Pore properties of the Golgi membrane from lactating-rat mammary gland

Effects of pH and temperature and reconstitution into phospholipid vesicles

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1. Golgi-membrane vesicle penetration was studied by osmotic lysis. 2. Pronounced temperature dependence of mannitol and mannoheptitol penetration over 0–37°C gave linear Arrhenius plots, with activation energies of 75 and 117 kJ/mol respectively. 3. Glucose penetration was constant over pH 5–9, but was respectively faster and slower at higher and lower pH values. 4. Solubilized, dialysed, heat-stable extracts of Golgi membrane were reconstituted into egg yolk phospholipid membranes with apparent recovery of specific permeability. 5. Penetration is interpreted in terms of a pore, for which the Renkin equation predicts a radius of about 0.54 nm.

Lactose formed in the lactating mammary gland is believed to be synthesized in the Golgi apparatus, on the membrane of which resides most of the $\beta(1\rightarrow4)$ -galactosyltransferase (EC 2.4.1.22). Homogenization of the gland pinches off Golgi membrane as small vesicles enriched in galactosyltransferase and nucleoside diphosphatase. In both crude and purified preparations such vesicles convert added glucose and UDP-galactose into lactose, most of which is retained within the lumen but can be readily released by osmotic rupture or by detergent lysis (Keenan *et al.*, 1970; Kuhn & White, 1975, 1977).

In exploring the differential permeability of this membrane to glucose and lactose we have observed a ready penetration of electrolytes and non-electrolytes possessing a molecular weight up to about 300, but little or no penetration by disaccharides including lactose and sucrose. So rapid is the penetration by most solutes that these experiments usually had to be carried out at 0–5°C (White *et al.*, 1980, 1981*a,b*). The present paper extends these studies to include the effects of varying temperature and pH, and the reconstitution into artificial phospholipid vesicles of solubilized Golgi material responsible for these permeability properties. The evidence for a pore protein is discussed, and the Renkin equation is applied to predict the pore diameter.

A preliminary report of some of this work has been made (Kuhn *et al.*, 1982).

Methods and materials

Preparations of lactating mammary gland

Experiments on the effects of osmotic protection, temperature and pH utilized [14 C]lactose-loaded vesicles in simple particulate preparations of mammary gland resuspended in 0.25 M-lactose containing 50 mM-glycylglycine buffer, pH 7.0 (White *et al.*, 1980).

For reconstitution experiments Golgi membranes (about 14 mg of protein) were purified from about 40 g of rat mammary tissue [12–19 days lactating (Kuhn *et al.*, 1980)] and extracted with 4 ml of 2% (w/v) Triton X-100 in 1 M-NaCl for 15 min at room temperature. The extract was centrifuged at 175 000 *g* for 1 h at 5°C and the supernatant was dialysed for 16 h at 5°C against 5 litres of solution containing sucrose (50 mM), NaCl (0.1 M), MgCl₂ (1 mM), β -mercaptoethanol (3 mM), sodium azide (2.3 mM), Triton X-100 (0.05%) and Tris/HCl buffer, pH 7.5 (10 mM). A portion of the dialysed extract was heated at 100°C for 5 min, cooled and centrifuged at 2000 *g* for 10 min to remove denatured protein.

Osmotic lysis

Particle preparations (20 μ l) containing [14 C]lactose-loaded vesicles were diluted into solutions (0.38 ml) of the appropriate sugar concentration and under the conditions given in the legends to the

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Figures. Lysis was stopped by the addition of ice-cold 0.3M-lactose (3.6ml) and the particles were then collected by filtration (White *et al.*, 1981b) and counted for ^{14}C radioactivity.

