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Relationship of the Donnan Potential to the Transmembrane pH Gradient in Tracheal Apical Membrane Vesicles

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Summary. Experiments were performed to determine the factors which contribute to the transmembrane pH gradient (ΔpH) and the potential gradient ($\Delta\psi$) in apical plasma membrane vesicles isolated from bovine tracheal epithelium. As indicated by the accumulation of ^{14}C-methylamine, the vesicles maintained a ΔpH (inside acidic) which was dependent upon the external pH. The ΔpH was also proportional to the ionic strength of the suspending medium, suggesting that the H^+ distribution was dictated by a Donnan potential. Measurements of the distribution of $^{86}Rb^+$ demonstrated an electrical potential gradient across the vesicle membrane, inside negative which was proportional to the medium ionic strength. ΔpH changed in parallel with $\Delta\psi$ in response to a variety of imposed conditions. These results are compatible with the existence of a H^+ conductance in the vesicle membrane. Thus the endogenous electrical and proton gradients may be manipulated and used as a general experimental tool to complement kinetic analysis in investigations of transport mechanisms using isolated vesicle preparations.

Key Words membrane potential · pH gradient · membrane vesicles · trachea

Introduction

Plasma membrane vesicles derived from the luminal and contraluminal surfaces of epithelial cells have been used with increasing frequency over the past two decades in the study of transepithelial transport processes. Epithelia are complex multicomponent systems performing transport functions which involve a wide variety of transcellular and paracellular pathways. The aim of studies with purified membranes is to reduce such a system into relatively simple components, to define the transport properties of those components under controlled conditions, and then to describe the behavior of the whole system on the basis of the properties of its constituent parts. The advantages and limitations of this experimental approach have been thoroughly reviewed by Sachs, Jackson and Rabon (1980) and Murer and Kinne (1980).

A purified membrane vesicle preparation derived from the apical face of bovine tracheal epithelial cells has recently been used to investigate tracheal ion transport mechanisms (Langridge-Smith, Field & Dubinsky, 1983, 1984). Transepithelial flux studies in intact tracheal mucosa have identified active Cl^- secretion and active Na^+ absorption in this tissue (Olver et al., 1975; Al-Bazzaz & Al-Awqati, 1979; Langridge-Smith, Rao & Field, 1984). According to current transport models based on flux measurements and the results of electrophysiological studies in the intact tissue, passage of both Na^+ and Cl^- across the apical membrane of tracheal epithelium occurs by passive diffusion along appropriate electrochemical gradients, which are maintained at the expense of cellular energy (Widdicombe & Welsh, 1980; Frizzell, Welsh & Smith, 1981; Dubinsky & Langridge-Smith, 1985). Studies of Na^+ transport in the apical membrane vesicle preparation revealed a dependence upon the counterion in the incubation medium which was consistent with an electrogenic transport mechanism for Na^+ (Langridge-Smith et al., 1983). Both the initial rate and the maximal extent of Na^+ uptake were twofold higher in the presence of SCN^-, a permeant anion, than in the presence of $gluconate^-$, an impermeant species. The difference observed in the extent of Na^+ uptake at apparent equilibrium (60-min incubation) was unexpected. Subsequently, an apparent transmembrane electrical gradient of −54 mV was demonstrated in these vesicles under conditions of low ionic strength (Dubinsky & Langridge-Smith, 1985; Langridge-Smith & Dubinsky, 1985). This gradient was abolished in the presence of 50 mM salt. In addition, a pH gradient of 0.7 units (inside acid) was measured at low ionic strength, and was similarly abolished by 50 mM salt. These observa-

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tions suggest the existence of a relatively high concentration of impermeant negative charge at the vesicle interior which, depending upon the imposed conditions, may be a major determinant of the transmembrane distribution of permeant ions. In the present study, we have extended these preliminary findings to clarify the relationship between the electrical potential and the pH gradient, and to determine the factors which contribute to the generation and maintenance of these endogenous gradients in the tracheal vesicles.

