

ELECTROPHYSIOLOGY OF K⁺ TRANSPORT BY MIDGUT EPITHELIUM OF LEPIDOPTERAN INSECT LARVAE

III. GOBLET VALVE PATENCY

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Summary

The midgut epithelium of lepidopteran insect larvae contains characteristic goblet cells possessing an apical cavity surrounded by goblet cell apical membrane (GCAM) and guarded from the gut lumen by a valve-like structure. The currently accepted model of active K⁺ secretion by the midgut of lepidopteran insect larvae locates the major active step at the GCAM, implying that actively transported K⁺ reaches the gut lumen by passing through the valve.

The major question for these studies was whether K⁺ could pass through the valve by diffusion in free solution. Using tetramethylammonium (TMA⁺) as a surrogate for K⁺, and exploiting the high sensitivity of standard K⁺-selective ion-exchange resin to quaternary amines, we used K⁺-selective intracellular electrodes to measure the rate of access of apically applied TMA⁺ to the goblet cavity.

TMA⁺ failed to gain access to more than half of the cavities. For those cavities to which it did gain access, the

influx and efflux rates and predicted equilibrium concentrations of TMA⁺ were too low to be consistent with diffusive exit of transported K⁺ along the same path. Upon superfusion with cytochalasin E, a disrupter of actin-based cytoskeleton, the TMA⁺ influx rates immediately increased for those cavities previously accessible to TMA⁺, but not for those previously inaccessible. Increases in TMA⁺ influx suggestive of spontaneous valve opening were not observed. The results are consistent with an indirect route of access of TMA⁺ to goblet cavities.

We conclude that goblet valves are closed *in vitro*. Assuming that the goblet cavity is part of the transport route, actively transported K⁺ must exit the cavity to the gut lumen by a mechanism that does not involve diffusion in free solution.

Key words: insect, midgut, goblet cell, active K⁺ transport, tetramethylammonium, K⁺-selective microelectrode, *Manduca sexta*.

Introduction

The goblet cells of the midgut of larval lepidopteran insects belong to a diverse group of epithelial cells with apical crypts or cavities. Other members of this group include parietal cells of vertebrate stomach (Ito, 1984), chloride cells of seawater fish gill (Sardet *et al.* 1980), accessory cells of insect antennal sensilla (Klein and Zimmermann, 1991) and gut cells of other insect orders (Peacock, 1979; Dimitriadis, 1991). Like that of the fish chloride cell, the cavity of the midgut goblet cell contains a gelatinous matrix, suggested by histochemical studies (Schultz and Jungreis, 1977; Schultz *et al.* 1981) and X-ray elemental microanalysis (Dow *et al.* 1984) to consist of sulfated mucopolysaccharide. Lepidopteran midgut goblet cells are unique in possessing a valve that forms a narrow passage between the apical cavity and the gut lumen. The valve passage has an apparent diameter of at most 0.5 µm in electron micrographs and in some cells appears to be sealed by fusion of apposing membrane surfaces (Smith *et al.* 1969; Flower and

Filshie, 1976). Recently, U. Klein (personal communication) has shown by immunohistochemical methods that the goblet valve is invested by a dense network of actin-based cytoskeletal fibers that presumably maintains its integrity.

The isolated short-circuited midgut of lepidopteran larvae secretes K⁺ at a high rate; isotopic experiments showed that essentially all of the short-circuit current could be accounted for by the active transport of K⁺ (Cioffi and Harvey, 1981). K⁺-selective microelectrode studies showed that goblet cavity K⁺ activity, (K⁺)_g, is above electrochemical equilibrium with both the gut lumen and the goblet cell cytoplasm (Moffett and Koch, 1988*a,b*), suggesting active K⁺ transport into the cavity.

Studies of GCAM vesicles revealed that the GCAM contains quantities of a vacuolar H⁺-ATPase and of an amiloride-sensitive K⁺/H⁺ antiporter (Wieczorek *et al.* 1989, 1991; Schweikl *et al.* 1989), suggesting that electrogenic H⁺ transport drives K⁺/H⁺ exchange. The association of the vacuolar-type

ATPase with electrogenic K^+ transport in this and other tissues is supported by immunohistochemical and inhibitor studies (Bertram *et al.* 1991; Gill and Ross, 1991; Klein *et al.* 1991; Klein and Zimmermann, 1991). Measurements of goblet cavity H^+ activity showed that the electrical gradient across the GCAM, but not the H^+ chemical gradient, could support K^+/H^+ exchange (Chao *et al.* 1991), suggesting that more than one H^+ must be exchanged for each K^+ .

It has been assumed that K^+ transported into the goblet cavity diffuses out of the goblet cavity into the gut lumen through the goblet valve. In the simple view, this valve must be open to allow K^+ to diffuse out of the goblet cavity into the lumen. For the purposes of testing this hypothesis, an 'open' valve is defined as having a passage that is large in comparison with ionic dimensions and through which ions diffuse as if in free solution.

This picture of active transport into the goblet cavity with subsequent diffusion of K^+ into the gut lumen through an aqueous pore is consistent with the K^+ electrochemical gradients measured by ion-selective microelectrodes (Moffett and Koch, 1988b). There are, however, several apparent anomalies. First, large molecular markers such as Lucifer Yellow, Ruthenium Red and Acridine Orange do not readily enter or leave the goblet cavity unless the cells are injured (Dow and Peacock, 1989; K. Baldwin, personal communication). Second, in spite of the fact that the activity of K^+ in the goblet cavity is about three times that in the lumen, whereas the activity of H^+ in the goblet cavity is about six times that in lumen (Moffett and Koch, 1988b; Chao *et al.* 1991), K^+ is transported without apparent H^+ transport. The latter observation would suggest that the goblet valve allows passage of K^+ without allowing appreciable passage of H^+ . Third, the measurements of Dow and Peacock of electrical resistance from goblet cavity to gut lumen were 5–6 times higher than their measurements of resistance from cell fluid to lumen. Fourth, Chao *et al.* (1989) followed the leach-out of Cl^- from cells and from goblet cavities when the tissue was exposed to Cl^- -free solution. Activities were followed with intracellular or intragoblet microelectrode penetrations. Although the half-time for leach-out from cells was about 10 min, the half-time for leach-out from goblet cavities was nearly 35 min. This seems extraordinarily long for transfer through a large aqueous channel. Finally, the goblet valve is anatomically much more complex than seems to be required for just an open aqueous channel. These findings are difficult to reconcile with the idea of an open goblet valve.

