Preparation and characterization of basolateral plasma-membrane vesicles from sheep parotid glands

Mechanisms of phosphate and D-glucose transport

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A procedure is described for the preparation of basolateral membrane vesicles from the acinar cells of the sheep parotid gland. The ouabain-sensitive K^+ -activated phosphatase activity was enriched 30-fold over the tissue homogenate; 45% of this activity was recovered in the final membrane fraction. The presence of membranes from other organelles was negligible. Evidence is presented for the location of Na^+ -dependent symporters for phosphate and D-glucose on the basolateral membrane.

INTRODUCTION

Phosphate plays a vital role in the homeostatic mechanisms of the ruminant. It is an essential nutrient for the rumen microflora and it acts, with bicarbonate, to buffer the pH of the rumen fluid. The phosphate that enters the rumen originates either from the diet (approx. 70–100 mmol/day) or from the saliva (approx. 320 mmol/day), i.e. the quantities of phosphate that are secreted in the saliva are greatly in excess of the dietary intake. Consequently, there is a requirement for a continuous recycling of the phosphate via absorption in the intestine and secretion from the parotid gland (Clark *et al.*, 1972). On the luminal face of sheep enterocytes, the uptake of phosphate is facilitated by a novel H^+ -driven phosphate carrier (Shirazi-Beechey *et al.*, 1989, 1991). The parotid glands of ruminants, e.g. sheep, are unique in that they secrete large volumes of iso-osmotic saliva which contains 10–40 mM-phosphate and 80–100 mM-bicarbonate. Although many of the physiological properties affecting phosphate secretion from sheep parotid glands have been investigated (Wright *et al.*, 1984, 1986), little is known of the mechanisms which facilitate and control the flux of phosphate from the blood, through the acinar cells of the sheep parotid gland and into the parotid primary saliva. The concentration of phosphate in sheep plasma is 1–2 mM (Compton *et al.*, 1980). The acinar cells of sheep parotid glands secrete daily 5–10 litres of iso-osmotic saliva, which contains 10–40 mM-orthophosphate. Hence there must be energy-requiring mechanisms associated with the acinar cells to facilitate this gradient. The long-term objective of the present studies is to characterize these processes.

In this paper we describe a method for the preparation of vesicles from the basolateral region of the plasma membrane of sheep parotid acinar cells. The properties of these membrane vesicles have been studied. Significantly, they contain a Na^+ -dependent carrier which can facilitate the energy-dependent concentration of both phosphate and D-glucose. This is the first report of the simultaneous presence of these Na^+ -dependent symporters on a basolateral membrane. A brief report of these results has appeared (Vayro *et al.*, 1990).

MATERIALS AND METHODS

Animals

Male and female sheep of various breeds were used for these studies. The animals, aged between 8 months and 4 years old, were obtained from local sources. Parotid glands from

phosphate-depleted sheep (Shirazi-Beechey *et al.*, 1991) were obtained from the Rowett Research Institute, Aberdeen, Scotland, U.K.

Removal and storage of tissue

Parotids were removed from the animal within minutes of slaughter. The glands were dissected from adhering adipose tissue, blood vessels, secretory ducts and lymph nodes, wrapped in aluminium foil and dropped into liquid nitrogen. The frozen samples were later removed and subsequently stored at -80°C .

Preparation of parotid membrane vesicles

Plasma-membrane vesicles were prepared from sheep parotid glands by a technique developed from those described by Dyer *et al.* (1989), Shirazi-Beechey *et al.* (1987) and Mircheff *et al.* (1980). Tissue samples (6–8 g) were cut into small pieces, suspended (10%, w/v) in buffered saline (12.5 mM- $NaCl$ /2.0 mM- $Tris/HCl$, pH 8.0) and homogenized (setting 5 for 60 s) with an Ystral Polytron homogenizer. The homogenate was diluted 10-fold with the buffered saline and centrifuged at 500 g for 10 min to give supernatant (S_1) and pellet (P_1). S_1 was centrifuged at 10000 g for 20 min. The supernatant (S_2) was discarded. The pellet (P_2) was suspended in 20 ml of buffer solution containing 100 mM-mannitol and 2.0 mM-Hepes/ $Tris$, pH 7.1. This suspension was further homogenized with the Polytron at setting 4 for 30 s. The volume was increased to 40 ml and 2.5 M- $MgCl_2$ was added to give a final Mg^{2+} concentration of 8 mM. The suspension was stirred for 20 min and then centrifuged at 2000 g for 10 min, giving supernatant (S_3) and pellet (P_3). P_3 was discarded. The plasma membranes retained in S_3 were collected by centrifugation at 30000 g for 30 min. The final pellet (P_4) was resuspended in 1–2 ml of a solution containing 300 mM-mannitol, 20 mM-Hepes/ $Tris$, pH 7.4, 0.1 mM- $MgSO_4$ and 0.02% (w/v) NaN_3 . Samples (50–100 μ l; 4–8 mg of protein/ml) were stored in liquid nitrogen. Vesicles prepared this way retained their ability to transport solutes for up to 30 days. All procedures were performed between 0 and 4°C .