Reconstitution into liposomes

Egg yolk phospholipid (15mg) was dried under N_2 in a 15 ml conical glass tube and then placed in an evacuated desiccator for 30min to remove traces of solvent. To this was then added 0.5 ml of dialysed Golgi extract [containing a mean ($\pm$ S.E.M.) protein content of 1.12 ± 0.08 mg] plus 1.5ml of dialysis solution containing also 67mM-glucose and tracer quantities of [^3H]sucrose ($3\mu\text{Ci}$) and [^{14}C]glucose ($3\mu\text{Ci}$). The phospholipid was suspended by vortex-mixing, and the mixture was sonicated for $1\frac{1}{2}$ min at 0°C with an MSE-Mullard sonicator. The clear suspension of liposomes thus produced was then gassed with N_2 and incubated at 45°C for 6h. A portion was taken for radioactivity determination and the remainder was passed down a column (10ml) of Sepharose 4B equilibrated with a non-radioactive, lipid-free, solution of the same composition. The liposomes with their trapped sugar eluted at the void volume within 4ml, 1ml of which was taken for radioactivity determination. Columns were washed thoroughly and re-utilized. Where appropriate, glucose was replaced by 67mM- ^{14}C -labelled mannitol or citrate.

Radioactive samples were made up to 1 ml with water, mixed with a scintillation fluid (10ml) containing xylene and Triton X-100 (Kuhn & White, 1975), and counted for radioactivity with a suitably programmed scintillation spectrometer.

Materials

Phospholipid was extracted from egg yolk (Folch *et al.*, 1957), precipitated from its solution in chloroform once or twice with acetone and stored in chloroform under N_2 at -18°C .

D-[U- ^{14}C]glucose, D-[1- ^{14}C]mannitol, [1,5- ^{14}C]citrate and [6,6'- ^3H]sucrose were from Amersham International.

Results

Osmotic protection against osmotic lysis

In previous studies the osmotic rupture of [^{14}C]lactose-loaded vesicles, caused by exposure of the vesicles to iso-osmotic solutions of penetrating solutes such as glucose, was employed to monitor the rate of penetration (White *et al.*, 1980, 1981b). For the experiments reported below it was necessary to show that osmotic rupture could be arrested by the superimposition of a suitable concentration of non-penetrating solute. In an experiment carried out for this purpose the [^{14}C]lactose retained for 15 min at 0°C by vesicles diluted into 0.25M-lactose (control, 100%) equalled that retained after dilution into 0.5M-lactose (105%). The decreased retention after dilution into 0.225M-glucose (34%) was prevented by dilution into a solution containing both 0.225M-glucose and 0.25M-lactose (98%). Thus there was complete osmotic protection by the superimposed lactose.

Effect of temperature on rate of solute penetration

Because of the great rate at which glucose penetrated the Golgi vesicles (82% lysis in 15s at 37°C),

Fig. 1. Effect of temperature on rate of solute penetration of Golgi-membrane particles (a) Rates of osmotic lysis, calculated as percentages of [^{14}C]lactose released/min, and (b) Arrhenius plot of these data for 0.225M-mannitol ($\bullet$) and 0.225M-mannoheptitol ($\circ$), pH 7, (final concentrations) are shown. Rates were determined from time courses of lysis carried out at each temperature over 15s to 10min as appropriate.

Fig. 2. Lack of temperature effect on osmotic fragility of Golgi-membrane vesicles

The retention of [^{14}C]lactose by vesicles exposed to various concentrations of buffered (pH 7) lactose for 5 min at 0°C (●) or 37°C (○) is shown.

Fig. 3. Effect of pH upon glucose penetration of Golgi-membrane vesicles

[^{14}C]Lactose-loaded vesicles were diluted into a solution containing either 0.25M-glucose (experimental) or 0.25M-lactose (control) and 50mM of either dimethylglutarate (pH 4–7 inclusive), glycylglycine (pH 7 and 8) or glycine (pH 8–10 inclusive) buffer at 0°C for 3 min. Rates of lysis in dimethylglutarate and glycine buffers were multiplied by 1.24 and 0.944 respectively to adjust them to the scale of rates measured in glycylglycine buffer. Values are means $\pm$ S.E.M. of six experiments.

the more slowly penetrating solutes mannitol and mannoheptitol were employed to scan the temperature ranges 0–27°C and 0–37°C respectively. The addition of 0.3M-lactose served to arrest lysis at the appropriate times before collection of the vesicles by filtration. Fig. 1(a) shows the rate of osmotic lysis, calculated as a percentage of contained [^{14}C]lactose lost per min, for these two solutes. The rate of lysis by mannoheptitol increased about 200-fold over 0–37°C. These data yielded linear Arrhenius plots (Fig. 1b) from which the apparent activation energies for penetration by mannitol and mannoheptitol were 75 and 117kJ/mol respectively.

