidly filtered under negative pressure through a prewetted 0.22 μm pore size cellulose nitrate filter (Millipore, Bedford, MA). The filter was rinsed with 10 mL of stop solution, dissolved in Filtron-X cocktail (National Diagnostics, Atlanta, GA) and counted for radioactivity in a Tri-Carb 2100 TR scintillation counter (Packard, Canberra, Australia).

Specificity and structural requirements of the transport system were investigated by examining the effect of different sugar analogues on D-glucose uptake, such as phlorizin (a well-known competitive inhibitor of Na^+- dependent sugar transport; Wright, 1993), D-glucose, D-galactose, α-methyl-D-glucose, 3-O-methyl-D-glucose (substrates for SGLT1 of higher vertebrates; Wright, 1993), 2-deoxy-D-glucose and D-fructose (substrates for the GLUT family of sugar transporters; Wright, Martin & Turk, 2003); L-glucose was used to test stereospecificity (Kimmich, 1981).

For time course studies, vesicles were incubated from 0 s to 90 min. For studies on kinetics and inhibition by different substrates, BBMV were loaded for 30 min with a medium containing K^+, and then incubated for 5 s under short circuit conditions, in the presence of 12 μM valinomycin.

Total D-glucose fluxes were analyzed by non-linear regression using the Enzfitter program (Biosoft, Cambridge, United Kingdom). As errors associated with experimental fluxes were roughly proportional to their values, it was considered appropriate to apply proportional weighing to the data.

Phlorizin Binding Measurements

Steady-state [^{3}H]phlorizin binding was assayed at 21°C according to Garriga et al. (1999). 10 μL of BBMV suspension were rapidly mixed with 40 μL of the incubation medium in the presence of NaCl or KC1, to determine the total and nonspecific binding, respectively. After 5 s of incubation, the process was stopped by addition of the stop solution. The resulting suspension was filtered and treated as described for the assays of D-glucose transport. Specific [^{3}H]phlorizin binding was calculated by subtracting the nonspecific from total phlorizin binding. The density of phlorizin binding sites was expressed as pmols of phlorizin bound per mg of protein.

Cytochalasin B Binding Measurements

Steady-state [^{3}H]cytochalasin B binding was assayed at 21°C according to Garriga et al. (1999). 10 μL of BBMV suspension were rapidly mixed with 40 μL of the incubation medium, to a final mixture composition of (in mM): 200 mannitol, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4), 0.1 μM LiN_3, 0.5 μM [^{3}H]cytochalasin B, and 100 mM of L-glucose or D-glucose, to determine the total and nonspecific binding, respectively. At 5 s, the reaction was stopped by addition of 1 mL of a stop solution containing (in mM): 300 mannitol, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.1 μM LiN_3. The resulting suspension was rapidly filtered and treated as described above. Specific binding was calculated by subtracting the nonspecific from total cytochalasin B binding.

All experiments described above were performed with at least three different membrane preparations, each in duplicate. Nonspecific radioactivity binding to the filters was obtained by adding the stop solution to reaction tubes in the absence of vesicles. This nonspecific binding was subtracted from the total radioactivity of each sample. The composition of preload, incubation and stop solutions used in each experiment is reported in the figure or table legends.

Western Blot Analysis

Aliquots of BBMV suspensions (equivalent to 50 μg of protein) from either proximal or distal intestine were solubilized in Laemmli sample buffer and resolved by 8% SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes for 1 h at constant voltage of 100 V. Immunoblotting and visualization of particular proteins by immunoreactivity were carried out as described previously by Garriga et al. (1999). Blots were incubated with a rabbit polyclonal antibody (donated by Dr. M. Kasahara) raised against the synthetic peptide corresponding to amino acids 564–575 of the deduced amino-acid sequence of rabbit intestinal SGLT1. Purified BBMV from rabbit jejunum possessing the SGLT1 co-transporter protein (Hwang, Hirayama & Wright, 1991) were used as a reference material throughout. In experiments carried out in parallel, the antibodies were preadsorbed with the corresponding antigenic peptide corresponding to amino acids 564–575 provided by Dr. E. M. Wright.

Immunohistochemical Localization of SGLT1

Small fragments of proximal and distal intestine were fixed in 70% ethanol for 36 h at 4°C and subsequently washed, dehydrated in graded ethanol series and embedded in paraffin wax at 60°C. Horizontal sections of 10 μM were obtained with a microtome and mounted on glass slides. Before primary antibody incubation, deparaffinized and rehydrated sections were treated with 0.1% sodium borohydride to minimize autofluorescence and subsequently permeabilized and blocked with 0.2% Triton X-100/12.5% normal goat serum in phosphate-buffered saline. The previously described rabbit polyclonal anti-SGLT1 antibody (1:600) was applied to the slides overnight at 4°C. On the next day, sections were incubated with the goat anti-rabbit Alexa Fluor 568 (Molecular Probes Inc., Eugene, OR) secondary antibody (1:500), and counterstained with 1 μM Hoechst-33258 to reveal nuclei. Primary antibody omission controls were used to confirm the specificity of the immunofluorescence obtained. Slides were dehydrated, mounted in MOWIOL® 4-88 Reagent (Calbiochem, San Diego, CA) and examined with a TCS SP2 laser scanning spectral confocal microscope (Leica, Wetzlar, Germany).

Preservation of D-Glucose Transport after BBMV Freezing

Vesicles were stored in 200 μL aliquots and frozen in liquid nitrogen until performance of the experiments. When needed, frozen BBMV suspensions were carefully thawed by a two-step process: first, by placing them for 15 min in ice, and then at room temperature until their complete thawing.