Materials and Methods

Preparation of Apical Membrane Vesicles

Apical membrane vesicles were prepared from bovine tracheal epithelium using the following procedure based on our previously published method (Langridge-Smith et al., 1983). The purity of the membrane vesicle preparation was routinely evaluated by analyzing homogenates and final membranes for the apical membrane marker, alkaline phosphatase. Enzyme activity was measured as described by Langridge-Smith et al. (1983). The specific activity of the enzyme in the vesicle preparations was 97.1 ± 11 μmol P_i per hr per mg protein (n = 14) which represents a 31-fold enrichment over the crude homogenate. Protein was assayed according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

Measurement of ΔpH and Δψ

Transmembrane ΔpH[1] was calculated from the intra- and extravesicular distribution of ^{14}C-methylamine. The distribution of $^{86}Rb^+$ in the presence of the ionophore valinomycin, was used to calculate the transmembrane potential (Δψ). These methods have been employed in a variety of organelles over a wide range of experimental conditions, and found to yield highly reproducible results (Goldman & Rottenberg, 1973; Johnson & Scarpa, 1976; Johnson, Scarpa & Salganicoff, 1978; Johnson et al., 1980; Russell & Holtz, 1981; Carty, Johnson & Scarpa, 1982; Hutton, 1982; Scherman & Nordman, 1982; Grinstein & Furuya, 1983). The underlying theoretical principles for both methods are discussed in detail by Rottenberg (1978) and Ramos and Kaback (1977). Uptake of the radiolabeled solutes was measured using either Sephadex (G50, fine) (Penesfky, 1977) or DOWEX 50W-X-8 cation exchanger columns (Gasko et al., 1976). For the Sephadex procedure disposable 1-ml tuberculin syringes were plugged with dacron wool and filled to the 0.9-ml mark with Sephadex, previously swollen in deionized water. The column was equilibrated with 2 mM HEPES-Tris, 100 mM mannitol (pH 7.5), then placed in a 13 × 100 mm glass test tube and centrifuged at 300 × g for 2 min (GLC-1 centrifuge, Sorvall Inc., Newtown, Conn.). This caused significant shrinkage of the column. The Sephadex was then rehydrated by immersing the tip of the syringe in the buffered mamnitol solution, bringing the final volume of the column to 0.8 ml.

Apical membrane vesicles were thawed at room temperature, homogenized in 2 mM HEPES-Tris, 100 mM mannitol (pH 7.5) and then incubated at 37°C for 30 min. Assays were initiated by the addition of 20 μl vesicles (3 to 4 mg ml^{-1}) to 80 μl appropriate incubation medium containing tracer quantities of the radiolabeled solute. In the case of methylamine the medium concentration was 0.2 to 0.4 mM and $^{86}Rb^+$ was present at <10 μM. Details of the composition of the incubation media are given in the text and Figure legends. After 60 min incubation at 20°C, 90 μl of the vesicle suspension was placed on to the column via a 25 gauge ($\frac{5}{8}$") hypodermic needle resting point-down on top of the syringe. The needle retained the sample until a second centrifugation at 300 × g for 2 min was performed. Recovery of protein in the resulting eluent was 100%. The eluent was collected in a clean test tube, diluted with 2 ml distilled H_2O and transferred to a scintillation vial. Samples were then mixed with 8 ml ACS scintillation fluid (Amersham Corp., Arlington Heights, Ill.) and the radioisotope content was determined in a liquid scintillation counter (Tracor Analytic, Elk Grove Village, Ill.). Background counts, obtained by passing matched reaction mixtures without vesicles through the column, were measured and subtracted for each assay. The specific activity of the tracer in the medium was determined from a 5-μl sample of the total reaction mixture. The intravesicular concentration of the tracer was calculated using vesicle volume values measured under matched assay conditions.

In some experiments the Sephadex columns were replaced by DOWEX 50 W-X8 ($Tris^+$ form, 50 to 100 mesh) using a modification of the procedure of Gasko et al. (1976). Columns were prepared in $5\frac{1}{4}$ in pasteur pipettes plugged with dacron wool and filled to the constriction with the ion exchanger. The column was mounted in a vacuum manifold (Millipore Inc.). To assist sample passage through the column immediately prior to the application of the reaction mixture a vacuum of 5 mM Hg was applied to the apparatus. The sample was applied directly to the column and washed with 1 ml of isosmotic mannitol. All other details of the assay are as described above for the Sephadex column procedure.

