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High-affinity phlorizin binding in *Mytilus* gill

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The gill of the marine mussel, *Mytilus*, contains a high affinity, Na-dependent D-glucose transporter capable of accumulating glucose directly from sea water. We examined the ability of the β-glucoside, phlorizin, to act as a high-affinity ligand of this process in intact gills and isolated brush border membrane vesicles (BBMV). The time course of association of nanomolar [^{3}H]phlorizin to gills and BBMV was slow, with t_{50} values between 10 and 30 min, and a half-time for dissociation of approx. 30 min. 1 mM D-glucose reduced equilibrium binding of 1 nM phlorizin by 90–95%, indicating that there was little non-specific binding of this ligand to the gill. In addition, there was little, if any, hydrolysis by the gill of phlorizin to its constituents, glucose and phloretin. Phlorizin binding to gills and BBMV was significantly inhibited by the addition of 50 μM concentrations of D-glucose and α-methyl-D-glucose, and unaffected by the addition of L-glucose and fructose. Binding to gills and BBMV was reduced by > 90% when Na^+ was replaced by K^+. Replacement of Na^+ by Li^+ effectively blocked binding to the intact gill, although Li^+ did support a limited amount of glucose-specific phlorizin binding in BBMV. The K_d values for glucose-specific phlorizin binding in intact gills and BBMV were 0.5 nM and 6 nM, respectively. We conclude that phlorizin binds with extremely high affinity to the Na-dependent glucose transporter of *Mytilus* gill, which may be useful in future efforts to isolate and purify the protein(s) involved in integumental glucose transport.

Introduction

The apical, brush border membrane of the gill of the common mussel, *Mytilus*, contains a Na-cotransporter for D-glucose capable of a net accumulation of this substrate directly from sea water [1]. This process, in conjunction with Na-dependent transporters for amino acid, has been suggested to play a role in the nutrition of bivalves and other soft-bodied marine invertebrates (e.g., Refs. 2–4). The characteristics of glucose uptake in the gill are qualitatively similar to those reported for Na-glucose cotransport in mammalian intestine (e.g., Ref. 5) and renal outer medulla [6]. In particular, the following are comparable for all these systems: the cation dependence; the pattern of inhibition of glucose transport by other sugars; and Hill coefficients which suggest a cotransport of two Na^+ ions with each glucose molecule.

Despite these qualitative similarities, a quantitative characteristic sets glucose transport in the gill apart from transport in mammalian epithelia. The Michaelis constant (K_t) for glucose uptake by the 'high-affinity' Na-glucose cotransporter of renal outer medulla and small intestine is on the order of 0.05 to 0.5 mM [7]. In contrast, the K_t for integumental glucose transport in the gill is 5 to 10 μM [1]. This extremely high affinity for substrate is, presumably, a reflection of the very low concentration of free sugars in the near shore sea water habitat of *Mytilus* (i.e., 20–50 nM; Manahan, D.T., personal communication). The high affinity of the gill glucose transporter is matched by similarly high affinities for substrate that are characteristic of the several amino acid transporters of the gill (i.e., K_t values of 1 to 10 μM; [4]).

The β-glucoside, phlorizin, is a potent nontransported inhibitor of Na-dependent D-glucose transport in mammalian kidney and intestine [7], with K_d values on the order of 0.07 to 7 μM (e.g., Refs. 8–10). Considerable evidence supports the contention that phlorizin binds to the Na-dependent glucose transporter (e.g., Ref. 11), and the high affinity of the transporter for this ligand has made it a useful tool for identifying sites of sugar uptake [12] and quantifying numbers of transporters [13] in epithelial tissues. Phlo-

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rizin binding has also proven to be a valuable assay in efforts to isolate and purify the glucose transporter in renal and intestinal tissues (e.g. Refs. 11,14–16). Recognizing the potential value of isolating and purifying the novel high-affinity glucose transporter of *Mytilus* gill, we undertook a study of the characteristics of glucose-specific phlorizin binding in intact gill tissue and in isolated brush border membrane vesicles (BBMV) from the gill.

