The Journal of Experimental Biology 205, 463–474 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB3596

Insect midgut K^+ secretion: concerted run-down of apical/basolateral transporters with extra-/intracellular acidity

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Accepted 27 November 2001

Summary

In lepidopteran larvae, three transport mechanisms are involved in the active and electrogenic K^+ secretion that occurs in the epithelial goblet cells of the midgut. These consist of (i) basolateral K^+ channels, allowing K^+ entry from the haemolymph into the cytosol, (ii) apical electrogenic $K^+/2H^+$ antiporters, which are responsible for secondary active extrusion of K^+ from the cell into the gut lumen *via* the goblet cavity and (iii) apical V-ATPase-type proton pumps. The latter energize apical K^+ exit by building up a large, cavity-positive electrical potential that drives the antiporters. Net K^+ secretion (I_K) can be measured as short-circuit current (I_{sc}) across the *in vitro* midgut mounted in an Ussing chamber. We investigated the influence of protons on the transepithelial I_K and the partial reactions of the basolateral K^+ permeability (P_K) and the apical, lumped 'K^+ pump' current (I_P) at various extra- and intracellular pH values. In particular, we wanted to know whether increased cellular acidity could counteract the reversible dissociation of the V-ATPase into its V_1 and V_o parts, as occurs in yeast after glucose deprivation and in the midgut of *Manduca sexta* during starvation or moulting, thus possibly enhancing K^+ transport.

When intact epithelia were perfused with high-[K^+] (32 mmol l^{-1}) salines with different pH values, I_K was reversibly reduced when pH values fell below 6 on either side of the epithelium. Attempts to modify the intracellular pH by pulsing with NH_4^+ or propionate showed that intracellular acidification caused a reduction in I_K similar to that obtained in response to application of external protons. Treatment with azide, a well-known inhibitor of the mitochondrial ATP synthase, had the same effect as pulsing with ammonium or propionate with, however, much faster kinetics and higher reversibility. Breakdown of the basolateral or apical barrier using the antibiotic nystatin allowed the intracellular pH to be clamped to that of the saline facing the nystatin-treated epithelial border. Cell acidification achieved by this manipulation led to a reduction in both apical I_P and basolateral P_K. The transepithelial I_K showed an approximately half-maximal reduction at external pH values close to 5 in intact tissues, and a similar reduction in I_P and P_K values was seen at an intracellular pH of 5 in nystatin-permeabilised epithelia. Thus, the hypothesized V_1V_o stabilization by cell acidity is not reflected in the pH-sensitivity of I_P. Moreover, all components that transport K^+ are synchronously inhibited below pH 6. The significance of our findings for the midgut *in vivo* is discussed.

Key words: *Manduca sexta*, tobacco hornworm, K^+ secretion, V-ATPase, vacuolar, pump current, K^+ channel, intracellular pH.

Introduction

The midgut of lepidopteran larvae, an insect model epithelium, exhibits a number of remarkable physiological transport properties. Most conspicuous is the active and electrogenic secretion of K^+ against a K^+ electromotive force of at least 200 mV (Wieczorek et al., 2000). The haemolymph-directed K^+ gradient is used (Dow and Harvey, 1988) to enable nutrient uptake into the columnar cells (CCs) *via* apical K^+/amino acid symporters. Active K^+ secretion in the midgut is unusual in that it is not powered by a Na^+/K^+-ATPase (Jungreis and Vaughan, 1977) like that of many vertebrate K^+-secreting epithelia with a low blood-side K^+ concentration. In the midgut, a basal passive K^+ influx against a K^+ concentration gradient is observed as a consequence of a large, cytosol-negative voltage established by the activity of an apical, plasma-membrane-bound, V-type H^+-ATPase (Zeiske, 1992). Moreover, the high haemolymph [K^+] (20–35 mmol l^{-1}) of plant-feeding larvae further reduces the driving force necessary for K^+ entry into the intestinal cells against an at least fourfold [K^+] gradient (Moffett and Koch, 1992).

While the route of basolateral K^+ entry is *via* Ba^{2+}-blockable channels (Zeiske et al., 1986), which appear to be distributed over syncytial, gap-junction-coupled functional units of CCs and goblet cells (GCs) (Moffett et al., 1982), the apical exit pathway of K^+ is strictly confined to the goblet cell apical

membrane (GCAM). This apical membrane is the location of both V-ATPase-driven proton transport and an electrogenic antiporter that moves $1K^+$ out of the cytosol in exchange for $2H^+$ (Azuma et al., 1995) derived from the goblet cavity water, as no pH gradient could be observed across the GCAM in artificial insect saline at pH 8 (Chao et al., 1991). The (cytosol-negative) electrical potential set up by the V-ATPase must be the driving force for this electrophoretic $K^+/2H^+$ exchange.

The finding that the apical membranes have a large fractional electrical resistance (Schirmanns and Zeiske, 1994a) suggests that these regions are an appropriate controlling site for the transepithelial K^+ flux. Given a possible regulation of the proton pump and antiporter by, for example, second messengers, voltage- and substrate-controlled activity of electrogenic transporters may seem trivial, but it is of considerable interest. While fluctuations in haemolymph or cytosol K^+ concentrations appear highly unlikely, a downregulation of, for example, gap-junctional or basal K^+ channel permeability could well reduce the K^+ transport pool and so curb K^+ secretion by slowing down the rate of $K^+/2H^+$ exchange. However, with H^+ as the substrate of both the V-ATPase and the exchanger, even a small shift in pH could alter their transport rates not only directly but also perhaps allosterically. Thus, it has been argued that the H^+ pump is stimulated by decreasing the (cytosol-side) pH below 6 (Parra and Kane, 1996).

In addition, cation/proton exchangers such as those of the Na^+/H^+ exchange (NHE) family (Wakabayashi et al., 1997) are allosterically activated by mild cytosolic acidity. However, in the case of midgut $K^+/2H^+$ antiport, cell acidification would seem counter-productive for efficient exit of K^+ from the cell. In addition, pH-sensitive gap-junctional (Francis et al., 1999) and K^+ channel (Harvey, 1995; McNicholas et al., 1998) permeabilities are well known. Thus, at first sight one would suspect that cell acidification in the caterpillar midgut would lead to a reduction in the activities of the apical antiporters and/or the basolateral K^+ channels, thereby directly impairing the two transporters relevant for K^+ movement, while the apical proton pump may be simultaneously stimulated to counteract a putative acid-induced inhibition of the K^+ transporters.

K^+ secretion by the caterpillar midgut is highly dependent on maximal mitochondrial function (Mandel et al., 1980; Chamberlin, 1987), so metabolically produced cytosolic acidity might not be a direct hazard to effective K^+ secretion. The pH of the intestinal cells appears to be well-controlled and is approximately 7.2 with artificial saline (pH 8) as the ambient medium (Chao et al., 1991). However, the ambient pH of the *in vivo* midgut is quite extreme. The lumen pH may rise above 11, while the blood pH is slightly above 6, and it has been demonstrated that the establishment of this enormous transepithelial pH gradient is closely related to, and generated by, active K^+ secretion (Dow and Harvey, 1988). As might be expected, *in vitro* changes in luminal pH between 11 and 7, and of haemolymph pH between 8.5 and 6, have no effect on K^+ transport (Haskell et al., 1965) (see also Fig. 1A). For the pH range investigated, this suggests surprisingly low proton and hydroxyl permeabilities of the apical and basolateral membranes in this epithelium. At high ambient CO_2 pressure, however, it has been shown that K^+ secretion is effectively, but reversibly, reduced (Haskell et al., 1965), which indicates that cell acidification after CO_2 entry impaired one or more of the transporters responsible for K^+ movement. A closer investigation into putative cell-pH-controlled transport mechanisms, therefore, seems warranted.