A possible alternative explanation of the results shown in Fig. 1 is that higher temperature simply induced greater osmotic fragility in the membrane. However, Fig. 2 shows that the retention of [^{14}C]lactose by vesicles exposed to hypo-osmotic lactose solutions ranging from 25 to 250mM was the same at 0°C as at 37°C. This would not be expected if osmotic fragility varied with temperature. Therefore the results in Fig. 1 are ascribed to temperature-dependent changes in the rate of solute penetration.

Effect of pH upon rate of solute penetration

Fig. 3 shows that the rate of osmotic lysis by 0.25M-glucose at 0°C was nearly constant over the pH range 5–9, but was greater at pH 10 and less at

pH 4. Whatever the interpretation of this curve, it suggests that no one ionizable group is critical for penetration by the solute.

Specific solute penetration in solubilized, reconstituted Golgi material

Experiments were carried out to solubilize Golgi membranes and to reconstitute the dialysed material into artificial phospholipid membranes with retention of solute permeability. Solubilized, dialysed extracts were sonicated with egg yolk phospholipid in the presence of [^3H]sucrose and [^{14}C]glucose, or other solute. They were kept at 45°C for 6h, during which time there was a time-dependent and temperature-dependent increase in the amount of sucrose retained by the vesicles. Possibly this was associated with the fusing of small vesicles into large vesicles. The vesicles were then passed down a Sepharose 4B column to remove extravascular sugar as well as any sugar able to leave the vesicles by means of pores in the membrane. Table 1 shows that phospholipid vesicles formed in the absence of extract eluted in the column void volume with retention of about 0.7% of the added sucrose and a sucrose/glucose ratio close to unity. By contrast, vesicles formed in the presence of extract retained less of the added sucrose but had a sucrose/glucose ratio of about 12. The decreased sucrose retention presumably rep-

Table 1. *Differential retention of solutes by Golgi material reconstituted into phospholipid vesicles*
Results are means $\pm$ S.E.M. of six separate experiments. Ratios of sucrose to glucose etc. are expressed relative to the ratios in which they were added.

Solutes	Conditions	Proportion of added sucrose eluted with vesicles (%)	Ratio of sucrose to glucose, mannitol or citrate in eluted vesicle fractions
Sucrose/glucose	No extract	0.71 ± 0.08	0.90 ± 0.03
Sucrose/glucose	Complete	0.13 ± 0.01	11.8 ± 1.6
Sucrose/glucose	Boiled extract	0.12 ± 0.01	10.2 ± 1.0
Sucrose/glucose	Sugars added just before elution	0.03 ± 0.01	7.4 ± 1.8
Sucrose/mannitol	Complete	0.16 ± 0.02	17.3 ± 4.4
Sucrose/citrate	Complete	0.15 ± 0.05	12.9 ± 6.3

Table 2. *Calculated effect of pore radius on the ratio of effective pore area for glucose/effective pore area for lactose ($A_{\text{GLU}}/A_{\text{LACT}}$) and the ratio of effective pore area for glucose/actual pore area (A_{GLU}/A_0)*

Pore radius (nm)	$A_{\text{GLU}}/A_{\text{LACT}}$	A_{GLU}/A_0
2.0	1.43	0.624
1.0	2.76	0.336
0.7	4.58	0.160
0.65	5.76	0.125
0.60	11.0	0.09
0.55	286	0.056
0.545	1169	0.052

resents a smaller internal vesicular volume or the presence of leaky vesicles. However, the high sucrose/glucose ratio indicates preferential loss of glucose from vesicles still retaining sucrose. A similar behaviour was observed when boiled extracts were used, or when [^{14}C]glucose was replaced by [^{14}C]mannitol or [^{14}C]citrate. Addition of radioactive sugars to vesicles just before loading on to the Sepharose 4B column resulted in a smaller retention of sucrose, showing that most of the retention described above could not be ascribed to mere adsorption. The small amount of sugar retained in this control experiment nevertheless had an unexpectedly high sucrose/glucose ratio. Since it is impossible that adsorptive phenomena could distinguish between the two sugars so effectively, there must have been opening and re-sealing of some vesicles during the mixing process. The apparent pore-forming ability of dialysed, boiled extracts was lost after storage for several weeks at -18°C .