Materials and Methods

Animals

Two species of the mussel, *Mytilus*, were used in these studies. Studies with intact gills used tissue from the California coastal mussel, *Mytilus californianus* (Bodega Marine Biological Laboratory, Bodega Bay, CA, USA), because the structural characteristics of the gill facilitate its *in vitro* manipulation. Studies with membrane vesicles isolated from gills used tissue from the blue mussel, *Mytilus edulis* (Bodega Marine Biological Laboratory; Sea Life Supply, Sand City, CA, USA), because the gill of this species has substantially less connective tissue than that of *M. californianus*. The characteristics of transport in isolated membranes and intact gills of *M. edulis* are very similar [17], as are the characteristics of transport in intact gills of these two species (e.g., Ref. 18), justifying the cross-species comparisons made in the current study. Animals were kept in refrigerated aquaria (8–13°C) containing circulating, filtered, aerated artificial sea water (Tropic Marin). Animals were not fed and were typically used within six weeks of collection.

Measurement of phlorizin binding in intact gills

Binding of phlorizin to gills of *M. californianus* used a procedure modified from that used to measure uptake of organic nutrients [19,20]. Briefly, pieces of tissue (10–20 mg) were cut from isolated gills and then held in artificial sea water (ASW; [21]) at room temperature (20–24°C) for 30 min prior to use. In a typical experiment, tissue was placed in 200 ml of ASW containing phlorizin, trace-labeled with [^{3}H]phlorizin. This ratio of tissue mass to medium volume was used because it assured that, following equilibrium binding of phlorizin, depletion of [^{3}H]phlorizin from the medium was less than 5%. Experimental solutions also contained 10 μM 5-hydroxytryptamine (5-HT) to activate lateral cilia and thereby reestablish perfusion of gill surfaces [22]. At different time intervals, tissue was removed from the experimental solution and then rinsed for 30 s in an ice-cold ASW bath. Tissue pieces were then blotted and weighed to the nearest 0.1 mg. Accumulated radioactivity was extracted in 80% ethanol (EtOH) for at least 2 h prior to adding 6 ml of scintillation cocktail (Betaphase) and measurement of radioactivity in a liquid scintillation counter (Beckman 3801). All samples were corrected for variable quench using an external standard. Uptakes were expressed in moles per unit wet tissue mass.

Chromatography of phlorizin

Tissue pieces (approx. 165 mg) were incubated for 30 min in ASW containing 1 nM phlorizin and 1 μCi of [^{3}H]phlorizin. Following a 30 s rinse in ice-cold ASW, accumulated radioactivity was extracted in 2 ml of 80% EtOH. 25 μl of the extracts were spotted onto silica gel thin-layer chromatography (TLC) plates and run in one-dimension in a chloroform/methanol/H_2O (32:12:2, v/v) solvent system. Migration of each sample was compared to the migration of stock [^{3}H]phlorizin and to the migration of unlabeled phlorizin and phloretin as visualized by exposure to iodine vapor. Radioactivity of each lane was assessed by scraping 5-mm sections into vials and counting in a liquid scintillation counter.

Isolation of brush border membrane vesicles (BBMV) from gills

As described previously [17], BBMV were prepared from gills of the mussel, *M. edulis*, using a combination of differential and sucrose density gradient centrifugation. Gill tissue isolated from approx. 50 mussels was preincubated for several minutes in a high K^+ ASW (115 mM, as an equimolar replacement for Na^+) containing 1 mM dithiothreitol to release and disperse mucus. The gills were then homogenized in a sorbitol buffer (500 mM sorbitol, 5 mM EGTA, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (Hepes) buffered to pH 7.6 with tris(hydroxymethyl)aminomethane (Tris). A crude plasma membrane fraction was obtained after several differential centrifugation steps. This fraction was further purified using a linear 30% to 50% sucrose density gradient. Membranes from separate fractions of this gradient were collected by differential centrifugation and resuspended in a buffer containing 600 mM mannitol, 10 mM Hepes-Tris (pH 7.6). The final BBMV fraction (fractions 8–11) was typically enriched approx. 18-fold with respect to the apical membrane marker, γ-glutamyltranspeptidase, and less than 2-fold with respect to the basolateral marker, K-dependent *p*-nitrophenylphosphatase.

