Biochimica et Biophysica Acta, 777 (1984) 84–92
Elsevier

BBA 72278

Cl^- TRANSPORT IN APICAL PLASMA MEMBRANE VESICLES ISOLATED FROM BOVINE TRACHEAL EPITHELIUM

JANE E. LANGRIDGE-SMITH [a,c,*], MICHAEL FIELD [b] and WILLIAM P. DUBINSKY [a]

[a] *Department of Physiology and Cell Biology, University of Texas Health Science Center at Houston, P.O. Box 20708, Houston, TX 77025 and* [b] *Departments of Medicine and of the Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637 (U.S.A.) and* [c] *Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JT (U.K.)*

(Received April 26th, 1984)

Key words: Membrane permeability; Cl^- transport; (Bovine tracheal epithelium)

The Cl^- transport properties of the luminal border of bovine tracheal epithelium have been investigated using a highly purified preparation of apical plasma membrane vesicles. Transport of Cl^- into an intravesicular space was demonstrated by (1) a linear inverse correlation between Cl^- uptake and medium osmolarity and (2) complete release of accumulated Cl^- by treatment with detergent. The rate of Cl^- uptake was highly temperature-sensitive and was enhanced by exchange diffusion, providing evidence for a carrier-mediated transport mechanism. Transport of Cl^- was not affected by the 'loop' diuretic bumetanide or by the stilbene-derivative anion-exchange inhibitors SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid). In the presence of the impermeant cation, tetramethylammonium (TMA^+), uptake of Cl^- was minimal; transport was stimulated equally by the substitution of either K^+ or Na^+ for TMA^+. Valinomycin in the presence of K^+ enhanced further Cl^- uptake, while amiloride reduced Na^+-stimulated Cl^- uptake towards the minimal level observed with TMA^+. These results suggest the following conclusions: (1) the tracheal vesicle membrane has a finite permeability to both Na^+ and K^+; (2) the membrane permeability to the medium counterion determines the rate of Cl^- uptake; (3) Cl^- transport is not specifically coupled with either Na^+ or K^+; and, finally (4) Cl^- crosses the tracheal luminal membrane via an electrogenic transport mechanism.

Introduction

Tracheobronchial secretions produced by the combined activities of the respiratory epithelial cells and submucosal glands are of major importance in protecting the airways from damage by inhaled foreign particles. Secretion of fluid and electrolytes across the lining epithelium of the trachea depends largely on the active transport of Cl^- from serosa to mucosa [1–3]. Studies of this mechanism in canine trachea [1,4–9] suggest that it conforms to the general model which has been proposed for a variety of Cl^--secreting epithelia [10]. According to the model, Cl^- enters the secretory cell across the basolateral membrane via an electrically neutral, furosemide-sensitive, Na^+-coupled transport process. Coupling of entry to the Na^+ gradient allows intracellular accumulation of Cl^-, and the electrochemical Cl^- gradient

* To whom correspondence should be addressed at (present address): Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JT, U.K.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; TMA^+, tetramethylammonium.

across the apical membrane provides the driving force for passive exit of Cl^- to the lumen. The energy for transepithelial Cl^- transport is provided indirectly by the basolateral Na^+,K^+-pump, which serves to maintain a low intracellular concentration of Na^+. Stimulation of Cl^- secretion by a variety of agents (e.g., epinephrine or prostaglandins) depends primarily on an increase in the Cl^- conductance of the apical membrane, an effect which appears to be mediated by the intracellular second messengers cAMP and/or Ca^{2+}. This model can accommodate most, if not all available data, but in order for it to be rigorously tested, it is necessary to define the transport properties of the individual surface membranes of the tracheal epithelial cell. One approach to this problem has been to use intracellular microelectrode techniques to determine the electrical potential profile of the secretory cells and the relative resistances of their limiting membranes in both resting and stimulated states. Equivalent circuit analysis of these electrical measurements in canine trachea suggests that stimulation of Cl^- secretion is accompanied by a primary increase in apical membrane Cl^- conductance, and also by a decrease in basolateral membrane resistance, which may be due to enhanced K^+ permeability [4,5,7,11–14]. However, the existence of the paracellular shunt pathway makes it impossible to simply determine the ionic permeabilities of the luminal and contraluminal membranes by this experimental method. A more direct approach is to physically isolate the apical and basolateral membranes and to use the purified membrane vesicle preparations to characterize the transport properties of the respective cell surfaces.