We set out to modify intracellular pH (i) by large external pH shifts, (ii) by the use of permeant weak bases and acids and (iii) by cell pH clamp after membrane permeabilisation with an ionophore. We found that procedures causing eventual acidification of the cytosol led to a concerted downregulation of both the apical and basolateral mechanisms that achieve transepithelial K^+ secretion. A preliminary short report of this work was given at a meeting of the German Zoological Society (Zeiske et al., 2000).

Materials and methods

Insects, tissue mounting and electrical measurements

Larvae (tobacco hornworm) of *Manduca sexta* L. (Lepidoptera, Sphingidae) were reared under long-day conditions (16 h:8 h light:dark) at 27 °C using a synthetic diet modified according to Bell and Joachim (1974). The posterior midgut of the tobacco hornworm was isolated and mounted in an Ussing-type chamber, modified after De Wolf and Van Driessche (1986), as described previously (e.g. Schirmanns and Zeiske, 1994a,b). Both chamber compartments (2 ml) were gravity-perfused ($4\,ml\,min^{-1}$) with the appropriate aerated saline. We used Ag/AgCl electrodes connected *via* $1\,mol\,l^{-1}$ KCl/agar bridges to achieve a four-electrode voltage-clamp. To avoid clamping errors due to the finite resistivity of the salines compared with that of the tissue (approximately $10\,mS\,cm^{-2}$ for control conditions), the voltage electrodes were placed less than 1 mm from the tissue surface since this has been shown to achieve a perfect midgut voltage-clamp (Zeiske et al., 1986; Schirmanns and Zeiske, 1994a,b).

The midguts were usually short-circuited, and the short-circuit current (I_{sc}) was recorded on a chart recorder. The transepithelial conductance was measured as the ratio of the current deflection elicited by a voltage pulse of 5 or 10 mV (departing from the short-circuited state). The I_{sc} recorded largely reflects the transepithelial potential difference, which is generated by active K^+ transport, or, in some experiments, by passive K^+ movements along a concentration gradient. All current values are given for a nominal tissue area of $0.25\,cm^2$ (chamber diameter). It should be borne in mind that the midgut epithelium, although a monolayer, is highly folded (folding factor approximately 20) (see Dow, 1992), so the current values reported would have to be divided by 5 to give the true current density for an ideally flat epithelial surface. Undisputedly, with the standard saline used in this study (see below), the short-circuit current (of positive sign) represents net K^+ secretion with high accuracy (e.g. Harvey and

Nedergaard, 1964; Cioffi and Harvey, 1981; Moffett and Koch, 1988, 1991, 1992; Dow, 1992; Schirmanns and Zeiske, 1994a,b). We therefore term I_{sc} a K^+ current, I_K.

When one membrane of an epithelial goblet cell is permeabilised using the antibiotic nystatin (see below), the opposite membrane can be considered not only to be rate-limiting for K^+ transport but also to be perfectly voltage-clamped (Schirmanns and Zeiske, 1994a,b). In the case of basolateral permeabilisation and salines containing symmetrical K^+ concentrations, an I_{sc} can still be measured, and this reflects the sole contribution of the two collaborating apical transporters, the V-ATPase and the parallel $K^+/2H^+$ antiporter. Conversely, when apical nystatin is used in salines containing symmetrical K^+ concentrations, the apical pump is short-circuited and its current (I_p) is not measurable. However, a basolaterally directed K^+ concentration gradient will evoke a net lumen-to-haemolymph K^+ flux. Because of the tightness of the paracellular pathway, this flux (I_K^{rev}) passes through the cells.

Salines

The salines were prepared according to standard procedures (Harvey and Nedergaard, 1964; Moffett and Koch, 1992; Schirmanns and Zeiske, 1994a,b) and contained (in mmol l^{-1}) 30 KCl, 2.5 $KHCO_3$, 5 $CaCl_2$, 5 $MgCl_2$ and 166 sucrose, equilibrated with air to pH 8.0 (standard saline, abbreviated to 32K in the figures). In cases where hydrolysing substances such as sodium propionate, sodium azide or ammonium chloride were added, 5 mmol l^{-1} Tris was included and titrated (with HCl) to pH 8. This procedure maintains a constant saline pH despite some hydrolysis of the compounds. For higher or lower extracellular acidities, the salines were simply titrated with HCl or Tris/NaOH, respectively. We avoided the use of organic buffers in acid solutions, since many organic acids and bases have been shown (Chamberlin, 1989) markedly to influence I_K, an effect that might be due to their metabolic utilization. Furthermore, the perfusion rate of the salines (approximately 4 ml min^{-1}; half-chamber volume of 2 ml) is so high that it is reasonable to assume that the cell surfaces are bathed in solutions of constant pH. Finally, we can see no way to equilibrate the pH of the cytosolic medium with externally applied buffer systems (with the possible exception of CO_2/HCO_3^-). Nystatin (Sigma: 4400 USP units mg^{-1}) was dissolved in dimethylsulphoxide and applied at a final concentration of 1500 units ml^{-1} saline. All organic chemicals were obtained from Sigma, inorganic chemicals from Fluka or Merck.

Results

External pH changes

Many epithelia show a marked membrane permeability for H^+ that is frequently localised to channels that are normally 'specific' for Na^+ or K^+ (Lyall and Biber, 1994; Lyall et al., 1995). Thus, small electrical driving forces, i.e. those in the physiological range, may elicit major proton fluxes and generate non-negligible and deleterious shifts in cell pH. Transmembrane H^+ concentration gradients will considerably increase this problem. For instance, in frog skin, a well-known model for tight vertebrate epithelia, a 5 min exposure to external acidity (pH 3.3) causes a small drop in cell pH from 7.2 to 7.0, which is nevertheless sufficient to cause complete closure of cation channels (Harvey, 1995; Onken et al., 1990). Cell pH must, therefore, be strictly controlled.

As mentioned in the Introduction, however, the cells of the caterpillar midgut tolerate very large pH gradients at both their apical and basolateral borders while the pH of the cells remains at approximately 7.2. In Fig. 1A, we show that a change in luminal (apical) pH from 8 to 10, and in the haemolymph-side (basolateral) pH from 8 to 6, has hardly any effect on K^+ secretion. Increasing basal alkalinity to pH 10, however, impairs the K^+ current. When the extracellular pH on either side is reduced below pH 6, the K^+ current is severely affected. Below pH 4 (Fig. 1B), I_K drops quickly but can be fully restored by a return to control conditions. Interestingly, a two-step response can be observed when the acid is applied to the basolateral side. For comparison, the inhibitory effect of 5 mmol l^{-1} basolateral Ba^{2+}, which blocks the K^+ channels in this tissue (Zeiske et al., 1986), is shown (Fig. 1B). In the absence of K^+ (replaced by Na^+), the short-circuit current is usually zero (Zeiske et al., 1986; Moffett, 1979; Moffett and Koch, 1992); thus, only K^+ is actively transported.