The present studies test the valve patency to small cations using tetramethylammonium (TMA^+) as an indicator. Three questions are posed. First, can TMA^+ enter the goblet cavity from the gut lumen? Second, if entry occurs, does it occur through an open goblet valve? For this question, an 'open' valve is defined as a passage in which small cations diffuse at rates determined by their diffusion coefficients in free solution. Third, if valves are open, are they open continuously or only intermittently?

The results of these studies indicate that, on average, more

than half of the valves of isolated midgut preparations are closed to entry of TMA^+ . Furthermore, the unreasonably slow rate of TMA^+ movement into those cavities that it did enter and the kinetics of entry are incompatible with diffusive entry of TMA^+ through the goblet valve. By implication, exit of K^+ from the cavity of such cells must occur by a mechanism other than free diffusion.

Some experiments described here have previously been reported in abbreviated form (Woods *et al.* 1991; Moffett and Koch, 1992a,b). The present results served as the basis for a model for distribution of TMA^+ in the midgut (Koch and Moffett, 1995).

Materials and methods

Animals and bathing solutions

Fifth-instar tobacco hornworms (*Manduca sexta*) were reared as in previous studies (Moffett and Koch, 1988a). The posterior midgut was excised, mounted, superfused and impaled with electrodes as in previous reports from this laboratory (Moffett and Koch, 1988b; Chao *et al.* 1991). Where indicated, the tissues were short-circuited by an automatic circuit, with compensation for solution resistance. In the bathing solution used in these studies, the short-circuit current corresponds closely to the rate of active K^+ transport (Cioffi and Harvey, 1981).

The standard superfusion solution was 32KS (Chao *et al.* 1991). Where indicated, tetramethylammonium (TMA^+) was added as the chloride salt to a final concentration of either 5 or 10 $mmol\ l^{-1}$. Cyclic AMP (Sigma) was administered bilaterally as a 1 $mmol\ l^{-1}$ solution of 8-bromoadenosine 3',5'-cyclic monophosphate in four experiments. Cytochalasin E (Sigma Chemical) was added from a stock solution in dimethylsulfoxide (DMSO) to the luminal perfusate of 32KS+ TMA^+ to achieve a final concentration of $10^{-5}\ mol\ l^{-1}$ or $10^{-4}\ mol\ l^{-1}$ as indicated. Control experiments showed no effect on TMA^+ uptake of the DMSO carrier alone.

Microelectrodes

Double-barreled glass microelectrodes were pulled from 1.8–2.2 mm o.d. theta glass (Glass Co. of America). The barrel destined to be ion-selective was silanized and filled with K^+ -selective resin (World Precision Instruments) as in previous studies (Moffett and Koch, 1988b). The outputs of the two barrels were amplified by separate differential electrometers (Keithley 604). The algebraic difference between the outputs of the K^+ and the reference barrels is the ion-specific voltage. The primary signals, their difference and the transepithelial potential (V_{oc}) or short-circuit current (I_{sc}) were recorded on a four-channel pen recorder. Electrode penetrations were made through the basal (hemolymph) side of the tissue.

A key aspect of the present studies is the fact that the K^+ -selective resin is highly selective for quaternary amines over K^+ (Cotton and Reuss, 1991). This enabled us to measure the rate of influx of the cationic marker TMA^+ into impaled goblet

cavities even though K^+ is the major cation in the cavity (Moffett and Koch, 1988b).

Throughout this paper, parentheses () will be used to denote ionic activity and brackets [] will be used to denote concentration. Functional dependence, as in the Nernst or Nikolsky equations, will be indicated by curly brackets { }.

Immediately before and after use, each microelectrode was calibrated for K^+ and for TMA^+ . For calibration of the K^+ response, four solutions of KCl were used ranging from 500 to 10 mmol l⁻¹. The data were normally fitted to the Nikolsky equation:

$$V = V_o + RT/F \times \log\{(K^+) + \beta\}, \quad (1)$$

where V is the potential difference, V_o is the value of V in 500 mmol l⁻¹ KCl⁻¹, R is the gas constant, T is absolute temperature and F is Faraday's constant, by a nonlinear regression estimating β , the apparent concentration of interfering ion. RT/F was taken to be 58.6 mV per decade. The mean value of β was 0.002±0.0003 mmol l⁻¹ and the average root mean square of the regression was 1.31±0.11 mV ($N=74$). For seven electrodes, β was so small that calibration was to the Nernst equation:

$$V = V_o + RT/F \times \log\{(K^+)\}, \quad (2)$$

where the regression was for the value of RT/F . The average value of RT/F was 60.59±0.6 mV for this group.

Initially four solutions were used to calibrate the same electrodes to TMA^+ . These were 10, 5, 2 and 1 mmol l⁻¹. Each solution also contained 100 mmol l⁻¹ KCl. In later experiments, the calibrating solutions were changed to 5, 2, 1.0, 0.3 and 0.1 mmol l⁻¹, each with 100 mmol l⁻¹ KCl. The data were fitted to the Nikolsky equation, estimating both the apparent value of RT/F and β . A representative calibration curve for TMA^+ is shown in Fig. 1. The electrodes were calibrated for (K^+) using a calibration program similar to that described previously (Chao *et al.* 1991) that approximated the functional relationship between (K^+) and [K^+] described by Shedlovsky and MacInnes (1937). The relationship between activity and concentration for TMA^+ is not given in standard references. Hence, we express TMA^+ values in terms of concentration. In terms of the electrode response, this corresponds to a (TMA^+) the same as that of a TMA^+ solution made up to a given concentration. Since the intragoblet [TMA^+] values were generally much less than 10 mmol l⁻¹, the error arising from the assumption of equality of concentration and activity was assumed to be negligible.

Electrodes were accepted only when RT/F was estimated to lie between 50 and 65 mV per decade. The average value of RT/F was 60.21±1.02 mV; the average root mean square error of the fit was 3.71±0.3 mV ($N=42$). Since the calibrating solutions contained 100 mmol l⁻¹ KCl, the value of β was an estimate of the selectivity of the electrode to TMA^+ over K^+ . The average value for β was 0.085±0.007 mmol l⁻¹. Thus, the electrodes were 100/0.085=1176 times more sensitive to TMA^+ than to K^+ . In addition, electrodes were accepted only if the reference barrel changed less than 15 mV for the range of calibration of both K^+ and TMA^+ .