A method has recently been described for obtaining a highly purified, efficiently transporting plasma membrane vesicle preparation derived from the luminal border of bovine tracheal epithelium [15]. Measurements of transepithelial ion fluxes in intact bovine tracheal mucosa [16,17] have shown that this tissue resembles its canine counterpart in exhibiting both active Cl^- secretion and active Na^+ absorption. Agents which inhibit or stimulate ion transport in canine trachea have similar effects on ion movement and electrical parameters in bovine tissue [16–18], suggesting that the fundamental mechanisms and regulation of transepithelial ion transport are the same in both species. In the present study, the Cl^- transport properties of the luminal border of bovine tracheal epithelium have been investigated using the purified apical membrane vesicle preparation. The results indicate an electrogenic transport mechanism for Cl^-, which is not affected by the 'loop' diuretic bumetanide, or by the stilbene-derivative anion-exchange inhibitors SITS and DIDS. In addition, the data suggest that the apical membrane is permeable to both Na^+ and to K^+, and that the conductance to these two cations is approximately equal.

Materials and Methods

Preparation of apical membrane vesicles. Cattle tracheae were obtained from a local slaughterhouse. The trachea was removed 10–15 min after each animal had been killed and immediately placed in ice for transport to the laboratory. Tissue from five animals was used for each preparation. The procedure for obtaining purified plasma membrane vesicles from bovine tracheal mucosa has recently been described in detail [15]. In the present work, this procedure was slightly modified by homogenizing tracheal epithelial scrapings in a Waring Blendor (2 min at top speed) instead of a glass-and-Teflon pestle apparatus. Subsequent isolation of the apical membranes was achieved by Mg^{2+} precipitation and differential centrifugation, as previously described. Membranes were isolated in a medium comprising 100 mM mannitol/2 mM Hepes-Tris (pH 7.5) and were stored at a protein concentration of 4–6 mg/ml in a $-80°C$ freezer for subsequent transport studies.

Assay procedures. The tissue fractionation procedure and the purity of the final membrane preparation were monitored by assaying standard biochemical markers for nuclei, mitochondria, endoplasmic reticulum, and apical and basolateral plasma membranes. The assay procedures employed have been described in a previous publication [15]. Protein was estimated according to Lowry et al. [19], using bovine serum albumin as standard.

Transport measurements. Radiolabelled Cl^- influx was assayed using Dowex 1-X8 (50–100 mesh, formate form) columns, according to the proce-

dure of Gasko et al. [20]. Disposable Pasteur pipettes (5.75 inch) were packed with 1.5 ml anion-exchange resin, supported by a Dacron wool plug. The columns were washed with ice-cold 100 mM mannitol/2 mM Tris-sulfate (pH 7.5). Apical membrane vesicles were homogenized in 100 mM mannitol/2 mM Tris-sulfate (pH 7.5), and the protein concentration of the vesicle suspension was adjusted to 2–2.5 mg/ml. The vesicles were then incubated at 37°C for 30 min. In preliminary studies, this preincubation step was found to increase the transport rates for both Na^+ and Cl^- (data not shown), presumably by enhancing vesicle resealing; 30 min proved to be an optimum period for preincubation.

The transport assay incubation medium comprised 2 mM Tris-sulfate/20 mM $K^{36}Cl$/40 mM potassium-Hepes (pH 7.5). Additions to, or modifications of the composition of the incubation medium in particular experiments are detailed in the text and figure legends. Reactions were initiated by addition of 22 μl vesicles to 198 μl incubation medium at 20°C. Any addition to the reaction mixture or to the vesicle suspension was made prior to the start of the reaction. At predetermined times, 200 μl of the reaction mixture was placed directly on to a column of Dowex and immediately washed into the column with 0.25 ml of the ice-cold buffered mannitol solution, then eluted with 1.75 ml of the same solution. Transit time through the column was less than 10 s. The eluent was dissolved in 8 ml scintillation fluid and counted for radioisotope content. The specific activity of ^{36}Cl was determined from a standard sample of the total reaction mixture, and averaged 1000 cpm/nmol Cl. Fluxes were calculated in terms of nmol Cl^- transported per mg protein. Background counts (130–170 cpm), obtained by passing matched incubation media without vesicles through the columns, were measured and subtracted in each experiment. Zero-time points were obtained in matched incubation media at 0°C. All experiments were done in duplicate or triplicate.

Materials. Amiloride hydrochloride was obtained from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ); bumetanide from Hoffman-La Roche (Nutley, NJ); SITS from Calbiochem-Behring (La Jolla, CA); DIDS from Pierce (Rockford, IL); dithiothreitol from Boehringer-Mannheim Biochemicals (Indianapolis, IA); and ^{36}Cl (12.1 mCi/g Cl in 1.25 M HCl) from ICN (Irvine, CA). Hepes, Tris and Dowex were obtained from Sigma (St. Louis, MO). All other chemicals were of the highest quality available.

