# A Chloride-Activated $Na^+/HCO_3^-$-Coupled Transport Activity in Corneal Endothelial Membranes

Jason Lane, Christopher G. Wigham, and Stuart A. Hodson
Laboratory of Ocular Biophysics, Department of Optometry and Vision Sciences, Cardiff University, Cardiff CF1 3XF, Wales

ABSTRACT Investigations of corneal endothelium were made to resolve the apparent contradiction of the presence of sodium/bicarbonate cotransporter (NBC) in fresh and cultured cells and NBC's reported absence in isolated plasma membrane vesicles. Gradient-driven ion fluxes into the vesicles were measured. Short-term incubations (0–30 s) showed the presence of a bicarbonate-dependent inward sodium flux (BDSF), which was active when the insides of the vesicles were preloaded with chloride ions. The BDSF was absent if chloride was present only externally to the vesicles. Chloride at concentrations between 30 and 40 mM inside the vesicle had its maximum effect on BDSF. Other anions (acetate, thiocyanate, or gluconate) inside the vesicles did not mimic the chloride effect. Associated with the net inward sodium flux was a net inward bicarbonate flux. Hill plots of sodium influx with respect to external bicarbonate concentrations indicated that the stoichiometry of the net transfer was 1.7 ± 0.2 (mean ± standard error, $n = 5$) bicarbonate ions for each sodium ion transported. There was no net chloride flux found across the membrane vesicles. The finding of a novel chloride-activated NBC activity fully resolves the apparent contradiction between whole-cell and membrane vesicle preparations.

## INTRODUCTION

Jentsch and Wiederholt suggested in a series of experiments (Jentsch et al., 1984, 1985, 1988; Wiederholt et al., 1985) that the movement of sodium into cultured corneal endothelial cells is mediated via a sodium/bicarbonate cotransporter (NBC) and via a sodium/hydrogen exchanger (NHE). Bonnano and Giasson (1992a,b) showed that, although cultured and fresh corneal endothelia have different transport properties, both NBC activity and NHE activity are also present in fresh endothelial cell preparations. Studies with isolated membrane vesicles isolated from corneal endothelium confirmed the presence of NHE activity, but attempts to demonstrate NBC activity in corneal endothelial vesicles have so far yielded negative results (Wigham et al., 1994; Lane et al., 1997).

NBC activity was first suggested to exist after studies made on whole renal cell preparations (Boron and Boulpaep, 1983), and, in contrast to corneal endothelium, NBC activity has frequently been demonstrated in renal membrane vesicles (Grassl and Aronson, 1986; Hagenbuch et al., 1987; Soleimani and Aronson, 1989; Seki et al., 1993). Renal NBC activity appears to require no cofactors, although the basal activity can be stimulated (Eiam-Ong et al., 1993).

Corneal endothelium actively transports bicarbonate ions from the stroma to the surrounding aqueous humour (Hodson and Miller, 1976; Huff and Green, 1983; Wigham and Hodson, 1985), and the presence of NBC in these cells could help to explain the underlying phenomena of bicarbonate transport in these cells. We decided to reexamine corneal endothelial vesicles for NBC activity, but this time we extended our search range to look for possible cofactors to activate sodium/bicarbonate coupled transport. An early study on whole endothelial cell layers proposed that chloride ions had no effect on bicarbonate-related transport in these cells (Hodson, 1971), but a more detailed study later showed that chloride was in a subtle way necessary for corneal endothelial bicarbonate transport (Winkler et al., 1992). Along with other potential cofactors, we attempted to determine whether chloride might be involved in corneal endothelial NBC activity.

Studies of Na ion uptake into corneal endothelial vesicles are complicated, under certain ionic conditions, by the presence of early latent periods of up to 7 s, where the transport processes seems to be effectively "frozen" (Lane et al., 1999). In the current study, we adopted the protocol of examining the kinetics of uptake during the first 30 s of exposure, when any new ions were added to the external solutions to which the vesicles were exposed.

