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CYTOPLASMIC pH AND GOBLET CAVITY pH IN THE POSTERIOR MIDGUT OF THE TOBACCO HORNWORM *MANDUCA SEXTA*

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Summary

In the larval lepidopteran midgut, the major energy-requiring step of transepithelial K^+ secretion occurs across the goblet cell apical membrane. Studies of vesicles of goblet cell apical membrane suggest that K^+ secretion across this membrane is a secondary active process in which electroneutral K^+/H^+ antiport is driven by primary electrogenic H^+ secretion. Transbasal K^+ movement is thermodynamically passive under standard conditions, but the presence of an active process is revealed in hypoxic solutions or at low extracellular K^+ concentration.

We measured the pH of cytoplasm and goblet cell cavities, together with the corresponding transmembrane voltages, using double-barreled pH and voltage-sensing microelectrodes. For short-circuited midguts in standard bathing solution (pH 8.0) the weighted mean of cytoplasmic pH was 7.14±0.06 (mean±S.E.M.), an average of 0.34 units more acid than expected for electrochemical equilibrium with the hemolymphal solution. The mean pH of goblet cavities was 7.23±0.11, 1.62 units more acid than expected for equilibrium with the luminal solution. The pH gradient across the goblet cell apical membrane is thus of the wrong polarity to drive K^+ secretion by electroneutral K^+/H^+ antiport; however, if the exchange ratio were two or more H^+ per K^+, the cavity-positive electrical potential could drive H^+ back to the goblet cell cytoplasm coupled to K^+ secretion from cytoplasm to the goblet cavity.

Insensitivity of the goblet cavity pH to the change in the transvalve voltage caused by open-circuiting suggests either that the goblet cavity pH is well regulated or that the valve connecting the cavity to the gut lumen poses a significant barrier to protons. Transbasal potential and cytoplasmic pH were insensitive to a decrease of hemolymphal pH to 6.8, suggesting that the basal membrane is relatively nonconductive to H^+. Intracellular pH was unaffected by a

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decrease of hemolymphal K^+ to 5 mmol l^{-1}, suggesting that K^+/H^+ exchange is not important for transbasal K^+ uptake.

Introduction

The isolated posterior midgut of the tobacco hornworm actively transports K^+ from hemolymphal to luminal side (Harvey and Nedergaard, 1964) and, at a lower rate, Cl^- from luminal to hemolymphal side (Chao *et al.* 1989). *In situ*, the midgut epithelium mediates a substantial pH gradient: the hemolymph pH is about 6.7 and the luminal pH is as high as 11.0 in the middle midgut, decreasing to about 9 in the posterior midgut (Dow, 1984). The roles of different parts of the midgut in generating and sustaining the pH gradient are still unclear, but recent reports suggest that the alkalization of midgut contents is carried out primarily by the anterior and middle regions (Chamberlin, 1990; Dow and O'Donnell, 1990).

The midgut epithelium contains two major cell types: columnar cells, typical of those found in many invertebrate absorptive epithelia, and specialized goblet cells. The goblet cells are characterized by mucoprotein-filled apical cavities that are represented as communicating with the luminal surface of the epithelium by valves each containing a single tortuous passage (Anderson and Harvey, 1966; Flower and Filshie, 1976; Schultz and Jungreis, 1977; Cioffi, 1979). In electron micrographs of thin sections, valves of different cells in the same tissue may appear to be closed or open (Flower and Filshie, 1976). The large electrochemical gradients across the valve and the retention of ionophoresed Ni^{2+} or Lucifer Yellow dye by the cavity suggest that in any case the valve characteristically represents a substantial barrier to diffusion of solutes (Moffett and Koch, 1988*b*; Chao *et al.* 1989; Dow and Peacock, 1989).

Goblet cells have long been implicated in active transepithelial transport of K^+ and other alkali metals (Anderson and Harvey, 1966; reviewed by Dow, 1986). Potassium is actively transported into the goblet cell cavity across the goblet cell apical membrane (GCAM) against an electrical gradient of about 40 mV (goblet cavity positive to cytoplasm) but essentially no concentration gradient (Dow *et al.* 1984; Moffett and Koch, 1988*a*,*b*). Studies of isolated membrane vesicles of GCAM suggest that there is an ATP-dependent electrogenic proton pump directed into the goblet cell cavity (Wieczorek *et al.* 1989). Wieczorek *et al.* (1989) proposed that a H^+ electrochemical gradient established by this pump between cytoplasm and goblet cavity drives K^+/H^+ exchange *via* an electroneutral antiporter.