Results

The purity of the vesicle preparation was routinely evaluated from the enrichment of the apical membrane marker, alkaline phosphatase. The specific activity of the enzyme in the final membrane suspension was 103.5 ± 15.4 μmol P_i liberated/h per mg protein ($n = 8$). This represented a 28-fold enrichment over the specific activity of the whole homogenate. A full biochemical characterization was performed on only one batch of vesicles. In keeping with our previous study [15], the apical membrane vesicles were essentially free of nuclear material, mitochondria and endoplasmic reticulum (data not shown). The specific activity of $(Na^+ + K^+)$-ATPase was 4.5-times that of the homogenate, indicating the presence of only a relatively small amount of basolateral membrane material in the vesicle preparation.

Time-dependent uptake of Cl^- by the tracheal apical membrane vesicles is demonstrated in Fig.

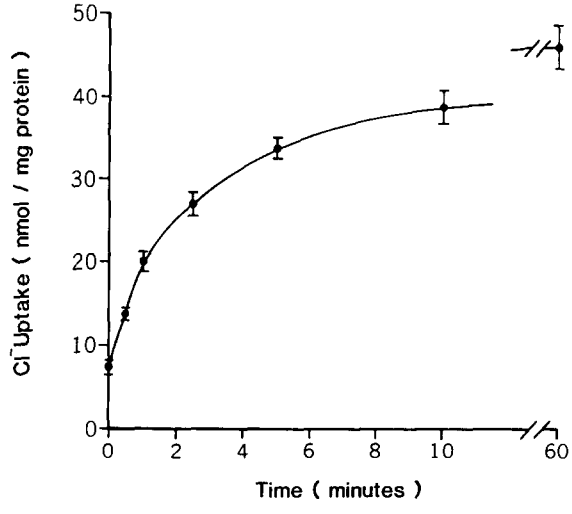

Fig. 1. Time-course of Cl^- uptake by tracheal apical membrane vesicles. Vesicles contained 2 mM Tris-sulfate, 100 mM mannitol (pH 7.5). The incubation medium was 20 mM $K^{36}Cl$ in 2 mM Tris-sulfate, 40 mM K-Hepes (pH 7.5), with valinomycin present at 5 μg/ml. Each value is the mean ± 1 S.E. of duplicate assays using five separate vesicle preparations.

1. Cl^- transport was measured in a K^+ medium in the presence of valinomycin in order to eliminate any rate-limiting effect of the membrane cation conductance. Under these conditions, the rate of Cl^- uptake by the vesicles was very rapid. However, due to limitations of the assay procedure, which will be discussed below, it was not possible to make accurate determinations of the initial rate of transport.

Uptake of a solute into vesicles may represent transport into the intravesicular space or binding to the exterior or interior of the vesicle membrane. Fig. 2 shows that the uptake of Cl^- at (or near) equilibrium was inversely related to the osmolarity of the incubation medium, varied by different concentrations of mannitol. The portion of uptake that is independent of the intravesicular space (i.e., binding) was calculated as 1.13 nmol/mg protein by extrapolation of the regression line to infinite

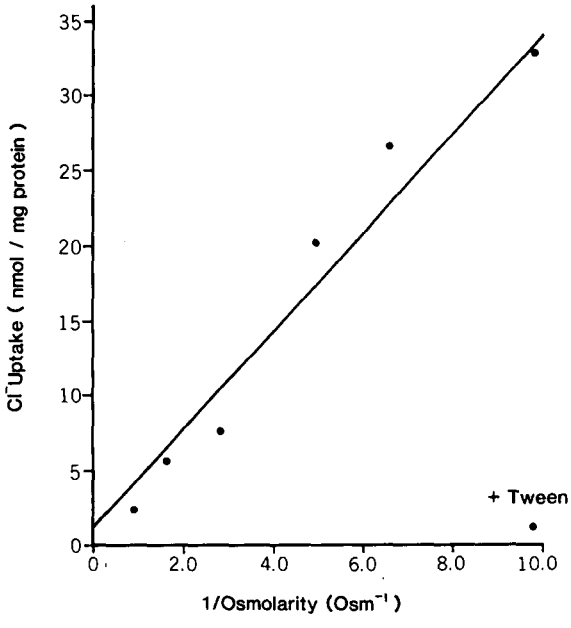

Fig. 2. Effect of medium osmolarity on Cl^- uptake by tracheal apical membrane vesicles. Vesicles contained 2 mM Tris-sulfate, 100 mM mannitol (pH 7.5). The incubation medium was 20 mM $K^{36}Cl$ in 2 mM Tris-sulfate, 40 mM K-Hepes (pH 7.5), with valinomycin present at 5 μg/ml. Medium osmolarity was increased by addition of mannitol. The incubation period was 60 min. The line, drawn by linear regression analysis, has a correlation coefficient $r = 0.98$. Also shown is the effect of addition of the nonionic detergent Tween-20 (0.1%).

osmolarity. This value represents only 2–3% of the total Cl^- uptake measured in the 100 mosM transport medium. A similar value of vesicle Cl^- (1.27 nmol/mg protein) was obtained following treatment of the membranes with the detergent Tween-20 (0.1%). Thus, complete release of accumulated Cl^- was observed upon detergent-induced disruption of vesicles, confirming that Cl^- is transported into the intravesicular space.