Protein assay

Protein was estimated by its capacity to bind Coomassie Blue, according to the Bio-Rad assay technique. Bovine γ -globulin was used as the standard.

Assay of marker enzymes

The assays were performed at 39°C . The basolateral marker

enzyme ouabain-sensitive K^+ -activated phosphatase was assayed by the method described by Colas & Maroux (1980); the substrate was *p*-nitrophenyl phosphate. Alkaline phosphatase, a luminal plasma-membrane marker enzyme was assayed at pH 9.0 by the method of Shirazi *et al.* (1981); the substrate was *p*-nitrophenyl phosphate. α -Mannosidase (Golgi) was determined as described by Tulsiani *et al.* (1977). Tris-resistant α -D-glucosidase (endoplasmic reticulum) was determined by the method of Peters (1976). Succinate dehydrogenase (mitochondria) was determined by the procedure described by Pennington (1961). Acid phosphatase (lysosomes) was assayed by the method of Hubscher & West (1965). DNA contents were measured by the method of Burton (1956).

Assay of transport activity

Na^+ -dependent phosphate transport. The uptake of phosphate was measured by a filtration-stop technique as described by Shirazi-Beechey *et al.* (1988). Vesicles (15–20 μ l, containing approx. 100 μ g of protein) were preincubated for 10–15 min at either 25 or 39 °C. Reactions were then initiated by addition of 100 μ l of prewarmed incubation medium, which contained 100 mM-NaCl, 100 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, 0.1 mM-MgSO₄, 0.02 % NaN₃ and 0.1 mM-[³²P]P_i (about 125 000 c.p.m.). After an appropriate period, the uptake was quenched by addition of 1 ml of ice-cold stop solution (150 mM-KCl, 20 mM-Hepes/Tris, pH 7.4, 10 mM-KH₂PO₄, 0.1 mM-MgSO₄, 0.02 % NaN₃). Then 0.9 ml of the quenched assay medium was filtered under vacuum through a 0.22 μ m-pore cellulose acetate/nitrate filter (Millipore GSWP 02500). The basolateral membrane vesicles were retained on the filter. The filter was then washed with 5 × 1 ml of stop buffer. Radioactivity remaining on the filter was measured with a scintillation counter. The uptake at 'zero time' was determined by adding stop solution to the vesicles before addition of incubation medium. Measurements of initial rates of transport were performed with a 10 s uptake time. Over this period the uptake was directly proportional to the incubation time.

Na^+ -dependent D-glucose transport. The procedure for measurement of D-glucose transport activity was similar to that for phosphate. The incubation medium contained 0.1 mM-D-[¹⁴C]glucose (about 25 000 c.p.m.), 100 mM-NaCl, 20 mM-Hepes/Tris, pH 7.4, 0.1 mM-MgSO₄ and 0.02 % NaN₃. The stop solution contained 250 μ M-phlorizin, 150 mM-KCl, 20 mM-Hepes/Tris, pH 7.4, and 0.02 % NaN₃. In experiments where phlorizin was used as an inhibitor, it was preincubated with the vesicles at a concentration of 500 μ M for 10–15 min.

Histology and histochemistry

Small pieces of freshly dissected parotid gland were fixed overnight in Bouin's reagent. Sections (5 μ m) were stained for 20–30 min with Harris's haematoxylin in 50 % (v/v) ethanol. After acid/ethanol differentiation, sections were counter-stained with 1 % eosin in 70 % ethanol.

Histochemical localization of alkaline phosphatase activity within the gland was examined. Small pieces of tissue were fixed overnight in 80 % ethanol before dehydration; 5 μ m sections were prepared and alkaline phosphatase activity was assayed by the Gomori method as described by Pearse (1968).