Protons, like Ba^{2+}, therefore appear specifically to inhibit K^+ secretion. With symmetrical Na^+ saline, both a $[H^+]$ gradient from lumen to haemolymph and a gradient in the reverse direction failed to elicit any deviation of I_{sc} from zero that could be interpreted as a passive proton current (data not

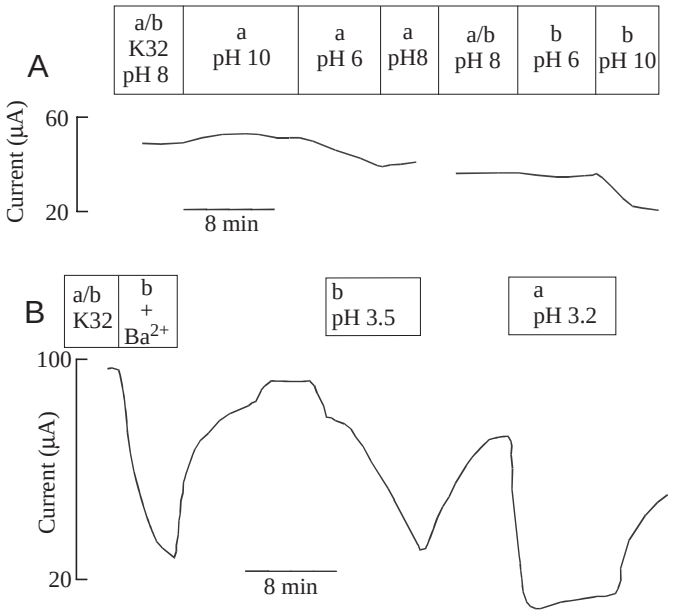

Fig. 1. (A) Effects of apical and basolateral pH shifts on the transepithelial secretory (positive sign) K^+ current. K32, standard saline (see Materials and methods); a, apical variations; b, basolateral variations. (B) Reduction in active K^+ transport induced by 5 mmol l^{-1} basolateral Ba^{2+} and basolateral/apical acidification in the absence of Ba^{2+}.

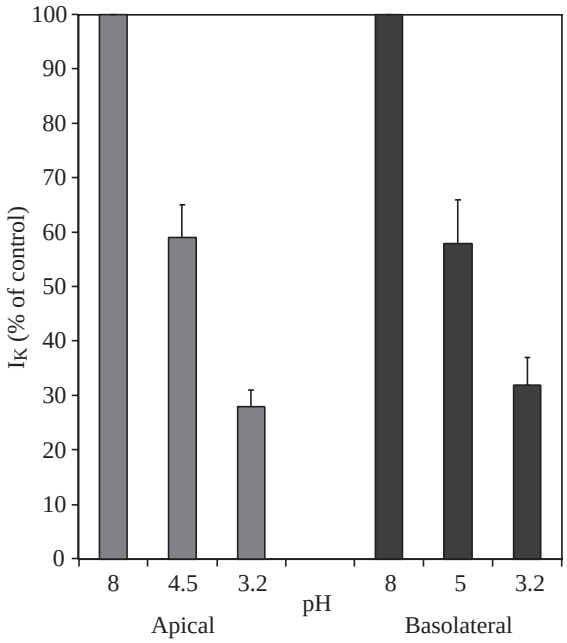

Fig. 2. Percentage of the control (pH 8) K^+ current (I_K) after external acidification on the apical or the basolateral side. Values are shown as means + standard error of the mean (S.E.M.; *N*=6).

shown). We may therefore conclude (i) that paracellular pathways are virtually proton-tight, and (ii) that no other passive transcellular H^+ pathways are present, judging from the absence of a current, within the range of pH gradients tested.

Because of the inherent drift in I_K (observed by virtually all experimenters) (e.g. Haskell et al., 1965; Moffett and Koch, 1988, 1991; Dow and Harvey, 1988; Schirmanns and Zeiske, 1994a,b), an unambiguous recording of a 'pH titration curve', using small (0.5–1 pH unit) steps, is largely blurred by the overlap of the I_K drift and the pH-induced current decrease. We therefore raised the acidity of the saline in larger steps. Fig. 2 illustrates that a decrease in the apical pH to 4.5 reduced I_K by 41 % and a further decrease to pH 3.2 reduced I_K by 72 %. Similar jumps in basolateral pH (Fig. 2) led to reductions in I_K of 42 % at pH 5 and 69 % at pH 3.2. Since the inhibition of current by acid salines is fast and appears to be rapidly and largely reversible, it is possible that membrane transporters involved in K^+ secretion, i.e. the apical antiporter or the basolateral K^+ channels, might be titrated at sites on the extracellular face of the cell membrane. The estimated (Fig. 2) half-maximal inhibition suggests that the titrated site may be a glutamate side-chain carboxylate (free solution pK_a 4.25). It is surprising to find that the pH-sensitivity of I_K was almost identical from both sides of the epithelium. Although not the only possible interpretation, this might mean that an intracellular rather than an extracellular site is titrated when I_K is inhibited by protons. Indeed, we show below that different methods known to acidify the cytosol at constant external pH lead to comparable results.

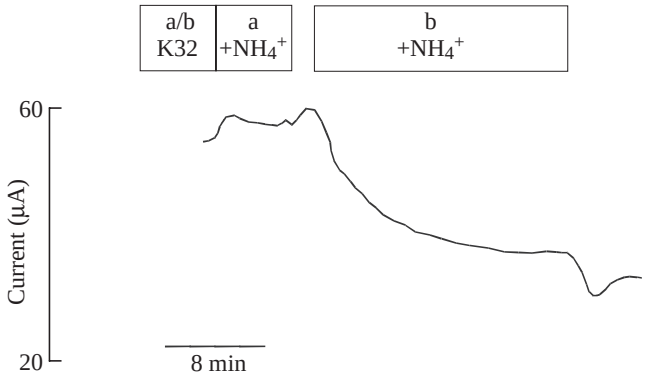

Fig. 3. The effect of 20 mmol l^{-1} ammonium chloride, replacing sucrose iso-osmotically on the apical (a) or basolateral (b) side, on K^+ current. K32, standard saline (see Materials and methods).

Cell pH shifts induced by external NH_4^+

A number of alkali-ion-transporting proteins, especially channels, are impaired by protons, and for renal or similar epithelia a complete loss of Na^+ or K^+ channel conductance was found when cell pH was reduced by a mere 0.3 pH unit (Onken et al., 1990; Harvey, 1995; McNicholas et al., 1998). External addition of ammonium salts has been widely used to alter the cytosolic pH (e.g. Boron and De Weer, 1976; Zeiske et al., 1999). A concentration of 10–20 mmol l^{-1} NH_4Cl has been shown to evoke cellular pH shifts of between 0.2 and 0.5 unit, depending on the internal buffering capacity and the activity of H^+-removing membrane transport proteins. Since a small percentage of NH_4^+ is present as NH_3 at neutral pH, the latter uncharged base is assumed to enter the cell (possibly *via* membrane lipid pathways) and to render the cytosol alkaline as a result of hydrolysis of NH_3. However, many K^+-transporting pathways have been shown to handle NH_4^+ as well, including the basolateral K^+ channel in vertebrate renal epithelia (Zeiske et al., 1999) and the midgut of *Manduca sexta* (Schirmanns and Zeiske, 1994b). Once inside the cell, NH_4^+ may hydrolyse and so induce cell acidification, often by more than 0.5 pH unit. The direction and amount of the final pH shift will, of course, depend on the relative membrane permeabilities for the corresponding acid–base pair, the buffering capacity of the cytosol and the capacity of pH-controlling membrane-bound H^+ or base transporters associated with the cell.