The calculated binding component for Cl^- is considerably lower than the zero-time value of 7.25 ± 0.85 nmol/mg protein obtained in the presence of K^+ and valinomycin (Fig. 1). This suggests that, at least under conditions of maximal transport activity, the initial uptake of Cl^- is too rapid to allow measurement of a true zero-time value by the present assay method. In practice, because of the time required to transfer the reaction mixture to the Dowex column, the 'zero-time' actually represents a 5–10 s time-point. To limit uptake during these few seconds, the measurement is made with the incubation medium at 0°C. However, the discrepancy between the value obtained and the Cl^- binding component (true zero uptake) suggests that even at 0°C, Cl^- uptake occurs at an extremely rapid rate. It was therefore of interest to examine the temperature-sensitivity of Cl^- transport in more detail. Fig. 3 shows the relationship between assay temperature and Cl^- uptake at 1.0 min. The plot demonstrates a clear temperature-dependence of the rate of Cl^- transport, despite the rapid uptake observed at 0°C.

Temperature-dependence of the Cl^- uptake rate does not of itself imply that transport occurs by a carrier-mediated process rather than by simple diffusion. Moreover, it was not possible to use the Arrhenius activation energy for transport as an indicator of the mechanism of membrane permeation, since calculation of this parameter was precluded by the inability to make accurate determinations of the initial rate of Cl^- uptake. In order to establish whether or not carrier function was involved in the transmembrane movement of Cl^-, experiments were performed to examine the effect on $^{36}Cl^-$ uptake of preloading the vesicles with Cl. Vesicles were loaded with either Cl ions or gluconate ions by preincubation with appropriate solutions for 4 h at 4°C. To eliminate the possibility of diffusion potentials being responsible for

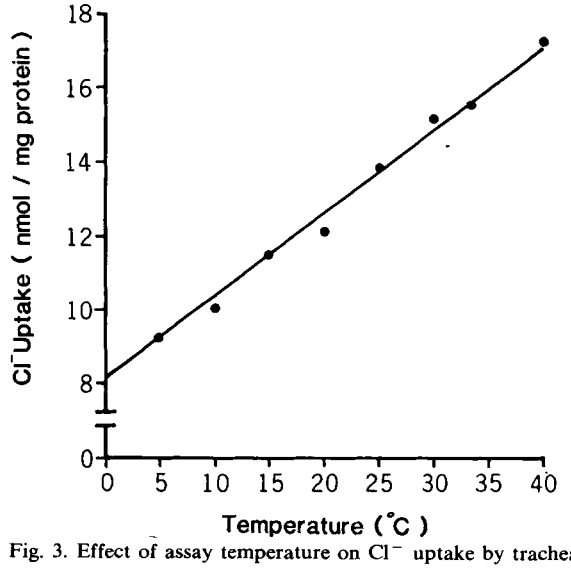

Fig. 3. Effect of assay temperature on Cl^- uptake by tracheal apical membrane vesicles. Vesicles contained 2 mM Tris-sulfate, 100 mM mannitol (pH 7.5). The incubation medium was 20 mM $K^{36}Cl$ in 2 mM Tris-sulfate, 40 mM K-Hepes (pH 7.5), with valinomycin present at 5 μg/ml. Cl^- uptake was assayed after 1.0 min, and the calculated Cl^--binding component of 1.13 nmol/mg protein (see text) was subtracted from uptake measurements to yield an approximation of the initial rate of transport. Each value represents the mean of duplicate assays using vesicles from a single preparation.

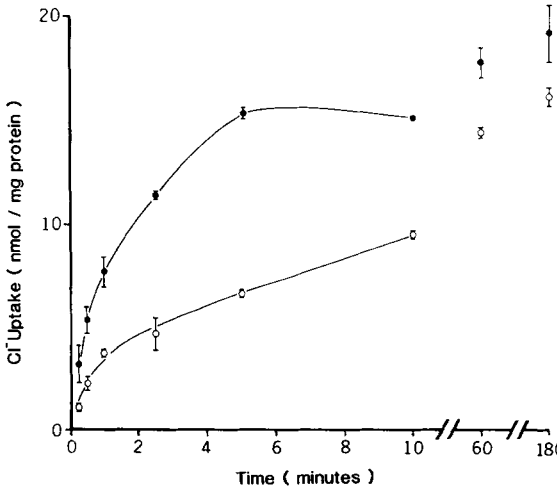

Fig. 4. Enhancement of $^{36}Cl^-$ uptake into tracheal apical membrane vesicles by exchange diffusion. Vesicles were loaded with 2 mM Hepes-Tris (pH 7.5), 100 mM mannitol, 100 mM potassium gluconate (○) or 100 mM KCl (●) and valinomycin (approx. 25 μg/mg protein) by preincubation on ice for 4 h. The transport medium contained 2 mM Hepes-Tris (pH 7.5), 100 mM mannitol, 80 mM potassium gluconate and 20 mM $K^{36}Cl$, with valinomycin present at 5 μg/ml. Each value is the mean ± 1 S.E. of triplicate assays using vesicles from a single preparation.