The present studies show that the electrochemical gradient of H^+ across the GCAM of intact goblet cells is consistent with active H^+ secretion from cytoplasm to goblet cavity. However, the concentration term of the H^+ electrochemical gradient (the transapical pH gradient) is small and in the wrong direction to drive K^+/H^+ exchange. Energization of K^+ secretion by the transapical electrical potential would require that more than one H^+ be exchanged for each K^+.

Materials and methods

Insects

Larvae of *Manduca sexta* were reared in continuous culture, supplemented at times by purchase of eggs from Carolina Biological Supply (Burlington, NC). The animals used in this study weighed between 5.9 and 8.6 g.

The morphologically distinct posterior midgut (Cioffi, 1979) was removed from cold-anesthetized fifth-instar larvae, mounted hemolymphal side up in a chamber patterned after that of Thompson *et al.* (1982), and gravity-superfused with bathing solutions equilibrated with 100 % O_2. Except as noted, the tissue was continuously short-circuited with an automatic voltage-clamp with solution resistance compensation. Electrode penetrations were made from the hemolymphal side with electrical reference to the hemolymphal-side bathing solution.

Bathing solutions

Except as noted, tissues were continuously superfused with oxygenated '32KS' solution containing 32 mmol l^{-1} KCl, 166 mmol l^{-1} sucrose and 5 mmol l^{-1} each of $CaCl_2$, $MgCl_2$ and Tris–HCl (pH 8.0). '5KS' solution contains 5 mmol l^{-1} KCl with 27 mmol l^{-1} NaCl substituted to maintain the electrical resistance of the solution similar to that of 32KS. In some experiments, the pH of the hemolymphal side solution was set at 6.8 with Mes substituted for Tris in this solution.

Electrodes

Double-barreled electrodes were pulled as previously described (Chao *et al.* 1989, 1990); the procedure of Ammann *et al.* (1981) was used to make one barrel H^+-selective. In brief, the barrel destined to become H^+-selective was silanized by exposure to hexamethyldisilazine vapor for 35 min. The tip of this barrel was filled with H^+-selective ion exchange resin (proton cocktail 1, Fluka). Resin-filled electrodes were equilibrated in an atmosphere of 100 % CO_2 at least 16 h. Before use, electrodes were removed from the CO_2 atmosphere and the H^+ barrel was backfilled with buffer solution containing 40 mmol l^{-1} KH_2PO_4, 15 mmol l^{-1} NaCl and 23 mmol l^{-1} NaOH adjusted to pH 7.0. The reference barrel was filled with 1 mol l^{-1} KCl. The calibration curves of acceptable electrodes had slopes of 53–60 mV pH unit^{-1} over the calibration range 6.7–8.4 pH units. The calibration curves were generated by a computer program that fitted them to either the Nicolsky equation or the Nernst equation. In most cases a fit to the Nernst equation was found to be more appropriate. H^+ barrels were calibrated at the beginning and at the end of each experiment. Data from experiments in which the two calibration curves differed by more than 2 mV per decade were rejected.

The reference barrel and H^+-selective barrel of the microelectrode were connected to the two inputs of a Keithley 604 differential electrometer. The corresponding outputs of the first-stage amplifiers are the transmembrane electrical potential and the uncorrected H^+-specific voltage. The output of the differential amplifier of the Keithley electrometer is the corrected H^+-specific