## MATERIALS AND METHODS

### Preparation and incubation of corneal endothelial cell plasma membrane vesicles

Ion uptake into membrane vesicles was based on our earlier published methodology (Wigham et al., 1994; Lane et al., 1997, 1999). Bovine eyes were obtained from a local abattoir 2–4 h post mortem, and the eyes were used immediately or refrigerated at 4°C for use within 24 h. For all of the experiments reported here, the scrapings from 15 ox corneal endothelia (~30 $cm^2$ of cell monolayer) made one preparation. The cell suspension was transferred to a 1-ml Potter-Elvehjem homogenizer and homogenized with four strokes of the plunger. This suspension was then centrifuged, using self-forming Percoll gradients under isopycnic centrifugation (Hodson and Hodson, 1988), for 30 min at 4°C at 29,000 rpm (80,000 × $g_{max}$; 60,000 × $g_{ave}$). After centrifugation three distinct bands were visible in the

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Address reprint requests to Dr. Stuart A. Hodson, Laboratory of Ocular Biophysics, Department of Optometry and Vision Sciences, Cardiff University, P.O. Box 905, Cardiff CF1 3XF, Wales. Tel.: +44-1222-874-374; Fax: +44-1222-874-859; E-mail: hodsonsa@cf.ac.uk.



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Percoll gradient, previously identified as band 1, the nuclear enriched fraction; band 2, the plasma membrane enriched fraction; and band 3, the mitochondrial enriched fraction (Hodson and Hodson, 1988). The plasma membrane fraction was removed and transferred into a clean centrifuge tube and centrifuged at 35,000 rpm (118,000 $\times$ $g_{max}$; 90,000 $\times$ $g_{ave}$) for 1 h to sediment out the membranes from the Percoll. After removal of the Percoll, the final pellet containing the membranes was resuspended in various solutions, which were buffered at pH 7.5 by 10 mM HEPES and 10 mM Tris and included 0.2 mM $CaSO_4$ and 10 mM $MgSO_4$. All solutions to which the vesicles were exposed also included 1 mM amiloride to inhibit sodiuma/hydrogen exchanger activity (Wigham et al., 1994; Lane et al., 1997). The resuspending solution also included either 250 mM sorbitol or isotonic substitutions of various salts, as described in the text, and acted as the initial intravesicular medium for each experiment. The plasma membrane suspensions were then passed rapidly through a 19-gauge hypodermic needle four times to form vesicles.

Radio tracer fluxes into or out of the vesicles were measured using a rapid filtration technique (Renner et al., 1989; Soleimani and Aronson, 1989; Lynch and McGivan, 1987, Lane et al., 1999). To initiate the transport reaction, 10 $\mu$l of vesicle suspension was rapidly mixed with 10 $\mu$l of bathing solution. The bathing solution always included 1 mM amiloride and was buffered at pH 7.5 by 10 mM HEPES and 10 mM Tris base and contained 0.2 mM $CaSO_4$ and 10 mM $MgSO_4$. There were also salts and sorbitol (specified for each experiment later) in the bathing solution, to give a final solution approximately isotonic to the internal vesicle solution. This bathing solution defines the extravesicular composition at the start of the experiment. The reaction was terminated after the appropriate time interval by the addition of 2 ml ice-cold stop solution containing (in mM) 250 sorbitol, 0.2 $CaSO_4$, 10 $MgSO_4$, 1 amiloride, 10 HEPES, and 10 Tris base at pH 7.5. The diluted suspension was quickly filtered through a prewetted membrane filter (HAWP 02500, 0.45-$\mu$m pore size) attached to a Millipore vacuum pump assembly under light suction. The filters were then washed with 3 $\times$ 3 ml of ice-cold stop solution, removed, and placed in 10 ml of scintillation fluid (Ecoscint). The activity of the solution was measured in a liquid scintillation counter. The activities were used to calculate uptake per milligram of protein. Vesicle protein was measured using the Coomassie blue protein assay reagent, with bovine serum albumin as the standard.