Antibodies

Monoclonal antibody (TK 3B5) raised against the pig kidney basolateral Na^+/K^+ -ATPase (Bourne *et al.*, 1989) was used to determine the presence of structurally related membrane proteins on the vesicles. Suspensions (3–4 μ l, containing approx. 18–25 μ g of protein) were 'dot-blotted' on to nitrocellulose and incubated for 1–2 h with the anti- Na^+/K^+ -ATPase antibody (1:100 di-

lution). Rabbit anti-mouse antibodies, conjugated to either fluorescein or peroxidase (1:1000 dilution), were used to detect primary antibody binding.

A polyclonal antibody raised in rabbits against a synthetic peptide corresponding to residues 402–420 of an extra-cellular peptide sequence of the rabbit enterocyte Na^+ -dependent D-glucose transporter, STLFTMDIYTKIRKKASEK (Hediger *et al.*, 1987), was used to identify a related symporter on the sheep parotid basolateral membrane. Vesicle proteins were separated by SDS/PAGE and electrotransferred on to nitrocellulose sheets for Western blotting.

RESULTS AND DISCUSSION

Development of the procedure for isolating sheep parotid basolateral membrane vesicles

Initially the procedure described by Dyer *et al.* (1989) for the preparation of basolateral membranes from enterocytes was used as the basis to develop the fractionation of parotid tissue. It was found that the fraction P₃ (see Table 1), corresponding to that used by Dyer *et al.* (1989) to prepare the basolateral membranes, contained little K^+ -activated phosphatase activity. Other fractions arising from this procedure were then examined for K^+ -activated phosphatase and alkaline phosphatase activities (Table 1): fraction S₂ contained 40–50 % of the former and 10 % of the latter. Centrifugation of this fraction yielded a pellet which contained little or no alkaline phosphatase activity and a high K^+ -activated phosphatase activity and recovery. This was the fraction used routinely in the following experiments.

It was noted that S₂ contained significant amounts of both these enzymes, and there was enrichment. This fraction was discarded on the basis of the observation (described below) that the alkaline phosphatase activity was associated mainly with the myoepithelial cells, with only a small amount of activity associated with the luminal membrane of the acinar cells. Also the volume of this fraction, approx. 700–900 ml, presented technical problems for further fractionation.

Table 1 shows that in the course of the fractionation of the parotid gland there was an activation of alkaline phosphatase and K^+ -activated phosphatase. This was seen consistently, and we ascribe it to the removal of endogenous inhibitory substances during the fractionation. The observed enhancement of K^+ -activated phosphatase activity was 30-fold, and there was a very high recovery. If the final values are manipulated to take into account the enhancement of activity, then there is still a 7-fold enrichment of K^+ -activated phosphatase activity with a recovery of 13 %.

Characterization of membrane vesicles isolated from sheep parotid gland

The specific activities of the following enzymes were measured in the homogenate and the final vesicle preparation: ouabain-sensitive K^+ -activated phosphatase, alkaline phosphatase, acid phosphatase, α -mannosidase, succinate dehydrogenase and Tris-resistant α -D-glucosidase. These enzyme activities were used to indicate the presence of membranes from the basolateral region of the plasma membrane, the luminal region of the plasma membrane, lysosomes, Golgi, mitochondria and endoplasmic reticulum respectively. The enrichments and percentage recoveries were calculated. The results are presented in Table 2.

In the final preparation there was a 30-fold enrichment of the specific activity of the ouabain-sensitive K^+ -activated phosphatase over that in the homogenate. Typically, 45 % of the total ouabain-sensitive activity was recovered in this fraction. Histochemical studies by Speight & Chisholm (1984) have shown that this enzyme is located on the basolateral membrane of the

Table 1. Distribution of ouabain-sensitive K⁺-activated phosphatase activity and alkaline phosphatase activity in the fractionation of sheep parotid gland

Sheep parotid glands were homogenized and fractionated as described in the Materials and methods section. The basolateral-membrane marker enzyme ouabain-sensitive K⁺-activated phosphatase, and the luminal marker enzyme alkaline phosphatase, were assayed at 39 °C as described in the Materials and methods section. Enzyme specific activity is expressed as nmol/min per mg of protein. The recoveries are expressed as percentages of the homogenate value. Values are representative of a typical fractionation procedure.