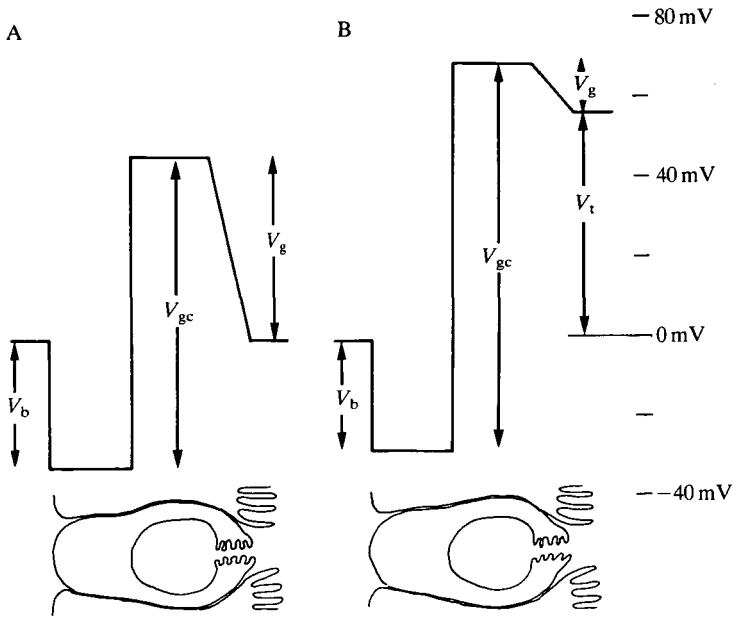

Fig. 1. The electrical potential profiles of short-circuited midgut (A) and open-circuited midgut (B) measured by advancing the microelectrode from the reference solution on the hemolymphal (left) side to the luminal solution on the right side. In each case, the horizontal axis reflects the position of the electrode in the epithelium as indicated by the goblet cell diagram below each trace. V_b, transbasal voltage step; V_{gc}, voltage step between cytoplasm and goblet cavity; V_g, voltage drop between goblet cavity and luminal solution; V_t, transepithelial potential. Under short-circuit conditions (A), V_t is zero. See Materials and methods for further discussion. The values of voltage steps shown are means determined in the present study.

voltage and was used to compute cytoplasmic pH (pHi) and goblet cavity pH (pHg) from the calibration curve of the H^+ barrel. The three outputs of the electrometer and the short-circuit current were recorded using a Gould Brush 2400 four-channel pen recorder. As in previous studies, penetrations that became unstable or did not return to within 2 mV of the baseline on withdrawal of the electrode were rejected.

Fig. 1 shows idealized potential profiles of the short-circuited and open-circuited midgut. Penetration of a midgut cell was signalled by a sharp deflection of the reference trace to at least −20 mV negative to the hemolymphal solution (Moffett *et al.* 1982). This step is the transbasal potential (V_b). As shown by Moffett *et al.* (1982) and Thomas and May (1984), V_b of goblet cells is indistinguishable from that of columnar cells, and other evidence suggests that the two cell types are effectively syncytial (Moffett and Koch, 1988*a*).

Following penetration of the basal membrane, penetration of a goblet cavity was indicated by a sharp deflection of at least 20 mV positive to the hemolymphal side reference electrode (Moffett and Koch, 1988*b*). The voltage differenc

Table 1. *Tissue electrical parameters, measured cytoplasmic and goblet cavity pH and differences between measured pH and computed equilibrium pH under three experimental conditions: short circuit, open circuit and open circuit with decreased hemolymphal pH*

V_b (mV)	pHi	$pHi_{eq}-pHi$	V_g (mV)	pHg	$pHg_{eq}-pHg$	I_{sc} ($\mu A\,cm^{-2}$)
Short circuit, pH=8.0 both sides						
−31.7±0.99	7.14±0.06	0.34±0.05	45.9±2.79	7.23±0.11	1.62±0.11	211±25.6
Open circuit, pH=8.0 both sides						
−27.4±1.09	7.02±0.05	0.53±0.07	12.4±10.82	7.21±0.39	0.09±0.78	56±7.9
Open circuit, hemolymphal pH=6.8; luminal pH=8.0						
−26.9±1.03	7.16±0.22	−0.83±0.23	−8.9±0.66	7.39±0.20	0.20±0.09	57±13.2

Equilibrium pH values are computed with respect to the extracellular solution.
Means are weighted averages (see Materials and methods).

between the luminal solution and the goblet cavity, referred to the goblet cavity, is designated V_g. Under short-circuit conditions, when the transepithelial potential (V_t) is zero, V_g is equal to the positive step seen when the electrode passes from cytoplasm into a goblet cavity (Fig. 1A). Under open-circuit conditions, the goblet cavity is typically positive to the lumen by 5–15 mV (Fig. 1B). For any individual goblet cell, the potential across the GCAM (V_{gc}) is equal to $V_t+V_g-V_b$.