Measurement of phlorizin binding in BBMV

The binding of phlorizin to gill BBMV was measured using a variation of the rapid filtration method used to measure solute transport in membrane vesicles [23]. 90 μl of 'binding buffer' containing 500 mM NaCl, 10 mM Hepes-Tris (pH 7.6) and [^{3}H]phlorizin (described in greater detail in the figure legends) were mixed with 10 μl of the BBMV suspension. Binding was allowed to proceed for between 1 min and 3 h.

Incubations were terminated by the addition of 1 ml of an ice-cold stop solution (500 mM NaCl, 10 mM Hepes-Tris (pH 7.6)). The vesicles and their accumulated radioactivity were collected by filtering through a 0.45 μm filter (Millipore, HAWP) and then rinsed with 4 ml of ice-cold stop solution. Radioactivity retained on the filter was determined using a liquid scintillation counter.

Data analysis

The kinetic constants of binding were calculated using microcomputer-based non-linear regression algorithms for two or three parameter kinetic equations (Enzfitter, BioSoft). For the purpose of gauging the statistical significance of observed differences in transport or binding, data were analyzed using ANOVA with comparisons made using Scheffe's F-test (Statview II by Abacus).

Chemicals

[^{3}H]Phlorizin (55 Ci/mmol) and D-[^{3}H]glucose (83 Ci/mmol) were purchased from New England Nuclear. All other chemicals were purchased from standard sources and were the highest quality available.

Results and Discussion

Time course of phlorizin binding in intact gills

Gills from *M. californianus* bound 1 nM phlorizin in a first-order fashion with a half-time (t_{50}) of between 10 and 20 min (Fig. 1), corresponding to an association constant (k_{+1}) of $6.9 \cdot 10^7$ min^{-1} M^{-1} [24]. Addition of

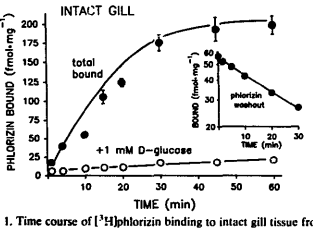

Fig. 1. Time course of [^{3}H]phlorizin binding to intact gill tissue from *Mytilus californianus*. Discs of gill tissue (≈ 10 mg each) were incubated for the time periods indicated in 200 ml of ASW containing 10 μM 5-HT (to activate lateral cilia), 0.25 μCi of [^{3}H]phlorizin and sufficient unlabeled phlorizin to produce a total phlorizin concentration of 1 nM. In one series (open circles), 1 mM D-glucose was added. Each point is the mean accumulation (±S.E.) in three discs of tissue. The inset shows the washout (dissociation) of [^{3}H]phlorizin from gill tissue into (substrate-free) ASW following a 10 min period incubation in 1 nM [^{3}H]phlorizin. Data shown are from single, representative experiments.

1 mM D-glucose reduced binding to the gill by 89% at 60 min (Fig. 1), suggesting that phlorizin binding in the gill is predominantly limited to a site(s) sensitive to glucose. For our study of the kinetics of phlorizin binding in the gill we used 60 min incubations (i.e., 3–6-times the t_{50}) to estimate equilibrium binding. Shorter incubations were used in some experiments examining the effect of different conditions on phlorizin binding.