differences in ^{36}Cl uptake, the preequilibration solutions contained potassium salt at the same concentration as the transport (outside) medium. In addition, valinomycin was added to allow rapid movement of K^+ to compensate for any differences in transmembrane charge. As shown in Fig. 4, there was a clear enhancement of ^{36}Cl uptake into the Cl-loaded vesicles as compared to the vesicles loaded with gluconate. This experimental demonstration of countertransport stimulation provides convincing evidence for carrier-mediated transport.

Fig. 5 shows the time-course of Cl^- uptake into apical membrane vesicles measured in a Na^+ and a K^+ transport medium in the absence of ionophores. The two curves are approximately superimposable, suggesting that Cl^- transport is not specifically dependent on either cation or, alternatively, that both Na^+ and K^+ are equally effective in supporting Cl^- uptake via a cation-coupled Cl^-

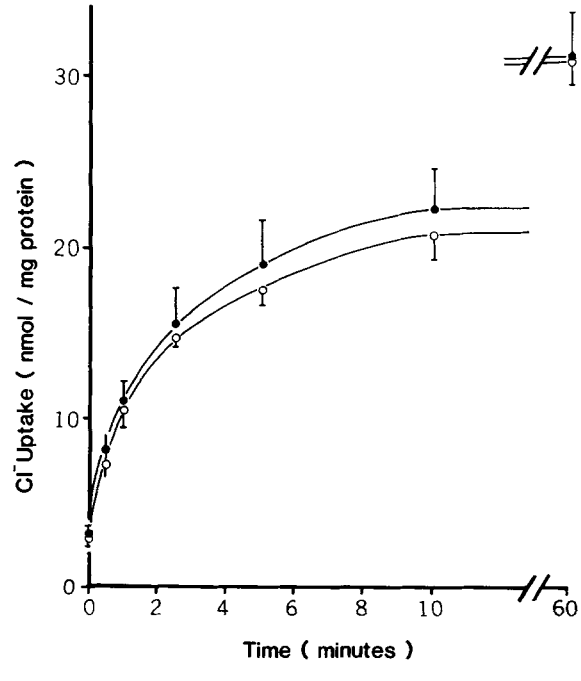

Fig. 5. Time-course of Cl^- uptake by tracheal apical membrane vesicles measured in the presence of Na^+ or K^+. Vesicles contained 2 mM Tris-sulfate, 100 mM mannitol (pH 7.5). The incubation medium was 20 mM $M^{36}Cl$ in 2 mM Tris-sulfate, 40 mM M-Hepes (pH 7.5), where $M = Na^+$ (●) or $M = K^+$ (○). Each value is the mean ± 1 S.E. of duplicate assays using two separate vesicle preparations.

transport mechanism. In order to investigate further the role of cations in the Cl^- transport process, uptake of Cl^- was measured in transport media containing (a) K^+, with or without valinomycin; (b) Na^+, with or without amiloride, and (c) TMA^+, a relatively impermeant cation. The results are presented in Table I. At 1.0 min, uptake of Cl^- in the presence of TMA^+ was minimal. Transport was stimulated by the substitution of either K^+ or Na^+ for TMA^+. The ionophore valinomycin in the presence of K^+ further enhanced Cl^- uptake. In contrast, addition of amiloride (1 mM) to the Na^+ medium reduced Cl^- uptake towards the level observed with TMA^+. Several conclusions may be drawn from these results. Firstly, Cl^- transport required the presence of either K^+ or Na^+, and each cation supported uptake equally. This would be consistent with a cation-coupled transport mechanism for Cl^-, in which either K^+ or Na^+ can act as cosubstrate. However, the further enhancement of Cl^- transport in a K^+ medium upon addition of valinomycin, a 'model' K^+ conductance, suggests that the rate of Cl^- uptake depends on the cation conductance of the membrane and that transport is not necessarily directly coupled to K^+. Stimulation of Cl^- uptake by Na^+ was inhibited by amiloride, which has been previously shown to block electrogenic Na^+ transport across the tracheal apical membrane [3,15]. The inhibition of Na^+-stimulated Cl^- transport by amiloride is therefore attributable to a decrease in membrane Na^+ conductance. Thus, it can be concluded that Cl^- uptake is not specifically coupled to either K^+ or Na^+, but instead is rate-limited by the membrane cation conductance. This, in turn, implies an independent, electrogenic mechanism for Cl^- transport.

