IDENTIFICATION OF ACTIVE CELL IN POTASSIUM TRANSPORTING EPITHELIUM

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SUMMARY

Epithelial ion transport regulates the environment of cells and modulates the environment inside them, making possible the evolution of eukaryotic organisms. Analysis of the transport process requires that the route taken by ions as they are actively transported across epithelia be known. The route can be determined by locating ion pumps electrically and by measuring transport pool sizes kinetically provided that the epithelial structure is simple enough to enable the results to be interpreted in cellular terms. The route by which potassium is actively transported across the lepidopteran midgut is demonstrated here by a combination of electrical and kinetic analyses.

Two sorts of cells, goblet and columnar cells, are distinguished by their electrical properties. The goblet cell is identified as the site of active potassium transport, the pump being localized in its apical membrane. The goblet cells are electrically coupled with columnar cells only when midguts that have been isolated from diet-reared larvae are short circuited. A corollary to this result is that the size of the pool of potassium which is involved in transport should be small when the goblet cells are not coupled with columnar cells and should become large when they are coupled. This corollary has been confirmed by direct measurement of the transport pools using isotope tracer kinetic analysis.

This is the first time that the cell type responsible for active ion transport in a polymorphic tissue has been identified directly by physiological means.

INTRODUCTION

Knowledge of epithelial ion transport is important for our understanding of cellular function because the ionic composition of the fluid in which eukaryotic cells are bathed is regulated by epithelial ion pumps. As Claude Bernard has stressed, the very evolution of eukaryotic organisms has been possible only because the internal environment is constant (Bernard, 1878). Thus, the ionic composition within cells, which modulates protein synthesis (Lubin & Ennis, 1964), is determined in part by the extracellular ionic composition. Before such epithelial ion pumps can be isolated chemically and their structures proven by reconstitution, the route taken by the ions during transport must be established. Specifically, the location of the ion pump and of the pool from which it transports must be known.

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If an ion pump is electrogenic (i.e. if its action produces an electrical potential difference) then the most direct way to locate the pump is to probe the tissue with microelectrodes and look for changes in electrical resistance as the pump is turned off and on (Blankemeyer, 1976a, b). The most direct way to locate an ion pool is to determine its size by tracer kinetic analysis and use this information to deduce the compartment within the epithelium which might contain it (Wood & Harvey, 1975). Both electrical probing of pump location and tracer measurement of pool size must be interpreted with respect to cell structure. Hopefully, the route can be confirmed by testing corollary hypotheses (e.g. that the pool size must increase if non-transporting cells become electrically coupled to the transporting cells).

Surprisingly, the transport route has not previously been established for any complex tissue. Thus the location of the sodium pump within the well known frog skin is still undecided with recent papers still trying to demonstrate which cellular layer within the skin contains the pump (Nagel, 1976). In toad urinary bladder the sodium pool is only a small fraction of the total sodium, making the deduction of its location difficult (MacKnight, Civan & Leaf, 1975).

In the present paper we establish the route taken by potassium ions as they are actively transported across the isolated midgut of lepidopteran larvae. The midgut's numerous columnar cells and less numerous goblet cells are arranged in a single layer with occasional replacement cells clustered basally, all of the cells being so large that interpretation of both electrical and tracer kinetic data is possible in cellular terms (Anderson & Harvey, 1966).

A summary of the little known midgut system follows (Harvey & Zerahn, 1972). When larvae of *Hyalophora cecropia* (L) are reared on foliage the high potassium level of the leaves is reflected by a high level, 250 mM, in the midgut contents whereas the haemolymph level is much lower, 26 mM (Harvey et al. 1975). The isolated midgut transports potassium actively from haemolymph to lumen sides. The transport process is electrogenic and renders the lumen side more than 100 mV positive to the blood side. The activity of the potassium pump (as measured by the potential difference, the short circuit current, or by isotopically measured fluxes) is quickly inhibited by anoxia or metabolic inhibitors but is unaffected by cardiac glycosides (Haskell, Clemons & Harvey, 1965; Jungreis & Vaughan, 1977).

