TRANSIENT OPENING OF BRUSH BORDER MEMBRANE VESICLES IN ALKALINE MEDIA

Preservation of D-glucose transport after removal of extrinsic proteins

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1. Introduction

As a preliminary stage in the isolation of the hexose carrier of small intestinal brush borders, we recently accomplished a partial purification of this component in the membrane [1]. This was achieved by selectively extracting some unrelated proteins from brush border membrane vesicles with deoxycholate and perturbants such as NaI. Because the resulting membranes are leaky, the presence of the carrier could only be assessed by measuring the binding of a potent competitive inhibitor, i.e., phlorizin [2]. Numerous attempts to reseal these purified membranes were unsuccessful. Thus, it is at present impossible to decide whether the complete hexose translocating machinery was preserved after the purification, or if only the inhibitory binding site remained intact.

Clearly, alternative fractionation procedures which allow subsequent resealing and sugar transport determinations would be advantageous. We report here that brush border vesicles incubated in highly alkaline media open transiently, releasing most of their loosely bound intra- and extravesicular proteins. Some of these vesicles then reseal and display a Na^+-dependent and phlorizin inhibitable uptake of D-glucose, analogous to that of untreated controls.

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2. Materials and methods

Brush border membrane vesicles from rabbit small intestines were prepared by the method in [3] as modified [4]. The vesicles (20–25 mg protein/ml) were resuspended in 300 mM mannitol, 10 mM Hepes/Tris (pH 7.0) with 1 mM dithioerythritol, frozen in an ethanol–dry ice mixture, and kept at −20°C for up to 1 week. The membranes were thawed at 25°C and used immediately.

For alkaline extraction, the vesicles were first washed once with 10 vol. unbuffered 300 mM mannitol and then resuspended by repeated passage through a 25 gauge needle in an ice-cold medium containing 300 mM mannitol, 1 mM dithioerythritol and enough NaOH to bring the pH to 12.2. The final protein concentration of the resuspended vesicles was 3–6 mg/ml. The suspension clarified remarkably upon alkalinization. The membranes were incubated for 10 min on ice and then centrifuged for 40 min at 100 000 × *g* (sedimentation under the conditions normally used for intact vesicles, i.e., 30 000 × *g* for 30 min, was often incomplete). The supernatant was stored and the pellet washed once with 300 mM mannitol, 10 mM Hepes/Tris (pH 7.0). The washed pellet was resuspended again in the same buffer (final conc. 10–20 mg/ml) and used for transport measurements, chemical analyses, or electron microscopy.

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D-Glucose uptake measurements and papain digestion were performed as described [4,5]. Polyacrylamide (8.5%) gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate was performed according to [6]. Protein was determined by the method in [7] using the Bio Rad reagent. P_i, mainly derived from phospholipids, was determined after perchloric acid digestion by the method in [8]. Unless otherwise indicated, the data are expressed as the mean ± 1 SE of the number of experiments shown in parenthesis. Duplicate determinations were made in each experiment.

3. Results

After exposure of brush border vesicles to alkaline media, stronger centrifugation was required to spin down the extracted membranes (see section 2). In addition, the pellets obtained appeared more translucent than those of control membranes. When vesicles treated at pH 12.2 were centrifuged at 100 000 × g for 40 min, 54.2 ± 3.5% (n = 9) of the protein and only 23.8 ± 1.6% (n = 9) of the phospholipid remained in the supernatant. The polypeptide composition of the extracted membranes and the supernatant was analysed by PAGE and compared to that of the original vesicles. The results are shown in fig.1, gels B, C and A, respectively. Protein extraction was selective, insofar as the composition of pellet and supernatant were remarkably different. As reported [9], alkaline treatment partially removes sucrase (which is a major component of band S) but leaves in the membrane virtually all of the band containing isomaltase (I), which is considered to be an integral membrane protein [10]. The band corresponding to actin (band A in fig.1) is solubilized to a great extent by the treatment. Since this protein is thought to be entrapped in the interior of the vesicles constituting their electron dense core [5,11], it is likely that the basic extraction opened the vesicles and released their contents.