Washout of phlorizin from binding sites on the gill was also examined. Following a 10 min incubation in a test solution containing 1 nM [^{3}H]phlorizin, a gill was placed into 1 liter of ASW at room temp (20–24°C) containing 10 μM 5-HT. A piece of tissue (20–40 mg) was immediately cut from the gill and placed for 30 s in ice-cold ASW, blotted, weighed and assayed for radioactivity; this was considered the time-zero washout point. At increasing time intervals, successive pieces of gill tissue were cut from the gill, rinsed in ice-cold ASW and assayed for radioactivity. Washout of [^{3}H]phlorizin from the gill under these conditions was adequately described by a single exponential (inset of Fig. 1) and, in two experiments, had a half-time of approx. 30 min (i.e., dissociation constant, k_{-1}, of $1.1 \cdot 10^{-2}$ min^{-1}). Consequently, there should have been little loss of bound phlorizin from the gills during the 30-s ice-cold rinse used to stop our binding assays. It is worth noting that, following the accumulation of radiolabeled amino acids into the gill, there is an insignificant washout of radioactivity over 30 min ([19]; unpublished observations). Therefore, the present results suggest that [^{3}H]phlorizin was bound to glucose-specific binding sites on the surface of gill cells and there was little, if any, internalization of radioactive label during the course of these experiments.

The time course of association and dissociation of phlorizin to intact mammalian epithelia is very different from that noted here. For example, Ferraris and Diamond [13] reported a half-time for association of 15 nM phlorizin to mouse jejunum of approx. 10 s, and a half-time for dissociation (at 0°C) that ranged from 1 to 10 min, depending on the bath conditions. The difference in the time course of phlorizin accumulation noted in gill integument compared to mammalian intestine probably stems from the difference in the apparent affinities for phlorizin of the glucose transporters of these two different tissues, which is discussed below.

Metabolism of phlorizin by intact gills

Phlorizin can undergo hydrolysis to form free glucose and the aglycon, phloretin [25] and this has been a significant problem in studies of phlorizin binding, particularly in intestinal tissues [13]. The apical surface of cells in *Mytilus* gill has been shown to contain hydrolytic enzyme activity [26]. It was, therefore, neces-

sary to establish whether metabolism of this compound occurred during the prolonged incubations required to achieve equilibrium binding of phlorizin to gill tissue. The tritium label on the phlorizin used in this study was in the phloretin, instead of the glucose, moiety. In our solvent system, phloretin migrated with an R_f of 0.77, whereas intact phlorizin migrated with an R_f of 0.56. Radioactivity extracted from gills exposed to [^{3}H]phlorizin for 30 min migrated in a single peak, representing 84% of the total activity applied to the TLC plate, with an R_f of 0.56; there was no peak corresponding to an R_f of 0.77. We concluded that negligible hydrolysis of phlorizin occurred during the time course of a typical experiment.

Phlorizin binding to brush border membrane vesicles isolated from Mytilus gill

We have previously shown [17] that there is a differential distribution through a sucrose density gradient of brush border and basolateral membranes from gill tissue, with distinct peaks of marker enzyme and transport activity being distributed at the bottom vs. top of the gradient, respectively. As shown in Fig. 2, glucose-specific phlorizin binding was clearly limited to single peak in fractions 8–11 of the sucrose density gradient, which corresponds to the distribution of Na-dependent D-glucose transport in these same membranes [1]. Thus, phlorizin binding and glucose transport appear to be limited to the brush border membrane of the gill.

Time course of phlorizin binding in BBMV

As with the intact gill, binding of phlorizin to BBMV occurred in a first-order fashion, approaching equilibrium after approx. 30 min (Fig. 3). Addition of 1 mM D-glucose reduced total binding by > 95%, indicating that, as with the intact gill, binding of phlorizin to purified apical membranes is effectively limited to substrate-specific sites.