The uptake of D-glucose was measured for 20 s in proximal and distal intestinal BBMV the day of isolation and after freezing in liquid nitrogen for 1 day and 1, 2, 3, 4, 6 and 8 weeks. D-Glucose influx decreased with freezing in proximal and distal intestine (33 and 76 %, respectively), and no significant differences were found among the different times studied (*data not shown*). Consequently,

Table 1. Homogenate and BBMV specific activities, enrichment factor and total recovery of enzymatic markers and protein of gilthead sea bream

	Segment intestine	Specific activity, nmol. mg^{-1} protein. s^{-1}		Enrichment factor	Yield, % of homogenate activity	Total recovery, % of homogenate activity
		Homogenate	BBMV			
Alkaline Phosphatase	Proximal	2.3 ± 0.2	19.5 ± 1.9[a]	7.7 ± 0.6	39.7 ± 4.7	92.4 ± 10.7
	Distal	1.9 ± 0.2	12.4 ± 1.3[b]	7.9 ± 0.6	38.5 ± 1.8	85.6 ± 6.3
Maltase	Proximal	1.3 ± 0.1	7.4 ± 0.3	5.9 ± 0.4	36.3 ± 2.4	90.0 ± 6.0
	Distal	1.4 ± 0.2	6.8 ± 0.8	6.3 ± 0.5	33.4 ± 1.1	84.0 ± 3.7
Sucrase	Proximal	0.23 ± 0.02	1.02 ± 0.07	5.0 ± 0.3	28.4 ± 2.3	90.4 ± 8.3
	Distal	0.26 ± 0.03	1.03 ± 0.09	5.3 ± 0.3	28.3 ± 1.5	84.0 ± 5.4
Na^+/K^+-ATPase	Proximal	0.21 ± 0.03	0.12 ± 0.02	0.67 ± 0.01	3.8 ± 0.7	93.9 + 6.0
	Distal	0.21 ± 0.01	0.16 ± 0.04	0.88 ± 0.03	4.7 ± 0.8	98.6 ± 5.3
Protein	Proximal				7.3 ± 0.8	95.6 ± 4.0
	Distal				6.5 ± 0.7	93.2 ± 2.0

Enzymes and protein were measured as indicated in Materials and Methods. Enrichment factor is the ratio of specific activity in BBMV fraction versus specific activity in starting homogenate. Yield represents amount of enzymatic activities measured in BBMV with respect to that in homogenate. Total recovery indicates the ratio between sums of enzymatic activity measured in each fraction of isolation procedure versus activity in homogenate. Data are expressed as means ± SE of at least 10 separate determinations. Different letters indicate significant differences ($P < 0.05$) for each parameter between proximal and distal intestinal BBMV.

intestinal BBMV frozen between 1 day and 1 month were used for further experiments of preservation of D-glucose influx in vesicles stored in liquid nitrogen.

Statistical Analysis

The statistical analysis between regions was performed by Student's *t*-test. Differences among more than two groups were tested by one-way analysis of variance and Tukey test. Statistical analysis was performed by means of a computerized package (SigmaStat 2.01, San Rafael, CA).

Results

Validation of the Isolation Procedure

The activities of enzymatic markers of apical and basolateral membrane were measured to ascertain the purity of brush border membrane vesicles isolated from proximal and distal intestine of the gilthead sea bream (Table 1). Specific activity of sucrase, maltase and alkaline phosphatase, generally accepted to be BBM-marker enzymes, were enriched between 5 and 7.9 times, both in proximal and distal intestine. On the other hand, the activity of Na^+-K^+-ATPase, a basolateral membrane marker, was lower in the final suspension than in the starting homogenate. No significant differences between the proximal and distal intestine were detected in homogenate and BBMV specific activities, enrichment factor, yield or total recovery for any of the enzymes studied, except for the specific activity of alkaline phosphatase in BBMV. Neither was any significant difference detected in yield and total recovery of protein between the two types of vesicles.

Characterization of D-Glucose Transport in Proximal and Distal Intestinal BBMV

The time course of the uptake of 0.1 mM of D-glucose in the presence or absence of sodium is shown in Fig. 1. With an inwardly directed Na^+ gradient, the D-glucose was rapidly transported into the vesicles. In both cases, maximal intravesicular sugar accumulation (overshoot) was reached after a short time of incubation (60 s in proximal and 30 s in distal BBMV). When sodium was replaced by potassium, the initial uptake rates were low and the overshoot disappeared. For each type of vesicles, uptake of D-glucose at equilibrium (90 min) was identical in the presence or absence of sodium. When transport was referred to vesicle protein (pmol D-glucose.mg^{-1} protein), as in Fig. 1*A*, the value at equilibrium was higher in distal BBMV, thus denoting larger vesicular volumes (Table 2). Overshoot in these uptake units was also higher in distal vesicles. When D-glucose uptake values were normalized to concentration units (μM D-glucose), as shown in Fig. 1*B*, proximal intestinal BBMV exhibited actually higher transport capacity. However, no significant differences were found between initial rates or accumulation ratios of the two types of vesicles studied (Table 2). D-Glucose uptake was linear with time for 6 s in both proximal and distal BBMV (Fig. 2); therefore, 5 s were used in subsequent kinetic experiments.

Figure 3 shows the effect of a wide range of D-glucose concentrations on the initial uptake rate of D-[^{14}C]-glucose in BBMV of proximal (Fig. 3*A*) and distal (Fig. 3*B*) intestine of gilthead sea bream. This experiment was carried out using short-circuit conditions to prevent undesirable changes in the mem-