We used 20 mmol l^{-1} NH_4Cl to induce such intracellular pH shifts in the midgut epithelium and, for osmotic balance, omitted 40 mmol l^{-1} sucrose from the salines. With respect to the possible pathways for NH_3 and NH_4^+ into the cells, we would expect cell alkalization from apical loading with ammonia but cell acidification from basal loading with NH_4^+. Of three epithelia tested, only one (shown in Fig. 3) responded clearly to apical ammonium with a tiny current increase of less than 10 %. Conversely, when basolateral ammonium was used on the same epithelium, an immediate and appreciable drop in K^+ current was induced, as expected if cell acidification results

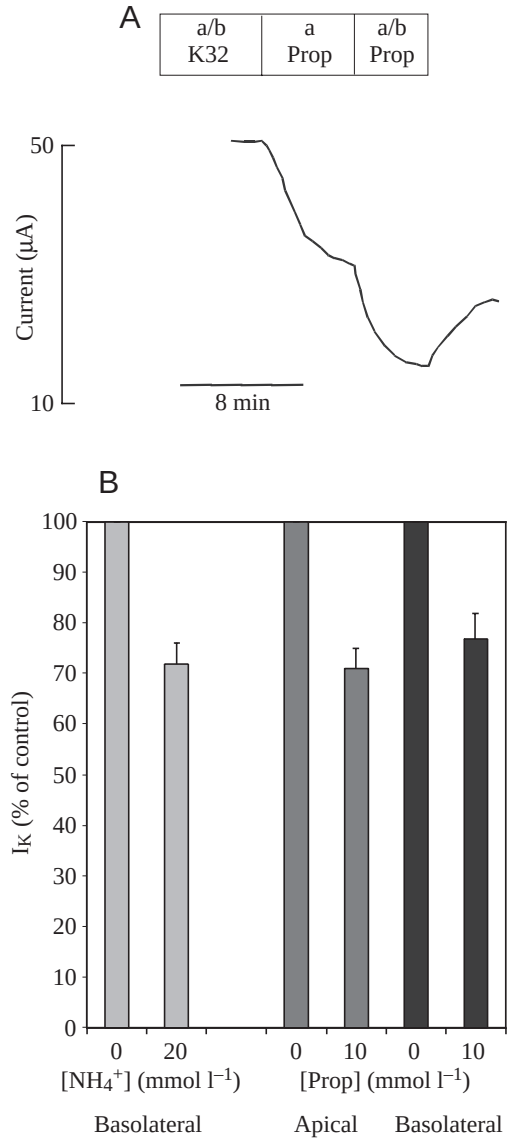

Fig. 4. (A) The effect of 10 mmol l^{-1} sodium propionate (Prop) applied first apically (a) then basolaterally (b), on K^+ current. K32, standard saline (see Materials and methods). (B) Percentage of control K^+ current (I_K) before and after treatment with 20 mmol l^{-1} basolateral ammonium chloride (left side, *N*=4) and before and after 10 mmol l^{-1} apical or basolateral propionate (Prop) (right side, *N*=4). Values are means + S.E.M.

from NH_4^+ entry *via* K^+ channels (Fig. 3). We conclude that a rise in cell pH increases, whereas a drop in pH decreases, K^+ transport, thus mimicking the effects of external pH changes. Upon removal of ammonium, the current dropped even further and then showed a very small reversal (see Discussion). Fig. 4B (left side) demonstrates the inhibitory effect (approximately 30 %) of 20 mmol l^{-1} basolateral NH_4^+ for four midguts.

Cell acidification after weak acid anion treatment: external propionate and azide

A lower cell pH can also be achieved by the external addition of alkali salts of weak acids such as sodium propionate (Keifer, 1981; Szatkowski and Thomas, 1989). The small amount of undissociated acid (pK_a=4.8) present at neutral external pH gains access to the cytosol *via* lipid pathways and so lowers cell pH after hydrolysis. Fig. 4A shows the results of such an experiment with 10 mmol l^{-1} sodium propionate (substituted for 20 mmol l^{-1} sucrose) given first in the apical saline and then, in addition, in the basolateral saline. As observed with a basolateral addition of ammonium, the K^+ current dropped quickly in an additive manner. During washout, there was a slow and incomplete reversal similar to that seen after ammonium removal (Fig. 3). Fig. 4B (right side) demonstrates the mean reduction in K^+ current obtained with 10 mmol l^{-1} propionate applied apically or basolaterally. Probably because of its stronger acidic nature, 10 mmol l^{-1} propionic acid is as effective as 20 mmol l^{-1} NH_4^+.

Another weak acid, with a pK_a almost identical (pK_a=4.7) to that of propionic acid, is HN_3, the anion of which (azide) is known specifically to inhibit the mitochondrial F-type H^+-ATPase (for references, see Syroeshkin et al., 1995; Vasilyeva and Forgac, 1998). Unexpectedly, a direct inhibitory effect of azide on H^+ transport, but not on ATP hydrolysis, of the V-ATPase from clathrin-coated vesicles has recently been demonstrated (Vasilyeva and Forgac, 1998). To test whether azide exerts an effect similar to that of propionate on K^+ secretion in the midgut seems, therefore, highly desirable. Our results show that apical or basolateral application of an osmotically balanced solution in which azide is substituted for some of the sucrose causes a reduction in the K^+ current. The reduction is very rapid, dose-dependent and rapidly reversible (Fig. 5A). Half-maximal current inhibition with azide is obtained at concentrations more than 10 times lower than with propionate (see Fig. 4). Most interestingly, and very different from the observations after propionate or ammonium removal, the washout of azide led to a fast but, within the frame of the inherent drift in I_{sc} (see above), quite good recovery of the current (Fig. 5A). Fig. 5B depicts the inhibitory effect of 1 or 10 mmol l^{-1} azide, given apically or basolaterally, the latter being slightly more effective. A 50 % current reduction was achieved at concentrations between 1 and 5 mmol l^{-1} from either side.

Clamping cell pH through the use of the ionophore nystatin

Basolateral application of nystatin and the pH-sensitive apical pump current

Nystatin is a polyene antibiotic that creates monovalent ion channels (preferring cations) when embedded in the lipid phase of plasma membranes (Lewis and Wills, 1989). To date, such substances have been widely used for equilibration of the cytosolic space with the external solution with respect to ions such as Na^+ and K^+ (Lewis and Wills, 1989) or H^+ (Eaton et al., 1984). The perforated epithelial tissues are then functionally reduced to ‘pseudo-mono-membrane’ preparations: the antibiotic-treated membrane has lost its barrier function for monovalent ions, has gained a high conductance and so allows a perfect voltage-clamp of the remaining intact, contralateral membrane.

