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REVERSIBLE ALKALINIZATION BY *MANDUCA SEXTA* MIDGUT

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Summary

1. Midguts of the larva of the tobacco hornworm larva, *Manduca sexta*, were isolated, pinned out and double perfused on the stage of an inverted microscope. The pH gradients across the anterior, middle and posterior regions of the gut were measured with a double-barrelled pH microelectrode, simultaneously with electrical potentials and the transepithelial potential difference (TEP).

2. The microenvironment surrounding the apical surface is more alkaline, and that surrounding the basal surface more acid, than either the perfusion medium or the intracellular pH. Under double perfusion, a stable gradient of 1.4 pH units is observed across the middle midgut. A similar gradient is found across the anterior midgut, but no significant pH difference occurs across the posterior midgut.

3. The pH gradient across the middle midgut is reversibly and symmetrically collapsed by anoxia, implying that it is sustained by a process requiring oxidative phosphorylation.

4. The time course of decay and reconstitution of the pH gradient matches closely the activity of electrogenic K^+ pumping, as measured by the TEP.

5. These results are consistent with a model for high pH generation which links electrogenic K^+ transport into the goblet cavities with net alkalinization of the lumen of the anterior and middle midgut regions.

Introduction

Larval Lepidoptera are voracious, rapidly growing phytophages. It is not surprising, therefore, to find that the midgut dominates the bodyplan of such insects (Dow, 1986). The midgut is endowed with a phenomenally active K^+-ATPase in the apical membrane of a unique goblet cell, pumping potassium from blood side to gut side (Harvey *et al.* 1981, 1983). A primarily excretory role for this pump has been rejected on several grounds, not least that potassium can be

Key words: *Manduca sexta*, K^+-ATPase, active transport, osmoregulation, pH transport, alkalinization.

rescued from the faeces (Dow and Harvey, 1988). However, several plausible roles for the K^+ pump have been suggested. The pump appears to regulate potassium levels in both blood and gut lumen (Dow and Harvey, 1988); it drives a K^+/amino acid cotransport (Nedergaard, 1977; Giordana *et al.* 1982, 1985), and it may help generate the massive pH gradient observed across the midgut (Dow, 1984).

There is now supporting evidence in favour of the latter model. The discontinuity in the pH profile at the junction of the middle and posterior midgut regions matches a conspicuous change in goblet cell morphology (Cioffi, 1979) and in carbonic anhydrase distribution (Ridgway and Moffett, 1986). There is also a systematic change in alkaline phosphatase levels along the length of the midgut (Azuma and Eguchi, 1989). The electrical isolation of goblet cavities, estimates of the trans-cavity potential difference (PD) at 270 mV (Dow and Peacock, 1989) and high proton permeability of the goblet cavity membrane (Wieczorek *et al.* 1989) were also predicted by the model (Dow, 1984).

However, clear demonstration of sustainable alkalinization by the midgut *in vitro* has proved elusive. Here, we investigate the microenvironment formed by the deep infoldings within the tissue, demonstrating that a significant pH gradient (>1 pH unit) exists in the microenvironment around the midgut, and that the gradient can be reversibly collapsed by exposing the tissue to anoxia. The results show that the pH gradient observed is consistent with a model linking luminal alkalinization (and basal acidification) to the electrical PD across the tissue.

Materials and methods

Fifth-instar larvae of *Manduca sexta*, weighing 3.5–6 g, were chilled, and the midguts were removed and pinned out on a microscope slide under double perfusion on the stage of an inverted microscope, as described previously (Dow and Peacock, 1989). The saline was based on earlier recipes (Dow and Peacock, 1989) and contained (in mmol l^{-1}) K^+, 32; Cl^-, 36; Mg^{2+}, 1; Ca^{2+}, 1; Tris–HCl, 5; sucrose, 200. Additionally, 5 mmol l^{-1} sodium succinate, 5 mmol l^{-1} caproic acid and 10 mmol l^{-1} glucose were added as metabolic substrates (Chamberlin, 1989). The saline pH was adjusted to 6.7 with either KOH or HCl, and the saline osmotic pressure was adjusted to 320 mosmol l^{-1} (isosmotic with the blood) with sucrose. Experiments were conducted at ambient temperature (21–23°C).