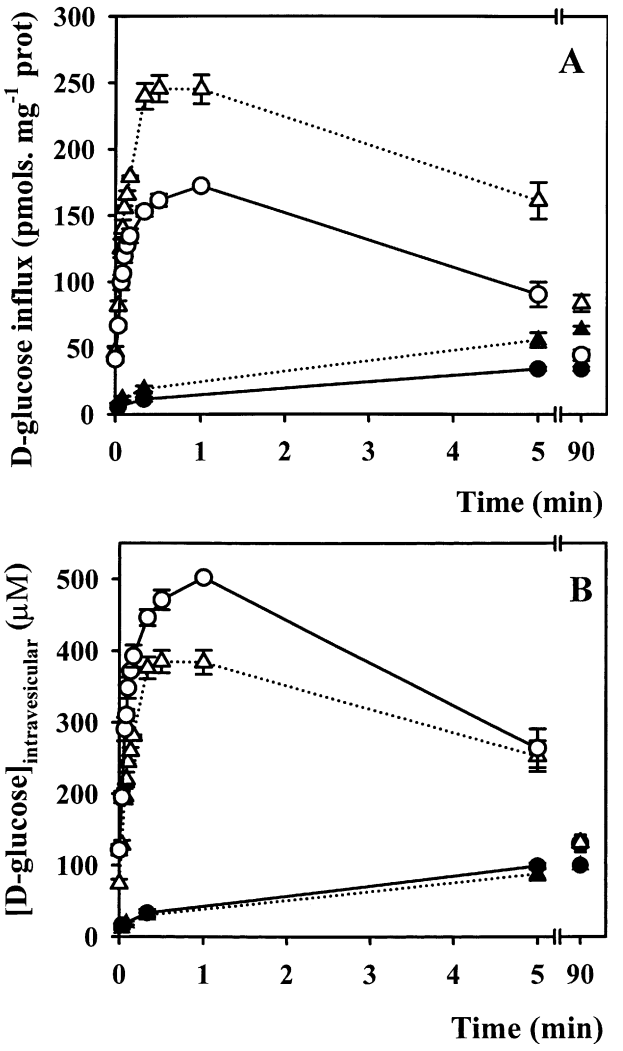

Fig. 1. Time course of 0.1 mM D-glucose uptake by proximal (*circles*) and distal (*triangles*) intestinal BBMV measured just after isolation. Vesicles were incubated for different times in the presence of buffers containing either sodium (*empty circles* or *triangles*) or potassium (*filled circles* or *triangles*). After addition of vesicles, final incubation mixture composition was (in mM) 100 mannitol, 0.1 D-glucose (0.01 D-[^{14}C]glucose), 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3, plus 100 mM NaSCN or KSCN. The composition of stop solution was (in mM) 300 mannitol, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. Uptake data are expressed as means ± SE of at least 3 separate experiments in front of protein (*A*) or in concentration units (*B*).

brane potential, caused by the electrogenic Na^+-glucose cotransport, that may affect transport rates. D-Glucose influx appears mainly non-saturable for both proximal and distal intestinal BBMV, indicating that at high concentrations most of the uptake is independent of carriers. The Eadie-Hofstee plot of D-glucose influx by proximal (Fig. 3*A*) and distal (Fig. 3*B*) intestinal BBMV suggests that only one transporter is involved in each case. Kinetic constants are summarized in Table 3. No significant differences were found between the K_ts of D-glucose carriers of proximal and distal intestine, whereas the J_{max} was 73% higher in proximal intestine. The K_d was higher in the distal segment.

To further characterize the transport system responsible for the uptake of D-glucose in each segment of the intestine, high concentrations of several D-glucose analogs (50 mM) and phlorizin (200 μM) were used to cis-inhibit the incorporation of 0.1 mM D-glucose by short-circuited proximal and distal intestinal BBMV (Fig. 4). No relevant differences were found in the pattern of inhibition between both intestinal segments. These data, together with the absence of significant differences between the K_ts of proximal and distal D-glucose transporters described above, suggest that the same type of carrier is present in the two segments of intestine. Figure 4 also shows that D-glucose influx was significantly reduced by phlorizin, D-glucose, D-galactose, α-methyl-D-glucose, 3-O-methyl-D-glucose and 2-deoxy-D-glucose. Transport was not inhibited by D-fructose. Furthermore, the negligible mediated uptake of 0.1 mM L-glucose by either proximal or distal intestinal BBMV (corresponding to 1.92 ± 0.02 % and 1.60 ± 0.25% of D-glucose uptake, respectively), together with a lack of inhibition of D-glucose uptake by L-glucose in both segments, proved the stereospecificity of this transporter. Figure 4 also confirms the need of sodium to carry the D-glucose to the inside of the vesicle.

The number of D-glucose carriers was quantified by means of labelled phlorizin binding (Ferraris & Diamond, 1986). Figure 5 shows that specific phlorizin binding was significantly ($P < 0.05$) higher (26.8 ± 4.1%) in proximal intestinal BBMV than in distal vesicles.

Figure 6*A* shows a representative immunoblot experiment carried out using a polyclonal antibody raised against rabbit intestinal SGLT1. In both proximal and distal intestine, the anti-SGLT recognized a single band of 75 kDa, which was blocked by preadsorbing the antibody in the presence of the antigenic peptide. The relative abundance of this anti-SGLT reactive protein was significantly higher in proximal than in distal BBMV (Fig. 6*B*), in accordance with the observed J_{max} and specific phlorizin binding.

Cytochalasin B is a specific inhibitor of GLUT-type D-glucose transporters (Garriga et al., 1999). Total and non-specific cytochalasin B binding were measured in proximal and distal BBMV. Both types of vesicles showed negligible specific cytochalasin B binding, suggesting that no GLUT2 transporters were present in brush border membranes of the intestine of the gilthead sea bream.

Immunohistochemical Localization of SGLT1

Immunohistochemichal localization of SGLT-type protein in cross sections of both intestinal regions of

Table 2. Vesicular volume, initial rate and accumulation ratio of D-glucose influx by proximal and distal intestinal BBMV of gilthead sea bream

	Vesicular volume μL. mg^{-1} prot	Initial rate, pmols. mg^{-1}prot. s^{-1}	Accumulation ratio
Just after isolation:			
Proximal intestine	0.40 ± 0.02^{a}	31.8 ± 1.3^{a}	4.0 ± 0.3^{a}
Distal intestine	0.77 ± 0.04^{b}	37.1 ± 1.8^{a}	3.2 ± 0.2^{a}
After vesicle freezing:			
Proximal intestine	0.29 ± 0.02^{c}	27.8 ± 1.8^{ab}	3.6 ± 0.3^{a}
Distal intestine	0.49 ± 0.11^{a}	19.4 ± 2.6^{b}	1.3 ± 0.2^{b}

The coefficients were measured for both proximal and distal intestinal BBMV just after the isolation of vesicles ($n = 5$) and after freezing in liquid nitrogen ($n = 3$). Freezing and thawing were performed as indicated in Materials and Methods. Vesicles were incubated for different times in the presence of buffers containing either sodium or potassium. After addition of vesicles, final incubation mixture composition was (in mM) 100 mannitol, 0.1 D-glucose (0.01 D-[^{14}C]glucose), 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3, plus 100 mM NaSCN or KSCN. The composition of stop solution was (in mM) 300 mannitol, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. Accumulation ratio is the relationship between maximal uptake and uptake at equilibrium. Data are expressed as means ± SE. Different letters indicate significant differences ($P < 0.05$) for each parameter among the four experimental conditions.