Statistics

The grand means of determinations of V_b, pHi, V_g and pHg given in Table 1 are weighted averages of the means of individual experiments; the variance of each individual experiment is the weighting factor.

Results

pHi and pHg under standard conditions

In most recent studies of K^+ transport, the midgut has been held under continuous short-circuit conditions in 32KS oxygenated with 100% O_2. Voltage and pH traces from a typical transbasal penetration made under these conditions are shown in Fig. 2A. The weighted mean of V_b was −31.7 mV and that of pHi was 7.14 in 71 penetrations from 10 midguts (Table 1). As in previous studies (Moffett and Koch, 1988*a*), there was neither a consistent trend in the values of V_b recorded from different cells of the same tissue at different times after mounting, nor a correlation of V_b and short-circuit current (I_{sc}) in pooled data. Similarly, we were unable to detect a relationship between the magnitudes of pHi and either V_b or I_{sc}.

For each penetration into cytoplasm, the corresponding equilibrium pH (pHi_{eq}) was calculated from the measured hemolymphal pH and V_b using the Nernst equation. For all the 71 cells penetrated, the cytoplasm was more acidic than predicted for equilibrium; the mean difference was 0.34 pH units (Table 1).

Representative voltage and pH traces from a penetration of a cell followed

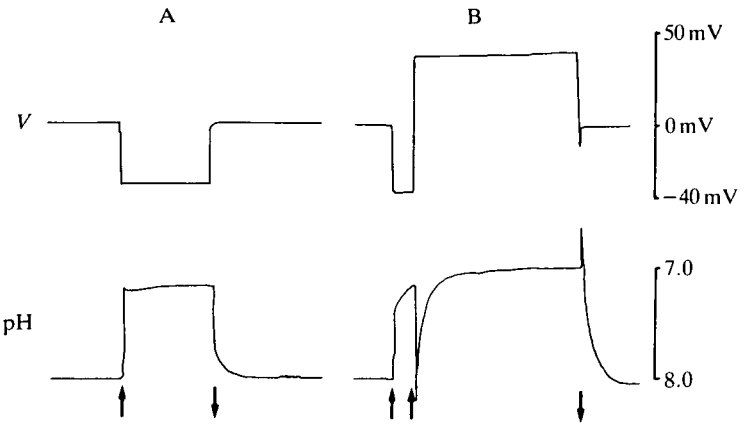

Fig. 2. Typical voltage and pH traces for a transbasal penetration (A) and a transbasal penetration followed by penetration of a goblet cavity (B). Upward-pointing arrows indicate advance of the electrode; downward-pointing arrows withdrawal of the electrode.

immediately by a penetration of a goblet cavity are shown in Fig. 2B. The weighted mean of V_g was 45.9 mV and that of pHg was 7.23 in 33 penetrations from 10 tissues (Table 1). For each penetration of a goblet cavity, the equilibrium pH of the cavity (pHg_{eq}) may be calculated with reference to the luminal solution, since under short-circuit V_g is equal to the voltage between the electrode in the goblet cavity and the luminal solution (see Fig. 1). Since the goblet cavity is electrically positive to the lumen, pHg_{eq} is higher than the pH of the luminal solution. According to this calculation, the mean measured pHg is more than 1.6 pH units lower than expected for equilibrium with the luminal solution (Table 1). This difference was consistent: all of the 33 goblet cavities penetrated in this study were more acid than the equilibrium value calculated with respect to the gut lumen.

As has been noted in many previous reports (reviewed by Dow, 1986), there was a steady decline in I_{sc} with time. This decline was accompanied by a decrease in V_g. Fig. 3 shows a representative experiment in which values of V_g, determined at different times after mounting the midgut, are plotted against corresponding values of normalized I_{sc}. Each value of V_b represents a different cell. The regression line is the weighted mean of the linear regressions of points from a total of four such experiments.

In Fig. 4, the values of pHg corresponding to the V_g values shown in Fig. 3 are plotted as a function of normalized I_{sc}; the regression line is the weighted mean of the regressions of the four experiments. This result shows that, in contrast to V_g and I_{sc}, pHg is relatively stable over the life of the isolated preparation. However, it should be noted that the decrease of V_g with time progressively lessened the difference between pHg and pHg_{eq}.