Discussion

A pore model for transport

The experiments reported in the present and in previous papers (see the introduction) combine to

give a consistent view of solute penetration through that part of the Golgi membrane associated with lactose synthesis. Penetration is achieved by all charged and uncharged solutes of molecular weight below about 300. Above this value penetration is extremely slow. Solute penetration is apparently non-saturable, non-competitive and not readily inhibited by the sort of reagents that poison glucose carriers in plasma membranes. Despite the remarkable catholicity of the transport process, rates of penetration do differ measurably between different solutes. Differences among differently charged solutes may reflect the radii of hydration spheres. Among uncharged solutes the rate of penetration appears to decrease with increasing possibilities of hydrogen bonding, but also exhibits certain steric preferences, as between 2-deoxyglucose and 6-deoxyglucose or between glucose and galactose. The large temperature dependence and calculated activation energy, measured for mannitol and mannoheptitol, would be consistent with the breaking of about four and seven hydrogen bonds respectively in the course of membrane penetration. These properties are not consistent with the participation of a carrier protein to which the solute must bind. For such a mechanism one would expect not only saturation kinetics but also a far greater specificity towards the solute. Moreover it is unreasonable to postulate a large number of separate carriers, each specific towards one of the solutes shown to be transported. On the other hand the present properties are fully consistent with the presence of water-filled pores or channels through which solutes can diffuse, breaking hydrogen bonds with their associated hydration sheaths in the process. However, attempts to find an altered rate of solute penetration in solutions made up in $^2\text{H}_2\text{O}$ were unsuccessful (results not shown). From the constancy of rate of penetration of glucose over a wide pH range it is unlikely that maintenance of the pore structure is associated with any one critical charged group, and

this accords with the ready passage of both anions and cations.

Estimate of pore size

The ability of a small pore to discriminate between differently sized penetrating molecules is a sensitive function of its own radius of aperture. Therefore, given a, say, 1000-fold discrimination between glucose and lactose (a conservative estimate, since the Golgi membrane *in vivo* probably separates about 200 mM-lactose in the lumen from about 0.2 mM-glucose in the cytosol) one can employ Renkin's (1954) equation to estimate the radius of a cylindrical water-filled pore. In this equation the effective pore area (A) for a solute is related to the actual pore area (A_0):

$$A/A_0 = (1 - a/r)^2 [1 - 2.104(a/r) + 2.09(a/r)^3 - 0.95(a/r)^5]$$

where a = effective hydrodynamic radius of the solute molecule, r = actual radius of pore. The initial term in parentheses is a geometric correction for solute molecules striking the edges of the pore mouth, whereas the term in square brackets is theoretically derived to correct for friction between the moving molecule and the internal walls of the pore. Given the effective hydrodynamic radii of glucose and lactose as 0.42 nm and 0.54 nm respectively, as determined from viscosity (Schultz & Solomon, 1961), the discrimination between glucose and lactose at different pore radii can be calculated as the effective pore area for glucose relative to that for lactose ($A_{\text{GLU}}/A_{\text{LACT}}$). Table 2 shows that the required discrimination is not achieved until the pore radius is set at 0.545 nm, a value about equal to that of the lactose molecule itself. At the same time the pore is unlikely to be much smaller, since the effective area for glucose would become too small for efficiency. Therefore insofar as the Renkin equation is applicable, the pore radius is expected to be close to 0.545 nm.

Pore proteins in other systems

A variety of other membranes are known to contain proteins, often multi-subunit and relatively heat-stable, that form non-specific pores or channels. These include the porins of *Escherichia coli* outer membrane (Schindler & Rosenbusch, 1978;

Nikaido, 1979; Chen *et al.*, 1979), *Staphylococcus aureus* α -toxin (Füssle *et al.*, 1981), yeast killer toxin (Kagan, 1983), Entamoeba toxin (Lynch *et al.*, 1982), gap-junction connexin (Staehlin & Hull, 1980), complement components C5b-C9 (Bhakdi & Trantum-Jensen, 1978) and a protein from the outer membrane of mitochondria (Colombini, 1980; Lindén *et al.*, 1982). It seems possible that the Golgi membrane contains a similar pore-forming protein.

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