The results shown in fig.2 are consistent with this view. The morphological aspect of control (A) and pH 12.2-extracted membranes (B) was analysed by electron microscopy in thin sections of samples fixed and stained by freeze-substitution. It is obvious that most of the core material of the original vesicles was

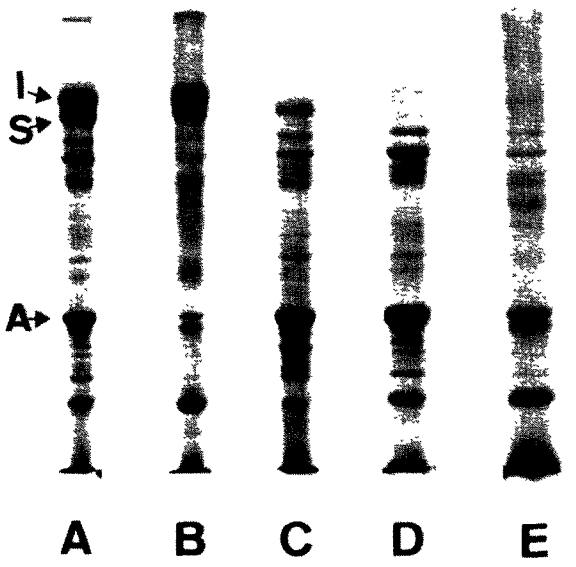

Fig.1. Polypeptidic pattern of control and alkali-extracted brush border membranes. Membranes were treated as indicated below and then analysed by sodium dodecylsulphate–PAGE. Gel A: control brush border vesicles. Gel B: pellet obtained after alkaline extraction. Gel C: supernatant from the alkaline extraction. Gel D: control vesicles treated with papain. Gel E: alkali-extracted membranes treated with papain. The major bands are labeled as follows: actin-like protein, A; sucrase, S; isomaltase, I. Direction of run was from top to bottom.

lost upon alkaline treatment. Yet, the size and shape of the extracted vesicles were not grossly altered. Round vesicles with apparently continuous membrane were the most abundant species, but small membrane fragments were also present. The number and size of the outward-facing knobs, some of which have been associated with the sucrase–isomaltase complex [10,12], decreased with the alkaline extraction, presumably as a result of the removal of a considerable fraction of sucrase (see fig.1).

We next determined whether the hexose transport system was still present in the extracted membranes in a functional state. D-Glucose uptake was measured in both extracted and control vesicles in the presence of a NaSCN gradient. Figure 3 shows that under these conditions glucose accumulates transiently (overshoot) inside control vesicles and eventually equilibrates with the external medium. A similar behaviour was displayed by extracted vesicles. In the experiment