Double-barrelled pH-sensitive microelectrodes were pulled from thick-septum theta glass (WPI). One barrel was filled with a 2–3 cm column of water before pulling. The other barrel was then silanized by exposing the stem to dichlorodimethylsilane vapour (Sigma) for 10–30 s, and heating the pipette for 1–2 h on a hotplate at 200°C. A small volume of a pH-sensitive neutral carrier based on tridodecylamine (hydrogen ionophore 1, cocktail B, Fluka) was introduced into the shank of the silanized barrel and ran to the tip by capillarity. Air bubbles were removed with glass whiskers and application of heat from a soldering iron, and the shoulder and stem of the pH-sensitive barrel were backfilled with a solution of 0.1 mol l^{-1} sodium citrate and 0.1 mol l^{-1} NaCl adjusted to pH 6. The reference

barrel was backfilled with 0.5 mol l^{-1} KCl. Acceptable electrodes had a slope of better than 50 mV per decade over the pH range 6–10.

The TEP developed by the tissue was monitored throughout the experiment *via* silver/silver chloride wires on either side of the tissue. It was found that this technique underestimated the true TEP at the tissue surfaces within the folds (measured by microelectrodes) by 25–40 %, but provided a linear measure of the transport status of the tissue.

Electrodes were positioned under visual guidance either in folds of the midgut epithelium, or in the cytoplasm of goblet or columnar cells. As the cytoplasms of the two cell types are coupled, and show no difference in electrical PD or pH (Dow and Peacock, 1989; Moffett and Koch, 1988*a*,*b*), no attempt was made to distinguish the two intracellular compartments. For apical folds, the electrode was advanced into the base of the fold until the base of the fold dimpled, then backed off slightly. For basal folds, the electrode was advanced completely through the epithelium, then withdrawn until the tip was nearly below the original puncture site. The electrode was then raised slightly to restore the fold to its normal, tightly folded morphology, before taking readings. Excessive trauma in these manipulations would be expected to reduce the magnitude of any electrical or pH gradients seen, and so results should be taken as underestimates (if anything) of true gradients. The electrodes used in this study were not fine enough to impale goblet cavities.

The effect of oxidative phosphorylation on the pH gradient sustained by the tissue was studied by switching the perfusion from saline saturated with O_2 to one saturated with N_2 for a period of 3 min. This is a well-documented procedure for inhibiting the electrogenic K^+ pump of the midgut by reduction of ATP levels (Mandel *et al.* 1980*b*; Dow *et al.* 1984), and produced a large and fully reversible decrease in electrogenic K^+ transport, as measured by the TEP.

Some experiments were performed with salines containing bicarbonate at 5 mmol l^{-1}, and adjusted to pH 6.7. No difference in the pH profiles or their oxygen sensitivity was observed, compared with bicarbonate-free saline, and bicarbonate-free saline was used for the results presented here.

Student's *t*-test was used, with a significance level of 0.05 (two-tailed), to test for differences between sample groups. Data are presented throughout as mean±S.E.M., with the number of determinations in parentheses.

Results

In all cases, the potential gradients and pH gradients across the middle midgut regions measured by microelectrodes within basal or apical folds were larger than those measured in the bulk perfusion medium (Table 1). The apical surface was more electropositive than the bulk apical fluid and of higher pH (pH 7.8) than either the perfusion medium (pH 6.7) or the cells (pH 7.0). Symmetrically, the tighter basal infoldings showed the opposite tendency, with a pronounced hyperpolarization of the extracellular microenvironment, together with a lower

Table 1. *Electrical and pH gradients across the isolated middle midguts of* Manduca sexta *larvae*

Compartment	PD	TEP	pH
Apical folds	8±3 (22)	−41±3 (21)	7.8±0.12 (24)
Cells	−89±5 (18)	−39±3 (17)	7.0±0.10 (18)
Basal folds	−85±4 (19)	−42±3 (19)	6.4±0.12 (20)

Electrical gradients are in mV relative to apical side.
Data are presented as mean±S.E.M. (*N*).