In its track through the epithelium, the electrode tip may well pass laterally into a goblet cavity after being inserted initially in the cytoplasm of an adjacent

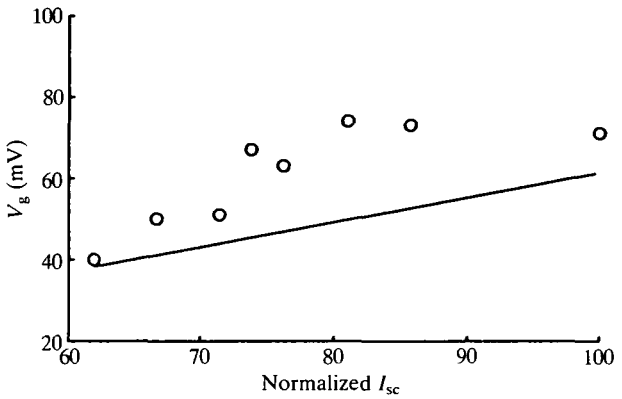

Fig. 3. The relationship of V_g to short-circuit current (I_{sc}). In this and the following plot, I_{sc} is normalized to its maximum value in each experiment. The individual points show values from a single representative experiment; the slope of the solid line (61.7±17.58 mV/100 %) is the weighted mean of the slopes of the individual regressions. The intercept is the mean of the intercepts of the individual regressions.

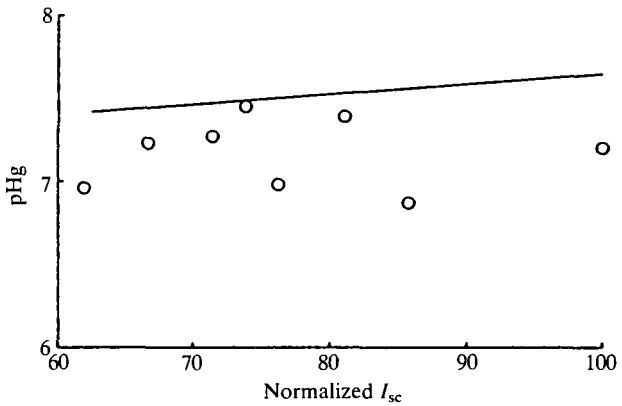

Fig. 4. The relationship of pHg to I_{sc}. The experiments are the same as in Fig. 3, and points from the same representative experiment are shown. The slope of the solid line (0.62±0.75 units/100 %) is the weighted mean of the slopes of the individual regressions. The intercept is the mean of the intercepts of the individual regressions.

columnar cell. However, previous studies did not find a difference in values of V_b between goblet cells and columnar cells (Moffett *et al.* 1982; Thomas and May, 1984) and suggested that goblet cells are effectively syncytial with adjacent columnar cells (Moffett and Koch, 1988*a*). Using the assumption of cytoplasmic uniformity between goblet and columnar cells, the mean transapical H^+ electrochemical gradient can be calculated using the mean values of V_g, V_b, pHi and pHg reported in the first row of Table 1. For mean $V_b = -32$ mV and mean $V_g = 46$ mV, the net V_{gc} is 78 mV. Using this voltage and a mean pHi of 7.14 (Table 1), the

calculated pHg for equilibrium with the cytoplasm is 8.47. The pH of goblet cavities is thus, on average, more than 1.2 units too low for electrochemical equilibrium with the cytoplasm.

Effects of open-circuiting and decreased hemolymphal-side pH

In three experiments, tissues were superfused with 32KS bathing solution at pH 8.0 on both sides, but were not short-circuited. In all, there were 22 intracellular and 8 intragoblet measurements. The second row of Table 1 summarizes the results. Under open-circuit conditions, the weighted mean of V_b was significantly less inside-negative than the mean recorded from short-circuited tissues. The mean pHi and pHg recorded under open-circuit conditions were not significantly different from those recorded under short-circuit conditions (compare the second row of Table 1 with the first row). The change in V_g with open-circuiting makes pHg equal to pHg_{eq} within measurement error.