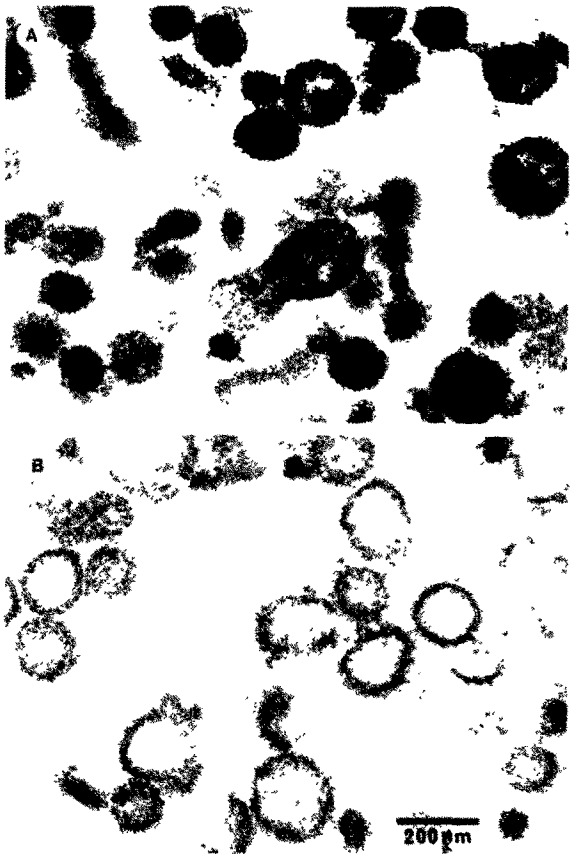

Fig.2. Electron micrographs of thin sections of control and extracted brush border membranes. The membranes were rapidly frozen using a propane-jet freezing method and stained by freeze-substitution with methanol containing osmium tetroxide and uranyl acetate (M. Müller et al., submitted). (A) Intact brush border membrane vesicles. Note the presence of considerable amounts of electron-dense material in the intravesicular space. (B) Alkali-extracted brush border membranes. The dense core of most vesicles has disappeared.

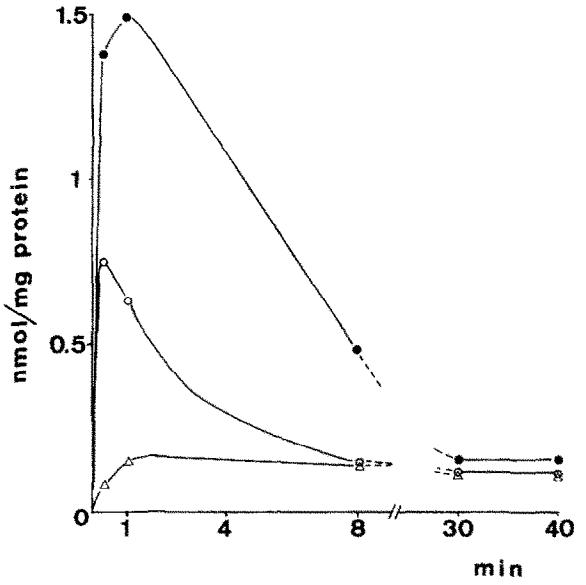

Fig.3. Uptake of D-glucose by control and alkali-extracted brush border vesicles. Concentrative [^{3}H]glucose transport was measured in the presence of an inwardly directed NaSCN gradient, at room temperature. Control vesicles: filled circles. Alkali-treated vesicles: open circles. Alkali-treated vesicles in the presence of 250 μM phlorizin: triangles. Abscissa: time course in min. Ordinate: nmol/D-glucose taken up/mg membrane protein. The points are the mean of duplicate determinations from a representative experiment.

illustrated, the overshoot in extracted membranes exceeded by 6-fold the equilibrium value, as compared to 10-fold in intact vesicles. In 6 different experiments, the overshoot values of extracted membranes ranged from 3–8-times the equilibrium level.

In extracted vesicles it was routinely observed that the overshoot peaked sooner and decayed faster than in control vesicles. This could be explained if the duration of the ionic gradient driving sugar uptake were reduced by an increased leakiness produced by the extraction. The decrease in overshoot height could be similarly interpreted.

The overshoot of the extracted membranes was almost completely abolished by 250 μM phlorizin (fig.3) as has been reported for control vesicles [13]. Figure 3 also shows that in extracted vesicles the equilibrium value (a measure of the secluded volume) was 77% of that of control membranes when expressed per unit protein. However, since proteins were selectively removed by the extraction, equilibrium values are better compared per milligram lipid. When computed this way, the volume trapped by extracted vesicles was 43.9 ± 1.9% (n = 11) of control membranes. Because vesicular size and shape were not grossly altered by the extraction (see fig.2), it is possible to conclude that ~44% of the vesicles are sealed.