Table 2. *pH of the cells, apical folds and basal folds in different regions of the isolated, double-perfused midgut of* Manduca sexta *larvae*

	Midgut region		
Compartment	Anterior	Middle	Posterior
Apical folds	7.7±0.2 (4)	7.8±0.1 (24)	7.1±0.1 (10)
Cells	6.8±0.2 (5)	7.0±0.1 (18)	6.8±0.1 (9)
Basal folds	6.6±0.2 (5)	6.4±0.1 (20)	6.8±0.2 (9)

Data are presented as mean±S.E.M. (*N*).
12 larvae were used.

pH (6.4) than that found in either cells or perfusion medium. A similar pattern for pH was observed for the anterior and posterior regions (Table 2), although the pH gradient sustained by the posterior midgut was not significant.

The shortfall of the gross TEP, compared with that measured directly with microelectrodes close to the tissue surface (Table 1), could be due either to electrical PD differences between the fold lumen and the bulk medium on each side of the tissue, or to current leakage around the sides of the tissue. This was investigated by placing the microelectrode relatively far from the tissue on the basal and apical sides. This suggested that the two effects contributed roughly equally to the shortfall. In detail, the apical folds of middle midgut were about 10 mV electropositive relative to the bulk apical lumen, and the tighter basal folds were about 20 mV electronegative relative to the bulk basal lumen.

The presence of a relatively stable pH gradient across the midgut does not, of itself, imply that the gradient is actively maintained; the infoldings might simply be poorly stirred, and still contain the fluid to which they were exposed *in vivo*. However, brief anoxia significantly and reversibly collapsed the pH gradient from 1.8 to 1.0 pH units; this effect was symmetrical on both basal and apical sides (Fig. 1; Table 3). The gradient recovered to the original level, or even overshot, within 5 min of restoration of oxygenated saline, and with a similar time course to the restoration of the electrical gradient by electrogenic K^+ pumping (Fig. 1). The recovery pH was significantly higher than the pH measured during anoxia, and di[illegible]

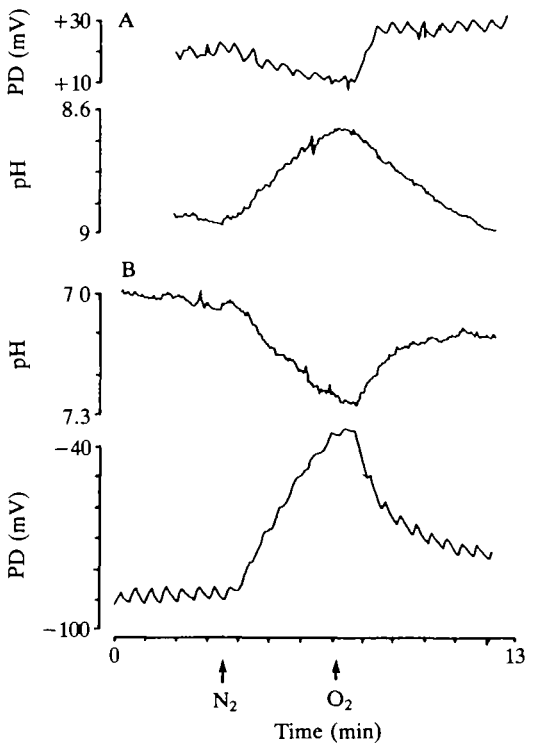

Fig. 1. Effect of anoxia and restoration of oxygenated saline (arrows) on electrical (relative to apical side) and pH gradients across the isolated middle midguts of *Manduca sexta* larvae. Data shown are chart recordings from a typical experiment; regular cycles in the potential difference (PD) traces are artefacts of the perfusion system. (A) Microelectrode in apical folds, (B) microelectrode in basal folds.

not differ significantly from the original level. Similar results were obtained in the anterior midgut (not shown); however, anoxia had no significant effect on the already small gradients in the posterior midgut.

Intracellular pH in the three regions is close to 7.0, in line with earlier data for posterior midgut (Moffett, 1988) and other systems (Roos and Boron, 1981). In middle midgut, the pH of cytoplasm under anoxia was found to be 6.9±0.1 (N=7), which did not differ significantly from normal values (Table 2). These values vindicate the extrapolation of posterior midgut intracellular pH (Moffett, 1988) to the middle and anterior midgut in calculations of proton gradients in the middle midgut (Dow and Peacock, 1989).