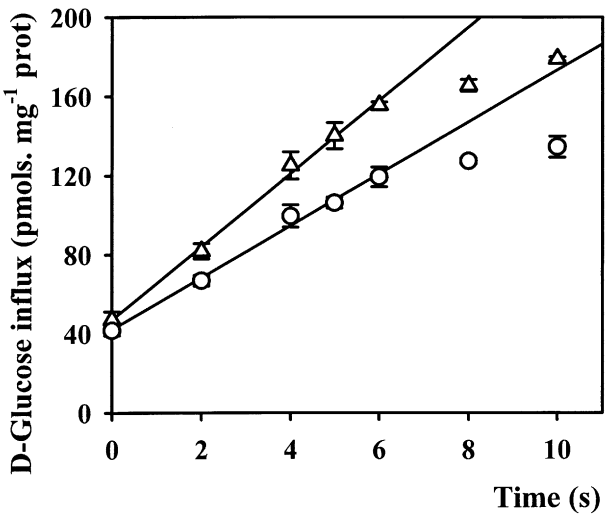

Fig. 2. Initial rate of Na-dependent 0.1 mM D-glucose uptake by proximal (*circles*) and distal (*triangles*) intestinal BBMV measured just after isolation. Vesicles were incubated for 0, 2, 4, 5, 6, 8 and 10 seconds in a buffer containing 100 mM of sodium. Final incubation mixture composition and composition of stop solution were the same as in Fig. 1. Uptake was found to be linear for the first 6 seconds in both proximal and distal BBMV, being the correlation coefficient 0.992 and 0.996, respectively. Lines represent the linear regressions. Data are expressed as means ± SE of at least 3 separate experiments.

gilthead sea bream was performed with the same rabbit polyclonal anti-SGLT1 antibody used for the Western blot analysis. A representative experiment is displayed in Fig. 7. Positive immunostaining (red fluorescence) was uniformly localized along the apical end of the entire brush border of proximal (Fig. 7*A*) and distal (Fig. 7*B*) intestine. As also shown, less intense immunoreaction was observed below the nuclei (blue fluorescence), as well as in other portions of the intestine, such as the submucosa. No specific immunofluorescence was detected in primary antibody omission controls (*data not shown*).

Effect on D-Glucose Transport Capacity of BBMV after Storage in Liquid Nitrogen

Freezing modified the profile of time courses of D-glucose uptake in proximal (Fig. 8*A*) and distal (Fig. 8*B*) intestinal BBMV. In the proximal segment, overshoot was reached after only 20 seconds in previously frozen BBMV, versus the 60 seconds needed in freshly isolated BBMV. In spite of a drop in D-glucose influx (Fig. 8*A*), no significant changes were found between frozen and freshly isolated proximal BBMV in initial rate and accumulation ratio, possibly due to the fall in vesicular volume that also occurred after freezing (Table 2). On the other hand, previously frozen BBMV from the distal segment showed lower uptake than those from the proximal intestine, without apparent overshoot (Fig. 8*B*). Initial rate, accumulation ratio and vesicular volume also decreased significantly in distal BBMV after freezing (Table 2).

Finally, the effect of D-glucose concentration on initial rate was measured in either proximal or distal intestinal BBMV after freezing in liquid nitrogen. In both cases, the Eadie-Hofstee plot of D-glucose uptakes (*not shown*) suggested that a single transporter was involved. Kinetic constants are shown in Table 3. In proximal intestine, J_{max}, K_t and K_d remained unchanged in frozen BBMV. Instead, a significant fall of J_{max} (of 55%) and a significant rise of K_t (of 200%) were found in distal BBMV after freezing. These data suggest a differential effect of liquid nitrogen in the preservation of D-glucose influx in proximal and distal intestinal BBMV, thus being effective as a