Vesicle Volume Determination

Two different methods were used to determine the volume of the tracheal apical membrane vesicles, in order to confirm the accuracy of the measurements. The first method was based on the equilibrium uptake of ^{14}C-urea, which was measured by the Sephadex column technique described above. As a freely permeable species, urea will achieve an intravesicular concentration equal to that in the medium under equilibrium conditions. The vesicle volume can therefore be calculated from the following equation:

$$\frac{\text{sample cpm}}{\text{medium cpm}/\mu\text{l} \times \text{mg protein/sample}} = \text{intravesicular space } (\mu\text{l/g protein}).$$

[1] The abbreviations used are: ΔpH, transmembrane proton concentration gradient; Δψ, transmembrane potential; $\Delta\psi_{H^+}$, millivolt equivalent of transmembrane proton gradient; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tris, Tris (hydroxymethyl) aminomethane; MES, 2(N-morphino)-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetracetate; PEG, polyethelene glycol; DTT, dithiothreitol; TMA, tetramethyl-ammonium; KSCN, potassium thiocyanate.

Table. Volume determinations in tracheal apical membrane vesicles[a]

Medium composition	Method A: Sephadex columns	Method B: Ultracentrifugation
100 mM mannitol	1.83 ± 0.18 (n = 3)	1.83 ± 0.19 (n = 12)
1 M mannitol	0.25 ± 0.01 (n = 3)	0.22 ± 0.05 (n = 4)
50 mM KSCN	1.91 ± 0.12 (n = 8)	1.60 ± 0.12 (n = 3)
50 mM KCl	1.58 ± 0.27 (n = 6)	2.19 ± 0.14 (n = 3)
50 mM K gluconate	1.75 ± 0.21 (n = 6)	1.80 ± 0.42 (n = 3)

[a] The assay medium contained 2 mM HEPES-Tris (pH 7.5) in addition to the indicated compounds. The methods of vesicle volume determination and calculation are described in Materials and Methods. Results are expressed in μl/mg protein and represent means ± 1 SE for n assays.

Parallel assays in which the osmolarity of the incubation medium was varied by addition of mannitol indicated that there was no significant binding of urea to the vesicle membrane.

In the second method of vesicle volume determination, 80 μl appropriate incubation medium containing tracer quantities of ^{14}C-o-methylglucose and ^{3}H-polyethylene glycol (PEG) was placed in small (5 × 20 mm) polyallome centrifuge tubes, and 20-μl vesicles (3 to 4 mg ml^{-1}) was added. After 60-min incubation at 20°C, the vesicle suspension was centrifuged at 178,000 × g for 5 min in an air-driven ultracentrifuge (Beckman Inst. Inc., Palo Alto, Calif.). The supernatant was carefully removed with a small hypodermic needle attached to a vacuum line, having first saved a 10-μl sample for calculation of the specific activity of the tracers. The pellet was denatured for 60 min in 50 μl NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.). The tube was then placed in a vial containing 8 ml scintillation fluid and counted for radioisotopic content. The intravesicular space volume was calculated as the total water space of the pellet measured with ^{14}C-o-methylglucose minus the extravesicular space determined with ^{3}H-PEG.

Binding of ^{14}C-o-methylglucose to the vesicle membrane was accounted for by making parallel volume determinations in matched incubation media in which the osmolarity was varied from 100 to 1100 mosmol by the addition of mannitol. The calculated vesicle volume was plotted against the reciprocal of medium osmolarity. The value obtained by extrapolation of the plot to infinite osmolarity was assumed to represent ^{14}C-o-methylglucose binding and was subtracted from the measured volume in all cases (Rottenberg, 1978).

Vesicle volumes calculated by both the Sephadex column and the centrifugation methods are expressed as μl intravesicular space/mg protein.

Data Analysis and Presentation

Membrane potential was computed from the Nernst equation as $\Delta\psi = -59 \log [\text{cation}]_i/[\text{cation}]_o$. Transmembrane H^+ gradient was calculated as $\Delta\text{pH} = \log [\text{methylamine}]_i/[\text{methylamine}]_o$, the validity of this calculation being based on the high pK_a of the amine. Each type of experiment was generally performed in duplicate or triplicate in at least three vesicle preparations. Results are given as the mean ± SE and, unless otherwise stated, standard errors are computed on the number of vesicle preparations.