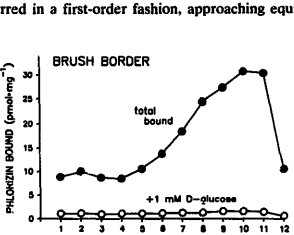

Fig. 2. Glucose-specific [^{3}H]phlorizin binding to isolated gill membranes distributed through a linear 30%–50% sucrose density gradient. Membranes were preincubated in 600 mM mannitol, 10 mM Hepes-Tris (pH 7.6). 10 min incubations were carried out in a binding buffer containing (final concentrations) 260 mM NaCl, 10 mM Hepes-Tris (pH 7.6), and 0.5 μCi of [^{3}H]phlorizin with (open circles) or without (solid circles) 1 mM D-glucose. Each point is the mean of a duplicate determination of binding in membranes from a single, representative preparation. For the other experiments reported here examining phlorizin binding to brush border membranes from the gill, membranes from fractions 8–11 were pooled.

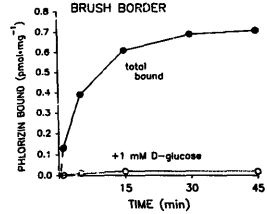

Fig. 3. Time course of [^{3}H]phlorizin binding to BBMV isolated from *Mytilus* gill. Vesicles were preequilibrated in a solution containing 600 mM mannitol and 10 mM Hepes-Tris (pH 7.6). Binding incubations were carried out in a binding buffer containing (final concentrations) 450 mM NaCl, 60 mM mannitol, 10 mM Hepes-Tris (pH 7.6), and 0.6 nM [^{3}H]phlorizin, with or without 1 mM D-glucose. Each point is the mean of 2–4 measurements of binding measured in membranes from a single, representative preparation.

Na-dependence of phlorizin binding

In a previous study [1], we reported that glucose uptake in the gill is Na-dependent and involves a direct coupling between the transport of Na^+ and the transport of glucose. Given our hypothesis that phlorizin binding to the gill involves an interaction with these Na-dependent glucose transport sites, we examined the effect on phlorizin binding in intact gills and BBMV of replacing Na^+ with either K^+ or Li^+. In the intact gill replacement of Na^+ with K^+ or Li^+ effectively eliminated binding of phlorizin (binding decreased by > 90%, compared to control; Table I), which is consistent with the inhibition of glucose transport into intact gills and isolated BBMV noted when Na^+ is replaced with either of these cations [1]. Similarly, when Na^+ was replaced by K^+, phlorizin binding to BBMV was reduced by > 90%. However, replacement of Na^+ by Li^+ reduced binding to BBMV by only 58%. Significantly, addition of 1 mM glucose blocked the Li-supported binding of phlorizin to BBMV (Table I). These data suggest that the isolation procedure used for the preparation of BBMV from the gill may influence the cation binding characteristics of the glucose-specific phlorizin binding site.

Substrate inhibition of phlorizin binding

Binding of 4 nM [^{3}H]phlorizin to intact gill was significantly inhibited ($P < 0.05$) by the presence of 50 μM concentrations of either D-glucose or α-methyl-D-

TABLE I

The effect on [^{3}H]phlorizin binding of replacing Na^+ in seawater with either K^+ or Li^+

For studies with gill tissue, gill discs were incubated for 10 min in 200 ml of ASW containing 10 μM 5-HT and 1.1 nM of ^{3}H-labeled phlorizin. Na^+ in ASW was isosmotically replaced with chloride salts of K^+ or Li^+, and binding was measured in the presence or absence of 1 mM D-glucose. For studies with gill BBMV, membranes were preincubated in a solution containing 600 mM mannitol and 10 mM Hepes-Tris (pH 7.6), and 30 min binding was measured in a binding buffer containing (final concentration) 60 mM mannitol, 10 mM Hepes-Tris (pH 7.6), 45 nM [^{3}H]phlorizin, and either 450 mM NaCl, KCl, or LiCl. In each case, binding was measured in the presence and absence of 1 mM D-glucose