Discussion

The technique of measuring standing gradients in unstirred layers provides little information about transport rates; however, it usefully measures the transport electromotive force, i.e. the maximum size of gradient a given transport process

Table 3. *Comparison between effects of anoxia on electrical and pH gradients across the isolated middle and posterior midgut regions of* Manduca sexta *larvae*

Compartment	Before axonia	Anoxia	Recovery
PD (mV relative to apical side)			
Middle midgut			
Apical folds	16±3 (9)	1±4 (9)	16±3 (9)
Basal folds	−90±5 (13)	−47±5 (13)	−84±5 (13)
Posterior midgut			
Apical folds	−3±7 (5)	−9±4 (8)	2±7 (5)
Basal folds	−67±6 (4)	−37±6 (4)	−59±8 (4)
pH			
Middle midgut			
Apical folds	8.2±0.2 (9)	7.8±0.1 (9)	8.3±0.1 (9)
Basal folds	6.4±0.1 (13)	6.8±0.1 (13)	6.5±0.1 (13)
Posterior midgut			
Apical folds	7.2±0.1 (5)	7.1±0.2 (5)	7.1±0.2 (5)
Basal folds	7.1±0.3 (4)	7.4±0.3 (4)	7.2±0.4 (4)

Data are presented as mean±S.E.M. (*N*).

can sustain (Harvey *et al.* 1983). This difference is analogous to the information supplied by short- and open-circuit experiments. In this case, the midgut can sustain a large standing pH gradient (frequently over 2 pH units), even under the suboptimal conditions pertaining *in vitro*. This gradient collapses when cellular ATP is depleted by anoxia, and recovers when oxygen is restored. These effects have a similar time course to the level of electrogenic K^+ transport, as measured by the TEP. This thus demonstrates that a process dependent on cellular metabolism is capable of generating and sustaining a pH gradient across the larval lepidopteran midgut.

The relative degree of folding of the three midgut regions (posterior>anterior> middle midgut) might be expected to affect the gradients measured within the folds, the more deeply convoluted tissues producing higher pH values through less efficient perfusion. However, the most highly folded region (the posterior midgut) generated the weakest pH gradient, whereas the anterior and middle midgut regions generated similar gradients (Table 2). This system of linear perfusion of three regions of a folded epithelium resembles the physiological condition of the gut, and might prove a useful model system compared with, for example, conventional short-circuit, which is confined to one sub-region of the gut.

The folds of the midgut tissue are at neither the same potential nor the same pH as the bulk fluid in which they are perfused. It has already been shown that the goblet cavity is not isopotential with the bulk apical compartment (Dow and Peacock, 1989; Moffett and Koch, 1988*a*,*b*), and that the peritrophic membrane is a significant barrier to diffusion (Wolfersberger *et al.* 1986; Santos *et al.* 1983); it is now clear that the microenvironment of the deep folds on both basal and apical

sides of the epithelium is very different in both electrical PD and ionic composition from the bulk basal and apical environments. Even in a stretched tissue under active perfusion with highly buffered saline, these differences are substantial; so in the more deeply folded *in vivo* midgut, it is reasonable to suspect that the environment within the folds would diverge even more markedly from the main blood and gut luminal volumes. Thus, despite the ostensibly simple structure of the midgut, it is clearly a multi-compartment system which is only imperfectly monitored from sites distant from the tissue. In this context, it is interesting to note that the existence of a substantial K^+ cycling between goblet cells, apical extracellular volume and columnar cells, inferred from *Bacillus thuringiensis* endotoxin intoxication studies (Harvey and Wolfersberger, 1979) and from earlier microelectrode studies (Dow and Peacock, 1989), would be consistent with these data, and would help to explain the complexity of earlier results of kinetic pool labelling experiments with radioactive K^+ and Rb^+ (Wood and Harvey, 1979; Dow, 1986).