Measurements of TMA^+ influx rate were conducted as follows. Voltage measurements were made with respect to the hemolymph side. Stable impalement of goblet cavities was established according to the criteria listed below. The value obtained for the ion-selective output was taken to be a measure of goblet cavity (K^+). Luminal superfusate only was then changed to 32KS with an additional 10 mmol l⁻¹ of TMA^+ . The perfusion system imposes a delay of about 20 s, so 0.01 % Evans Blue dye was included in the TMA^+ bathing solution to allow the actual time of arrival at the midgut surface to be noted visually. A rise in the output of the ion-selective barrel indicated entry of TMA^+ . The increase in the ion-selective voltage was applied to the TMA^+ standard curve to estimate [TMA^+]. An increase of 2 mV in the ion-selective trace was easily detectable. This normally represented a [TMA^+] of 7 μ mol l⁻¹. For the shortest impalement of 4 min, we were thus able to detect a rate of TMA^+ entry of less than 2 μ mol l⁻¹ min⁻¹. A more typical holding time was 10 min, but some were stable for up to 90 min. In the 10 min impalements, the minimum detectable rate of TMA^+ entry was 0.7 μ mol l⁻¹ min⁻¹. In several experiments which showed TMA^+ entry, the efflux of TMA^+ was subsequently measured by changing the superfusate back to 32KS with no TMA^+ while the impalement was held.

Data were rejected from impalements in which either the electrode response or the biological parameters were unacceptable. The electrode requirements were as follows: (1) After the experiment, the K^+ -selective electrode must recalibrate acceptably as described above. (2) The impalements must be sharp and stable in both the voltage and K^+ electrodes. (3) Both electrode recordings were required to return to within 2 mV of baseline after removal from the cell. The biological requirements were as follows: (4) The voltage difference between the K^+ -selective and the reference electrode must be at least 20 mV prior to exposure to TMA^+ , which implies that (K^+)_g is greater than 63 mmol l⁻¹. (5) In short-circuit conditions (SC), V_g , the potential difference between goblet cavity and lumen, is greater than +25 mV or V_b , the potential difference across the basal membrane, is more negative than

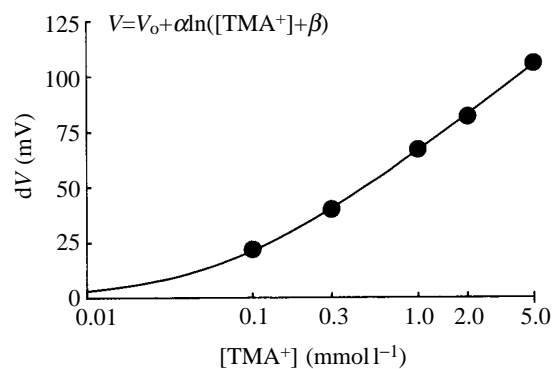


Fig. 1. TMA^+ calibration curve. All solutions contained 100 mmol l⁻¹ KCl. The voltage plotted (dV) is the change in K^+ voltage with [TMA^+]. $\alpha=RT/F$; for this electrode, $\alpha=57.4$ mV per decade, $\beta=0.07$ mmol l⁻¹ and root mean square=0.67 mV.

-20 mV. In open-circuit conditions (OC), $V_g > -10$ mV. An additional criterion derived from the double sampling techniques was applied somewhat more loosely. It was noted that many impalements that failed the criteria listed above had entry rates of TMA^+ 20–100 times higher than impalements that were acceptable. Impalements that were barely acceptable according to the five criteria listed above but that had extremely high entry rates were tentatively discarded. This practice is dangerous in terms of biasing the data and, the two cases in which this occurred were looked at individually and then discarded.

Statistics

All values are given as mean \pm standard error of the mean.

Results

If TMA^+ can pass through an open valve into the goblet cavity, then the highest rate of influx in response to a step function of luminal $[\text{TMA}^+]$ would occur initially, when the concentration in the goblet cavity is zero. We therefore examined influx rates from the earliest times after luminal $[\text{TMA}^+]$ had reached 10 mmol l^{-1} , at a time when $[\text{TMA}^+]$ in the goblet cavity ($[\text{TMA}^+]_g$) was less than 2% of its luminal concentration. A representative result is shown in Fig. 2.

The analysis of initial influx rates assumes that a change of superfusate causes $[\text{TMA}^+]$ to change as a step function at the luminal side of the tissue. This assumption would be invalidated by a large unstirred layer lining the luminal side of the preparation. The tissue is normally folded into large rugae, and for goblet cavities deep in these rugae the luminal fluid could be expected to exchange very slowly with the bulk solution on the luminal side. To reduce this problem, we selected impalement sites where the apical side was most

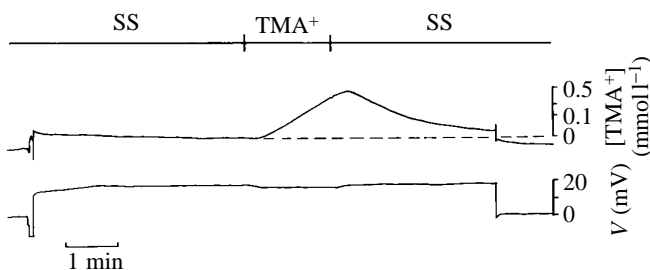


Fig. 2. Example of a TMA^+ influx and efflux experiment showing the ion-specific electrode trace ($[\text{TMA}^+]$) and reference voltage trace (V). The experiment begins with the tissue superfused with 32KS bathing solution. Establishment of a goblet cell penetration in 32KS bathing solution (SS) is indicated by a positive deflection of the V trace and simultaneous measurement of a high K^+ activity by the ion-specific barrel (see penetration criteria in Materials and methods). After addition of 5 mmol l^{-1} TMA^+ , an increase in ion-specific voltage without a corresponding change in the reference voltage reflects influx of TMA^+ ; note that the electrode response is logarithmic. A return to 32KS without TMA^+ leads to TMA^+ efflux. In the following figures, the influx curves are based on values of $[\text{TMA}^+]_g$ computed from such data.

directly exposed. Control experiments were conducted in four different tissues. A goblet cavity was impaled and then the electrode was advanced the minimal distance required to make it emerge from the goblet cavity to the other side. Unilateral TMA^+ superfusion was instituted and the time course for elevation of $[\text{TMA}^+]_g$ was recorded for this 'push-through' experiment. The results were the same in all cases. Fig. 3 shows $[\text{TMA}^+]_g$ as a function of time for a 'push-through' experiment and for the goblet impalement that was taken immediately afterwards in the same general location. In terms of the time course of TMA^+ influx into the goblet cavity, luminal $[\text{TMA}^+]$ at such selected sites is essentially a step function.

TMA influx measurements

Of the 68 impalements obtained from 21 preparations exposed to luminal TMA^+ under OC, 35 were absolutely closed (less than 2 mV increase in the ion-selective electrode) and an additional 12 had initial influx rates of less than $10 \mu\text{mol l}^{-1} \text{ min}^{-1}$. This rate represents a time constant for influx in excess of 600 min. Combining these, 69% of the impalements were effectively closed under open circuit. Including those impalements for which the initial influx was zero, the mean initial influx rate was $0.021 \text{ mmol l}^{-1} \text{ min}^{-1}$. The distribution was not normal.