The midgut epithelium of *H. cecropia* is composed of numerous columnar cells, with apical microvilli and basal infoldings of the plasma membrane lined with mitochondria, and less numerous goblet cells, with an apical cavity ringed with cytoplasmic projections each containing a mitochondrion and, uniquely, bearing spicules on the inner leaflet of the plasma membrane (Anderson & Harvey, 1966). In other Lepidopteran insects such as *Manduca sexta*, there are small numbers of a third cell type, the replacement cell, (T. W. Schultz, personal communication), but these are virtually absent in *H. cecropia* (Anderson & Harvey, 1966), and therefore cannot be the pumping cells.

The first attempt to locate the potassium pump electrically was by Wood, Farrand & Harvey (1969) who impaled the isolated midgut with Ling-Gerard microelectrodes and described a single type of two-step potential profile across the epithelium. On the average, as the microelectrode penetrated the tissue from the basal side it became 27 mV negative with respect to the basal bathing solution. This basal PD step was
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insensitive to anoxia but sensitive to changes in the potassium concentration of the bathing solution. As the microelectrode emerged from the tissue to the lumen side it became 98 mV positive with respect to the basal bathing solution, i.e. it measured the transepithelial PD. Later Schultz (1972) showed that PD measurements alone are not sufficient to characterize epithelia electrically.

The first attempt to measure the transport pool size was by Harvey & Zerahn (1969) who used $^{42}$K influx kinetics to measure the K transport pool. The report of Wood et al. (1969) that there was only one type of basal step in the impalement profiles implied that all of the epithelial cells were coupled electrically and must share a common ion pool. The small pool size found by Harvey & Zerahn (1969) suggested an extracellular or non-mixing pool and an extracellular K pump location. However, a subsequent kinetic analysis by Wood (1972) and Harvey & Wood (1972) revealed a large transport pool which amounted to two thirds of the tissue potassium. Later, Harvey & Wood (1973) demonstrated that the loading pool of potassium was approximately equal to the kinetically measured transport pool and concluded that the potassium transport pool and active potassium transport site were located intracellularly. This pool size controversy remained extant with Zerahn (1973, 1975) finding evidence for a small isotope loading pool and Wood & Harvey (1975) finding a large pool size from both influx and loading experiments.

We will show that the potassium pump is located in the apical membrane of the goblet cells because the resistance of that membrane increases uniquely when the electrogenic potassium pump is turned off by removing its oxygen supply. We will show that the potassium pool size is so small that it can be contained in just the goblet cells under conditions when these cells are uncoupled from columnar cells but that the pool size is so large that it must occupy the columnar cells as well under conditions when these two cell types are coupled.

We are able to conclude that potassium ordinarily enters the goblet cells across their basal and lateral borders and forms a cellular pool from which it is actively transported across the apical membrane to the goblet cavity which is confluent with the lumen side of the tissue. When midguts are isolated from larvae fed on artificial medium and are short-circuited the columnar cells become electrically coupled to the goblet cells while the size of the potassium pool increases accordingly.

MATERIALS AND METHODS

All experiments were performed on feeding fifth instar larvae. Leaf-fed H. cecropia were reared on wild black cherry trees; diet-fed H. cecropia were reared on Riddiford’s diet (1968); and diet-fed M. sexta were reared on Yamamoto’s diet (1969). Midguts were isolated from larvae and mounted as flat sheets on perspex chambers (Wood, 1972). The bathing solutions were composed of 32 mM-KCl, 1 mM-CaCl$_2$, 1 mM-MgCl$_2$, 5 mM-TrisCl, and 166 mM-sucrose. The transepithelial potential difference was measured with calomel electrodes and a Keithley 602 electrometer (Keithley Instruments). The midgut was short-circuited by a device which automatically passes current (through Ag-AgCl electrodes) sufficient to maintain the PD at the asymmetry PD of the calomel electrodes (close to zero) (Wood, 1972). The potassium influx was measured with $^{42}$K or $^{88}$Rb and was corrected for decay in the short circuit current.
yielding a single exponential time course. The kinetic influx pool size, $S_p$, was calculated as the steady state influx, $J^*$, divided by the mixing time constant, $\alpha$ (Wood & Harvey, 1975).