Fraction	K ⁺ -activated phosphatase		Alkaline phosphatase	
	Specific activity	Recovery (%)	Specific activity	Recovery (%)
Homogenate	4	100	35	100
Supernatant, S ₁ from low-speed centrifugation (10 min at 500 g)	11	225	18	36
Pellet, P ₁ from low-speed centrifugation (10 min at 500 g)	13	10	44	2
Supernatant, S ₂ from centrifuging S ₁ (20 min at 10000 g)	9	105	22	32
Pellet, P ₂ from 20 min centrifugation at 10000 g	15	32	65	4
Supernatant, S ₃ from P ₂ which was resuspended, treated with MgCl ₂ and centrifuged for 20 min at 2000 g	85	43	59	2
Pellet, P ₃ from 20 min centrifugation at 2000 g	11	3	13	1
Supernatant, S ₄ from high-speed centrifugation of S ₃ (30 min at 30000 g)	Not detected		Not detected	
Pellet, P ₄ from high-speed centrifugation (30 min at 30000 g)	142	67	35	1

Table 2. Enzyme properties associated with the homogenate and final plasma-membrane fraction isolated from sheep parotid glands

The preparation of plasma-membrane vesicles from sheep parotid glands and the procedures for the assay of enzyme activity are described in the Materials and methods section. The membrane region with which each marker enzyme is associated is denoted by the following abbreviations: BLM, basolateral membrane; LM, luminal membrane; ER, endoplasmic reticulum. Enrichments are expressed as the ratio of specific activities (basolateral membrane vesicles/homogenate). The recoveries are expressed as percentages of the homogenate values. Values are means $\pm$ S.D., where the numbers in parentheses denote the numbers of experiments performed.

Marker enzyme	Specific activity (nmol/min per mg of protein)		Enrichment	Recovery (%)
	Homogenate	Final membrane preparation		
K ⁺ -activated phosphatase (5) (BLM)	4 $\pm$ 1	122 $\pm$ 25	30 $\pm$ 4	45 $\pm$ 12
Alkaline phosphatase (5) (LM)	48 $\pm$ 13	50 $\pm$ 15	1 $\pm$ 0.1	1.5 $\pm$ 0.5
Acid phosphatase (3) (lysosomes)	23 $\pm$ 4.5	54 $\pm$ 5	2 $\pm$ 0.3	3 $\pm$ 0.3
α -Mannosidase (4) (Golgi)	3 $\pm$ 1	3.5 $\pm$ 1	1 $\pm$ 0.2	1 $\pm$ 0.3
Succinate dehydrogenase (4) (mitochondria)	21 $\pm$ 3	7.5 $\pm$ 4.5	0.3 $\pm$ 0.2	0.4 $\pm$ 0.3
Tris-resistant α -D-glucosidase (3) (ER)	2 $\pm$ 1	6 $\pm$ 3	3.5 $\pm$ 3	4 $\pm$ 3

acinar cells in the rodent parotid gland. There was little or no enrichment of any of the other enzymes that were assayed. It was concluded that the isolated membranes originated from the basolateral region of the plasma membrane of the parotid cells, and that they were substantially free of membranes from subcellular organelles and from the luminal surface of the plasma membrane. The activity of the ouabain-sensitive K⁺-activated phosphatase was not enhanced by the addition of 0.1 % Triton X-100. We conclude that the inner face of the basolateral

membrane forms the exterior face of the vesicles and that the vesicles are of uniform polarity. The results of the transport studies that are reported below confirm that the isolated membranes have formed sealed vesicles.

Cellular origin of the isolated membranes

The sheep parotid gland is homocrine (Young & Schneyer, 1981). It is made up of two main types of cells. The acinar cells, which are organized into lobules around a central lumen, are

Fig. 1. Time course of Na^+ -dependent phosphate transport into sheep parotid basolateral membrane vesicles

Preparation of the vesicles and the procedure used to measure phosphate uptake are described in the Materials and methods section. Vesicles were incubated for different time periods in the presence of reaction media that contained 100 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, 0.1 mM- MgSO_4 , 0.02% NaN_3 , 0.1 mM- $[\text{P}^{32}\text{P}]_i$ and either 100 mM- NaCl or 100 mM- KCl . The effect of temperature on uptake in the presence of either a Na^+ or a K^+ gradient at 39 °C (●, ■ respectively) and at 25 °C (○, □ respectively) is shown. Values shown are means $\pm$ S.D. for 4 assays.

polar and responsible for the active secretion of the serous saliva. The non-polar myoepithelial cells surround the acini; their function is to enhance the secretion of saliva (Blair-West *et al.*, 1969).