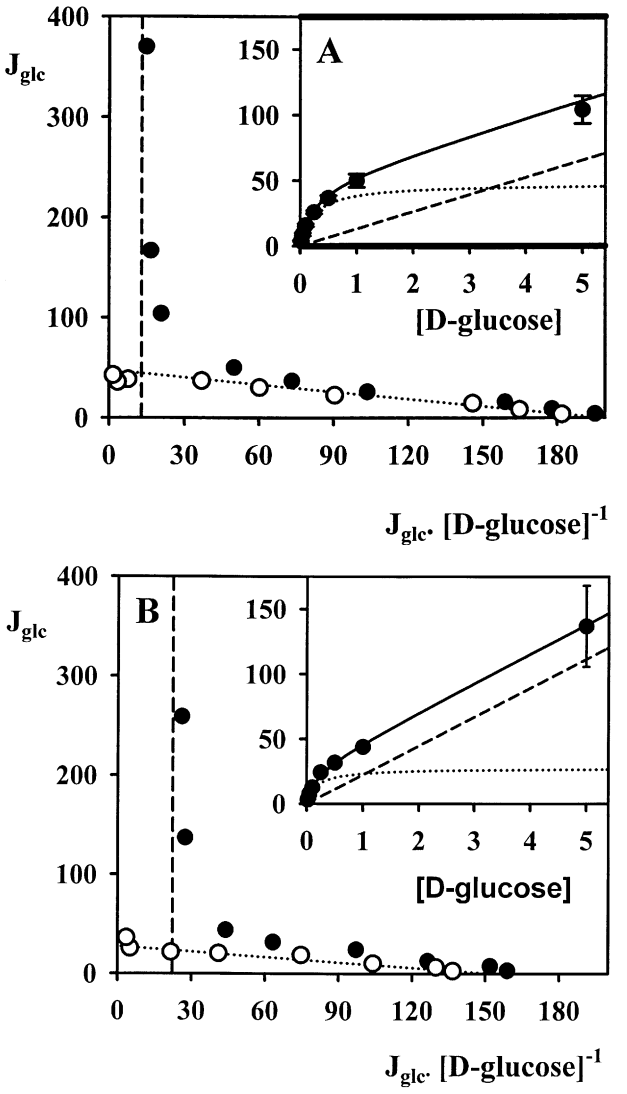

Fig. 3. Concentration dependence and Eadie-Hofstee plot of D-glucose by short-circuited proximal (*A*) and distal (*B*) intestinal BBMV measured just after isolation. Initial uptake rates were measured for 5 s in the presence of increasing concentrations of extravesicular D-glucose, from 0.02 to 25 mM. BBMV were preloaded to a final intra- and extravesicular composition of (in mM) 300 mannitol, 50 KCl, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. For D-glucose concentrations of 0.02, 0.05, 0.1, 0.25, 0.5, 1 and 5 mM, final incubation mixture composition was (in mM) 100 mannitol, 100 NaCl, 50 KCl, 0.02 D-[^{14}C]glucose, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. In incubation media for D-glucose concentrations of 10 and 25 mM, mannitol was reduced in order to keep osmolality of intra- and extravesicular media constant. Composition of stop solution was (in mM) 400 mannitol, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. *Inset* shows data plotted according to the Michaelis-Menten equation plus a non-mediated process. Data reported in the figure were plotted using an Eadie-Hofstee plot before (*filled circles*) and after (*empty circles*) subtracting estimated diffusional component. Total uptake (*continuous line*), mediated uptake (*dotted line*) and diffusional component (*dashed line*). Maximal D-glucose influx (J_{max}) is referred to vesicle protein (pmols D-glucose.mg^{-1} protein), and D-glucose concentration is given in mM. Data are expressed as means ± SE of at least 4 separate experiments.

Table 3. Kinetic constants of D-glucose uptake by short-circuited BBMV from proximal and distal intestine of gilthead sea bream.

	*J*max, pmols. mg^{-1} prot. s^{-1}	K_t, mM	K_d, nL. mg^{-1} prot. s^{-1}
Just after isolation:			
Proximal intestine	47.3 ± 2.2^a	0.24 ± 0.03^a	13.1 ± 2.4^a
Distal intestine	27.3 ± 3.6^b	0.18 ± 0.03^a	22.3 ± 2.3^{ab}
After vesicle freezing:			
Proximal intestine	45.1 ± 1.3^a	0.23 ± 0.02^a	12.3 ± 0.2^a
Distal intestine	12.3 ± 1.3^c	0.53 ± 0.12^b	30.2 ± 3.0^b

The coefficients were measured for both proximal and distal intestinal BBMV after the isolation of vesicles and after freezing in liquid nitrogen. Freezing and thawing were carried out as indicated in Materials and Methods. J_{max}, maximal rate of influx; K_t, apparent affinity; K_d, non-mediated uptake. Data are expressed as means ± SE of at least 4 separate determinations. Different letters indicate significant differences ($P < 0.05$) for each parameter among the four experimental conditions.

preservative method for proximal but not for distal vesicles.

Discussion

The present study proves that the magnesium precipitation technique, widely used in the purification of vertebrate brush border membranes (Storelli et al., 1986; Maffia et al., 1996; Vázquez et al., 1997), is adequate for the preparation of intestinal BBMV from the gilthead sea bream. Thus, the final fraction obtained from either proximal or distal intestine was enriched in brush border marker enzymes and impoverished in Na^+-K^+-ATPase, and was suitable for the performance of D-glucose transport studies.

Although the majority of authors report the use of two magnesium precipitation steps (Storelli et al, 1986; Maffia et al., 1996), only one was carried out to obtain intestinal BBMV from the gilthead sea bream, as after a second step the D-glucose influx was drastically reduced (*data not shown*). The performance of a single precipitation step led to a lower enrichment in specific brush border marker enzymes than those obtained in other fish with the double precipitation method (Storelli et al, 1986; Maffia et al., 1996). However, BBMV were not significantly contaminated with basolateral membrane, in view of negligible specific cytochalasin B binding, and allowed the measurement of D-glucose uptake.

Freezing in liquid nitrogen affected D-glucose transport in proximal and distal BBMV in a different manner. Uptake was well preserved in proximal BBMV, as reported for intestinal brush border and basolateral membrane vesicles from *tilapia* (Reshkin et al., 1988), but was not maintained in distal BBMV. Differences in vesicular volume between proximal