All data were corrected for nonspecific trapping of $^{22}Na$, $^{36}Cl$, or $H^{14}CO_3$ by the membranes and filters by subtracting the uptake observed at zero time. Zero time uptake was determined in the presence of radioactive substrate when the vesicle and stop solutions were added simultaneously. Viability of the vesicle population was determined by $Na^+$- versus $K^+$-dependent L-alanine accumulation (Van Amelsvoort et al., 1978; Sanchis et al., 1994; Lane et al., 1997). Orientation of the vesicles was determined by ouabain inhibition studies (Lane et al., 1999).

## Uptake of sodium into the vesicles

All solutions prepared for use both inside and outside the vesicles were made isotonic to 250 mM sorbitol by the appropriate addition of sorbitol in concentrations not given explicitly below.

Vesicles were preloaded with 40 mM KCl, 40 mM K acetate, 40 mM K gluconate, or 40 mM K thiocyanate, and $Na^+$ uptake was measured from external solutions including trace amounts of $^{22}Na$ acetate (11.5 kBq) plus either 25 mM $NaHCO_3$ or 25mM Na acetate by the protocol described above. Five determinations were made for 10-, 20-, or 30-s incubations on three separate preparations, and each preparation was pooled from 15 ox corneal endothelia.

Vesicles were preloaded with 0, 20, 30, 40, 50, or 75 mM KCl and exposed to 25 mM $NaHCO_3$, including 11.5 kBq $^{22}Na$ acetate for 15 or 30 s (five determinations $\times$ three preparations).

Vesicles were preloaded with sorbitol, and $Na^+$ uptake was measured from external solutions, including 11.5 kBq $^{22}Na$ acetate plus 25 mM $NaHCO_3$ plus 0, 4, 8, or 30 mM KCl for 15 s (5 $\times$ 3 preparations).

Vesicles were preloaded with 40 mM KCl, and $Na^+$ uptake was measured from external solutions including 11.5 kBq $^{22}Na$ acetate plus 1 mM NaCl plus 4, 8, 16, 25, or 40 mM $KHCO_3$ for 15 s (5 $\times$ 3 preparations). For these experiments, to maintain a pH of 7.5 at high bicarbonate concentrations the strength of the buffer was doubled to 20 mM HEPES and 20 mM Tris.

Vesicles were prepared in the presence of 7 $\mu$M valinomycin and depolarized with 40 mM KCl on each side of the membrane. The inside solutions contained the basal solutions plus 40 mM KCl, and $Na^+$ uptake was measured from an external solution including 11.5 kBq $^{22}Na$ acetate plus 25 mM $NaHCO_3$ plus 40mM $KHCO_3$ for 15 s (5 $\times$ 3 preparations). The uptake rates were compared to vesicles that were not pretreated with valinomycin but were otherwise treated with an identical protocol.

## The fluxes of chloride into and out of the vesicles

Inward flux studies were made with internal solutions including 40 mM KCl and external solutions including 12 kBq of tracer ($^{36}Cl$) plus 1 mM NaCl plus either 25 mM $NaHCO_3$ or 25mM Na acetate for 15 s (5 $\times$ 3 preparations).

Efflux studies were made with internal solutions including 12 kBq of tracer ($^{36}Cl$) plus 40 mM KCl and external solutions including 25 mM total sodium salt divided as 0 mM bicarbonate plus 25 mM acetate, or 4 mM bicarbonate plus 21 mM acetate, or 8 mM bicarbonate plus 17 mM acetate, or 25 mM bicarbonate. Fluxes with a duration of 15 s were investigated five times on three separate preparations.

## Bicarbonate movements into the vesicle

Influx studies were conducted using either 40 mM KCl or 40 mM K acetate inside the vesicles and 25 mM $NaHCO_3$, including 50 kBq of $H^HCO_3$ outside the vesicle (5 $\times$ 3 preparations; 15-s uptakes).

Determinations were also made with 40 mM KCl or 40 mM K acetate inside the vesicles and 25 mM $KHCO_3$ with 50 kBq of $H^HCO_3$ outside the vesicle to check for the reported $Cl^-/HCO_3^-$ activity found in cultured (Jentsch et al., 1988) and fresh (Bonnano and Giasson, 1992b) corneal endothelial cells. Single time point determinations of 15 s duration were made five times on three separate preparations.