Of the 47 impalements from 21 preparations under SC exposed to luminal TMA^+ , 18 were absolutely closed and 14 had initial influx rates of less than $10 \mu\text{mol l}^{-1} \text{ min}^{-1}$. This rate represents a time constant for influx in excess of 200 min. Combining these, 68% of the impalements were effectively closed. Including those impalements for which the initial influx was zero, the mean initial influx rate was $0.018 \text{ mmol l}^{-1} \text{ min}^{-1}$. The distribution of influx rates was not normal in these preparations either. Although there was no change in V_{oc} in the OC experiments, unilateral exposure to TMA^+ led to a small increase in I_{sc} in about one-third of the exposures in the SC series. The increase was not accompanied by any consistent change in the potential difference between

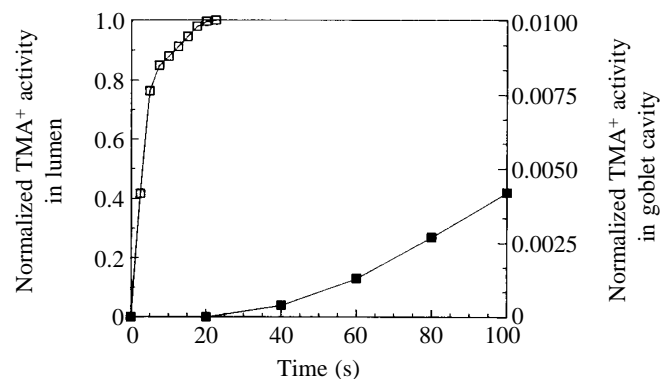


Fig. 3. TMA^+ influx curves recorded from a goblet cavity (filled symbols) and subsequently after advancing the electrode until it just emerged into the gut lumen (open symbols). The values were normalized to the fraction of their equilibrium values.

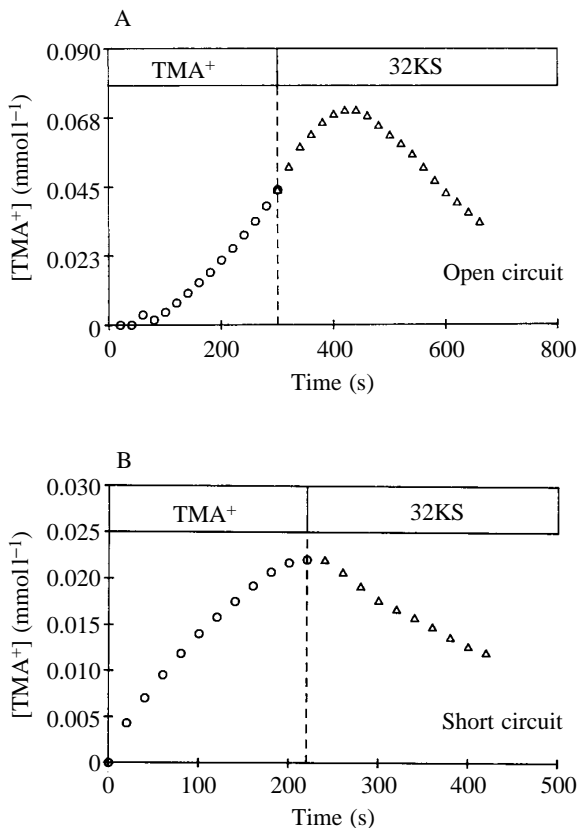


Fig. 4. Time courses of $[TMA^+]_g$ in an open-circuit influx–efflux experiment (A) and a short-circuit influx–efflux experiment (B). In this and subsequent figures, TMA^+ solution arrives at the tissue surface at time zero and the vertical dashed line indicates the time of replacement of TMA^+ solution by standard solution. Values obtained during uptake are plotted as circles; those obtained after replacement with standard solution are plotted as triangles. The data shown in this figure were smoothed by a three-window moving average.

goblet cavity and lumen (V_g), nor was it correlated to the rate of TMA^+ influx.

Fig. 4A,B shows the changes in $[TMA^+]_g$ as a function of time from representative OC and SC experiments. Several features characteristic of the entire series of experiments are best seen in Fig. 5, wherein the values of $[TMA^+]_g$ from the same experiments are normalized to the concentration they had attained at the end of TMA^+ superfusion. First, influx followed a sigmoid rather than an exponential time course. The initial concave upwards behavior can be seen in Figs 3, 4A, 5 and 7. It is not apparent in Fig. 4B because the inflection point was so early that it was lost in the moving window averaging. In the SC preparations, the inflection point occurred very early and most of the influx curve was convex upwards. In contrast, the inflection point tended to occur relatively late in the exposure of OC preparations and most of the influx curve was concave upwards (see Fig. 4, for example). Second, $[TMA^+]_g$ continued to rise for a period after the luminal solution had been switched back to TMA^+ -free solution (overshoot). In SC preparations, this continued rise lasted less than 1 min and the

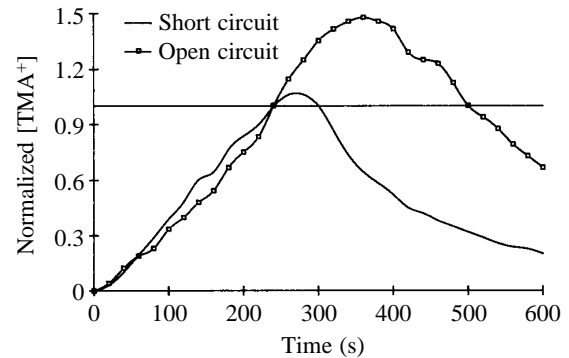


Fig. 5. Unsmoothed values from the two experiments shown in Fig. 4 normalized to the maximum value obtained during influx.

concentration of TMA^+ never rose far above the value it had attained at the end of exposure (Fig. 4B). In OC preparations, $[TMA^+]_g$ continued to rise for several minutes after the switch to TMA^+ -free solutions, and the peak value of $[TMA^+]_g$ was normally more than 30% higher than at the end of TMA^+ superfusion (Fig. 4A). A further point is also important. In those preparations that showed a significant overshoot, that is in the OC preparations, the rate of rise in $[TMA^+]_g$ did not change until some time after luminal TMA^+ exposure had been stopped. This is evident in Figs 4A and 5 as well as in Fig. 9, which is from an OC preparation to which cytochalasin E had been added.