Addition of bicarbonate to the medium had no discernible effect in these experiments. In view of the presence of carbonic anhydrase in the tissue and the sensitivity of short-circuit current to carbon dioxide and to some carbonic anhydrase inhibitors (see Dow, 1986), this might seem surprising, but the results do not exclude a role for bicarbonate. The tissue itself generates a reasonable volume of carbon dioxide, which could in turn be converted to bicarbonate by carbonic anhydrase within the midgut epithelium. Given that the K^+:O ratio for *Hyalophora cecropia* midgut tissue is 1.22 (Harvey and Zerahn, 1972) and that the total midgut K^+ transport rate in *Manduca sexta* is 150 μmol h^{-1} (see Dow and Peacock, 1989), the midgut O_2 consumption is around 60 μmol h^{-1}. This compares with values for *Manduca* midgut strips of 125–240 μmol O_2 g^{-1} dry mass h^{-1} (Mandel *et al.* 1980*b*). Using the author's measured midgut wet masses of 86, 46 and 110 mg for anterior, middle and posterior midguts, respectively, of 5 g larvae, and a tissue water content of 75 %, yields a range of 7–14 μmol O_2 h^{-1}. With a 1:1 O_2:CO_2 ratio from oxidative metabolism, the midgut could generate 10–60 μmol HCO_3^- h^{-1} *via* carbonic anhydrase.

The nature of the process of midgut luminal alkalinization is not yet clear; there is likely to be active transport of some acidic or basic species, such as H^+, OH^- or HCO_3^-. The existence of carbonic anhydrase in the goblet cells of only the anterior and middle midgut regions (where the pH gradient is maximal: Dow, 1984) argues strongly for this (Ridgway and Moffett, 1986). However, it should be noted that no bicarbonate-based transport alone could attain pH values approaching 12, as reported in lepidopteran midgut (Dow, 1984). There is evidence that the primary transport event in *Manduca* midgut may be a vacuolar-type H^+-ATPase in the goblet cavity membrane, acting from cytoplasm to cavity (Wieczorek *et al.* 1989; Schweikl *et al.* 1989); however, this would tend to *acidify* the midgut lumen. To reconcile these data, one would need to hypothesise that net K^+ transport occurs *via* a K^+/H^+ exchanger, tightly coupled to the original H^+ flux, and without a significant pH signature, and that, ultimately, an alkaline secretion can

occur through further exchange processes. However, it is important to note that, to avoid mitochondrial contamination, only the posterior midgut is used for protein purification studies (Cioffi and Wolfersberger, 1979). Our results confirm the earlier prediction from *in vivo* pH gradients (Dow, 1984) that the posterior midgut is the only region of the midgut to transport K^+ *without* concomitant luminal alkalinization. It thus seems likely that membrane studies on the posterior midgut will be a more useful tool for studies of K^+ transport than for studies of alkali transport. In particular, the tight apposition of mitochondria to goblet microvillar membranes in anterior and middle (but not posterior) midgut (Cioffi, 1979) may prove critical in modelling alkalinization.

Although primary or secondary active transport seems likely in the generation of an alkaline lumen, there is a simpler physical explanation – that the pH gradient represents a Nernstian proton equilibration across the tissue, and possibly across the goblet cell apical membrane, polarised by electrogenic K^+ transport (Dow, 1984). There is now supporting evidence from microelectrode studies, which show that the PD across the goblet cell apical membrane may be as large as 270 mV, sufficient to account for a luminal pH as high as 11.6 (Dow and Peacock, 1989). In addition, it can be seen from Table 1 that the pH gradient sustained across the gut *in vitro* (1.4 units) closely matches that predicted from the electrical PD by the Nernst equation (93 mV, equivalent to 1.6 pH units). Similarly, under anoxia the pH gradient fell from 1.8 to 1.0 units, then recovered to 1.8 units, as the PD across the gut fell from 103 to 41 mV, then recovered to 98 mV (Table 1), again in good agreement. However, the model for alkalinization suggests (Dow, 1984) that it is the PD across the goblet apical membrane, rather than across the whole epithelium, which directly determines the size of the pH gradient, and so the agreement reported here may be fortuitous. In particular, the differing nature of the goblet cavities in the three regions might explain why the posterior midgut in this study fails to maintain a pH gradient, despite a substantial electrical PD.

It is worth emphasising that this model does not exclude active acid or base transport, and that the formidable electrical gradient sustained by the electrical activity of the K^+ pump would act not only to reduce the energy required to transport acid/base actively across the tissue but would also reduce markedly the tendency for the gradient to dissipate *in situ*.

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