The distribution of alkaline phosphatase activity on parotid gland membranes was determined by the histochemical technique of Pearse (1968). An overwhelming proportion of the activity was associated with the myoepithelial tissue surrounding the basolateral membrane. No alkaline phosphatase activity was detected on the basolateral membrane of the acinar cells, and only a slight activity was seen on the luminal surfaces. This distribution of activity closely parallels that described by Silver (1954) and Speight & Chisholm (1984).

The absence of polarity in the myoepithelial cells would lead to similar recoveries and enrichments of alkaline phosphatase and ouabain-sensitive K^+ -activated phosphatase activities in plasma-membrane preparations from these cells. The data presented in Table 2 show that in the final membrane preparation only 1.5% of the initial alkaline phosphatase activity was recovered; in contrast, 45% of the initial ouabain-sensitive K^+ -activated phosphatase activity was present. By this criterion the membranes in the final preparation are not likely to be derived from the myoepithelial cells.

The ouabain-sensitive K^+ -activated phosphatase activity is regarded as a partial reaction of the Na^+/K^+ -ATPase (Skou, 1975). The presence of the latter enzyme on the membranes of the final preparation was confirmed by the use of the monoclonal antibody TK 3B5, raised to the pig kidney basolateral membrane Na^+/K^+ -ATPase (Bourne *et al.*, 1989). Samples of the vesicles were dot-blotted on to nitrocellulose sheets, and the reaction of the monoclonal antibody was detected by either fluorescent or peroxidase conjugates of rabbit anti-mouse antibodies. There was a strong reaction. We conclude that there are proteins which are structurally related to the pig Na^+/K^+ -ATPase on the vesicle membranes isolated from sheep parotid.

Table 3. Cation specificity of phosphate uptake into sheep parotid basolateral membrane vesicles

Vesicles were prepared and assayed for phosphate transport as described in the Materials and methods section. Cation specificity was determined, at 39 °C, pH 7.4, in reaction media that contained 100 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, 0.1 mM- MgSO_4 , 0.02% NaN_3 , 0.1 mM- $[\text{P}^{32}\text{P}]_i$ and 100 mM concentrations of Na^+ , Li^+ , Cs^+ , Rb^+ , K^+ or choline ions. Chloride was the counter-ion. Values shown are means $\pm$ S.D. for 4 assays.

Cation	Phosphate uptake (nmol/min per mg of protein)
Na^+	7.33 ± 0.33
Li^+	2.22 ± 0.07
Rb^+	0.35 ± 0.03
K^+	0.34 ± 0.04
Cs^+	0.28 ± 0.01
Choline $^+$	0.21 ± 0.01

Na^+ -dependent transport of phosphate

One of the main functions of the parotid gland is to facilitate the movement of phosphate from the blood and to concentrate it in the saliva. We investigated the ability of the vesicles to transport phosphate. Parotid basolateral membrane vesicles were incubated with 0.1 mM- $[\text{P}^{32}\text{P}]_i$ in the presence of either a Na^+ or a K^+ gradient, 100 mM outside, nil inside. The amount of phosphate transported into the vesicles in each condition was measured by stopping the reaction at different time periods. The time course of phosphate transport at 39 °C was measured at pH 7.4. A typical result is shown in Fig. 1. In the presence of a Na^+ gradient there was a rapid uptake of phosphate; the initial rate was 8.50 nmol/min per mg of protein, which is more than 10 times greater than the rates recorded for phosphate transport into enterocyte brush-border membrane vesicles. After 10 s the intravesicular concentration then fell. Within 60 min there was no further loss of phosphate from the vesicles. When the Na^+ gradient was replaced with a K^+ gradient, there was little phosphate uptake over the whole time period. The initial rate of transport was 0.72 nmol/min per mg of protein, considerably less than the initial Na^+ -driven phosphate uptake. There was no overshoot accumulation of phosphate. After 60 min the concentrations of phosphate inside and outside the vesicles were equal. The volume of the vesicles was calculated to be 2.5 μl /mg of protein. This is higher than the value 1.1 μl /mg of protein calculated from the data of Takuma & Baum (1985) for rat parotid basolateral membrane vesicles and 0.8 μl /mg of protein for rabbit enterocyte basolateral membrane vesicles (Dyer *et al.*, 1990). Since the intravesicular concentration of phosphate reached a value which was 5 times greater than the extravesicular concentration, we conclude: that phosphate transport into the vesicles was driven by the Na^+ gradient, the only source of energy that is available; that the basolateral membrane vesicles are sealed; and that there are functional phosphate transporters present on the membrane.