Because TMA^+ is a cation, its distribution would be expected to be affected by V_g . Mean values of V_g differ between SC and OC conditions. In the present series, including impalements in which no TMA^+ was given, V_g averaged $+42.0 \pm 2.76$ mV ($N=51$) during SC and $+11.9 \pm 2.30$ mV ($N=88$) during OC. On average, therefore, the concentration of TMA^+ in equilibrium with 10 mmol l^{-1} in the lumen was 1.9 mmol l^{-1} during SC conditions and 6.2 mmol l^{-1} during OC conditions. This difference can be corrected for by examining the influx turnover time; that is, the equilibrium concentration of TMA^+ in the goblet cavity divided by the initial influx rate. The mean influx turnover time was 218 min for SC and 237 min for OC preparations. The frequency distributions of turnover times are shown in Fig. 6.

Two impalements from a single tissue were retained even though the tissue was apparently not healthy. This tissue was an OC preparation in which V_{oc} was only 20 mV. Two impalements were made in which V_g was greater than 50 mV. Thus, this was a tissue in poor condition in which two healthy cells were found. Values from these impalements are not included in the statistics given above, but the results of these impalements are of particular interest. They show the operation of cells in an OC preparation when the transepithelial voltage and the transvalve potential are near the values found during SC. Influx and efflux characteristics in these impalements were similar to those found in the SC preparations. That is, influx was convex upwards from very early in the exposure, and the overshoot was small and occurred very shortly after cessation

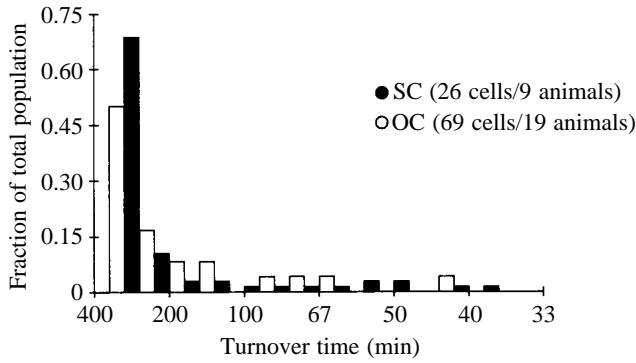


Fig. 6. Frequency distribution of turnover times for TMA⁺ in goblet cavities of open-circuited (OC) and short-circuited (SC) tissues calculated from initial influx rates and equilibrium concentrations.

of TMA⁺ superfusion. Hence, the properties of a late inflection point during exposure and a large and delayed overshoot depend on the value of V_{oc} or V_g rather than on whether the tissue is SC or OC.

Comparison of TMA⁺ influx and efflux

In four impalements from the SC series and in six from the OC series, the superfusing solution was changed back to 32KS from the TMA⁺ solution while the impalement was held. These experiments thus gave information about TMA⁺ efflux rates as well as influx rates and were used to estimate flux ratios of TMA⁺. For these comparisons, fluxes were estimated from two concentrations near the highest values of $[TMA^+]_g$ obtained during exposure. Since the two concentrations were the same for both uptake and leach-out, the reciprocal of the time required to rise from the lower to the higher concentration was the measure of the influx rate from lumen to goblet cavity (J_{l-g}) and the reciprocal of the time required to fall was the measure of the efflux rate (J_{g-l}). The ratio of these measures is the flux ratio. In the experiment depicted in Fig. 4A, the two TMA⁺ concentrations were 0.033 and 0.0395 mmol l⁻¹. It took 23 s for $[TMA^+]_g$ to rise from the lower to the higher concentration and 37.5 s for it to fall; the flux ratio was therefore 1.6. For the experiment depicted in Fig. 4B, the two concentrations were 0.018 and 0.021 mmol l⁻¹ and the flux ratio was 1.1. Note that these flux rates were determined near the highest $[TMA^+]_g$ attained during exposure, rather than when it was near zero,

and thus were different from the initial influx rates. With the exception of a single impalement in which the values of $[TMA^+]_g$ were near equilibrium, all influx rates measured in this way were higher than the initial rates from these same tissues. The column in Table 1 labeled ' J_{late}/J_{early} ' is the ratio of those uptake fluxes measured near equilibrium to those measured immediately after TMA⁺ had been introduced.

The $[TMA^+]_g$ in the gut lumen was 10 mmol l⁻¹ during exposure and 0 mmol l⁻¹ during leach-out. The mean of the two chosen values of $[TMA^+]_g$ ('mean $[TMA^+]_g$ ' in Table 1) was taken as the concentration in the goblet cavity. The concentrations chosen were close and the arithmetic mean was a reasonable approximation of the true mean between the two points. These values were inserted into the Ussing equation (Ussing, 1949) as a test of passive free diffusion. The form of the equation used was:

$$\log(J_{g-l} \times [TMA^+]_l / J_{l-g} \times [TMA^+]_g) - V_g / 58.6 = F, \quad (3)$$

where F is a dimensionless term that equals zero in the case of free ionic diffusion. Table 1 shows that F was significantly different from zero under both OC and SC.

TMA⁺ entry into cytoplasm

Initially we assumed that TMA⁺ would not readily cross cell membranes. This assumption was tested in experiments in which transbasal penetrations of cytoplasm were made and the same procedure followed as in the cavity influx experiments. In four of eight experiments, TMA⁺ entered the cytoplasm. Fig. 7 shows the time course of influx and efflux from one of these, taken from an SC preparation. The results for influx are similar to those obtained from the goblet cavity in SC preparations. Although efflux is far from complete in this experiment, there is the suggestion that $[TMA^+]_g$ is not approaching zero as a first-order (single exponential) process.

Modification of influx rates

The results above show that there was no entry of TMA⁺ into nearly half of the goblet cavities and extremely slow entry into another 25%. Furthermore, in those impalements in which influx was followed for sufficient time to allow an estimate of the final value, $[TMA^+]_g$ was approaching an equilibrium value less than 0.1 of the predicted equilibrium value computed from the Nernst equation. In an attempt to resolve the conflict between these results and our initial assumptions, we carried

Table 1. Parameters for the Ussing flux ratio equation

	Mean $[TMA^+]_g$ (mmol l ⁻¹)	V_g (mV)	J_{l-g}/J_{g-l}	F	J_{late}/J_{early}
Short circuit ($N=4$)	0.023±0.006	45.8±11.9	1.65±0.26	3.5±0.39	5.12±2.35
Open circuit ($N=6$)	0.130±0.045	17.3±11.8	2.67±0.50	2.31±0.38	12.46±7.29

$[TMA^+]_g$, concentration of TMA⁺; V_g , potential difference between goblet cavity and lumen; J_{l-g} , influx rate from lumen to goblet cavity; J_{g-l} , efflux rate from goblet cavity to lumen; F, see equation 3; J_{late}/J_{early} , ratio of uptake flux measured near equilibrium to that measured immediately after TMA⁺ addition.