Although there was no carbonic anhydrase activity detected in these purified membrane preparations, the spontaneous conversion of bicarbonate to $CO_2$ in the open environment of the filtration procedure used here would still probably result in a systematic underestimate of any bicarbonate influx due to outgassing. This error would act to reduce the signal.

## Reagents and materials

Percoll was obtained from Pharmacia LKB (Uppsala, Sweden); membrane filters were from Millipore (UK); $^{22}Na$ acetate was from NEN Dupont (USA); $Na^{36}Cl$ and $NaH^{14}CO_3$ were from the Radiochemical Centre, Amersham (UK); Ecoscint was from National Diagnostics (USA). All other reagents were obtained from Sigma (Poole, England).

## RESULTS

In all of the procedures reported here the vesicle populations maintained a constant profile with respect to activity and orientation. They showed a consistent and higher uptake of L-alanine in the presence of $Na^+$ compared to $K^+$ inward gradient (Lane et al., 1997), and ouabain inhibition studies showed the consistent and usual orientation (Lane et al., 1999) of the vesicles to be ~90% outside out.

Table 1 shows the effect of anions upon the initial sodium influx rates into the plasma membrane vesicles. In all of the experimental protocols whose results are illustrated in Table 1, the initial sodium gradient was 25 mM (0 outside and 0 mM inside). In general anions had no significant effect upon the influx of sodium, except in the single experimental condition, when there was simultaneously bicarbonate outside the vesicle and chloride inside the vesicle. Then the rate of sodium influx was significantly increased by ~0.61 nmole/s·mg protein.

Fig. 1 shows the time courses of sodium accumulation downhill into the vesicles, which are more rapid when there is, simultaneously, bicarbonate outside the vesicles and chloride inside the vesicles. When there is only bicarbonate outside the vesicle and no chloride inside, or when there is only chloride inside the vesicle and no bicarbonate outside, then the downhill $Na^+$ fluxes revert to a basal rate that was always present in our preparations but which we have not investigated further.

Further investigations indicated that the roles of the two anions, chloride and bicarbonate, in stimulating sodium transport were different. The vesicles exhibited a bicarbonate-dependent sodium influx of 0.61 ± 0.04 nmole/s·mg protein (mean ± SEM, $n = 5$ replicates on each of five preparations; Table 2) and a Na-dependent bicarbonate influx of 0.69 ± .07 nmole/s·mg protein (mean ± SEM, $n = 5$ replicates on each of three preparations). These data were taken with an initial gradient into the vesicle of 25 mM $NaHCO_3$ and 40 mM KCl inside the vesicle. Bicarbonate flux in the absence of Na was not enhanced (Table 2). In the absence of Na, we found no Cl-stimulated bicarbonate flux (Table 2), indicating no significant $Cl/HCO_3$ exchanger activity under the conditions we tested, although the bicarbonate influx activity was possibly (but not statistically significantly) higher in the presence of chloride. The stoichiometry of $Na^+$:$HCO_3^-$ from the direct flux data appears to be ~1:1, but, as pointed out in Materials and Methods, the bicarbonate flux is likely to be underestimated because of $CO_2$ outgassing from the vesicles. We also estimated stoichiometry of the fluxes by using Hill's equation (see later). The (what might be termed) nonstimulated basal fluxes for the two ions under similar conditions were (for $Na^+$ in 25 mM Na acetate) 0.99 ± 0.07 and (for bicarbonate in 25 mM $KHCO_3$) 0.58 ± 0.02 nmole/s·mg protein (Tables 1 and 2). In contrast to the findings for bicarbonate, we found no corresponding stimulated efflux of chloride from vesicles preloaded with 40 mM KCl (a total flux with external 25 mM sodium bicarbonate of 0.31 ± 0.7 and a basal flux with external 25 mM sodium acetate of 0.20 ± 0.6; mean ± SEM, $n = 5$ replicates on each of three preparations). It seemed possible that the small amount of external chloride that would wash over from the vesicle preparation as it was diluted by the bathing medium might have some effect, but when we tested for external chloride stimulation of the enhanced sodium and enhanced bicarbonate flux effect (chloride outside the vesicles only in concentrations up to 30 mM) we found none (Table 1 illustrates the sodium data). We also found no significant influx of chloride.