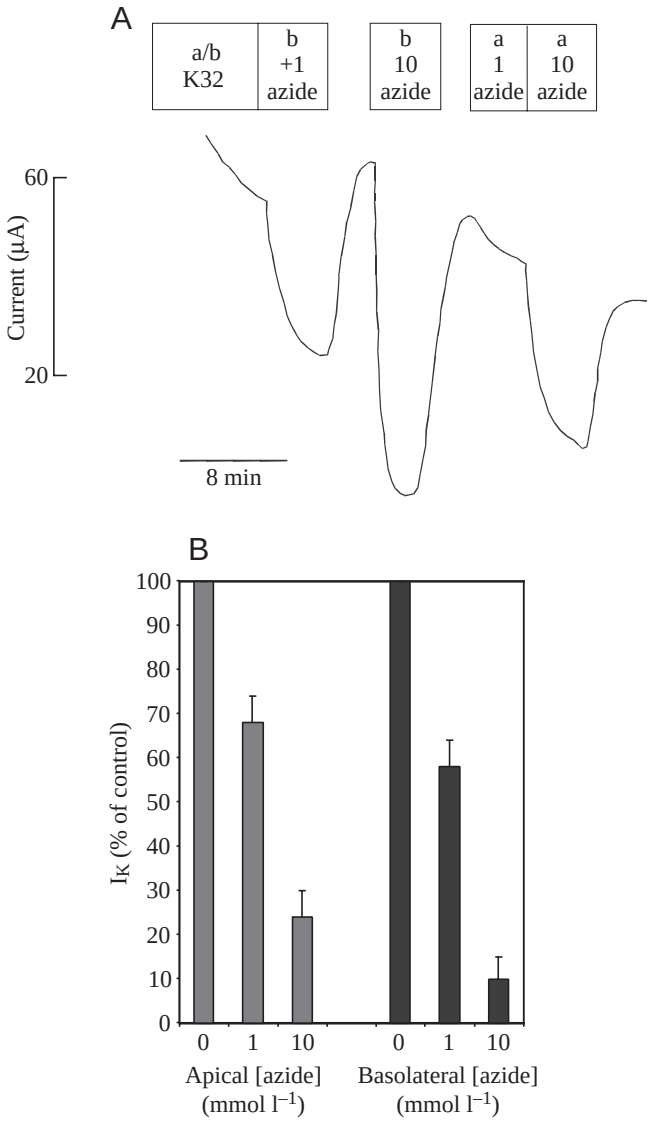

Fig. 5. (A) The effect of apical (a) or basolateral (b) sodium azide on K^+ current. K32, standard saline (see Materials and methods). Azide concentrations are given in mmol l^{-1}. (B) Percentage of control K^+ current (I_K) after apical or basolateral addition of 1 or 10 mmol l^{-1} sodium azide. Values are means + S.E.M. (N=4).

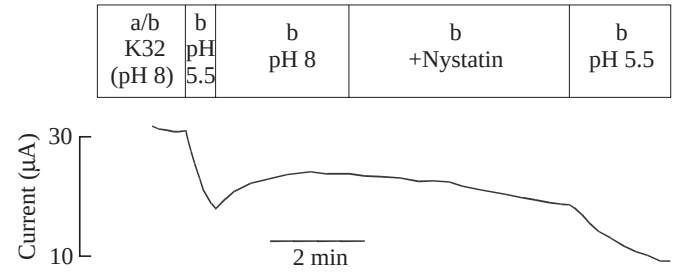

Fig. 6. Inhibition of K^+ current induced by basolateral acidification before and after basolateral membrane permeabilisation using nystatin. K32, standard saline (see Materials and methods); a, apical variations; b, basolateral variations.

Thus, in our case, basolaterally applied nystatin will functionally eliminate the Ba^{2+}-sensitive and specific basolateral K^+ permeability and allow a selective focus on apical ion-transport mechanisms (Schirmanns and Zeiske, 1994a,b). Here, we use the excellent K^+ and proton permeability induced by nystatin to 'clamp' cell [K^+] and cell pH to the values of the 'nystatin-side' extracellular solution. Incidentally, any apical voltage will vanish because the apical membrane alone can now be properly short-circuited (Schirmanns and Zeiske, 1994a).

Fig. 6 depicts the inhibition of I_K caused by reducing basolateral pH to 5.5, before and after application of basolateral nystatin. The rapid drop in I_K with acidic saline is partially reversible. After nystatin treatment, the basolateral barrier disappears permanently and becomes insensitive to Ba^{2+} blockade (not shown here) (but see Schirmanns and Zeiske, 1994a). This is without visible consequences for I_K because the fractional resistance of the basolateral membrane is, even in control conditions, only 5 % of the total transcellular resistance (Schirmanns and Zeiske, 1994a). The observed transepithelial short-circuit current is no longer rate-limited by the basolateral membrane and may, therefore, be termed the apical 'K^+ pump current', I_P. Under these conditions, when the cell pH is clamped from 8 to 5.5, the pH of the acidic basolateral saline, the 'K^+ pump' current (I_P) inhibition clearly indicates (i) that the intact apical epithelial membrane is strongly proton-sensitive with respect to the apical (lumped) K^+ transport machinery (Fig. 6) and (ii) that half-maximal inhibition of I_P occurs (see also Fig. 7B) at approximately the same intracellular pH as was observed with extracellular acidification (see Fig. 2).

The possibility that the K^+ current decrease observed with external acidification in non-nystatin-treated tissues may reflect to some degree an intracellular pH drop cannot, therefore, be completely ruled out. This would mean we would have to assume some finite proton permeability of the intact membranes, and the prime candidate for apical H^+ influx is the antiporter. Basolaterally, K^+ channels or a still hypothetical K^+/H^+ exchanger could allow access of protons to the cytosol (see Discussion).

Apical nystatin and the pH-sensitive basolateral K^+ channel permeability

It has been recognized that many epithelial or other K^+ channels in blood-facing membranes are strongly inhibited by only small shifts in the intracellular pH to weakly acidic values, and in some tight epithelia, a concerted decrease in apical and basolateral cation channel permeabilities was observed upon lowering the pH of the cytosol (Harvey, 1995). To lower cell pH by manipulating extracellular acidity, the latter generally has to be markedly below pH 7 (Hille, 1992; Van Driessche and Zeiske, 1985; Onken et al., 1990). To study not only the pH-dependence of the apical K^+-transporting mechanisms (I_P; see preceding section) but also to focus on the pH-sensitivity of the basolateral K^+ channels, we used apical instead of basolateral nystatin to remove the limiting function of the

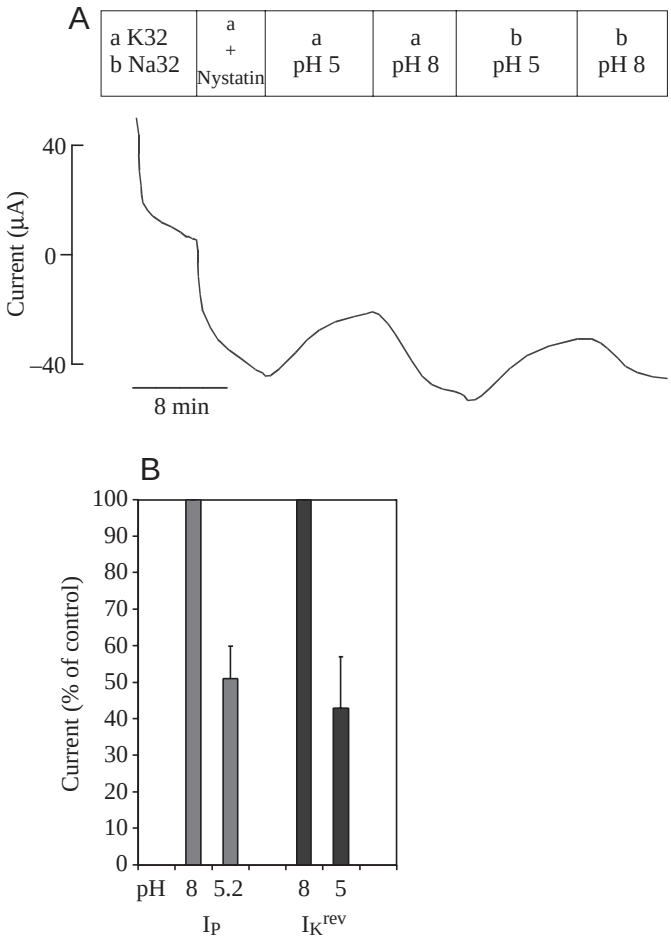

Fig. 7. (A) Establishment of a reversed (negative sign) current (abbreviated as I_K^{rev} in the text) after apical (a) permeabilisation with nystatin and its inhibition by apical or basolateral (b) acidification. K32, standard saline (see Materials and methods); Na32, all K^+ in the saline replaced by Na^+. (B) Percentage of control pump current (I_P, left side, N=4) and reversed current (I_K^{rev}, right side, N=4) following intracellular acidification *via* basolateral (left side) or apical (right side) nystatin channels. Values are means + S.E.M.