Values are means ± S.E.M.

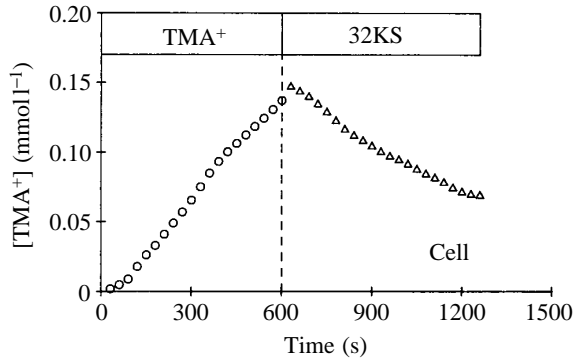


Fig. 7. TMA⁺ influx and efflux curves recorded from a cell penetration. The data shown in this figure were smoothed by a three-window moving average.

out experiments in which we tried either to control the valve or to show that we could detect TMA⁺ if it entered. The procedure in these experiments was to establish a stable cavity penetration, initiate a TMA⁺ influx measurement and then switch to 32KS+TMA⁺ together with the putative modifying agent.

Neither a reduced O₂ level (which inhibits net K⁺ transport) nor an elevated CO₂ level had any effect either in changing a pre-existing influx rate or in initiating influx into a goblet that had previously been closed. Cyclic AMP, a weak stimulant of net K⁺ transport (Moffett *et al.* 1983), had no effect in seven impalements in which measurable influx was established before application of cyclic AMP, but it clearly increased influx in five others. Fig. 8 shows results from one of two penetrations in which there were multiple brief applications of cyclic AMP. Each application was followed by a sudden increase in the rate rise of [TMA⁺]_g, indicating an increase in influx rate. This increase was reversed upon return to 32KS with TMA⁺ but without cyclic AMP. Note that there were no



Fig. 8. Effect of cyclic AMP on TMA⁺ influx in a short-circuited preparation. The tissue was bathed initially in 32KS. TMA⁺ (5 mmol l⁻¹) was added during the interval indicated by the upper horizontal bar; measurable TMA⁺ influx is indicated by the increase in ion-specific voltage (upper trace) without a change in reference voltage (lower trace). Two brief applications of 1 mmol l⁻¹ cyclic AMP (lower horizontal bar) were followed by increases in influx rate that were reversed by a return to 32KS+TMA⁺ solution without cyclic AMP. A return to 32KS without TMA⁺ resulted in the efflux of TMA⁺; a cyclic AMP pulse had no effect on efflux rate.

sudden changes in slope during leach-out; that is, efflux was unaffected by cyclic AMP.

In seven impalements in which there was measurable appearance of TMA⁺ in the cavity during the control interval, administration of cytochalasin E increased the influx rate by a mean factor of 18.1±4.7. There was no clear difference between results obtained with 10⁻⁴ mol l⁻¹ (three experiments) and 10⁻⁵ mol l⁻¹ cytochalasin E (four experiments), nor was there an apparent effect of SC *versus* OC. Fig. 9 shows the results of a representative experiment. Four of the experiments conducted with 10⁻⁴ mol l⁻¹ cytochalasin E and five of the experiments conducted at 10⁻⁵ mol l⁻¹ cytochalasin E were on impalements for which there was no initial uptake of TMA⁺. No TMA⁺ uptake was observed after cytochalasin E treatment in any of these.

The stimulation of TMA⁺ uptake by cytochalasin E was taken initially as evidence of valve opening, but measurements of tissue electrical properties did not support this supposition. If cytochalasin E were opening goblet valves, V_g would be expected to become less positive, reflecting a closer approach to the Donnan equilibrium between the goblet matrix and the gut luminal solution. The Donnan potential of goblet cavities is expected to be negative with respect to the gut lumen, because of the fixed negative charge of the goblet matrix (Moffett and Koch, 1988b). However, in five OC experiments, the change in V_g ranged from 0 to +6 mV, with a mean change of +2 mV; in two SC experiments, the changes were -8 and -2 mV. In some of the cytochalasin E experiments, current pulses were delivered between the goblet cavity and the lumen before, during and after its administration. The resistance from cavity to lumen was estimated to have fallen by 10%; this magnitude of change is within measurement error.

After the administration of cytochalasin E, regardless of whether the particular cavity impaled had allowed entry of TMA⁺ or not, subsequent impalements suggested that TMA⁺ had gained entry into many goblet cavities. That is, the initial readings from the K⁺-selective electrode of the next impalements were much too high to represent (K⁺) and must have reflected the presence of TMA⁺. The elevated readings

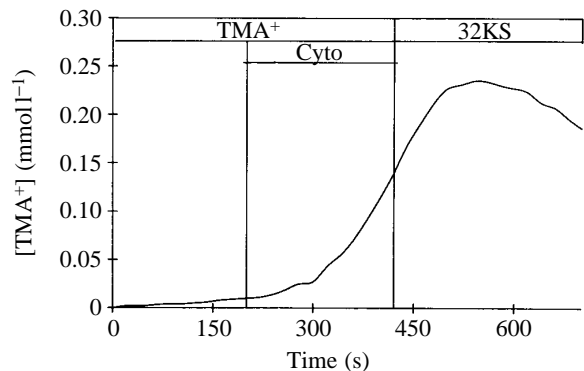


Fig. 9. Rapid increase in TMA⁺ influx during application of cytochalasin E (Cyto) (10⁻⁵ mol l⁻¹) and the start of the subsequent efflux.