The concentration of chloride in the vesicles had a biphasic effect upon the stimulated sodium transport (Fig. 2). Maximum stimulation was observed at internal chloride concentrations in the range of 30–40 mM. This pattern of activation/inhibition cannot easily be explained by a straightforward kinetic analysis and will require further study. It could be that intracellular chloride levels in corneal endothelium act to regulate NBC activity in these cells. The

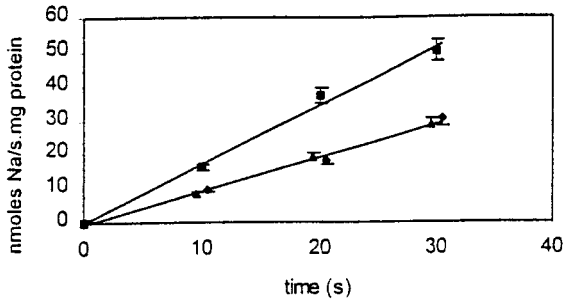


FIGURE 1 The uptake of sodium into corneal endothelium plasma membrane vesicles with 25 mM $NaHCO_3$ outside the vesicle and 40 mM KCl inside the vesicle (♦); 25 mM $NaHCO_3$ outside the vesicle and 40 mM K acetate inside the vesicle (▲); or 25 mM Na acetate outside the vesicles and 40 mM KCl inside the vesicles (●).

**TABLE 1 The effect of anions upon the initial accumulation rate of sodium into plasma membrane vesicles from corneal endothelia**

| | $Cl_o$ | $A_o$ | $Bic_o$ | $Bic_o + Cl_o$ |
|---|---|---|---|---|
| $Cl_i$ | 0.99 ± 0.07 | 0.94 ± 0.05 | 1.60 ± 0.04 | |
| $A_i/G_i/T_i$ | 1.01 ± 0.03 | 0.91 ± 0.09 | 0.89 ± 0.11 | |
| None in | | | 0.99 ± 0.06 | 1.05 ± 0.06 |

$Cl_i$, $A_i$, $G_i$, and $T_i$ represent the potassium salts at 40 mM chloride, acetate, gluconate, or thiocyanate, respectively, within the vesicles. $Cl_o$, $A_o$, and $Bic_o$ represent their sodium salts at 25 mM outside the vesicles, and ($Bic_o$ + $Cl_o$) represents an external solution including 25 mM sodium bicarbonate and 30 mM KCl. All solutions were made isotonic with 250 mM sorbitol by adding sorbitol and were buffered at pH 7.5. Units: nmole/s · mg protein.

**TABLE 2 The effect of various ions upon the initial accumulation rate of bicarbonate into plasma membrane vesicles from corneal endothelia**

| | $Na_o$ | $K_o$ |
|---|---|---|
| $Cl_i$ | 1.27 ± 0.06 | 0.58 ± 0.02 |
| $A_i$ | 0.52 ± 0.03 | 0.50 ± 0.05 |

$Cl_i$, and $A_i$ represent the potassium salts of chloride and acetate, respectively, at 40 mM within the vesicles. $Na_o$ and $K_o$ represent sodium and potassium salts of bicarbonate at 25 mM outside the vesicles. All solutions were made isotonic with 250 mM sorbitol by adding sorbitol and were buffered at pH 7.5. Units: nmole/s · mg protein.