For impalement with microelectrodes midguts were mounted as flat sheets in a chamber modified to allow access to one side of the midgut through the bathing solution (see Fig. 1). Micropipettes were pulled from borosilicate glass (1-2 mm o.d., 0.5 mm i.d.) on a horizontal puller (Industrial Science Associates, Model M-1) and were filled with 3 M solutions of KCl by the method of Tasaki (1968). The microelectrodes had a typical resistance of 10 MΩ. For the measurement of resistance and of cell to cell coupling external current was applied either through Ag, AgCl electrodes in the bathing solutions or through Ag, AgCl wires in KCl filled microelectrodes. Potential differences which were measured with microelectrodes were impedance matched with a high impedance ($10^{13}$ ohms) voltage follower and measured on a Tektronix 5114 oscilloscope or a Keithley 602 Electrometer. Bipolar, constant-current pulses of 0.5 s duration and typically 100 nA current for microelectrode impalements and 10 μA current for transepithelial measurements were used to measure transmembrane and transcellular resistance. The transepithelial resistance is calculated from the voltage change produced by the current pulse. The transcellular resistance is calculated from the transcellular voltage change produced by the constant current pulse through the microelectrodes. The voltage change was referenced to the haemolymph side solution although either side of the midgut could have been used for reference (transepithelial resistance of midgut is ~ 200 Ωcm²).
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RESULTS AND DISCUSSION

Microelectrode PD profiles of the midgut

An impalement of the midgut of H. cecropia with a microelectrode reveals the PD profile shown in Fig. 2 (left). The microelectrode was advanced toward the tissue from the haemolymph (basal) side. In the first PD step encountered, the basal PD, the microelectrode voltage becomes negative with respect to the haemolymph side bathing solution. After further movement of the microelectrode toward the lumen (apical) side of the tissue a second PD step is encountered, the apical PD, in which the voltage becomes positive to the haemolymph side solution yielding the trans-epithelial PD. The basal PD in Fig. 2 (left) has a value of $-27$ mV in good agreement with the values reported by Wood et al. (1969). The value of the apical PD is the algebraic difference between the transepithelial PD and the basal PD and is therefore dependent on the value of both. In this case it amounted to $+132$ mV.

Less frequently such an impalement of the midgut reveals a second type of PD profile which is shown in Fig. 2 (right). This profile has a lower (closer to zero) basal PD than that in Fig. 2 (left). The value of this second type of basal PD varies from preparation to preparation, often being positive to the basal solution, and amounting to $-1$ mV in this case. At first it seemed that this second profile was an artifact of
Table 1. Cellular resistance of LPD and HPD impalements in paired experiments in oxygen and nitrogen

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<tr>
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<th>LPD</th>
<th>HPD</th>
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<tr>
<td>O₂</td>
<td>7.6 ± 1.5 x 10⁶ (5)</td>
<td>2.3 ± 0.33 x 10⁶ (4)</td>
</tr>
<tr>
<td>N₂</td>
<td>2.0 ± 0.4 x 10⁶</td>
<td>2.4 ± 0.6 x 10⁶</td>
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* The resistance was measured as the voltage change from the tip of the intracellular microelectrode to the bathing solution divided by the constant microelectrode current and is presented in ohms. All results are paired (in the same cell) between oxygen and nitrogen. (*M. sexta*, diet.)

impalement or of cellular damage. However, the second type of profile was seen repeatedly in previously unimpaled as well as impaled regions and its basal PD remained constant for as long as 20 min. Clearly a previously unseen type of profile has been discovered. The profile showing the -27 mV basal step, as in Fig. 2 (left), will be referred to as an HPD (High Potential Difference) profile and the one showing the -1 mV basal step, as in Fig. 2 (right) will be referred to as an LPD (Low Potential Difference) profile. The original profile described by Wood et al. (1969) is clearly an HPD profile whereas the new one just described is an LPD profile. These are the only types of profile found in midguts from *H. cecropia*. A third type of profile with an intermediate basal step appears in impalements of midguts from *M. sexta* (Fig. 3) and will be referred to as an IPD (Intermediate Potential Difference) profile. These profiles have been confirmed repeatedly in midguts isolated both from *H. cecropia* and from *M. sexta*.