The zero-time measurements shown in Table I exhibit a cation-dependence pattern identical to that seen at 1.0 min. In a TMA^+ medium, the zero-time value of 1.27 ± 0.43 nmol/mg protein is not significantly different from the nonspecific binding of Cl^- (Fig. 2), and probably represents true zero uptake. However, the introduction of K^+ or Na^+ doubled the zero-time measurement, and valinomycin in the presence of K^+ further increased the value, while addition of amiloride to the Na^+ medium lowered the value towards the level of nonspecific binding. These results indicate that the 'zero'-time measurements in reality reflect extremely rapid uptake of Cl^-, provided that conditions are favorable for Cl^- transport (i.e., that a permeant counterion is present).

At 60.0 min, the Cl^- influx measurements still show the same pattern of cation dependence (Table I). The value of Cl^- uptake at equilibrium would be expected to be the same regardless of the nature of the medium cation composition. The differences in the 60.0 min values suggest, therefore, that this time period is insufficient for Cl^- influx to have attained the level of equilibrium, especially under conditions where the membrane cation conductance limits the rate of Cl^- uptake. Studies of ion transport in other plasma membrane vesicle preparations have also shown that intervals of considerably longer than 60 min may be required before equilibrium is achieved. For

TABLE I

EFFECT OF MEDIUM CATIONS ON Cl^- UPTAKE BY TRACHEAL APICAL MEMBRANE VESICLES

Vesicles contained 2 mM Tris-sulfate, 100 mM mannitol (pH 7.5). Transport media contained 20 mM $M^{36}Cl$ in 2 mM Tris-sulfate, 40 mM M-Hepes (pH 7.5), where (1) $M = TMA^+$; (2) $M = K^+$; (3) $M = K^+$, with valinomycin present at 5 μg/ml; (4) $M = Na^+$; and (5) $M = Na^+$, with amiloride present at 1 mM. For assays in the presence of amiloride, vesicles were preincubated with 1 mM amiloride for 15 min at 20°C prior to the start of the reaction. Data tabulated are Cl^- uptake measurements in nmol/mg protein made at zero time, 1.0 and 60.0 min, and are means ± 1 S.E. of duplicate assays using three separate vesicle preparations.

Medium cation	Zero time	1.0 min	60.0 min
(1) TMA^+	1.27 ± 0.43	5.57 ± 0.35	23.70 ± 1.06
(2) K^+	2.58 ± 0.26	10.93 ± 1.02	34.10 ± 0.85
(3) K^+ + valinomycin	4.02 ± 0.59	14.90 ± 0.81	39.25 ± 1.05
(4) Na^+	2.68 ± 0.45	10.64 ± 0.81	34.25 ± 1.05
(5) Na^+ + amiloride	1.89 ± 0.45	7.66 ± 0.82	28.70 ± 0.61

example, Fan and colleagues [21] found that incubation periods of at least 150 min at 22°C were required before the rates of Na^+ uptake into ileal brush-border vesicles reached similar levels in the presence of different anions.

The mechanism of Cl^- transport across the tracheal apical membrane was investigated further by examining the effects of agents which inhibit specific modes of Cl^- transport in other tissues. The agents tested included the 'loop' diuretic bumetanide, and the disulfonic stilbene derivatives, SITS and DIDS. The inhibitors were preincubated with vesicles for 15 min at 20°C in addition to being present in the transport medium. Cl^- uptake at 1.0 min was measured in triplicate assays in a K^+ medium in the presence of valinomycin (5 μg/ml). In the absence of inhibitors, Cl^- uptake was 12.59 ± 0.59 nmol/mg protein. In the presence of bumetanide (100 μM), SITS (200 μM) and DIDS (100 μM), uptake was 12.20 ± 1.40, 12.80 ± 1.90 and 12.99 ± 1.75 nmol/mg protein, respectively. Therefore, at concentrations which give maximal inhibition in sensitive systems, all three agents were without effect on Cl^- transport into the tracheal apical membrane vesicles.

Discussion

The model for active Cl^- secretion in trachea and other secretory epithelia predicts that the uphill step in transepithelial Cl^- transport is at the basolateral or serosal membrane. The mechanism envisaged involves coupling of Cl^- and Na^+ entry across the basolateral membrane, with the Na^+ gradient providing the driving force for accumulation of Cl^- in the cell above electrochemical equilibrium. A Cl^- conductance in the apical membrane would then allow Cl^- to leave the cell in a secretory direction, and secretagogue control of such an apical membrane Cl^- conductance would provide a point for regulation of the rate of transepithelial secretion. This model is consistent with data obtained from studies in trachea [1,6,8,9], dog fish rectal gland [22] and frog cornea [23]. Intracellular microelectrode techniques have recently been applied to investigate the apical membrane Cl^- permeability of secretory epithelia. This experimental approach only permits a direct measure of apical membrane resistance in very tight epithelia (where the contribution of the paracellular shunt pathway to overall transepithelial conductance is negligible). However, electrical measurements in leaky epithelia can be interpreted by equivalent circuit model analysis. Studies of this kind in both trachea [4–7,11] and cornea [24,25] strongly suggest that stimulation of Cl^- secretion involves secretagogue-induced increase in apical membrane Cl^- conductance.