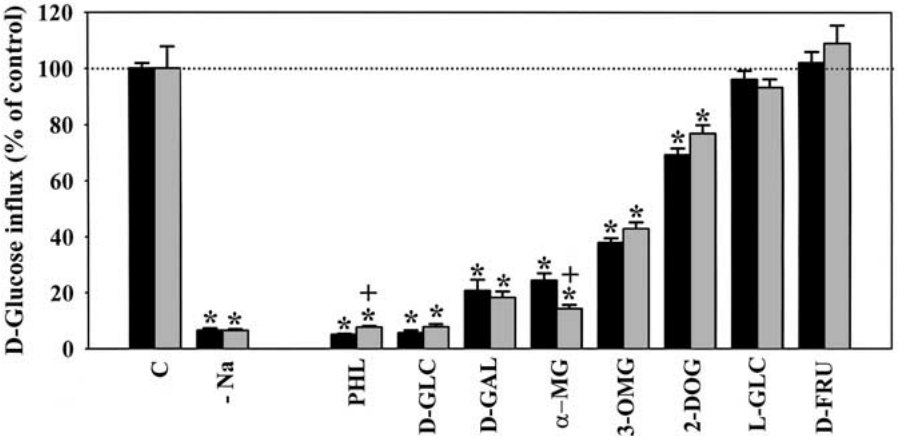

Fig. 4. Effects of external inhibitors on 0.1 mM D-glucose influx by short-circuited proximal (*black bars*) and distal (*gray bars*) intestinal BBMV measured just after isolation. Vesicles were preloaded to a final intra- and extravesicular composition of (in mM) 300 mannitol, 50 KCl, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. BBMV were incubated for 5 s in a medium containing (in mM) 50 mannitol, 100 NaCl, 50 KCl, 0.1 D-glucose (0.02 D-[^{14}C]glucose), 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3, plus 50 mM mannitol or 50 mM mannitol and 200 μM phlorizin or 50 mM inhibitory sugar. A negative control was made by incubating the vesicles in a medium containing (in mM) 300 mannitol, 50 KCl, 0.1 D-glucose (0.02 D-[^{14}C]glucose), 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. Composition of stop solution was the same as in Fig. 3. Bars represent percentage of control D-glucose influx. PHL, phlorizin; D-GLC, D-glucose; D-GAL, D-galactose; α-MG, α-methyl-D-glucose; 3-OMG, 3-O-methyl-D-glucose; 2-DOG, 2-deoxy-D-glucose; L-GLC, L-glucose; D-FRU, D-fructose. Asterisks(*) denote significant differences from control ($P < 0.001$). Crosses denote significant differences between proximal and distal intestinal BBMV ($P < 0.05$). Data are expressed as means ± SE of at least 3 separate experiments.

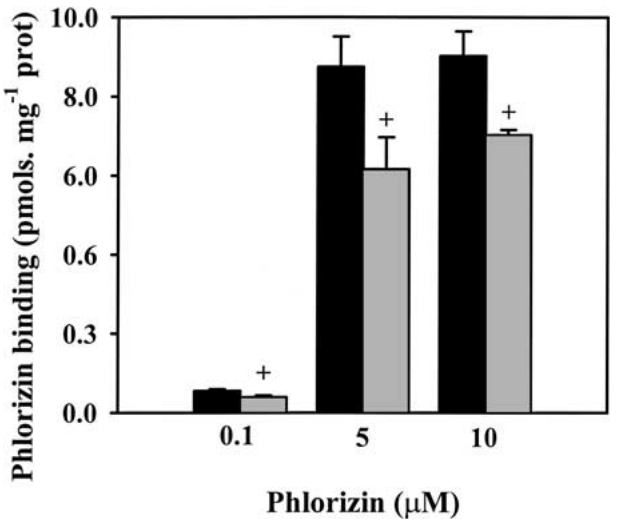

Fig. 5. Specific phlorizin binding by proximal (*black bars*) and distal (*gray bars*) intestinal BBMV measured just after isolation. BBMV were incubated for 5 s in a medium containing (in mM) 100 mannitol, 100 NaCl or KCl, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3, plus 0.1, 5 or 10 μM phlorizin (0.1 μM [^{3}H]phlorizin). Composition of stop solution was the same as in Fig. 1. Crosses denote significant differences between proximal and distal intestinal BBMV ($P < 0.05$). Data are expressed as means ± SE of at least 5 separate experiments.

and distal vesicles, suggesting variations in the lipid composition of brush border membrane of both intestinal segments, could be the cause of dissimilarities in preservation of D-glucose transport capacity after freezing. Therefore, to allow comparisons between proximal and distal intestine, D-glucose transport was further characterized in freshly isolated vesicles.

The mechanisms of solute transport by fish intestines have been reviewed (Smith, 1983; Collie & Ferraris, 1995) and are similar to those of mammals, though rates are lower (Reshkin & Ahearn, 1987a,b). The transfer of D-glucose from the lumen of the intestine to the blood of fish requires two types of carriers, one Na^+-dependent (in the apical membrane) and another Na^+-independent (in the basolateral membrane) (Casirola, Vinnakota & Ferraris, 1995). These allow the enterocytes to accumulate D-glucose, which can then enter the systemic circulation.

D-Glucose is rapidly taken up by proximal and distal intestinal BBMV from gilthead sea bream, reaching overshoot after a short time (60 and 30 seconds, respectively), as it has been described for other fish (Storelli et al, 1986; Ahearn et al., 1992; Maffia et al., 1996). The period of linearity found for D-glucose influx in intestinal BBMV from sea bream is similar to that found in intestinal vesicles of other fish species (Storelli et al, 1986; Ahearn et al., 1992; Maffia et al., 1996).

Affinity constant and maximum rate of D-glucose transport in freshly prepared BBMV from gilthead sea bream were in the same range as those measured in other fish (Ahearn & Storelli, 1994), and significantly lower than those measured in fish gut using