In two experiments on open-circuited tissues, the pH of the hemolymphal solution was 6.8 and that of the luminal solution was 8.0. These conditions approximate those expected *in situ*. In all, 18 intracellular and 18 intragoblet penetrations were made. Comparison of these results, summarized in the third row of Table 1, with those of the second row of Table 1 shows that V_b was unaffected by the decreased pH of the hemolymphal side, the means of pHi and pHg were actually slightly, but not significantly, more alkaline. As in the open-circuit experiments in which the bathing solution pH was 8.0 on both sides, pHg was not significantly out of equilibrium with the luminal solution. However, the reduction of hemolymphal-side pH reversed the transbasal H^+ electrochemical gradient, since both V_b and pHi remained unchanged. The significance of the reversal of the polarity of V_g in response to a decrease in hemolymphal-side pH is unclear.

The effect of low extracellular K^+ concentration

In two experiments totalling nine measurements, transbasal penetrations were established under short-circuit conditions in 32KS and then the hemolymphal superfusate was changed to 5K (Na^+ substituted for K^+ to maintain the conductance of the solution constant). A typical set of traces is shown in Fig. 5; the results are summarized in Table 2. As expected from previous studies (Moffett and Koch, 1988*a*), the decreased extracellular K^+ concentration led to hyperpolarization of the basal membrane and decreased I_{sc}. These changes were accompanied by a small but significant reduction in pHi.

Discussion

In standard bathing solution buffered to pH 8.0, pHi was significantly lower than predicted for electrochemical equilibrium with the hemolymphal-side bathing solution. The magnitude of the transbasal electrochemical difference for H^+ was increased by open-circuiting and by decreased extracellular K^+ concentration; this

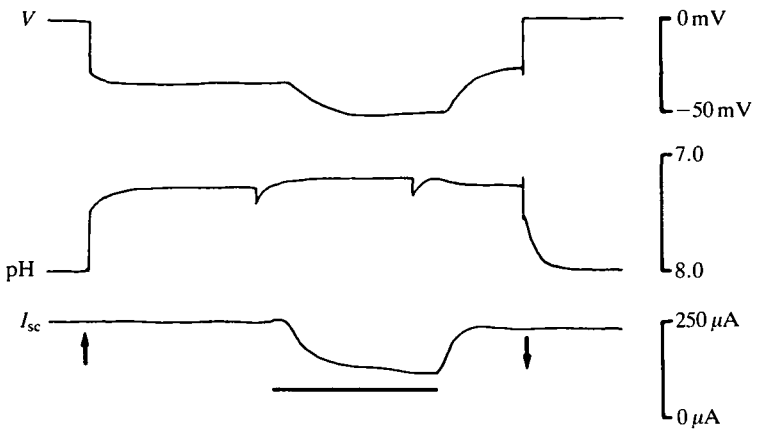

Fig. 5. Typical voltage, pH and I_{sc} traces during a change of hemolymphal K^+ from 32KS to 5KS. Advance and withdrawal of the electrode are indicated by the arrows. The horizontal bar indicates the period of superfusion with 5KS. The duration of exposure to 5KS was 4 min.

Table 2. V_b, *pHi and* I_{sc} *in 32K and changes of these variables upon changing hemolymphal side* $[K^+]$ *to 5 mmol* l^{-1}

V_b (mV)	ΔV_b (mV)	pHi	ΔpHi	I_{sc} ($\mu A\,cm^{-2}$)	ΔI_{sc} (%)
−29.8±0.03	−16.7±4.46	7.21±0.05	−0.04±0.004	125.0±89.0	−57.4±11.37

Values are mean±S.E. (N=9).

effect was entirely due to the resulting changes in V_b. However, when the pH of the hemolymphal-side solution of open-circuited tissues was 6.8 instead of 8.0, the transbasal H^+ electrochemical gradient was reversed. The insensitivity of V_b to these changes in the transbasal H^+ electrochemical gradient suggests that the basal membrane has a low conductance to H^+. Since passage of K^+ through the basal membrane is at least partly by way of K^+ channels (Zeiske *et al.* 1986; Moffett and Lewis, 1990), this conclusion implies that the K^+ channels can exclude protons. However, the slight decrease in pHi in 5KS is commensurate with relatively small decreases in intracellular K^+ activity reported for similar experiments with K^+-sensitive electrodes (Moffett and Koch, 1988*a*), consistent with replacement of some cytoplasmic K^+ by H^+ when extracellular K^+ concentration is reduced. In 5KS most of the transbasal K^+ uptake must proceed by a thermodynamically active pathway (Chao *et al.* 1990). The relatively small effect of 5KS on pHi suggests that the active uptake is not a countertransport process driven by the transbasal H^+ activity gradient.