Materials

^{14}C-urea (7 mCi/mmol), ^{3}H-PEG (4.5 mCi/mol), ^{14}C-o-methylglucose (55 mCi/mol), and ^{86}RbCl (1.6 mCi/mg) were all obtained from New England Nuclear (Boston, Mass.). ^{14}C-methylamine (40 to 60 mCi/mmol) was obtained from ICN (Irvine, Calif.). Valinomycin was purchased from Calbiochem (La Jolla, Calif.), Sephadex G-50 fine from Pharmacia Fine Chemical Chemicals (Piscataway, N.J.), methylamine from Eastman Organic Chemicals (Rochdale, N.Y.), and HEPES, Tris, dithiothreitol and tetramethylammonium hydroxide from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of the highest quality available.

Results

Determinations of Vesicle Volumes

In order to calculate the intra- to extravesicular concentration ratio of a probe species, the vesicle volume was determined under identical assay conditions. Two independent experimental techniques were employed to determine the volume of the tracheal apical membrane vesicles. Measurements based on the equilibrium distribution of ^{14}C-urea indicated osmotically active vesicles since the intravesicular space decreased when the medium osmolarity was increased by the addition of mannitol (Table). Extrapolation to infinite mannitol concentration yielded a vesicle volume which was not significantly different from zero (0.07 ± 0.01 μl/mg protein). In contrast, measurements made by the centrifugation technique with ^{14}C-o-methylglucose and ^{3}H-PEG suggested that the limiting internal space at infinite osmolarity was not zero (2.26 ± 0.48 μl/mg protein). Since the osmolarity was varied by the addition of mannitol in both assay methods, this latter observation cannot be attributed to some permeability of the vesicle membrane to mannitol. Instead, the apparent space is most likely to be due to nonspecific binding of ^{14}C-o-methylglucose to the vesicle membrane. This "background" value was therefore subtracted in all cases from the volumes measured by the centrifugation technique.

Vesicle volumes determined by both methods under a variety of assay conditions are presented in the Table. Varying the ionic strength and composition of the incubation medium had no effect on the volume when the osmolarity of the medium was kept constant. There was no significant difference

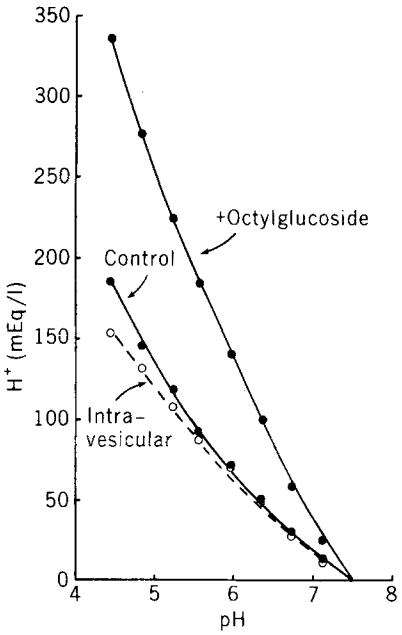

Fig. 1. Titration of tracheal membrane vesicles. Tracheal membrane vesicles (1.48 mg protein × ml^{-1}) in 100 mM KCl were adjusted to pH 7.5 with a stock solution of 100 mM KOH. The suspension was titrated with aliquots of a standard 50 mM H_2SO_4 solution. The pH was recorded as a function of the amount of added acid. A buffer blank (-protein) was also titrated and subtracted from the curves obtained for control vesicles and those in the presence of 1% octylglucoside. The intravesicular curve (open symbols) is the difference of the octylglucoside-control titration curve. Values are expressed as the amount of added acid in milliequivalents H^+/liter of intravesicular volume.