Effects of oxygen-lack on resistance of LPD and HPD profiles

An electrogenic ion pump should show a resistance change upon changes in pump rate (Thomas, 1972). K active transport in insect midgut is electrogenic since all of the resistance change occurring during the typical decay is due to changes in active transport (Blankemeyer, 1976b, 1978a). Since a change in active transport is associated with a change in resistance, identification of the source of the resistance change will locate the site of K active transport or pump.

Resistance of cells — A midgut was impaled with a microelectrode until a stable LPD profile or a stable HPD profile was identified. Then current was passed from the microelectrode in the cell to the bathing solution and the resistance was measured by the change in voltage due to the applied current (see Methods). No corrections are necessary for the spread of current to other cells because these measurements were made under open circuit conditions (see section on Intercellular Communication). The stirring gas was then changed from oxygen to nitrogen and these cellular resistances were measured again (in the same cell). The experiment was conducted five times on LPD cells and four times on HPD cells. The resistance of the LPD cells increased in nitrogen whereas the resistance of the HPD cells was unchanged in nitrogen (Table 1). Further experiments (Blankemeyer, 1976a, b) show that the change in resistance occurs shortly after the stirring gas is changed from oxygen to nitrogen and that it parallels a change in transepithelial resistance.

Apical to basal resistance ratios — In a separate series of experiments the ratio of
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Table 2. Ratio of apical to basal resistances in LPD and HPD cells in nitrogen and oxygen*

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<tr>
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<th>LPD</th>
<th>HPD</th>
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<tr>
<td>O₂</td>
<td>4.31 ± 0.53 (13)</td>
<td>23.5 ± 4.6 (11)</td>
</tr>
<tr>
<td>N₂</td>
<td>20 ± 1.75 (6)</td>
<td>30.4 ± 4.1 (5)</td>
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* The resistance ratio was calculated from the ratio of apical PD change to basal PD change when a current pulse was passed across the epithelium. (M. sexta, diet.)

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The finding of the primary resistance change in the LPD cell (Table 1) identifies this cell type as the source of the increase in transepithelial resistance in anoxia and therefore as the source of the transepithelial active potassium transport. The values of cellular resistance (Table 1) and of the apical to basal resistance ratios (Table 2) can be substituted into simultaneous equations which show that the resistance change in the LPD cell is localized in its apical membrane identifying the apical membrane of the LPD cell as the location of the potassium pump (Blankemeyer, 1976b).

Identification of LPD profile with goblet cell and HPD profile with columnar cell

Having demonstrated that the active potassium transport site is located in the apical region of the cell yielding the LPD profile it remains to identify the cell. In H. cecropia there are essentially only two cell types: the goblet cell and the columnar cell, replacement cells being so infrequent that Anderson & Harvey (1966) could find none for electron microscope analysis. Only LPD and HPD profiles are found in H. cecropia (Blankemeyer, 1976b). Therefore, the LPD profile must arise in either goblet or columnar cells in impalements from H. cecropia. In M. sexta there are replacement cells as well (T. W. Schultz, personal communication). In M. sexta there are three types of impalement profile, the third being the IPD profile which is infrequent (Fig. 3) and must arise from the infrequent replacement cells. Therefore the LPD profile must arise in either goblet or columnar cells in impalements from M. sexta as well as in those from H. cecropia.

Indirect but convincing evidence points to the goblet cell rather than the columnar cell as the source of the LPD profile. In impalements from H. cecropia LPD profiles are about one-half as frequent as HPD profiles (see Fig. 3). Analysis of the micrographs of Anderson & Harvey (1966) reveals that the goblet cells occur only half as often as columnar cells and that there is scant goblet cell cytoplasm in which a microelectrode tip may rest, so it is clear that the LPD profiles must come from goblet cells in H. cecropia. This hypothesis was tested in M. sexta by making 227
Fig. 3. Frequency distribution of 227 impalements of midguts showing three populations. We designate the left hand population (mode at $-28 \text{ mV}$) as HPD, the middle population (mode at $-17 \text{ mV}$) as IPD, and the right hand population (mode $-9 \text{ mV}$) as LPD profiles respectively. The HPD and LPD profile populations do not overlap being separated by the IPD profile population. (*M. sexta*, diet.)

random impalements under as nearly identical conditions as possible (Fig. 3). The most frequent profile was the HPD profile which clearly must arise in the numerous large columnar cells in *M. sexta* as well as in *H. cecropia*. The least frequent profile was the IPD profile which must arise from the infrequent replacement cells as discussed in the previous paragraph. By elimination, then, the LPD profile must arise from the goblet cells in *M. sexta* as well as in *H. cecropia*, a conclusion which is supported by the relatively low frequency of both LPD profiles and goblet cells in *M. sexta*.