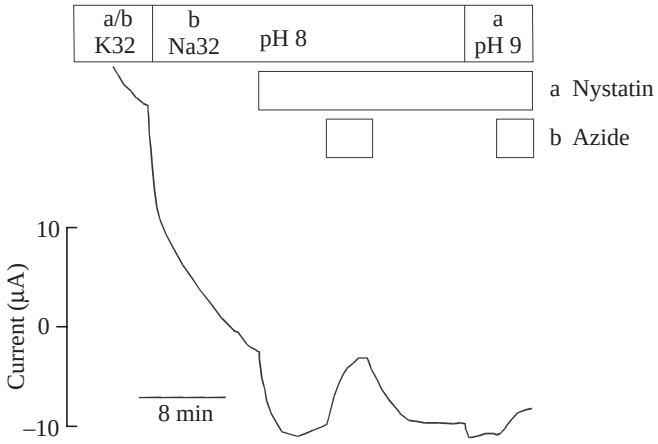

Fig. 8. Establishment of a reversed (negative sign) current (abbreviated as I_K^{rev} in the text) after apical (a) permeabilisation with nystatin, and the effect of 1 mmol l^{-1} sodium azide in K^+-free basolateral (b) saline at different cell pH values. K32, standard saline (see Materials and methods); Na32, all K^+ in the saline replaced by Na^+.

apical membrane. In the presence of nystatin, the apical membrane becomes permeable to monovalent cations (Lewis and Wills, 1989; Schirmanns and Zeiske, 1994b). Under these conditions, a lumen-to-haemolymph [K^+] gradient (see Materials and methods) will drive a passive, transcellular K^+ current (I_K^{rev}) in the absorptive direction, which is the opposite direction to I_K with intact membranes and to I_P in the presence of basolateral nystatin. Assuming that the apical, permeabilised membrane is no longer rate-limiting, I_K^{rev} becomes a direct measure of basolateral K^+ permeability, P_K (Schirmanns and Zeiske, 1994b).

Fig. 7A shows an experiment in which, after exposure to normal K^+ saline on both sides, basolateral K^+ was initially substituted by Na^+, which abolished active K^+ secretion and reduced the current to almost zero. Nystatin was then introduced into the apical chamber compartment and the reversed, negative current (blood-side-directed) increased. A subsequent lowering of the apical (and, therefore, the intracellular) pH from 8 to 5 led to an immediate decrease in I_K^{rev} that was readily reversible upon acid washout. Not surprisingly (see Fig. 2), a simultaneous reduction in the basolateral pH to 5 produced an almost identical, roughly half-maximal, reversed current inhibition. For four such experiments (Fig. 7B), I_K^{rev} was more than half-maximally impaired by a cell pH of 5. We may conclude that intracellular H^+ inhibits not only the apical K^+ transport mechanism (I_P) but also the basolateral passive K^+ permeability (I_K^{rev}), and both to a comparable degree with an apparent pK_a close to 5.

In a preliminary experiment analogous to that mentioned above, we showed that, in the presence of apical nystatin (Fig. 8), basolaterally applied azide is able almost to abolish the reversed current, I_K^{rev} (and thus basolateral P_K), just as it had decreased I_P. Most interestingly, when we titrated the cell pH from 8 to 9 (Fig. 8), the potency of the I_K^{rev} block by azide was greatly reduced. This again suggests that our interpretation of azide acting *via* HN_3 (now strongly buffered at a cell pH of 9) is correct, at least for the passive I_K^{rev}.

Discussion

At this point, we can state that moderate changes in the acidity of the extracellular pH (<6) will lead to a quantitatively similar blockade of K^+ secretion as when acidification is exclusively confined to the cytosol. Obviously, in the absence of nystatin, external protons can reach H^+-sensitive sites on the membrane proteins involved in K^+ transport. A possible intracellular action of externally applied protons would not, however, seem unlikely since there is a reduction in the K^+ current following presumed cytosolic acidification by mobile protonophores, such as basolateral ammonium or the bilaterally effective corresponding weak acids of propionate and azide. Further convincing support for an intracellular site

of action of protons comes from the nystatin studies, which were able to differentiate the apical (I_P) from the basolateral (I_K^{rev}) contributions to the H^+-induced I_K block.

Methodological aspects: salines

We used a 'pseudo-physiological' saline composed of the principal inorganic ions at approximately haemolymph concentrations (Harvey and Nedergaard, 1964; Cioffi and Harvey, 1981). Sucrose, which is virtually absent from the haemolymph, served as an external osmolyte and also, according to Harvey and Nedergaard (1964), as a possible source of metabolic energy. These putative roles are, however, questionable since removal of sucrose severely impairs K^+ secretion in a manner that cannot readily be understood (Moffett, 1979; Meyer et al., 2000). In our experiments using NH_4Cl, sodium propionate or sodium azide as putative intracellular pH-modifiers, we substituted no more than 40 mmol l^{-1} sucrose with these salts. The omission of 40 mmol l^{-1} sucrose from the salines *per se* had, in the absence of propionate or azide, no deleterious consequences for I_K (W. Zeiske, H. Meyer and H. Wieczorek, unpublished observations) (Meyer at al., 2000). Nevertheless, the conspicuous and well-known (Haskell et al., 1965; Moffett, 1979; Cioffi and Harvey, 1981) drift in I_K after mounting the tissue in the Ussing chamber could be due to a non-appropriate supply of specific nutrients (Chamberlin, 1989).

As the I_K drift is largely unavoidable (Chamberlin, 1989), we could not determine absolute mean values for the transport parameters; instead, we had to normalise the observable effects (e.g. of protons) to the recognisable underlying drift of I_K, which explains why our data on pH effects are presented as percentages of the control current, i.e. the current immediately before a modification of pH. Although claims have been made to the contrary (Chamberlin, 1989), close inspection of the available data shows that the composition of salines alone, even if complex, does little to alleviate the I_K drift. We must probably accept that internal 'fuels' (i.e. from reservoirs within the tissue) play a major role in maintaining a constant rate of K^+ transport. On the basis of the subsequent discussion, it might even be that an intracellular accumulation of H^+ during long-term experiments is the cause of the major I_K drift.

The intact epithelium and external protons

In contrast to a previous conclusion by Dow and Harvey (1988), the experimental evidence collected to date favours the assumption of a negligible junctional permeability to H^+ (Moffett and Cummings, 1994). This is also suggested by the finding of a large, 4–5 pH unit, H^+ gradient between the haemolymph and gut lumen *in vivo* (Dow, 1992). In line with this is our finding that, in the absence of external K^+, I_{sc} is zero even in the presence of large transepithelial pH gradients (W. Zeiske, H. Meyer and H. Wieczorek, unpublished observations). It thus appears reasonable to disregard not only a paracellular route but also transcellular passive and conductive H^+ routes across the midgut epithelium (unless they become closed only at ambient acidity). Even with a high perfusion rate, the extracellular pH might not be uniform: the epithelium is highly folded, so that the intercellular basolateral space may be a restricted region for effective acidification, and effective access of apical solutions through the goblet cavity to the apical membrane of goblet cells may be especially difficult. These factors are intrinsic properties of the midgut structure and cannot, therefore, be circumvented.

Fig. 1B (experiment with Ba^{2+}) and Fig. 5A (experiment with azide) show that basolateral effects can be quite fast. In addition, labelling experiments (Koch and Moffett, 1987) with the comparably sized sucrose molecule revealed that the entire extracellular space is accessible from the basolateral compartment within a few minutes. We feel, therefore, that we are readily able to equilibrate the basolateral membranes with a given solution of defined pH, unless the cells secrete acid or base into the lateral spaces, a situation for which there is no current evidence. While the microvillar brush border of columnar cells may equilibrate quickly with the apical solution compartment (leaving aside the folding factor), access to the goblet cell apical membrane from the luminal side may pose a severe problem.