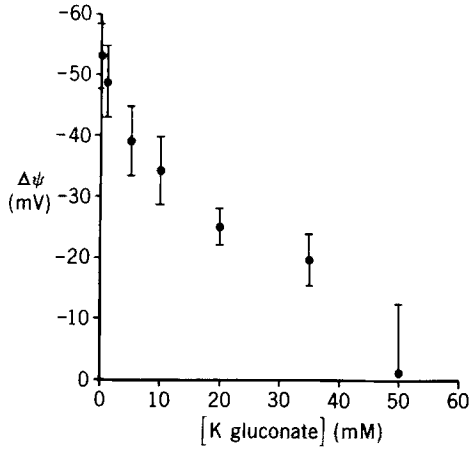

Fig. 2. $\Delta\psi$ as a function of medium K^+ concentration. Vesicles were incubated for 60 min at 20°C in the presence of $^{86}Rb^+$ in media containing 2 mM HEPES-Tris (pH 7.5) and K-gluconate at the indicated concentrations. Solutions were balanced for osmolarity by appropriate concentrations of mannitol. Valinomycin (10 $\mu g\ ml^{-1}$) was present in all assays. $\Delta\psi$ (in millivolts, inside negative) was calculated from the transmembrane distribution of $^{86}Rb^+$ using the Nernst equation. The distribution of $^{86}Rb^+$ was measured as described in Materials and Methods. Results represent mean ± SE for determinations in triplicate using vesicles from three separate peparations (SEM based on number of vesicle preparations, $n = 3$)

between the intravesicular volumes determined by the two different assay procedures, lending confidence to the accuracy of the results. The overall mean of the volume determinations, made under conditions identical to those used in subsequent measurements of Rb^+, and methylamine concentration ratios, was 1.79 ± 0.07 μl/mg protein ($n = 53$). This value was used as a standard volume in all of the following determinations of transmembrane solute distribution.

Buffer Capacity of Membrane Vesicles

The buffering capacity (β) of the membrane vesicles was determined by titrating the vesicle suspension over the range of 7.5 to 4.0. The titration was performed in the presence of the nonionic detergent octylglucoside in order to expose all ionizable groups (Fig. 1). In a control titration the vesicles were also titrated in the absence of the detergent to measure extravesicular or leaky membrane buffering (Fig. 1, control). Subtraction of the control from the total titration obtained in the presence of octylglucoside yields an estimate of the titration curve of the intravesicular compartment. The calculated titration curve for the intravesicular space was roughly linear over the range of 7 to 4.5 with a buffering capacity of 51 meq $H^+ \times liter^{-1}$ pH $unit^{-1}$ as determined from the slope (open symbols, Fig. 1).

Measurement of $\Delta\psi$ and ΔpH

Figure 2 shows the results of determinations of $\Delta\psi$ in the presence of increasing concentations of medium K gluconate. As predicted of a Donnan potential, $\Delta\psi$ varied inversely with the concentration of the salt. At 50 mM K gluconate, $\Delta\psi$ was not significantly different from zero. The minimal quantities of intravesicular $^{86}Rb^+$ measured in the presence of high salt concentrations were barely above background and thus are near the limit of detection possible with the assay technique employed; this accounts for the relatively large standard error of the $\Delta\psi$ determined under these conditions.

The $\Delta\psi$ was also determined as a function of the pH of the assay medium. With increasing acidity of the external solution there was a decline in the membrane potential from −59.9 ± 6.7 mV at pH 8.0 to −22.5 ± 1.7 mV at pH 5.0 (Fig. 3).

The transmembrane distribution of ^{14}C-methylamine was used as a measure of ΔpH. As previ-

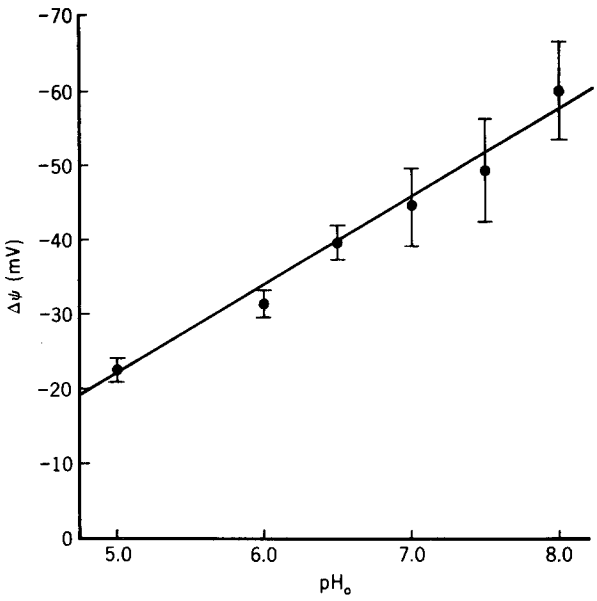

Fig. 3. Determination of $\Delta\psi$ as a function of external pH (pH_o). Vesicles were incubated for 60 min at 20°C as described in the legend to Fig. 1 in media containing 100 mM mannitol and 5 mM MES-Tris adjusted to the indicated pH. $\Delta\psi$ (in millivolts, inside negative) was calculated from the transmembrane distribution of $^{86}Rb^+$ using the Nernst equation, as described in Materials and Methods. Results represent means ± SE for determinations in duplicate using vesicles from three separate preparations