Intact brush border vesicles are > 95% sealed and retain the configuration originally found in the

intestine [4,5], exposing the digestive enzymes to the extravesicular milieu. This disposition renders isomaltase susceptible to proteolytic solubilization by papain, a property that has been used to ascertain membrane sidedness. Conversely, the internally trapped actin is not affected by the protease [5]. In an attempt to determine their orientation, alkali-extracted membranes were exposed to papain and the fate of individual polypeptides was analysed by PAGE and compared with results obtained with control membranes. Figure 2 shows that, as reported, isomaltase (but not actin) was removed from control vesicles by papain (gel D). An essentially identical behaviour was observed with alkali-extracted vesicles (gel E), i.e., only traces of isomaltase remained after proteolysis whereas a substantial amount of actin was preserved. Precise quantitation of the data by scanning is difficult due to the low intensity of the residual isomaltase band, but it seems safe to say that the fraction of the vesicles which reseal with an inverted configuration, if any, is very small.

4. Discussion

Brush border membrane vesicles prepared by the Ca^{2+}-precipitation method were found to open and release more than half of their protein when treated at pH 12.2. Alkaline extraction had been shown to effectively remove extrinsic proteins from a variety of membranes, including red cell ghosts [14], adipocyte membranes [15], and electroplax membranes enriched in acetylcholine receptors [16]. In most of these studies, at least a fraction of the extracted membranes resealed and could be used for measurements of solute transport. In the case of brush border vesicles, the possibility of resealing was initially hinted at by the electron micrographs (fig.2) and then confirmed by the recovery of hexose transport into a secluded compartment (fig.3). The latter does not represent unbroken vesicles inasmuch as:

(i) The minor fraction of small vesicles retaining the electron-dense core after alkaline extraction cannot account for the trapped volume, which amounts to 43.9% of the original intravesicular volume.

(ii) $< 10\%$ of the intravesicular marker actin is left in the extracted vesicles; hence, provided actin is homogeneously distributed among the vesicles, $> 90\%$ of them must have ruptured upon alkalinization.

The remaining actin could represent either a population of particularly resistant vesicles, a more tightly bound form of the protein, or both. The existence of a different polypeptide that comigrates with actin in the gels but is not extracted at pH 12.2 could also explain this observation.

The sidedness of the resealed vesicles cannot be at present established with certainty, but preliminary results suggest that they are largely in the right side-out configuration. This is consistent with the observation that the size and shape of most vesicles were not drastically altered by the extraction. Thus it is conceivable that membrane breakdown occurs in the form of small discontinuities rather than involving a major disruption and rearrangement of the bilayer.

Na^+-dependent and phlorizin sensitive D-glucose uptake was observed in the resealed membranes after removal of substantial amounts of several polypeptides. However, it must be noted that even after correcting the data for the fraction of unsealed vesicles, the specific uptake did not increase proportionally to the amount of protein removed. This is probably due to a deleterious effect of the alkaline treatment on the carrier. Indeed, long incubations at pH 12 were found to progressively reduce phlorizin binding to these membranes (S.G. et al., unpublished observations).

The electrophoretic pattern of alkali-extracted vesicles resembles that of deoxycholate-treated brush border membranes [1], in which the phlorizin receptor sites were found to be enriched several-fold. In combination, these data suggest that those proteins which are largely or entirely removed with both extractions, play no essential role in hexose translocation or glucoside binding.

In summary, incubation of brush border vesicles in alkaline media induces their transient opening with the concomitant release of the dense core and of extrinsic polypeptides which are unrelated to glucose transport. This provides a simple initial step for the purification of the hexose carrier in situ, even if partial inactivation occurs. In addition, the momentary breakdown of membrane continuity could also be used for loading the intravesicular space with substances that cannot otherwise traverse the mem-

brane. This experimental approach has proved extremely useful in other membrane systems, such as red cell ghosts [14].

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