Effect of temperature on phosphate uptake

A time course of phosphate uptake at 25 °C over a period of 60 min showed levels of phosphate uptake which were considerably less than those observed at 39 °C (Fig. 1). There was no overshoot accumulation of phosphate in the presence of a Na^+ gradient. There was a steady increase in phosphate accumulation over the first few minutes, which began to level off towards 60 min. Na^+ did, however, activate the initial flux of phosphate.

Table 4. Anion specificity of phosphate uptake into sheep parotid basolateral membrane vesicles

The rates of Na⁺-dependent phosphate uptake in the presence of different anions were measured. Incubations were carried out at 39 °C, pH 7.4, in reaction media that contained 100 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, 0.1 mM-MgSO₄, 0.02 % NaN₃, 0.1 mM-[³²P]P_i and 100 mM concentrations of Cl⁻, SCN⁻, isethionate⁻, SO₄²⁻ (50 mM) or gluconate⁻ ions. Na⁺ was the counter-ion. Values shown are means ± S.D. for 4 assays.

Anion	Phosphate uptake (nmol/min per mg of protein)
Cl ⁻	7.92 ± 0.63
SCN ⁻	7.88 ± 0.45
SO ₄ ²⁻	6.85 ± 0.47
Isethionate ⁻	7.22 ± 0.34
Gluconate ⁻	5.74 ± 0.31

Fig. 2. Time course of Na⁺-dependent D-glucose transport into sheep parotid basolateral membrane vesicles

Preparation of the vesicles and the procedure used to measure glucose uptake are described in the Materials and methods section. Vesicles were incubated for different time periods in the presence of reaction media that contained 100 mM-NaCl or 100 mM-KCl, 100 mM-mannitol, 20 mM-Hepes/Tris, pH 7.4, 0.1 mM-MgSO₄, 0.02 % NaN₃ and 0.1 mM-D-[¹⁴C]glucose. The effect of temperature on uptake in the presence of either a Na⁺ or a K⁺ gradient at 39 °C (●, ■ respectively) and at 25 °C (○, □ respectively) is shown. Values shown are means ± S.D. for 6 assays.

The presence of a Na⁺-dependent phosphate transporter in the basolateral region of the plasma membrane has been reported previously in non-ruminant animals (Kikuchi & Ghishan, 1987; Schwab *et al.*, 1984).

Mechanism of Na⁺-dependent phosphate transport

The substrate-binding affinity (K_m) and the maximal velocity (V_{max}) of phosphate transport were measured with basolateral membrane vesicles prepared from the parotid glands of sheep fed on either normal or phosphate-deficient diets. Phosphate depletion had little effect on the K_m , 0.34 ± 0.10 mM (normal) and 0.41 ± 0.13 mM (deficient), and the V_{max} , 520 ± 67 and 400 ± 90 pmol/s per mg of protein respectively; values are mean ± S.D. for 4 assays. On the basis of these data we conclude that the mechanism and capacity of Na⁺-dependent phosphate transport in the parotid gland is not affected by depletion of dietary phosphate. This observation contrasts with the findings of Shirazi-Beechey *et al.* (1991), who noticed a dramatic increase in V_{max} of the H⁺-dependent transport of phosphate in luminal

membrane vesicles prepared from the small intestine of the same phosphate-deficient sheep.

In sheep the kidney plays a minor role in phosphate homeostasis (Scott *et al.*, 1985). It appears that phosphate homeostasis in the sheep is regulated at the level of intestinal absorption rather than the modulation of phosphate secretion by the parotid gland.

Cation specificity

The initial rates of phosphate transport into basolateral membrane vesicles in response to gradients of univalent cations were measured in reaction media that contained 100 mM concentrations of Na⁺, Li⁺, Cs⁺, Rb⁺, K⁺ or choline ions. Chloride was the counter-ion. The results are presented in Table 3; Li⁺ was 30 % as effective as Na⁺ in energizing the movement of phosphate. Rb⁺, Cs⁺, K⁺ and choline were relatively ineffective.