Condition	Phlorizin binding (% of control)	
	intact gill (±S.E.; $n = 3$)	BBMV (±S.E.; $n = 3$)
Na-ASW (control)	100	100
+1 mM D-glucose	7.8±1.48	3.6±0.96
K-ASW	7.8±1.17	5.2±1.32
+1 mM D-glucose	6.8±0.99	2.6±0.55
Li-ASW	12.9±1.8	35.7±3.49
+1 mM D-glucose	6.4±0.87	8.4±2.25

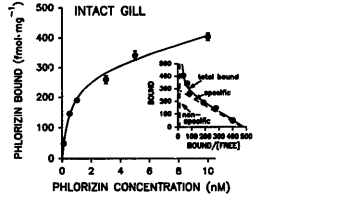

Fig. 4. Effect of increasing phlorizin concentration on the binding of phlorizin to intact gills. Gill discs were incubated for 60 min in 200 ml of ASW containing 10 μM 5-HT, 0.03 nM [^{3}H]phlorizin and unlabeled phlorizin to provide the indicated concentration of ligand. Each point is the mean (±S.E.) of binding measured in three discs of gill tissue from a single animal. The line describing total binding was calculated from the kinetic constants B_{max}, K_d and N which were determined from Eqn. 1 using a non-linear regression algorithm (see text). The inset shows a Woolf-Augustinsson-Hofstee transformation of these data. The dashed lines show the specific and non-specific components of total phlorizin binding (solid line).

glucopyranoside (α-Me-D-glucose) (Table II). D-Galactose and β-D-fructose had little effect on binding of phlorizin, and 3-*O*-methylglucose, 2-deoxy-D-glucose and L-glucose were without effect. A qualitatively similar pattern of inhibition was obtained in two experiments with isolated BBMV from the gill (Table II). These observations are in general agreement with the pattern of substrate inhibition of D-glucose uptake in intact gills and isolated BBMV reported previously: (in order of descending inhibitory effect) D-glucose ≈ α-Me-D-glucose > D-galactose > β-D-fructose = 3-*O*-Me-D-glucose = 2-deoxy-D-glucose = L-glucose [1]. This pattern of inhibition of phlorizin binding is also similar to that reported to occur in mammalian kidney [27].

TABLE II

Effect of different sugars on the binding of [^{3}H]phlorizin to intact gills and BBMV

For experiments with intact gill tissue, gill discs were incubated for 10 min in 200 ml of ASW containing 10 μM 5-HT and 0.05 nM [^{3}H]phlorizin with or without (control) a potential inhibitor (unless otherwise indicated, inhibitor concentration was 50 μM). For experiments with gill BBMV, membranes were preincubated in a solution containing 600 mM mannitol and 10 mM Hepes-Tris (pH 7.6), and 30 min binding was measured in a binding buffer containing (final concentration) 450 mM NaCl, 60 mM mannitol, 10 mM Hepes-Tris (pH 7.6) and 8 nM [^{3}H]phlorizin with or without (control) a 100 μM concentration of potential inhibitor

Inhibitor	Phlorizin binding (% of control)	
	intact gill (±S.E.; $n = 3$)	BBMV (±S.E.; $n = 2$)
None (control)	100	100
D-Glucose (5 mM)	6.8±0.85	–
D-Glucose	26.9±1.52	17.3± 1.19
α-Me-D-Glucose	38.4±2.45	55.1±12.93
D-galactose	82.6±2.12	89.6± 2.08
β-D-Fructose	84.8±1.95	106.7± 2.24
3-*O*-Me-D-glucose	94.2±6.14	–
2-Deoxy-D-glucose	98.2±3.44	–
L-Glucose	105.3±8.2	94.3± 1.72