To provide an alternative and more direct approach to investigating the ion-transport properties of the tracheal luminal membrane, we recently developed a procedure for isolating highly purified apical membrane vesicles from bovine tracheal epithelium [15]. The value of membrane vesicle studies in defining the mechanisms of epithelial transport has been amply demonstrated, particularly in the case of Na^+-dependent processes, and the advantages and limitations of this experimental approach have received comprehensive review by, amongst others, Hopfer [26] and Murer and Kinne [27]. The present investigations of Cl^- transport in bovine tracheal apical membrane vesicles extend those previously reported [15], in which assays of Na^+ transport demonstrated the suitability of this vesicle preparation for transport studies. The efficient transport of Cl^- by the isolated tracheal vesicles was confirmed according to standard criteria. The demonstration of countertransport stimulation of $^{36}Cl^-$ uptake (Fig. 4), in addition to being a rigorous criterion for transport versus binding, provides strong evidence for carrier-mediation of Cl^- movement across the vesicle membrane.

Transport of Cl^- into the tracheal apical membrane vesicles was too rapid to permit accurate determinations of initial rates of uptake. Furthermore, uptake was insensitive to bumetanide and to the stilbene derivatives, SITS and DIDS. Without an adequate inhibitor to quench the transport reaction, measurements of uptake for periods of less than 10 s could not be made. The discrepancy between the apparent zero-time measurement of 7.25 nmol/mg protein (Fig. 1) and the calculated value of 1.13 nmol/mg protein for Cl^- binding (Fig. 2) suggests that the 'zero'-time measurement also includes a very rapid initial rate of transport. This conclusion is supported by the variatoin of

apparent zero-time measurements under different assay conditions (Table I). The most rapid rates of Cl^- transport were observed in a K^+ transport medium in the presence of valinomycin. An approximation of the initial rate of transport under these conditions obtained by subtracting the zero-time measurement from the uptake measured at 1.0 min yields a value of 13.13 nmol/min per mg protein, which is clearly a significant underestimate. If, instead, the calculated Cl^- binding component of 1.13 nmol/mg protein is subtracted as a more accurate measure of true 'zero uptake', the estimated initial rate of transport is 25.30 nmol/min per mg protein. It also becomes clear that by 1.0 min, the transport rate has already begun to deviate from linearity. In future studies, it might be possible to establish initial transport rates by measuring influx or efflux by a rapid reaction technique (e.g., a flow-quench apparatus) or by employing alternative assay procedures such as assessment of vesicle swelling or the use of membrane potential-sensitive dyes.

The effect of medium cations on Cl^- uptake (Table I) suggests a conductive mode for Cl^- transport into the tracheal vesicles. The insensitivity of Cl^- uptake to bumetanide and to SITS and DIDS provides support for this view. These agents are classically associated with inhibition of two modes of Cl^- transport in epithelia and other tissues: the stilbene derivatives inhibit anion-exchange mechanisms [28,29], while bumetanide and related 'loop' diuretic agents have been shown to interfere with NaCl, KCl and Na-K-Cl cotransport mechanisms in a variety of cell types [10,30]. The lack of effect of these agents in the present study is consistent with the conclusion that the principal mechanism of Cl^- transport across the tracheal apical membrane involves neither a specific coupling to cations nor an electroneutral anion exchange, but rather occurs via an independent, electrogenic process.

It is of interest to note that the presence of Na^+ or K^+ produced approximately equal stimulation of Cl^- transport. Recent electrophysiological studies in intact canine tracheal mucosa have yielded conflicting results concerning the K^+ conductance of the apical membrane of tracheal epithelium. Changes in the electrical parameters of tracheal epithelial cells in response to ion substitutions in the mucosal bathing medium led Welsh [12] to conclude that there was no appreciable apical conductance to K^+. However, from an analysis of electrical measurements made under similar conditions, Shorofsky and co-workers [6] concluded that the apical membrane is 40% more permeable to K^+ than to Na^+, in both resting and stimulated states ($P_{Na^+}/P_{K^+} = 0.7$). The results of the present study suggest that the apical membrane possesses both a K^+ conductance and an amiloride-sensitive Na^+ conductance, and that the permeability to these two cations is approximately equal. However, such indirect measures of membrane permeabilities in isolated vesicles must be viewed with caution, and further studies are clearly required to define the cation conductances of the tracheal apical membrane.