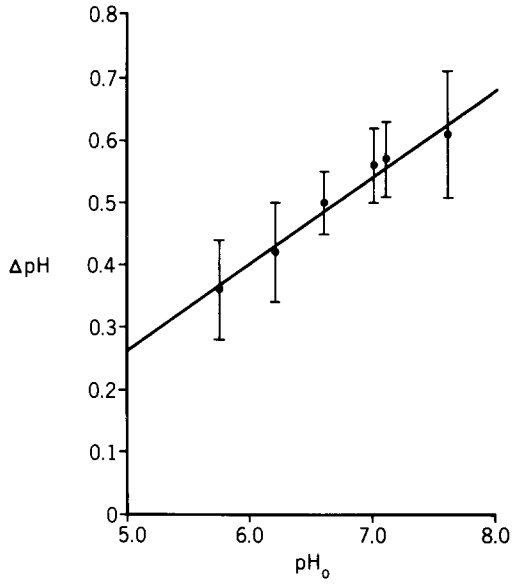

Fig. 4. Determination of ΔpH as a function of external pH (pH_o). Vesicles were incubated for 60 min at 20°C in the presence of ^{14}C-methylamine in media containing 100 mM mannitol and 5 mM MES-Tris adjusted to the indicated pH. ΔpH was calculated from the transmembrane distribution of ^{14}C-methylamine, as described in Materials and Methods. Results represent means ± 1 SE for determinations in triplicate using vesicles from two separate preparations

ously reported, vesicles incubated in a buffered 100 mM mannitol solution accumulated the probe to concentrations substantially higher than that of the medium (Dubinsky & Langridge-Smith, 1985; Langridge-Smith, & Dubinsky, 1985). Under control conditions, when the incubation medium was identical to the vesicle suspension buffer (pH 7.5), a ΔpH of 0.75 ± 0.07 (n = 6) was measured, corresponding to an intravesicular pH of 6.75. The accumulation of ^{14}C-methylamine inside the vesicles was dependent on the external pH (pH_o). The measured ΔpH declined as the acidity of the external medium was increased over the range from 7.6 to 5.5 pH units. The plot of ΔpH *vs.* pH_o (Fig. 4) exhibits a slope which is significantly less than one (0.14), indicating a finite permeability of the vesicle membrane to H^+, OH^- or a buffering species. Extrapolating this line to zero pH yields a value of 3.14, which represents an estimate of the lower limit for the intravesicular pH.

Relationship of $\Delta\psi$ to ΔpH

In order to determine the nature of the relationship between $\Delta\psi$ and ΔpH, the transmembrane distributions of ^{14}C-methylamine and $^{86}Rb^+$ were determined, using the same batch of vesicles in the presence of increasing concentration of K-gluconate. The results were calculated in terms of millivolt equivalents ($\Delta\psi$ and $\Delta\psi_{H^+}$) using the Nernst equation to compare directly the concentration ratio of both tracer solutes. A plot of $\Delta\psi_{H^+}$ *vs.* $\Delta\psi$ is shown in Fig. 5. The two parameters are clearly linearly related, with a slope of 0.96 (or 1 pH unit/57 mV) suggesting that protons are passively distributed according to the membrane potential.

Discussion

Tracheal apical membrane vesicles prepared for the present studies showed a high degree of purification. The enhancement of alkaline phosphatase specific activity (31-fold) compared favorably with enrichments of the apical marker enzyme (10- to 15-fold) commonly reported for luminal membrane preparations from other epithelia (Kessler et al., 1978; Turner & Silverman, 1978; Burckhardt et al., 1980). Tracheal luminal membranes isolated by the present procedure have previously been shown to be free of mitochondria, endoplasmic reticulum and nuclear constituents, and to have minimal contami-