Kinetics of phlorizin binding

To determine the total number of glucose-specific phlorizin binding sites in the gill and their affinity for this ligand, we examined the effect of increasing phlorizin concentration on equilibrium binding of phlorizin to isolated gill tissue and BBMV. In preliminary experiments with intact gills (data not shown) we examined phlorizin binding up to a concentration of 100 nM. Binding was a curvilinear function of concentration at low (< 5 nM) concentrations of phlorizin, and a linear function of concentration thereafter. For studies of the kinetics of phlorizin binding, attention was focused on binding over the concentration range of 0.2 to 10 nM phlorizin (Fig. 4). Analysis of these data indicated that the kinetics of total phlorizin binding were adequately described by a relationship which included a single class of substrate-specific (i.e., saturable) phlorizin binding sites, and a second class of apparently non-specific (i.e., non-saturable) sites:

$$B = \frac{B_{max}[\text{PHLOR}]}{K_d + [\text{PHLOR}]} + N[\text{PHLOR}] \qquad (1)$$

where B is total binding of phlorizin in equilibrium with a free substrate concentration of [PHLOR]; B_{max} is the total number of substrate-specific phlorizin binding sites; K_d is the affinity of these binding sites for phlorizin; and N is a coefficient describing the first-order binding of phlorizin to nonspecific binding sites. The inset of Fig. 4 shows a Woolf-Augustinsson-Hofstee plot of these data showing the calculated specific and non-specific components of phlorizin binding to intact gill tissue. Analysis of the data from three separate experiments resulted in a calculated value for B_{max} of 190 fmol/mg wet wt of gill tissue (±51.5; S.E.), a K_d of 0.47 nM (±0.10), and a value for N of 16.6 ml/mg (±1.85). The value of K_d determined by direct kinetic analysis was in reasonable agreement with that calculated from the ratio of dissociation and association constants (k_{-1}/k_{+1}) of 0.2 nM, determined from the time courses of accumulation and washout of phlorizin (Fig. 1).

The K_d for phlorizin binding observed here is substantially lower than the apparent K_i of 21 nM for phlorizin as an inhibitor of D-glucose uptake in intact gills [1]. However, this discrepancy is probably a reflection of the very large association constant (k_{+1}) for phlorizin binding to the gill glucose transporter. In our earlier study, we did not preincubate the gill with phlorizin, and the incubation period for glucose uptake was 5 min. Recall that the half-time for binding of nanomolar concentrations of phlorizin to the gill is on the order of 10–30 min (Figs. 1 and 3). It is likely, therefore, that phlorizin binding (and, hence, inhibition of glucose transport) was not near to being complete at the lower range of concentrations used in our earlier study, thereby leading to an overestimate of the concentration of phlorizin required to achieve half-maximal inhibition of glucose uptake.

We also examined the kinetics of glucose-specific phlorizin binding to BBMV isolated from *Mytilus* gill. Fig. 5 presents the data for binding over a concentration range of approx. 0.3 nM to 80 nM. Consistent with the behavior noted in the intact gill, total phlorizin binding to BBMV appeared to involve two components, one saturable and the other non-saturable (over the concentration range examined). In three experiments, the K_d was 6.0 ± 0.83 nM, the B_{max} was 19 ± 3.6 pmol/mg protein, and N (determined in two of the three experiments) was 0.1 ml/mg. As with the intact gill, this K_d was lower than the K_i of 153 nM we reported [1] for phlorizin inhibition of glucose uptake in BBMV from gill, and this also is probably a reflection of the low association rate of phlorizin onto the glucose transporter.

The difference between the apparent K_d values for the major glucose-specific phlorizin binding sites of the intact gill (0.6 nM) and isolated BBMV (6 nM) is reminiscent of the differences noted for the apparent

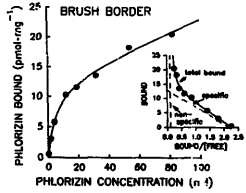

Fig. 5. Effect of increasing phlorizin concentration on phlorizin binding to gill BBMV. Membranes were preincubated in a solution containing 600 mM mannitol and 10 mM Hepes-Tris (pH 7.6), and 30 min binding was measured in a binding buffer containing (final concentration) 450 mM NaCl, 60 mM mannitol, 10 mM Hepes-Tris (pH 7.6) and increasing concentrations of labeled and unlabeled phlorizin (concentration range of 0.3 to 80 nM). Results presented show means of triplicate measures of total phlorizin binding to a representative BBMV preparation (the graphical representation of mean uptake is larger than the standard errors of the means). The curve was calculated by fitting Eqn. 1 to the data using a non-linear regression algorithm. The inset shows a Woolf-Augustinsson-Hofstee transformation of these data. The dashed lines show the specific and non-specific components of total phlorizin binding (solid line).