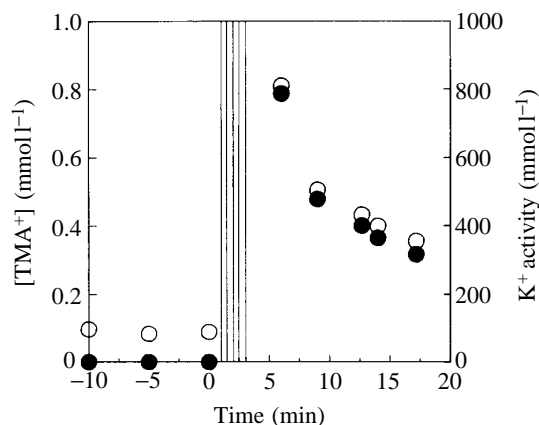


Fig. 10. K^+ microelectrode measurements from goblet cavities of a single tissue before and after a brief superfusion with 10 mmol l^{-1} TMA^+ + $10^{-5} \text{ mol l}^{-1}$ cytochalasin E (indicated by vertical lines) begun at time zero. Each pair of points is from a different penetration, with results expressed in two forms: assuming that the electrode is measuring $[\text{TMA}^+]$ (filled circles) and assuming it is measuring (K^+) (open circles). For the three pre-cytochalasin penetrations, (K^+) values are in the range of previously reported measurements; for the five post-cytochalasin measurements, the electrode must be detecting TMA^+ because the values are too high to reflect (K^+) .

declined over time after cytochalasin E treatment had been terminated, in a pattern suggestive of TMA^+ washout. In the example of Fig. 10, the initial reading from the first impalement at 5 min after TMA^+ + cytochalasin E treatment is equivalent to a value of (K^+) of more than 800 mmol l^{-1} , and after 18 min the last impalement still gave a reading equivalent to more than 300 mmol l^{-1} . In one case, we recorded an apparent (K^+) after cytochalasin E treatment of more than 3 mol l^{-1} . This phenomenon was also evident in successive impalements in the normal TMA^+ experiments. However, without cytochalasin E, it was much less obvious and simply led to apparent values of (K^+) between 150 and 200 mmol l^{-1} .

Discussion

The original hypothesis of these studies was that the goblet cell valve was a large (in ionic dimensions) water-filled hole. If so, then both ions (K^+ and TMA^+) should cross such a valve by free diffusion. The limiting conductivity of TMA^+ is 61 % of that of K^+ (Robinson and Stokes, 1959) so the flux rates for TMA^+ should be within a factor of two of those for K^+ . Equilibration times for isotopic K^+ applied to the hemolymph side of the tissue range from 2 to 8 min (Zerahn, 1975; Wood and Harvey, 1979). Hence, equilibration rates would be expected to be in the range 4–15 min for TMA^+ . Furthermore, one would expect the rate of uptake to be highest at the beginning of exposure to TMA^+ since the concentration gradient is greatest then. During the overshoot, when $[\text{TMA}^+]_g$ continues to rise even though the luminal concentration is zero, a direct path through the valve would have led to a sudden reduction in the rate of rise when luminal TMA^+ exposure was

ended. Figs 4A, 5 and 9 clearly show that there is no sudden change in the rate of TMA^+ accumulation early in the leach-out period of OC preparations. Finally, one would expect free movement of TMA^+ to comply with the Ussing flux ratio.

The data clearly fail to agree with all four of these predictions. The immediate conclusion is that TMA^+ cannot cross the goblet valve in either direction and that the goblet valve is therefore not a large water-filled hole. If K^+ is pumped into the goblet cavity and passes from there into the gut lumen, it must use a pathway that is small enough to have ionic selectivity.

Possible sources of error

There are four different circumstances that might lead us to err in concluding that the goblet valve is not patent. These are as follows. (1) There might be a thick unstirred layer between the bulk luminal fluid and the tissue. Its presence would lead to abnormally low influx rates as well as to second-order behavior such as concave upwards influx curves and overshoot. (2) There might be a large endo-osmotic water flow through the valve secondary to the active extrusion of K^+ . Its presence would also retard TMA^+ influx and thus lead to a failure of the data to fit the Ussing relationship. (3) The valves might open or shut intermittently. (4) The relative sensitivity of the electrode to K^+ versus TMA^+ might be altered in the glycoprotein matrix of the goblet cavity. These possibilities are examined below.

Unstirred layer

There are three reasons to exclude the presence of a thick unstirred layer. The first is that the 'push-through' did not show a large unstirred layer. The time constant for influx in the experiment depicted in Fig. 3 was approximately 4 s for the 'push-through' and approximately 2500 s for the subsequent goblet impalement in the same tissue.

The second reason for rejecting a thick unstirred layer comes from the measured response times. An estimate of the thickness of an unstirred layer can be made from the relationship:

$$x^2 = Dt, \quad (4)$$

where x is the thickness of the layer, D is the diffusion coefficient and t is the time in seconds. D for TMA^+ is of the order of $10^{-5} \text{ cm}^2 \text{ s}^{-1}$. The smallest estimate of the average phase lag for the overall set of data is about 4000 s. An unstirred layer would have to be 2 mm thick to produce this lag. Since the apical side of the chamber is 4 mm deep, and fluid is flowing through the chamber at a rate that gives a chamber turnover time of less than 0.5 min, a 2 mm unstirred layer seems unreasonable. For the experiment shown in Fig. 3, the estimated thickness of the unstirred layer in the goblet cavity would have been calculated as approximately 1.6 mm, whereas the luminal value estimated from the electrode response in the 'push-through' experiment was approximately $63 \mu\text{m}$. The latter value does seem reasonable.

The third argument comes from the absence of overshoot in

the SC preparation. A large unstirred layer could produce an overshoot such as that seen in OC preparations, but one would also expect to see comparable overshoots in SC preparations. Overshoots were not prominent either in SC preparations or in the two 'bad' OC preparations (tissues with low V_g from which two good impalements were found).

Bulk water flow through the valves

Net secretion of water by the midgut has been reported (Nedergaard, 1972; Zerahn, 1985); presumably this occurs by endo-osmotic movement in response to net K^+ secretion (Zerahn, 1985). Two arguments suggest that this process is not important for the results presented here. First, the system of an open valve with bulk water flow would still be a first-order system. The equation describing direct entry of TMA^+ from lumen to goblet in the absence of bulk water flow is:

$$d[TMA^+]_g/dt = K\{[TMA^+]_l \times q - [TMA^+]_g\}, \quad (5)$$

where K includes both the permeability and the cavity volume, $[TMA^+]_l$ refers to the concentration of TMA^+ in the lumen and q is the weighting factor for the goblet-to-lumen voltage difference (Φ). $q = \exp(-F\Phi/RT)$. The solution of this equation is:

$$[TMA^+]_g = [TMA^+]_l \times q \times \{1 - \exp(-Kt)\}, \quad (6)$$

a single exponential with rate constant K . If bulk flow from goblet to lumen carries TMA^+ with it, the differential equation becomes:

$$d[TMA^+]_g/dt =$$

$$K\{[TMA^+]_l \times q - [TMA^+]_g\} - \nu \times [TMA^+]_g, \quad (7)$$

where ν is the volume flow from the goblet cavity in $ml\ s^{-1}$ for a goblet cavity divided by the volume of the goblet cavity. The solution of this equation is:

$$[TMA^+]_g = [TMA^+]_l \times q \times \frac{K}{K + \nu} \{1 - \exp(-\{K + \nu\}t)\}. \quad (8)$$

This is still a single exponential function, but the equilibrium value is lower and the rate of approach to equilibrium is higher. That is, the initial rate of influx should be higher than any later influx rates. The last column in Table 1 shows that this was not the case. Also, in a first-order system, there should be no overshoot and $[TMA^+]_g$ should start to fall as soon as the lumen is cleared of TMA^+ . There was a significant overshoot in all of the OC experiments. There was a much smaller, but definite, overshoot in most of the SC experiments (see Fig. 4B, for example).