Under standard conditions, pHg was more than 1 unit lower than expected for electrochemical equilibrium with the gut lumen and was not clearly affected by

changes in the magnitude of V_g. In both sets of open-circuited tissues, pHg was close to electrochemical equilibrium with luminal pH. Thus, under open-circuit conditions there is little or no driving force for movement of H^+ or OH^- between goblet cavity and gut lumen. These results are consistent with reports by Chamberlin (1990) and Dow and O'Donnell (1990) that the isolated posterior midgut is less active in alkalization of the lumen than the anterior and middle regions.

In the short-circuited condition, V_g is about 40 mV more positive than in the open-circuited state (Fig. 1; Table 1). A higher rate of efflux of H^+ from the goblet cavity to the gut lumen ought to accompany short-circuiting, yet the mean pHg of short-circuited tissues is not higher than that of open-circuited tissues (Table 1). One possible interpretation of this result is that the ability of H^+ or OH^- to traverse the goblet valve is limited at best. A similar conclusion was reached by Dow and Peacock (1989) for middle midgut.

In summary, these studies suggest that both the basal membrane and the goblet valve are significant barriers to H^+. These conclusions rest on the expectation that changes in rates of H^+ movement into and out of the cytoplasmic and goblet cavity compartments should be reflected in changes in their pH. This expectation must be tempered by the possibility that the pH of cytoplasm and goblet matrix are so powerfully buffered or well regulated that changes in H^+ fluxes across the cell membranes and the goblet valve induced by our experimental manipulations had immeasureably small effects on compartment pH.

Wieczorek *et al.* (1989) hypothesized that K^+ transport across the GCAM is an electroneutral exchange secondary to active H^+ transport into the goblet cavity. The lower pH of the goblet cavity relative to the luminal solution is consistent with this hypothesis. However, the present results are most compatible with a H^+:K^+ exchange ratio greater than 1. If the exchange were electrically neutral, the chemical, but not the electrical, component of the H^+ electrochemical gradient would determine whether net K^+ secretion was energetically favorable. Previous studies with K^+-specific microelectrodes (Moffett and Koch, 1988*a*,*b*) showed essentially no K^+ chemical activity gradient across the GCAM, while the mean pHg measured in the present studies is slightly less than the mean pHi. Thus, the pH gradient across the GCAM is in the wrong direction to drive K^+ transport from cytoplasm to goblet cavity. If the exchange ratio were greater than $1H^+$:$1K^+$, the electrical component of the H^+ electrochemical gradient would enter into the energetic computation, and would provide sufficient energy to make net K^+ secretion favorable. Generally such secondarily active exchanges are electroneutral, but there are recent reports of electrogenic amiloride-sensitive Na^+/H^+ exchange in vesicles from crustacean tissues (Ahearn and Franco, 1989; Towle and Baksinski, 1989).

Harvey *et al.* (1981) estimated a ratio of $2K^+$ transported per ATP hydrolyzed. In this case, a stoichiometry for the H^+ ATPase of $4H^+$/ATP would be necessary to support a $2H^+/K^+$ exchange ratio. However, in other systems, such as turtle bladder, the vacuolar-type proton pump has a stoichiometry of at most only 3 (see

Steinmetz and Andersen, 1982). Further characterization of the goblet cell proton pump and K^+/H^+ antiporter are needed to resolve the questions raised by these studies.

Under standard conditions with the epithelium short-circuited, sizable electrochemical gradients for H^+ exist across the basal membrane and goblet valve. Efficient coupling of K^+ secretion to active proton transport under these conditions would require recycling of protons between cytoplasm and goblet cavity. In turn, this would require that the basal membrane and goblet valve should be effective barriers to protons while permitting a high rate of K^+ flux. As noted above, the present studies suggest that this requirement is met. The ability to select for K^+ over smaller, more mobile, H^+ would be unexpected in ion channels of the basal membrane, but remarkable in the case of the goblet valve, which in electron micrographs appears to have a passage of much larger than ionic dimensions.

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