affinity of phlorizin sites in intact intestine and isolated intestinal BBMV. Ferraris and Diamond [8] reported a K_d of 60–70 nM for phlorizin binding to intact mouse intestine. This is some 100 times lower than the K_d of 4.6 μM measured by Toggenburger et al. [10] using rabbit intestinal BBMV, and consistent with the difference in K_d values noted here for intact tissue vs. isolated membranes.

Collectively, the observations reported here suggest that phlorizin binds with very high affinity to the Na-glucose cotransporter of *Mytilus* gill. Consequently, the B_{max} for glucose-specific phlorizin binding (190 pmol/g gill tissue) represents a measure of the number of glucose transporters in this tissue. Two interesting numbers can be derived from this measure. First, the number of glucose transporters per gill cell can be calculated. Given the relationship between cell surface area and wet tissue weight (550 cm^2/g [28]), and the average apical area of a gill cell (≈ 37 μm^2, ignoring the contribution of microvilli, Ref. 28 and unpublished results), we estimate that there are approx. $8.7 \cdot 10^4$ transporters per cell. This is lower than estimates of the number of glucose transporters in cells of the small intestine, which vary from 10^5–10^6 (rabbits [29]) to 10^6–10^7 (mouse [8]), and is consistent with the comparatively low maximal rates of glucose transport in the gill (J_{max} of $7 \cdot 10^{-12}$ mol cm^{-2} s^{-1}; calculated from data in Refs. 1 and 28) vs. the mammalian intestine (e.g., ≈ $8 \cdot 10^{-9}$ mol cm^{-2} s^{-1}; [30]).

The second parameter that can be derived from the present data on phlorizin binding in the gill is the

turnover number for the glucose transporter. The J_{max} for glucose in the gill is $3.9 \cdot 10^{-9}$ mol $g^{-1} s^{-1}$ [1]. Dividing that number by the B_{max} for glucose specific phlorizin binding reported here gives a turnover number of 21 s^{-1} (at 20–24°C). In gill BBMV, the turnover number is substantially slower. The J_{max} for glucose uptake in BBMV is $8 \cdot 10^{-12}$ mol $mg^{-1} s^{-1}$ [1]. Dividing that number by the B_{max} for glucose-specific phlorizin binding in BBMV gives a turnover number of 0.4 s^{-1}. We are unsure of the basis of this discrepancy in the apparent turnover numbers measured in intact tissue vs. isolated membranes; it suggests that a substantial percentage of the transport protein in the isolated membranes may not be functional, although we cannot eliminate the possibility that it simply reflects a species difference (i.e, *M. californianus* vs. *M. edulis*). It is, however, interesting to note that the turnover number for the glucose transporter measured in intact intestine (mouse; 270 s^{-1} [8]) is also much higher than that estimated for intestinal BBMV (rabbit; 5.6 s^{-1} [29]). The relevant point is, however, that the range of apparent turnover numbers of molluscan Na-dependent glucose transporters is rather similar to that noted for the mammalian counterpart.

In conclusion, epithelial cells from the gill of the marine mussel, *Mytilus*, possess an extremely high-affinity binding site for the β-glucoside, phlorizin. Binding is Na-dependent, and the pattern of inhibition of binding produced by sugars parallels that of the Na-dependent glucose transporter of the gill. It is likely that this glucose-specific phlorizin binding site is a part of the Na-glucose cotransporter, and the very high affinity of the transporter for this ligand may prove useful in future efforts to isolate and purify the gill glucose transporter.

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