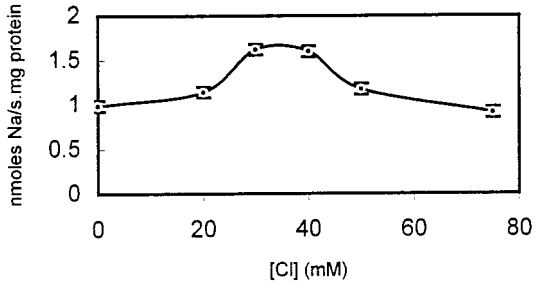


FIGURE 2 The effect of varying the concentration of intravesicular chloride (as KCl) on the influx of sodium, which was maintained at 25 mM $NaHCO_3$ outside the vesicle in all determinations. At zero chloride, sodium influx was ~1 nmole/s·mg protein and rose to a maximum of ~1.6 nmole/s·mg protein when the internal Cl concentration was at ~30–40 mM.

presence of chloride was necessary to stimulate the coupled transport of $Na^+$:$HCO_3^-$ , but chloride itself appeared not to be transported across the membrane. Other anions (acetate, thiocyanate, or gluconate), when substituted inside the vesicles, did not mimic the chloride effect and did not stimulate sodium and bicarbonate transport.

Fig. 3 shows a Hill plot in which the data on sodium fluxes were collected under conditions of constant inside chloride of 40 mM and constant outside sodium of 1 mM and variable external bicarbonate between 4 and 40 mM. $J$ represents the enhanced inward sodium flux, and $J_{max}$ represents its maximum value. The best fit line has a slope of 1.71 and a correlation coefficient of 0.92.

As the Hill plot indicated that the $Na^+$:$HCO_3^-$ coupling was electrogenic, whereas the direct flux method indicated that the process would not be electrogenic, we voltage-clamped the membranes by incorporating valinomycin in the membranes and adding 40 mM KCl to either side of the vesicle membrane. This strategy would inhibit the build-up of negative electrical potentials within the vesicles and act to promote the influx of $Na^+$ if the process were electrogenic and have no effect on the influx rates if the process were electroneutral. When we compared the influxes in valinomycin-treated and untreated membranes we found a significantly stimulated $Na^+$ transport rate when the vesicles were depolarized, from 0.95 ± 0.07 (untreated) to 1.62 ± 0.24 nmole/s·mg protein (valinomycin incorporated) (means ± SEM, $n = 15$; $p < 0.05$; one-sided $t$-test).

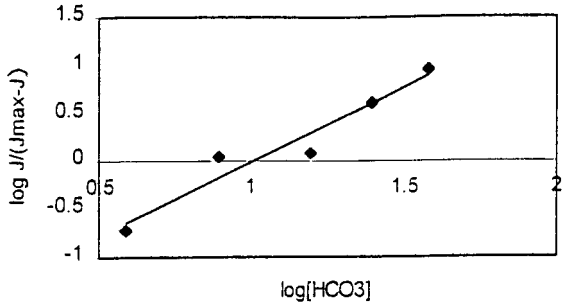


FIGURE 3 A Hill plot of the effect of external bicarbonate concentration on the influx of sodium into corneal endothelial vesicles, where $J$ represents the influx of sodium and $J_{max}$ is its greatest value.