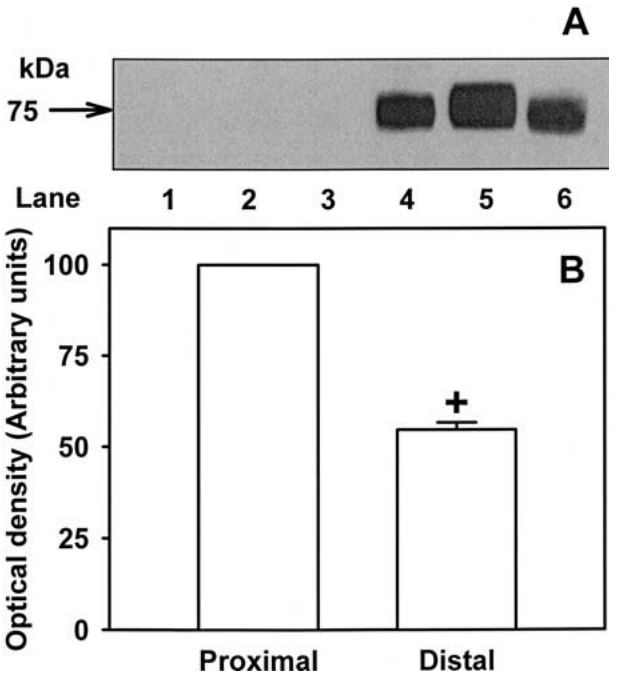

Fig. 6. (*A*) Western blot analysis of SGLT1 in BBMV from rabbit jejunum (lanes *1* and *4*) and in BBMV from proximal (lanes *2* and *5*) and distal (lanes *3* and *6*) intestine of the gilthead sea bream. Samples (50 μg of protein per lane) were blotted using a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 564 to 575 of the deduced sequence of rabbit intestinal SGLT1, in the presence (lanes 1 to 3) or in the absence (lanes *4* to *6*) of the antigenic peptide. Molecular weight standard is shown on left. (*B*) Relative abundance measured by optical densitometry. Data are expressed as means ± SE of 3 separate experiments Crosses denote significant differences between proximal and distal intestinal BBMV ($P < 0.05$).

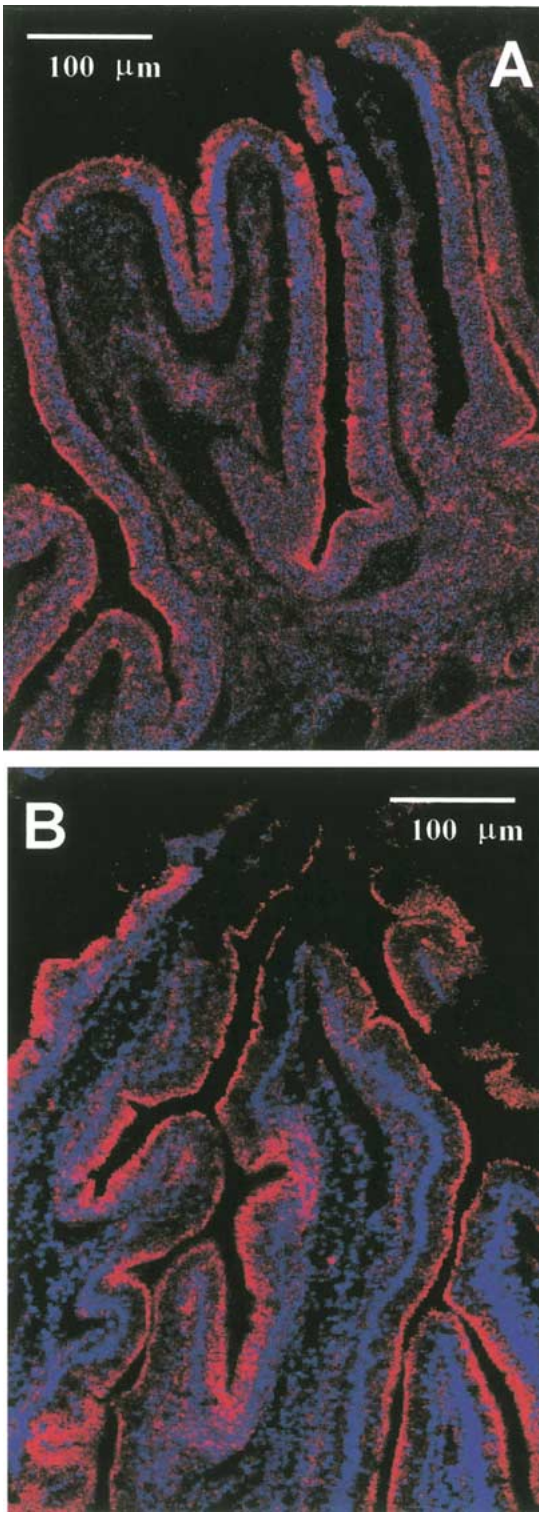

Fig. 7. Immunolocalization of SGLT1 protein in proximal (*A*) and distal (*B*) intestine of gilthead sea bream. Sections (10 μm) were incubated in the presence of the same rabbit anti-SGLT1 antibody as in Fig. 7 (primary antibody) and of goat anti-rabbit Alexa Fluor 568 (secondary antibody), and stained with Hoechst-33258 to reveal nuclei (shown in blue). SGLT-type protein is uniformly localized along the apical end of the brush border of both intestinal segments.

other experimental techniques such as intestinal sacs or sheets mounted in flux chambers (Ferraris & Ahearn, 1984). The use of membrane vesicles allows to eliminate the unstirred layer next to the transporting membrane present in intestinal sacs or sheet preparations (Dietschy, Sallee & Wilson, 1971) and therefore provides a more accurate assessment of the true kinetic constant values of transport.

Natural history and diet of the different fish species could influence transport along the intestine. In herbivorous fish, the proximal intestine has been proven to exhibit a significantly lower affinity for D-glucose transport than the distal segment, either in BBMV (Reshkin & Ahearn, 1987a) or in isolated intestine (Ferraris & Ahearn, 1984) preparations. Regarding carnivorous fish, the present is the first study to compare the D-glucose transport characteristics of BBMV isolated from proximal and distal intestine. Thus, in gilthead sea bream, no significant differences in K_ts have been found for the D-glucose transporter of both regions studied. These results are in agreement with those found in eel using isolated