The investigations described in this report, together with those of a previous publication [15], have shown that isolated membrane vesicles constitute a valuable experimental tool for the study of ion-transport mechanisms in tracheal epithelium. In summary, our studies of Cl^- uptake in tracheal apical membrane vesicles provide evidence for a carrier-mediated, electrogenic Cl^- transport mechanism, which is in agreement with the findings reported for the intact epithelium. Further studies are underway to directly assess the relationship between Cl^- transport and membrane potential and also to investigate the possibility of coexisting pathways for Cl^- transport, such as electroneutral Cl^--OH^- or Cl^--HCO_3^- exchange. In addition, the vesicle preparation may be used to examine the effects of secretagogues and of intracellular mediators of secretion, such as cAMP and Ca^{2+}, in order to determine their influence on the permeability of the tracheal apical membrane to Cl^- and to other ions. Isolated membrane vesicles will therefore provide a simplified system in which to investigate the overall mechanism and regulation of tracheal epithelial secretion.

Acknowledgements

These investigations were supported by NIH grants AM 21345, AM 27377 and HL 26241. J.E.L.-S. was the recipient of a postdoctoral research fellowship from the Cystic Fibrosis Foundation and a Royal Society University Research Fellowship (held currently).

References

1 Al-Bazzazz, F.J. and Al-Aqwati, Q. (1979) J. Appl. Physiol. 46, 111–119
2 Olver, R.E., Davis, B., Marin, M.G. and Nadel, J.A. (1975) Am. Rev. Respir. Dis. 112, 811–815
3 Widdicombe, J.H. and Welsh, M.J. (1980) Fed. Proc. 39, 3062–3066
4 Shorofsky, S.R., Field, M. and Fozzard, H.A. (1982) Phil. Trans. R. Soc. Lond. B. 299, 597–607
5 Shorofsky, S.R., Field, M. and Fozzard, H.A. (1983) J. Membrane Biol. 72, 105–115
6 Shorofsky, S.R., Field, M. and Fozzard, H.A. (1984) J. Membrane Biol., in the press
7 Welsh, M.J., Smith, P.L. and Frizzell, R.A. (1983) J. Membrane Biol. 70, 227–238
8 Widdicombe, J.H., Basbaum, C.B. and Yee, J.A. (1979) J. Cell Biol. 82, 380–390
9 Widdicombe, J.H., Ueki, I.F., Bruderman, I. and Nadel, J.A. (1979) Am. Rev. Respir. Dis. 120, 385–392
10 Frizzell, R.A., Field, M. and Schultz, S.G. (1979) Am. J. Physiol. 236, F1–F8
11 Frizzell, R.A., Welsh, M.J. and Smith, P.L. (1981) Ann. N.Y. Acad. Sci. 372, 558–570
12 Welsh, M.J. (1983) Am. J. Physiol. 244, C377–C384
13 Welsh, M.J. (1983) Am. J. Physiol. 244, F639–F645
14 Smith, P.L. and Frizzell, R.A. (1984) J. Membrane Biol. 77, 187–199
15 Langridge-Smith, J.E., Field, M. and Dubinsky, W.P. (1983) Biochim. Biophys. Acta 731, 318–328
16 Langridge-Smith, J.E., Rao, M.C. and Field, M. (1984) Pflügers Arch., in the press
17 Vulliemin, P., Durand-Arczynska, W. and Durand, J. (1983) Plfügers Arch. 396, 54–59
18 Durand, J., Durand-Arczynska, W. and Haab, P. (1981) Pflügers Arch. 392, 40–45
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Gasko, O.D., Knowles, A.F., Schertzer, H.G., Suolinna, E-M. and Racker, E. (1976) Anal. Biochem. 72, 57–65
21 Fan, C.C., Faust, R.G. and Powell, D.W. (1983) Am. J. Physiol. 244, G375–G385
22 Welsh, M.J., Smith, P.L. and Frizzell, R.A. (1981) Clin. Res. 29, 480a
23 Zadunaisky, J.A., Spring, K.R. and Shindo, T. (1979) Fed. Proc. 38, 1059
24 Klyce, S.D. and Wong, R.K.S. (1977) J. Physiol. (Lond.) 266, 777–799
25 Nagel, W. and Reinach, P. (1980) J. Membrane Biol. 56, 73–79
26 Hopfer, U. (1978) Am. J. Physiol. 234, F89–F96
27 Murer, H. and Kinne, R. (1980) J. Membrane Biol. 55, 81–95
28 Cabantchik, Z.I. and Rothstein, A. (1970) J. Membrane Biol. 10, 311–330
29 Villereal, M.L. and Levinson, C. (1977) J. Cell Physiol. 90, 553–564
30 Palfrey, H.C. and Greengard, P. (1980) Ann. N.Y. Acad. Sci. 341, 134–138