Intermittent valve opening

It has been suggested that the valves in this tissue open and close (Smith *et al.* 1969), and this hypothesis is supported by the finding that the valve is surrounded by a dense network of actin-based cytoskeletal elements (U. Klein, personal communication). However, no spontaneous transitions in TMA^+ influx or efflux suggestive of valve opening or closing

were noted in the recordings from more than 100 penetrations. We can estimate the frequency with which we would have expected to see an open goblet valve close or a closed valve open, assuming that the transition between the two states is random and asynchronous. If the average period for a hypothetical valve cycle is defined as the duration that a valve stays closed plus the duration of the subsequent opening, the closed state would account for 70% of this period, because only 30% of the goblet cavities were accessible to TMA^+ . The shorter the period, the more frequently would we expect to observe transitions. Taking the average time for observation as about 10 min, 17 transitions would be expected to be observed per 100 impalements if the cycle period were 2 h. We should still expect four transitions per 100 impalements if the cycle period were 8 h. Because we found no transitions, our data do not support spontaneous intermittent opening and closing of the goblet valves in the isolated tissue. This result does not rule out valve opening *in vivo*, but it does show that valve opening is not a necessary condition for active K^+ transport, which continues at a high rate for several hours in isolated tissues.

Reduced electrode sensitivity

The problem of reduced electrode sensitivity is one common to all studies using intracellular ion-specific microelectrodes. We note that if TMA^+ uptake rates were really comparable to those expected in the case of an open valve, the sensitivity of the electrode to TMA^+ must have been reduced by more than a factor of 10. Since the resin is 1000 times more sensitive to TMA^+ than to K^+ , one would certainly also expect to see a reduction in sensitivity to K^+ . However, the values of $[K^+]$ we measured in goblet cavities generally agreed with those obtained using X-ray microanalysis (Dow *et al.* 1984). Finally, if the resin sensitivity had been reduced by a factor of 10, the cytochalasin E data illustrated in Figs 9 and 10 would imply that $[TMA^+]_g$ had reached values of more than $2\ mmol\ l^{-1}$ in one case and more than $8\ mmol\ l^{-1}$ in the other. Both of these values exceed the Nernst equilibrium value between the lumen and goblet cavity. Since we do not believe that there was a bulk flow of fluid into the goblet cavity from the lumen or that there was active transport of TMA^+ into the cavity, we suggest that these data indicate that the sensitivity of the electrodes to TMA^+ within the tissue was not very different from that determined in the calibrating solutions.

The results with cyclic AMP could be taken to suggest that its stimulation of K^+ transport is accompanied by an increase in the diameter of some already-open valves, but this suggestion is contradicted by the failure of cyclic AMP to stimulate TMA^+ efflux (Fig. 8). Similarly, the results with cytochalasin E would seem to support the possibility of valve opening. However, the large increase in TMA^+ access in the presence of cytochalasin E was not accompanied by commensurate changes in V_g or transvalve electrical resistance. Furthermore, compartmental analysis of the TMA^+ efflux curves after cytochalasin E treatment (Koch and Moffett, 1995) revealed a substantial post-cavity compartment much too large to be accounted for by any plausible volume of the valve

passage itself. This compartment is visible as a substantial overshoot in the TMA⁺ influx curve shown in Fig. 9. Finally, we submitted tissue samples from these experiments to another laboratory for ultrastructural examination; no difference in valve ultrastructure was detected between control and experimental tissues (R. S. Hakim and K. M. Baldwin, personal communication).

Pathways of TMA movement – evidence for an indirect entry route

Although in 70% of the penetrations TMA⁺ did not gain access to the goblet cavity, it undeniably appeared in the other 30%. How did it get there? As discussed above, any passive movement through a direct connection between lumen and goblet cavity would lead to first-order kinetics for both influx and efflux. That is, the influx must start sharply and the curve must be convex. Efflux must start as soon as TMA⁺ has been cleared from the luminal solution and the curve must be concave and without an overshoot. These statements are valid even if endo-osmotic convective flow is present. The last column of Table 1, which gives the ratio of late to early fluxes, shows that the early uptake flux rates are not the highest. Furthermore, out of more than 100 recordings of TMA⁺ exposure, only one fulfilled the requirements for first-order behavior. In all of the others, initial influxes were lower than those determined later in the exposure – this implies concavity of the influx curve. In all of the OC preparations, there was a large overshoot and in most of the SC preparations, there was a small overshoot.

The presence of an overshoot and concavity in the influx curve (see Fig. 4) implies that [TMA⁺] did not rise as a step function in the compartment feeding the goblet cavity. Since, for the purposes of these experiments, [TMA⁺] did rise and fall as a step function in the lumen, there must be at least one mixing compartment between the lumen and the goblet cavity. From the perspective of the goblet cavity, this is a pre-compartment and the system is of an order greater than one. This result could be explained if TMA⁺ entry into the goblet cavities occurred by way of one or more pre-compartments within the tissue. The finding that the overshoot is larger under open-circuit conditions implies that this pre-compartment experiences the large transapical potential present when the tissue is open-circuited.

An obvious way to reconcile these results with the standard model of goblet cell function is to hypothesize that the valve itself is the pre-compartment for the goblet cavity. However, a compartmental model of the results (Koch and Moffett, 1995) fitted best when the volume of the pre-compartment was 25% of the total tissue volume, whereas the most liberal estimate of valve volume from electron micrographs (e.g. Smith *et al.* 1969) would be only a few per cent of the total tissue volume. This forced us to consider possible indirect routes. In a separate paper (Koch and Moffett, 1995), we propose a model in which there are two pre-compartments, the first being the columnar cell cytoplasm and the second being the goblet cell cytoplasm.

In summary, these studies confirm the suggestion by Dow

and Peacock (1989) that the goblet cavity is physically and electrically isolated from the gut lumen. Our results point to two possible hypotheses for future work: either there is a non-diffusional mechanism for K⁺ exit from the goblet cavity, or the goblet cavity apical membrane is not the site of the K⁺ transport measurable by the I_{sc} of the isolated preparation.

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