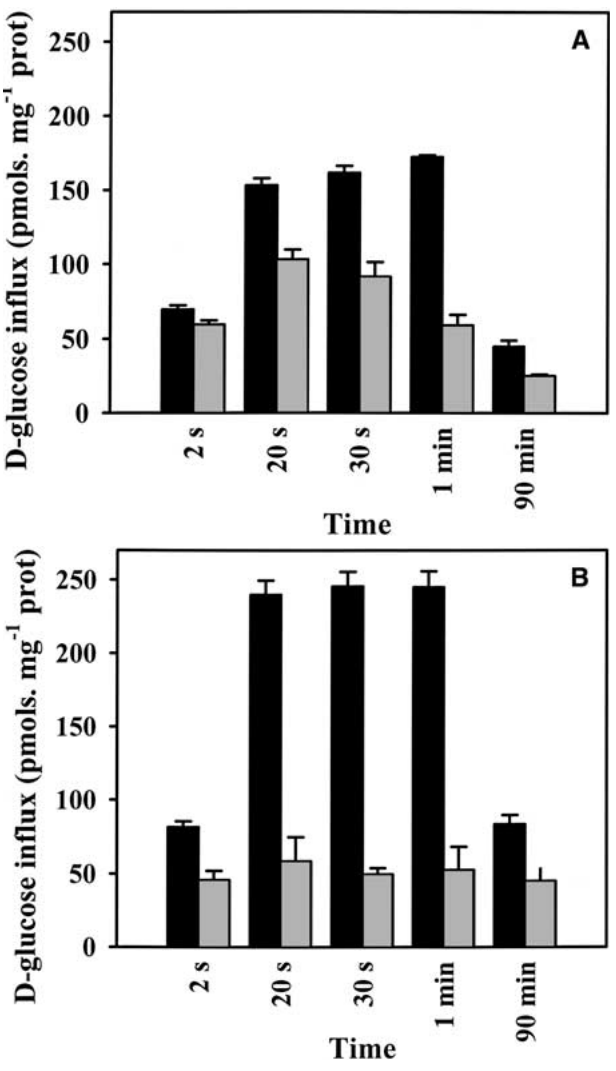

Fig. 8. Time course of 0.1 mM D-glucose uptake by proximal (*A*) and distal (*B*) intestinal vesicles measured just after isolation (*black bars*) or after freezing in liquid nitrogen (*gray bars*). Freezing and thawing were performed as indicated in Materials and Methods. Vesicles were incubated for different times in a buffer containing 100 mM of sodium. After addition of vesicles, final incubation mixture composition was (in mM) 100 NaSCN, 100 mannitol, 0.1 D-glucose (0.01 D-[^{14}C]glucose), 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. The composition of the stop solution was (in mM) 300 mannitol, 0.1 $MgSO_4$, 20 HEPES/TRIS (pH 7.4) and 0.41 μM LiN_3. Uptake data are expressed as means ± SE of at least 3 separate experiments.

intestine preparations (Ferraris & Ahearn, 1984). On the other hand, BBMV isolated from the pyloric ceca of copper rockfish exhibited a four-fold lower apparent affinity for the sugar than those isolated from proximal intestine (Ahearn et al., 1992).

The maximal capacity of D-glucose uptake was higher in the proximal than in the distal intestine, as assessed from J_{max}, specific phorizin binding data and Western blot analysis. A fall in D-glucose uptake from proximal to distal intestine has been previously described in other fish (Buddington, Chen & Diamond, 1987). These data suggest that the proximal segment of the gastrointestinal tract of fish, which receives the highest luminal load of nutrients, presents the highest capacity for D-glucose uptake. On the other hand, Lorenzo et al. (1987) measured net transepithelial flux of D-glucose in the intestine of the gilthead sea bream, reporting an active absorption in the posterior intestine that was not observed in the anterior. These results are in disagreement with those shown in the present work, but one should note that those authors used only the first 2 cm of the proximal intestine. It is possible that, at this first portion of proximal intestine, digestion of carbohydrates is not yet complete and thus availability of glucose is still low, which would require little or no active sugar uptake.

The most potent inhibitors of D-glucose influx in gilthead sea bream were phlorizin, D-glucose, D-galactose and α-methyl-D-glucose. In contrast, L-glucose and D-fructose did not modify D-glucose transport. Additionally, specific cytochalasin B binding was negligible. Equivalent inhibition profiles have been reported for other D-glucose carriers of fish intestinal brush border membrane (Storelli et al, 1986; Ahearn et al., 1992; Maffia et al., 1996) and SGLT1 carrier of mammal intestine (Brot-Laroche & Alvarado, 1983), suggesting a highly conserved steric requirement by the glucose carrier throughout the vertebrates. In this sense, a polyclonal antibody raised against amino acids 564 to 575 of the deduced sequence of rabbit intestinal SGLT1 (Hwang et al., 1991) recognized, in BBMV from both proximal and distal intestine of the gilthead sea bream, the same band of 75 kDa than in BBMV from rabbit jejunum. Furthermore, the antibody was successfully used to reveal the presence of an immunoreactive protein along the brush border of proximal and distal intestine of this fish, by means of immunolocalization. To our knowledge, the present work is the first in which an anti-rabbit SLGT1 antibody has been proven to recognize a peptide in fish intestine, as shown by Western blot and immunohistochemichal analyses.

Functional modifications in response to environmental dissimilarities of life style have been described among carnivorous, herbivorous, and omnivorous fish (Ahearn & Storelli, 1994). Thus, rates of sugar transport by fish intestine could be programmed to match the composition of the natural diet (Ferraris & Ahearn, 1984; Buddington et al., 1987). Specially, sugar uptake by carnivores is lower compared to herbivorous and omnivorous fish (Buddington et al., 1987). On the other hand, rates of sugar transport by the intestines of carnivorous fish like gilthead sea bream are exceedingly low, even compared to carnivorous mammals (Buddington, Chen & Diamond, 1991). The lower site densities of transporters and/or absorptive surface area relative

to mammals could be the cause (Collie & Ferraris, 1995). This coincides with the difficulties that carnivorous fish have in processing even moderate dietary loads of carbohydrate (Buddington et al., 1997).

In summary, a Na^+-dependent D-glucose transport system was characterized in brush-border membrane vesicles of proximal and distal intestine from gilthead sea bream. Vesicles from both parts of the intestine showed each one has only one type of D-glucose carrier, with kinetic characteristics and steric requirements compatible with those of the SGLT1 of higher vertebrates. The density of transporters was higher in the proximal segment, as indicated by specific phlorizin binding and Western blot analysis, performed using a polyclonal antibody against rabbit intestinal SGLT1. A peptide recognized by the same anti-rabbit SGLT1 antibody was localized along the brush border of proximal and distal intestine of this fish, by means of immunohistochemistry.

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