Efforts to confirm this deduction by iontophoretic injection of Niagara Sky Blue dye via microelectrodes in *M. sexta* revealed in whole mounts that dye injected into HPD cells appears in columnar cells (Blankemeyer, personal observation). Attempts to recover the dye in sectioned midguts have been unsuccessful.

In summary, then it is virtually certain that the LPD profiles arise in goblet cells and that the HPD profiles arise in columnar cells. It should be noted in passing,
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however, that the validity and significance of finding the K pump in the apical membranes of the LPD cells does not require that these cells be identified as goblet cells.

Pool size controversy

Although these electrical and cytological results clearly identify the apical region of the goblet cell as the active K transport site, a transport route through just the goblet cells cannot be regarded as established until the controversy regarding pool size is resolved. A goblet cell pathway would explain the small pool size found by Harvey & Zerahn (1969) and confirmed in loading experiments by Zerahn (1973, 1975) but it is inconsistent with the large pool size found by Wood & Harvey (1975). An answer was suggested when communication between goblet and columnar cells was found only in short circuited preparations from diet-fed larvae and was confirmed by comparing pool sizes from leaf- and diet-fed larvae under both open circuit and short circuit conditions.

Intercellular communication – corollary hypothesis

Extensive communication between goblet and columnar cells had been suggested by the uniform PD profiles obtained with microelectrodes in the original study by Wood et al. (1969) and seemed to be confirmed because the kinetically measured pool size was so large that it must involve all of the epithelial cells (Wood, 1972). However the microelectrode impalements illustrated in Fig. 2 and summarized in Fig. 3 show that the PD profiles are not uniform but that there are three types of profile, namely, LPD, IPD, and HPD profiles. These results imply that normally there is no electrical coupling between goblet and columnar cells. To resolve this discrepancy intercellular communication was studied in midguts from both leaf- and diet-reared larvae under both open circuit and short circuit conditions. The results of this study, which are shown as Table 3, reveal that there is no coupling between LPD and HPD cells in midguts from leaf-fed larvae or in those from diet-fed larvae under open circuit conditions. However, preparations from diet-fed larvae under short circuit conditions show extensive electrical coupling between LPD and HPD cells beginning less than one minute after the onset of short circuiting. This result suggests the corollary hypothesis that large pool sizes will be found in short circuited preparations from diet-fed larvae but under no other conditions.

Potassium influx pool under open circuit conditions

So long as the midgut is maintained under open circuit conditions the pool size is small whether the preparation is from leaf-fed or diet-fed larvae. This result is illustrated in Fig. 4 in which the steady state influx was measured with $^{86}$Rb and the time course of mixing was measured with $^{42}$K. The size of the influx pool, which is the area between these two curves (Wood, unpublished), is small, less than 10 μequiv K g$^{-1}$ wet weight, in both leaf-fed preparations and in diet-fed preparations. Using the value of 65 μequiv K g$^{-1}$ wet weight of tissue for a midgut mounted on a chamber (Harvey & Zerahn, 1969) the influx pool is 15% of the tissue K. These results confirm the small pool size initially reported by Harvey & Zerahn (1969) and subsequently
Table 3. Coupling ratio \( \frac{V_{1}}{V_{2}} \) between epithelial cells of isolated midgut*

<table>
<thead>
<tr>
<th>Open circuit</th>
<th>Short circuit</th>
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<tr>
<td>Leaf-fed</td>
<td>Diet-fed</td>
</tr>
<tr>
<td>CC</td>
<td>CC</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.46 ± 0.09</td>
<td>(3 midguts)</td>
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* A microelectrode was placed in each of two adjacent cells. A current was passed to ground from the first electrode and the ratio of PD between this cell to ground and the adjacent cell to ground \( \frac{V_{1}}{V_{2}} \) was calculated. In columns headed CC both electrodes were in columnar cells and in those headed CG one was in a columnar cell and the other in a goblet cell. The 'N' for all entries was 10 or more impalements/midgut and at least 3 midgut/entry. The measurements showing coupling in 3 midguts under short circuit conditions were paired with measurements showing no coupling under open circuit conditions, with the microelectrodes in the same cell. (H. cecropia and M. sexta, diet and H. cecropia, leaf.)