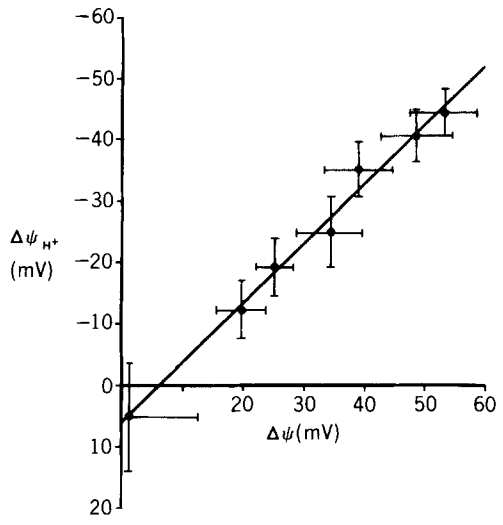

Fig. 5. Relationships between $\Delta\psi$ and $\Delta\psi_{H^+}$ at different concentrations of medium K^+. Vesicles were incubated for 60 min at 20°C in the presence of $^{86}Rb^+$ or ^{14}C-methylamine in media containing 2 mM HEPES-Tris (pH 7.5) and varying concentrations of K-gluconate from 0 to 50 mM. Solutions were balanced for osmolarity by appropriate concentrations of mannitol. Valinomycin (10 μg ml^{-1}) was present in all assays. $\Delta\psi$, and $\Delta\psi_{H^+}$ (in millivolts, inside negative) were calculated from the transmembrane distribution of $^{86}Rb^+$ and ^{14}C-methylamine, respectively, using the Nernst equation. The distribution of radiolabeled tracers was measured as described in Materials and Methods. Results represent means ± 1 SE for determinations in triplicate using vesicles from three separate preparations (SEM based on number of vesicle preparations, $n = 3$). $\Delta\psi_{H^+}$ *vs.* $\Delta\psi$; slope = 0.964 ($r^2 = 0.995$)

nation with basolateral membranes (Langridge-Smith et al., 1983).

Factors Controlling $\Delta\psi$ and ΔpH

The purified membrane vesicles have an acidic interior which was indicated by the intravesicular accumulation of a weak base. Methylamine accumulation in these studies resulted from a transmembrane ΔpH and not from intravesicular binding, as deduced from the following observations: (i) the measured ΔpH was much larger at an alkaline than at an acidic pH_o; (ii) increasing the ionic strength of the medium to 50 mM salt abolished ΔpH; and (iii) disruption of the vesicles with detergent caused complete release of accumulated amine. The observed effects of ionic strength on amine accumulation support previous studies suggesting that the ΔpH represents a passive distribution of H^+ dictated by a Donnan potential (Langridge-Smith & Dubinsky, 1985).

Estimates of the total buffering capacity of these membranes of 99 mg × liter^{-1} pH unit^{-1} are comparable to that measured in renal brush-border membranes in the presence of a detergent (Ives, 1985). In the present study of trachea vesicles, the titration reveals a range of titratable groups in both the vesicle interior and at the outer surface (Fig. 1). There was no discernable difference between the titration curves of the inner and outer surfaces except at the extreme of pH 5 and below. The buffer capacity of the intravesicular compartment is of greatest interest here since at the largest measured pH gradients the concentration of methylamine in the intravesicular compartment is 1 to 2 mM, considerably higher than the measured $[H^+]_i$. Assuming, even at the extreme of 2 mM intravesicular concentrations of protonated methylamine observed with a ΔpH of 0.7 pH units, from the measured buffering capacity of 51 meq liter^{-1} pH unit^{-1} we can calculate the change in the free H^+ concentration due to interaction with the probe to be about 0.04 pH units (β = dbase/dpH) well within the experimental error of the measurement. Thus the probe does not interfere with the determination.

The transmembrane ΔpH was linearly related to pH_o (Fig. 4). If the membrane were impermeable to H^+, then the internal H^+ should not vary and a slope of unity would be expected. Instead, a slope of 0.14 was obtained implying the movement of H^+ into the vesicle as the pH of the suspending medium was decreased. Over the range of pH_o examined, the calculated pH_i was always less than pH_o. Despite an increase in medium $[H^+]$ of over two orders of magnitude, a concentration gradient for H^+ was still evident. The maintenance of a proton gradient under these conditions is consistent with the existence of fixed negative charges on the vesicle interior comprised of ionizable groups which are titratable over the range of pH values tested. The presence of a finite proton permeability in the membrane would permit the titration of those groups with decreasing pH of the external medium.