## DISCUSSION

$Na^+$ influx into the vesicles is increased by the presence of external bicarbonate when chloride is inside the vesicles. Simultaneously there is an enhanced influx of bicarbonate. The enhanced sodium influx is not present if external bicarbonate is replaced by acetate or chloride. The stoichiometries of the sodium and bicarbonate fluxes were estimated in two ways, directly by comparing the sodium and bicarbonate flux magnitude under the same incubation conditions and indirectly by the use of a Hill plot. The direct method suggested a stoichiometry of 1.13 ± 0.08 ($HCO_3^-$ transported/$Na^+$), and the Hill plot suggested a stoichiometry of 1.7 ± 0.2 ($HCO_3^-$ transported/$Na^+$). The Hill plot data correspond well with the estimate of the stoichiometry suggested by Bonnano and Giasson (1992b). Our two estimates of the stoichiometry were significantly different, but it seems likely that the direct determination of stoichiometry may be underestimated because of potential losses of bicarbonate outgassing from the vesicles while they were transiently exposed to atmosphere through the filter for a few seconds. On the other hand, the stoichiometry for $Na^+$:$HCO_3^-$ could be 2:2 from the Hill plot, and this would not require the transport process to be electrogenic. We examined this possibility further by adding 7 $\mu$M valinomycin to the membranes and depolarizing them with 40 mM KCl on each side of the membrane when we found that the presence of valinomycin did not affect the basal sodium transport rate but did increase the uptake rate for $Na^+$ in the presence of bicarbonate. Overall the data suggest that the $Na^+$- and bicarbonate-coupled flux is probably electrogenic and support the data of Bonnano and Giasson (1992b). The data also indicate that NBC activity in corneal endothelium plasma membrane vesicles requires the presence of chloride. Jentsch et al. (1985) reported that the overall amplitude of intracellular voltage changes induced by $[HCO_3^-]_o$ changes were reduced when $Cl^-$ was replaced with other anions. The single exception to this phenomenon was that when bromide was substituted for chloride the amplitude reduction was markedly lessened. Winkler et al. (1992) also pointed out that in whole-cell studies, the chloride effect could be substituted well by nitrate. It will be interesting to note in future studies whether bromide or nitrate has an effect similar to that of chloride in activating NBC.

These studies do not prove that the activity we report here has a relationship to the known properties of these cells in translocating bicarbonate, but certain features appear to correlate. Most obvious is the common property of chloride dependence. Another correlation concerns the observation that the bicarbonate pump in vivo may be stimulated by exposure to higher concentrations of bicarbonate than are

found in the aqueous humor. Corneal endothelium bicarbonate pump activity is stimulated by transferring whole corneas from 25 to 45 mM bicarbonate (Riley et al. 1997). We noted during preliminary studies of our Hill plot that the maximum $Na^+$ transport activity of the NBC was also surprisingly high at ~50 mM bicarbonate. Another possible correlation between the NBC activity reported here and the pump was the $K_m$ for the bicarbonate concentration, which generated a half-maximum $Na^+$ flux. We found the value to be 10 ± 2 mM (the *intercept* in Fig. 3), which agrees well with the $K_m$ given for NBC in rabbit renal cortical cells of 9.7 mM (Grassl and Aronson, 1986), and which is near the figure of bicarbonate concentration, which causes half-maximum inhibition of transendothelial short-circuit current (Wigham and Hodson, 1981). As the relationship between short-circuit current and net bicarbonate flux through the cells is not fully understood, however, this apparent correlation is not convincing. Further experiments investigating the net bicarbonate flux across the monolayer while NHE activity is inhibited, which appear not to have been done yet, would provide better evidence for any possible relationship between vesicle NBC activity and transendothelial bicarbonate fluxes. This activity corresponds well with the experiments, which indicate two pathways for sodium reentry into the cells, of approximately equal magnitude, one of which utilizes NBC and the other of which utilizes NHE (Jentsch et al., 1985; Wigham et al., 1996). The NBC activity reported here is approximately equal in magnitude to the previously reported NHE activity in these preparations (Wigham et al., 1994). There remains the question of the identity of the basal sodium flux across these vesicles shown in Fig. 1, which is similar in magnitude to the basal sodium fluxes reported when the vesicles have their transport activities inhibited with the simultaneous presence of 1 mM each of amiloride + furosemide + 4,4′-diisothiocyanatostilben-2,2′-disulfonic acid (Wigham et al., 1994). This resilient sodium flux remains unidentified.

While this paper was being reviewed Usui et al. (1999) report the presence of NBC cotransporter mRNA in cultured human corneal cells of two types: kNBC-1 and pNBC-1. In contrast to the data presented by the studies of Jentsch et al. (1985) on tissue-cultured bovine cells, this report proposes that the NBC activity is independent of $Cl^-$ in human tissue-cultured cells.

Our own unpublished data suggest that NBC mRNA in fresh human and bovine corneal endothelial cells is neither kNBC-1 nor pNBC-1. Clearly, further studies are needed to resolve the current discrepancies.

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