Effect of anions on rates of phosphate transport

It is generally accepted that SCN⁻ can readily cross membranes, whereas isethionate and gluconate are thought to be relatively membrane-impermeant. The data in Table 4 show that the initial rates of phosphate transport were not affected by the membrane permeability of the anions which were present in the uptake reaction medium. These results suggest that the mechanism of Na⁺-dependent transport of phosphate is electroneutral, as reported by Cheng & Sacktor (1981) for renal luminal membranes, but in contrast with the electrogenic mechanism in brush-border membrane vesicles isolated from the rabbit duodenum (Danisi *et al.*, 1984; Shirazi-Beechey *et al.*, 1988). The minor difference in phosphate uptake in the presence of gluconate ions could be due to it having a slight inhibitory effect.

Inhibition of phosphate transport

The Na⁺-phosphate symporter was inhibited by increasing concentrations of phosphonoformate (Szczepanska-Konkel *et al.*, 1986): maximum inhibition (70–80 %) was reached at 5 mM. Decreases of 97 % and 93 % in transport were observed by using 1 mM concentrations of HgCl₂ and mersalyl respectively. The phosphate analogues, vanadate and arsenate (1 mM concentrations), did not inhibit the initial rate of uptake in parotid basolateral membrane vesicles. In contrast, phosphate transport in rabbit enterocyte brush-border membrane vesicles was inhibited by these anions (Shirazi-Beechey *et al.*, 1988).

Na⁺-dependent transport of D-glucose

As was observed for phosphate transport, a Na⁺ gradient energized the transcellular flux of D-glucose into parotid basolateral membrane vesicles (Fig. 2). The time course of D-glucose uptake at 39 °C, pH 7.4, showed similar properties to that of phosphate movement (Fig. 1), reaching a peak uptake rate (2.32 nmol/min per mg of protein) before declining. It is clear that D-glucose was accumulated inside the vesicles above and beyond its equilibrium value, resulting in the characteristic overshoot. There was little, if any, uptake of glucose when 100 mM concentrations of Li⁺, K⁺, Rb⁺, Cs⁺ or choline ions were substituted for Na⁺.

The initial rate of Na⁺-dependent D-glucose transport was decreased from 2.32 to 0.31 nmol/min per mg of protein by changing the assay temperature from 39 to 25 °C; Cl⁻ was the counter-ion. The rate of uptake was not affected by replacing Cl⁻ with either Br⁻ or I⁻ ions. When Cl⁻ was replaced with gluconate ions, the rate of transport was decreased by 60–70 %. We conclude that the Na⁺-dependent uptake of D-glucose into the vesicles is an electrogenic process. This uptake was completely inhibited by addition of 500 μM-phlorizin, an inhibitor of the

Na⁺-dependent D-glucose symporter (Alvarado & Crane, 1962). The existence of such a protein in the basolateral membrane has not been reported previously.

In conclusion, we have developed a technique for the isolation of intact plasma-membrane vesicles from the basolateral region of the parotid acinar cells. These membranes contain the ouabain-sensitive K⁺-activated phosphatase and the proteins which facilitate the Na⁺-dependent transport of phosphate and D-glucose. In the pre-ruminant lamb a Na⁺-dependent D-glucose carrier, with antigenic properties similar to those of the parotid basolateral membrane carrier, is found located in the enterocyte luminal plasma membrane (Shirazi-Beechey *et al.*, 1989), and there is a Na⁺-dependent phosphate carrier in the luminal plasma membrane of kidney tubule cells (J. I. Penny & S. Shirazi-Beechey, unpublished work). Thus in the same animal proteins with identical functions are located in opposite poles of the epithelia in different organs. This raises interesting questions as to the mechanisms of sorting of the nascent proteins, and the mechanisms whereby they are directed to the appropriate pole of the cell.

The high concentrations of phosphate found in the serous saliva demand a high flux through the acinar cell. This could be facilitated by the Na⁺-dependent uptake of phosphate through the basolateral membrane into the acinar cell. The subsequent secretion of phosphate into the saliva can be rationalized as a response to the membrane potential, approx. 90 mV positive externally, across the plasma membrane.

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