In electron micrographs, most goblet valves appear to be closed and to possess an interdigitated structure (Flower and Filshie, 1976). Controlled filling of goblet cavities with fluorescent compounds led to the conclusion that relatively small molecules, such as an organic Ni^{2+} complex (Moffett and Koch, 1988) or Lucifer Yellow (Dow, 1992), cannot pass through the goblet valve. Goblet cavity puncture with K^+-/tetraethylammonium (TMA^+)-sensitive microelectrodes yielded the surprising result (Moffett et al., 1995) that the valves of the cavities have a negligible permeability for TMA^+, whose radius is close to that of hydrated K^+. However, there is no doubt that the apical machinery for active K^+ extrusion resides in goblet cell apical membranes, so the valves must be K^+-permeable, which makes it likely that they are also permeable to protons.

This is also a prerequisite for the proposed K^+-transport-dependent alkalization of the gut lumen *in vivo* (Azuma et al., 1995). Nevertheless, using artificial saline (pH 8) with the *in vitro* midgut, the approximately neutral goblet cavity pH proved to be insensitive to voltage-clamping, which indicates that the goblet valve is a less H^+-permeable structure, at least with an applied driving force of less than 50 mV (Chao et al., 1991; Moffett and Koch, 1992). At high luminal acidity (Fig. 2), however, there is a good chance that lumen-side protons will equilibrate with the goblet cavity space. If so, the polyanion matrix within the cavity with its strongly acidic end groups will sequester K^+ (Gupta, 1989), but not H^+, and so prevent significant buffering of luminally applied protons at low external pH. Given the similarity to our results when only the intracellular pH is lowered (see below), external protons may also enter cells, possibly at a slower rate. The antiport pathway in the goblet cell apical membrane (Wieczorek et al., 1991) is one natural candidate pathway.

Basolaterally, protons experience a definite cell-directed gradient even at the physiological haemolymph-side pH of 6.8,

whereas, at pH 8, their driving force is haemolymph-directed (Chao et al., 1991). Within this pH range, both membrane voltage and cell pH were invariant. This, however, may not be the case at pH values below 6, as shown by our experiments. In the presence of an increased gradient, therefore, H^+ may well enter the cells, perhaps *via* K^+ channels, as observed elsewhere (Lyall and Biber, 1994; Lyall et al., 1995), and gradually decrease cell pH (Onken et al., 1990) until one of the K^+-transporting membrane proteins, together with gap junctions (Francis et al., 1999; Spray et al., 1985), is blocked by rising intracellular [H^+] (see Figs 7, 8). Finally, a, still speculative (Moffett and Koch, 1992; Klein et al., 1996), basolateral K^+/H^+ antiporter could lower cell pH, if working in reverse mode (e.g. in the presence of a pH gradient).

It is clear from our data that the quantitative outcome of our experiments (extracellular *versus* selective intracellular acidification; see below) is consistent with the view that titration of intracellular membrane sites, possibly in addition to extracellular ones, contributes to the depression of I_K at low external pH. In contrast, the very easy washout of the effect of pH 3 solutions applied from either side (Fig. 1B) suggests that only extracellular membrane sites are involved in the rapid action of extracellular acidity.

Focus on experiments with ammonium

In *Hyalophora cecropia* (Zerahn, 1971), active secretion of ammonium across the midgut (like the secretion of Na^+ and Cs^+) is observed only in the absence of divalent cations from the medium. In *Manduca sexta* (Schirmanns and Zeiske, 1994b), no I_{sc} can be recorded when ambient K^+ is replaced by NH_4^+, Na^+ or Cs^+. Even in the presence of divalent cations, however, the *Manduca sexta* midgut becomes passively permeable for monovalent cations such as NH_4^+ as soon as the apical membrane is permeabilised with amphotericin. This NH_4^+ permeability is inhibited by basolateral Ba^{2+}, thus showing that the K^+ channels are the route for ammonium. With respect to NH_4^+ permeability, the K^+ channel in *Manduca sexta* is no exception among K^+ channels (Hille, 1992; Van Driessche and Zeiske, 1985) and shows a similar permeability (P_{NH_4}=0.3P_K) to Rb^+ (Schirmanns and Zeiske, 1994b). Ammonium will, therefore, enter the cells from the basolateral side, which presumably leads to cell acidification (Boron and De Weer, 1976; Zeiske et al., 1999) provided that NH_3 entry *via* the lipid pathway is much less important than the high ammonium loading *via* K^+ channels. Once inside the cell, not only will NH_4^+ acidify the cytosol, but NH_3 (dissociating from NH_4^+) will escape to the goblet cavity and so reduce the proton-motive force (set up by the V-ATPase) that drives the antiporter, as shown for GCAM vesicles (Wieczorek et al., 1991). This process will stop K^+ transport, as evident here and in earlier experiments (Schirmanns and Zeiske, 1994b).

Our observation (see Results) that apical NH_4^+ has little influence on I_{sc} indicates that NH_3 cannot easily gain access to the cytosol from that side, a property that the midgut seems to share with apical membranes from the stomach (Boron et al., 1994) and the thick ascending limb of Henle (Kikeri et al., 1989). Lastly, the presumed post-acidification after NH_4^+ removal (Boron and De Weer, 1976; Zeiske et al., 1999) might overload the H^+ extrusion capacity of the cell, thus delaying the recovery of I_{sc} (see Fig. 3).

Focus on propionate and azide

Propionic acid produced by hydrolysis of propionate is known to be an acidifier of the cytosol after lipid permeation (Keifer, 1981; Szatkowski and Thomas, 1989). As expected, therefore, propionate treatment reduced I_K, as did basolateral NH_4^+ treatment. In both cases, there was very little recovery of current after washout of the agents (see also Zeiske et al., 1999).

Azide at submillimolar concentrations has long been used as a specific, reversible inhibitor of F-ATPases (e.g. Syroeshkin et al., 1995). Like the rapid fall in K^+ current observed after depriving the midgut of oxygen (Mandel et al., 1980), azide is fast-acting and its effects are reversible (Fig. 4B), so the current inhibition could simply be due to ATP deficit. In contrast to its effects on the clathrin-coated-vesicle V-ATPase (Vasilyeva and Forgac, 1998), azide concentrations up to 2 mmol l^{-1} affected neither H^+ transport nor the hydrolytic activity of the V-ATPase from *Manduca sexta* midgut (Wieczorek et al., 1986, 1991). An additional effect relating to the putative protonophoric action of HN_3, the corresponding acid of azide, might play a role here. HN_3 has a pK_a that is almost identical to that of propionic acid. The small size of HN_3 suggests that it can enter the cells quickly *via* the membrane lipid phase and that it does this more easily than propionic acid, a conclusion which would seem likely on the basis of their different rates of action (cf. Figs 4A and 5A). Indeed, in early experiments (Boron and De Weer, 1976) on squid axon, exposure to 3 mmol l^{-1} azide led to an immediate and reversible drop in cell pH by 0.1 unit. After a longer exposure (>15 min), a secondary slow cell acidification by another 0.2 pH unit occurred. This was ascribed to a build-up of metabolic acids following mitochondrial inhibition.