Fig. 4. Pool sizes in open circuit preparations. The upper traces record the time course of the transepithelial PD (left axis; mV) and the lower traces record the time course of \(^{48}\)potassium-measured influx (right axis, \( \mu A \)). The influx pool sizes were calculated as the area between the extrapolated steady state \(^{86}\)Rb influx (straight horizontal) lines and the \(^{48}\)K influx curves. For leaf-fed larvae (left) the pool size was 0.47 \( \mu \)equiv of potassium and for diet-fed larvae (right) the size was 0.38 \( \mu \)equiv. The latter determination was at 120 min when the transport rate as indicated by the PD had decayed somewhat accounting for the slightly smaller pool size. (H. cecropia).

Confirmed by Zerahn (1973, 1975). However both this initial report and the challenge to it by Wood & Harvey (1975) were based on pool size determinations under short circuit conditions.

Potassium influx pool under short circuit conditions

So long as the midgut is isolated from leaf-fed larvae this same small pool size is found even under short circuit conditions (Fig. 5, horizontal hatching). This result confirms the small pool size found by Harvey & Zerahn (1969) in short circuited preparations from leaf-fed larvae. However, if the midgut is isolated from diet-fed larvae and is short circuited, then the pool size is large, amounting to 60 \( \mu \)equiv K g\(^{-1} \) wet weight (Fig. 5, vertical hatching). This is the same large pool size reported by
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Harvey & Wood (1972) and Wood & Harvey (1975). In both of these latter papers the pool size was measured under short circuit conditions in midguts from diet-fed H. cecropia larvae. A detailed account of the pool sizes obtained with H. cecropia and M. sexta is in Blankemeyer (1976b) and is being prepared for publication (Blankemeyer, 1978b).

The corollary hypothesis is proven to be correct and the influx pool size controversy is resolved: only short circuited preparations from diet-fed larvae have large pool sizes; and only short circuited preparations from diet-fed larvae have coupling between columnar and goblet cells. Clearly the large pool size is the result of the addition of the columnar cell potassium pool to the goblet cell potassium pool in short circuited preparations from diet-fed larvae. In all other cases the cells are not coupled; only the goblet cell potassium pool is involved in transport; and the pool size is small.

Conclusion

The chain of evidence demonstrating that the potassium transport route lies through the goblet cells is strong and unbroken. Electrical evidence demonstrates that the transporting cells give rise to the LPD profiles and that the pump is in the apical border of the LPD cells. The histogram of basal PD’s strongly indicates that the LPD profiles are from goblet cells. Therefore, the site of active, transepithelial, potassium transport is in the apical membrane of the goblet cells. In open circuited preparations and in short circuited preparations from leaf-fed larvae the goblet cells are not coupled to columnar cells and the influx pool sizes are correspondingly small. However, in short circuited preparations from diet-fed larvae the goblet cells are coupled to columnar cells and the influx pool sizes are large. The transport route is demonstrated to be through the goblet cells with the pump in their apical border. In short circuited
preparations from diet-fed larvae potassium ions reach the goblet cells both from their basal surfaces and also from the interior of the columnar cells.

A powerful tool for the study of cellular coupling becomes available because for the first time coupling can be altered reversibly by the simple application of external current. Perhaps of greater significance, however, is the success of straightforward electrical resistance analysis and of straightforward influx kinetic analysis in this simple insect epithelium whose structure and ion pumping characteristics made possible the identification of the transporting cell type and transport route. The success of this approach in locating the transporting cell type in this simple polymorphic epithelium may establish a rationale for locating the transporting cell type in more complex polymorphic epithelia.

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