A transmembrane potential in the tracheal vesicles was demonstrated by the accumulation of $^{86}Rb^+$ in the presence of valinomycin. As in the case of methylamine, Rb^+ accumulation was dependent on pH_o, was decreased by increasing medium ionic strength, and was abolished in the presence of a detergent. The linear relationship between $\Delta\psi$ and pH_o (Fig. 3) is further evidence for the presence of titratable ionizable groups at the vesicle interior. Clearly the predominant driving force for H^+ accumulation is the Donnan potential, since the H^+ distribution is in equilibrium with $\Delta\psi$ (Fig. 5). However, as pH_o is decreased, protons are taken up, and neutralize titratable anion with a resultant decline of $\Delta\psi$. Thus, the magnitude of both the Donnan potential and the pH gradient under these conditions is dependent not only upon the pH_o, but also upon the

protonic dissociation constant (pK_a) of internal ionizable groups.

The ionic strength of the suspending media is the second major determinant of the magnitude of the membrane potential. Under low-salt conditions (2 mM HEPES-Tris) the measured membrane potential approaches −60 mV. Increasing the medium ionic strength results in a decline in the measured $\Delta\psi$ (Fig. 4). At 50 mM salt and $pH_o = 7.5$, the membrane potential approaches zero. The relative dependence of the $\Delta\psi$ on ionic strength would be a function of the amount of internal fixed negative charge. In the tracheal apical membrane vesicles, the amount of negative charge was remarkably consistent from preparation to preparation. In all likelihood the presence of fixed internal charge is a general property of plasma membrane vesicles. However, the concentration of negative charge may vary in different types of tissue preparations. For example, Liedtke and Hopfer (1982) report a $\Delta\psi$ of −11 mV in intestinal brush-border vesicles at a salt concentration of 100 mM indicating a considerably greater concentration of fixed negative charge than in the present preparation.

The parallel changes in $\Delta\psi$ and ΔpH observed in response to a variety of imposed conditions, in conjunction with the evidence for a H^+ conductance in these vesicles discussed above, point to the dependence of ΔpH on the Donnan potential across the vesicle membrane. The existence of a proton gradient determined by a Donnan potential has also been demonstrated in lysosomes (Goldman & Rottenberg, 1973), and in neurohypophyseal granules (Scherman & Nordman, 1982). In platelet secretory α-granules Grinstein and Furuya (1983) found that only 50% of the measured ΔpH was collapsed at physiological ionic strength, and concluded that they were observing the co-existence of two types of granules—one in which H^+ passively equilibrates according to a Donnan potential, and another which retains a H^+ gradient generated *in vivo* and is preserved *in vitro* by a low membrane H^+ permeability. The fact that, in the tracheal vesicles, ΔpH was completely collapsed in the presence of 50 mM K-gluconate or KSCN, and that $\Delta\psi$ was abolished under the same conditions, implies that the pH gradient in this preparation is entirely attributable to a Donnan potential.

In membrane vesicle studies, mechanistic information about a given transport system is generally extracted from analyses of the driving forces and the kinetic properties of the particular system. The driving forces are investigated by varying the composition of the intravesicular and extravesicular fluid; kinetic parameters have been determined either on the basis of initial velocity data obtained under "optimal" driving force conditions (Turner, 1983; Kessler, Tannenbaum & Tannenbaum, 1978) or from measurements of radioisotope transport under equilibrium conditions (isotope exchange) (Hopfer, 1977; Liedtke & Hopfer, 1982). The results of the present study demonstrate the existence of significant transmembrane gradient of both the electrical potential and pH which are maintained without decline over a period of at least 60 min. Under appropriate experimental conditions, these endogenous gradients are the major determinant of the equilibrium distribution of H^+ and other permeant ionic solutes. Several parameters have been identified (i.e., impermeant negative charge, ionic strength, pH_o) which contribute to the generation and maintenance of a membrane potential and a pH gradient in tracheal vesicles. Both gradients can be adjusted over a wide range of values and held constant, or "clamped." Flux analysis can therefore be conducted under conditions of controlled membrane potential and/or pH gradient.

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