The inhibition of I_K by azide in the midgut is as fast and reversible (Fig. 5A) as that observed with high bilateral H^+ concentrations (Fig. 1B). A significant recovery of current is, however, not observed after washout of ammonium or propionate (Fig. 4A). If azide leads to acidification of the cytosol, the fast recovery of the K^+ current after removal of azide could be related to the neutrality and the small size of HN_3 allowing a much faster exit from the cells compared with the weak, but charged, acid NH_4^+ and the neutral, but large, propionic acid. So, in addition to its expected blocking of the ATP supply, azide could, in the form of HN_3 (Boron and De Weer, 1976), be a rapid acidifier of the cell. As the cytosolic buffer contains 10 mmol l^{-1} phosphate (Mandel et al., 1980), this might counteract the presumed drop in cell pH. However, azide clearly inhibits the passive, reversed K^+ current (see Fig. 8 and below), an effect that appears to be completely abolished in nystatin-treated preparations when the cell pH is made more alkaline. This finding would be consistent with our assumption that HN_3 is also a plasma membrane protonophore.

Nystatin-permeabilised membranes

In two former studies (Schirmanns and Zeiske, 1994a,b), we have shown that amphotericin B, a close chemical relative of nystatin, was very effective in eliminating the apical/basolateral functional properties of *Manduca sexta* midgut so that the epithelium could be treated as if it were a pseudo 'mono-membrane' preparation. In the present study, we used nystatin at 10-fold higher concentrations, with the result that the effect was almost immediate (see Fig. 8) and approximately 10 times faster than previously observed using the low amphotericin B concentrations. With the nystatin method, we set out to distinguish the K^+-transporting capacity of the GCAM (antiporter plus H^+ pump) from that of the basolateral passive K^+ permeability (channels). In the case of basolateral application of nystatin, we would predict that, with 32 mmol l^{-1} K^+ saline, the cells would lose more than half their K^+. Experiments on intact tissues (Moffett et al., 1982), however, have shown that omission of basolateral K^+ (replaced with Na^+) leads to only a 10 % loss of cellular K^+ (usually around 100 mmol l^{-1}). The reason for this finding may be that bulk electroneutrality must not be violated when there is no other cation for bulk exchange with K^+ or no conductive pathway for small anions (Chao et al., 1989) to accompany any K^+ leaving the cell. The cellular negativity, generated by the V-ATPase, means that cells should maintain their high K^+ concentration in the presence of basolateral nystatin, a monovalent ionophore with only a very low Cl^- permeability. Very small movements of H^+ across the nystatin channels would introduce major intracellular pH changes, thus helping to equilibrate the cytosol with the basolateral pH.

In any case, basolateral permeabilisation using the ionophore will enable us to record apical K^+ transfer (Schirmanns and Zeiske, 1994a), called the 'pump current', I_P. This is a lumped parameter describing the cooperation between antiport and V-ATPase, but is also exactly equal to the net apical charge transfer of K^+. So far, we cannot use this parameter to distinguish the specific influence of protons on these two transporters; that is, a reduction in I_P might reflect an impact on the antiporter, on the V-ATPase or on both.

Using apical amphotericin, we were able to show that basolateral K^+ channels are also permeable to Tl^+, Rb^+ and NH_4^+ (Schirmanns and Zeiske, 1994b). In the present study, apical application of nystatin was used to measure the reversed K^+ current (I_K^{rev}), through apical nystatin and basolateral K^+ channels, along a $[K^+]$ gradient from 32 mmol l^{-1} in the lumen to 0 mmol l^{-1} on the haemolymph side. In this case, we have to assume some loss of K^+ from the cells (presumably 32 mmol l^{-1} as the final value in the cell) since the apical voltage (cell-negative) generated by the H^+ pump will be eliminated because of the short-circuit achieved by nystatin (Schirmanns and Zeiske, 1994a,b). Application of basolateral Ba^{2+} (Schirmanns and Zeiske, 1994b) virtually eliminates I_K^{rev}. Blockage of I_K^{rev}, which now reflects the permeability of the basolateral K^+ channels as the rate-limiting component, is almost complete in the presence of azide at pH 8 but is practically abolished at a cell pH of 9 (Fig. 8) and is approximately half-maximal at a cellular pH of 5 (Fig. 7). Our conclusion is that both I_P and P_K are decreased to a comparable degree by acidic cellular pH.

Targets of protons and the problem of physiological relevance

Our results indicate that I_P and P_K are under parallel control by cellular pH, but surprisingly not within the neutral, presumably physiological, pH range. This is unlike observations made in other systems (Harvey, 1995; McNicholas et al., 1998). Epithelial K^+ channels often display a steep activity-dependency on cell pH (Harvey, 1995), with histidine as a candidate for titration by intracellular protons (McNicholas et al., 1998; Tang et al., 2000) and glutamate and aspartate as candidates for titration by extracellularly applied protons (Hille, 1992). Here, both extra- and intracellular titration seem to be consistent with the assumption of Asp/Glu as H^+ sites.

Cation/proton antiporters, such as those of the NHE family, underlie in action and direction the applied ion gradients and are activated when the intracellular pH reaches a sharply defined acidic set point (Wakabayashi et al., 1997). However, the chemical nature of any stimulating or inhibitory H^+ site on the midgut $K^+/2H^+$ exchanger has yet to be determined.

In vivo, the V-ATPase from tobacco hornworm midgut (Sumner et al., 1995) as well as that from yeast (Parra and Kane, 1996) can dissociate into a soluble V_1 unit and a V_o complex that remains membrane-bound. This process is induced in yeast after deprivation of glucose and in *Manduca sexta* during starvation or moulting of the larvae. Surprisingly, this mechanism is reversible in both cases when 'normal' conditions (glucose for yeast, food or after-moult instar for the caterpillar) are restored. In the case of the *in vitro* yeast V-ATPase (Parra and Kane, 1996), incubation at pH 5 favours the reassembly of V_1V_o following chaotropic ion treatment to induce disassembly. However, below pH 5, the restored V_1V_o complex begins to lose ATPase activity. The *in vitro* GCAM V-ATPase of *Manduca sexta* displays quite a broad pH optimum over the range pH 7–9 (Wieczorek et al., 1986).

These findings are corroborated both by our results when we controlled cell pH using basolateral nystatin and also by our experiments with ammonium and propionate. For apical, extracellularly acting protons, inhibition of V-ATPase may simply be due to an opposing driving force; intracellular inhibition by H^+ should then be allosteric. From the chemical point of view, the glutamate residue in the supposedly H^+-transporting c subunit in the membrane-embedded V_o part of the complex (Harrison et al., 2000) could play a role in the inhibition by protons.

Another, more indirect, mechanism might also play a role in V-ATPase arrest. When K^+ channels are blocked by protons below pH 6 (present results), the apical cell membrane potential should hyperpolarise. It has been shown that V-ATPases in plant (Davies et al., 1996) and in amphibian (Andersen et al., 1985) epithelia respond to hyperpolarisation (in the direction of the proton-motive force) of the H^+-pump-containing membrane by a decrease in pump activity. We therefore have to envisage

that contralateral K^+ channel blockade by H^+ effects a similar voltage sensitivity of the apical V-type H^+ pump in the midgut. This hypothesis has already been put forward in previous reports of H^+-independent hyperpolarisations (Dow, 1992; Moffett and Koch, 1988).

On the basis of the properties of the membrane transporters involved in midgut K^+ secretion, the 'synchronized' changes in apical I_P and basolateral P_K elicited by H^+ occur over a seemingly unphysiological pH range and may be fortuitous. However, we can come to the important conclusion that acid-stimulated V_1V_o assembly, if it occurs in the midgut, does not play a protective role in maintaining midgut K^+ transport under acidic conditions. Nonetheless, given that the midgut has to face external/internal environments that are, with respect to pH or K^+, extreme compared with those experienced by vertebrate epithelia, the evolution of relatively pH-insensitive transporters might have been a critical factor for making this spectacular K^+-secreting system in the caterpillar midgut viable.

This research was supported by Deutsche Forschungsgemeinschaft grant SFB 431.

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