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A PRIMARY CATION TRANSPORT BY A V-TYPE ATPase OF LOW SPECIFICITY

J. KÜPPERS AND I. BUNSE

Institut für Neuro- und Verhaltensbiologie der Wilhelms-Universität, Badestraße 9, 48149 Münster, Germany

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Summary

The enzyme involved in outward K^+ transport in insect epithelia belongs to the family of V-ATPases. Evidence has been reported relating the generation of the K^+ gradient to a primary electrogenic proton transport *via* a distinct electrophoretic nH^+/K^+ antiport.

The subject of this paper is the transport of K^+ at a thread hair sensillum of the cockroach *in situ*. We recorded changes in the voltage and resistance of the ion-transporting membrane and of shifts in pH caused by inhibition of energy metabolism and by putative inhibitors of a proton/cation exchanger. The results are supplemented by previous determinations of the K^+ activities in the same preparation.

1. In cockroach hair sensilla, the ion transport generates a membrane voltage of 105 mV. We found that the transport rendered the positive output compartment alkaline with respect to the cytoplasm by 1.0 pH unit compared with the pH at equilibrium distribution, and we infer that proton transport cannot be the process that energizes the generation of the K^+ gradient.

2. The ion transport created an electrochemical potential difference for protons, $\Delta\eta_H$, of approximately 4.5 kJ mol^{-1}, while the potential difference for K^+, $\Delta\eta_K$, amounted to approximately 11 kJ mol^{-1}. Both potential differences are directed to the cytosol. It follows from $\Delta\eta_K/\Delta\eta_H$ that an antiport would have to be electrophoretic to drive K^+ by $\Delta\eta_H$ and it should, therefore, contribute to the membrane conductance. Amiloride and harmaline did not significantly change the pH in the adjacent spaces and did not affect the voltage or the resistance of the transporting membrane. Previous determinations of the impedance have shown that the ATP-independent conductance of this membrane is small, supporting the conclusion that it lacks an electrophoretic antiport.

From these results, we deduce that K^+ transport in cockroach sensilla is not secondary to a proton transport and an electrochemical proton gradient. The phenomena observed match the performance of a primary, electrogenic, cation-translocating ATPase of the type deduced from analyses of the short-circuit current at the midgut epithelium of lepidopteran larvae. The validity of the H^+ transport/antiport hypothesis is discussed.

Key words: K^+ transport, vacuolar ATPases, transporters, membrane impedance, insect sensilla, *Periplaneta americana*.

Introduction

Across the epithelia constituting the Malpighian tubules, the gut, the salivary glands and the epidermal sensilla of insects, there is an electrochemical K^+ gradient directed from the outside or the lumen of the organs to the haemolymph. The electrochemical potential difference (PD) of K^+ energizes a range of physiological functions such as excretion, absorption and signal transmission (summarized by Harvey *et al.* 1983; see also Küppers and Thurm, 1982). At least some of the cells of these epithelia are characterized by an apical membrane that is studded with particles, and it is this region of the membrane that transfers K^+ from the cytosol to the outside. *In vitro*, the K^+ transport, both at the midgut and at epithelial sensilla, was shown to be a cation transport of low specificity, preferring Rb^+ over K^+ and other monovalent cations (Zerahn, 1971; Thurm, 1974). The function of Malpighian tubules reflects the low ion specificity *in vivo* (Maddrell, 1980). There is a fundamental similarity in K^+ transport in the various organs, as indicated by a similarity in physiological performance, by the presence of characteristic 'portasomes' (Harvey, 1980; Harvey *et al.* 1981) and by the poor ion selectivity of the process. The transport at insect sensilla and that in the gut of *Manduca sexta* have been shown to possess further features in common: they are insensitive to the F_1F_o-ATPase inhibitors oligomycin and NaN_3 (Wieczorek *et al.* 1986; J. Küppers, unpublished observation), and antibodies to the *M. sexta* ATPase label the sites of K^+ transport at epidermal sensilla of other insect species (Klein, 1992). Cloning and sequencing of the subunits of the transporting enzyme of the midgut and of Malpighian tubules revealed that it belongs to the family of vacuolar-type ATPases (Schweikl *et al.* 1989; Meagher *et al.* 1990; Novak *et al.* 1992; Dow, 1992; Pietrantonio and Gill, 1993; Nelson, 1992; Gogarten and Taiz, 1992).

Cioffi and Harvey (1981) analysed the transepithelial short-circuit current, I_{sc}, at the midgut of *Manduca sexta*. The I_{sc} was

carried exclusively by K^+, and they identified the transporting enzyme as an electrogenic cation-ATPase mediating the primary transport of K^+.

Wieczorek and associates proposed a concept differing from that deduced from I_{sc} analysis. They observed changes in the fluorescence of voltage- and pH-sensitive dyes induced by harmaline and amiloride and by other experimental procedures in vesicle preparations of the apical membrane of the goblet cells of *M. sexta*. From the interpretation of the fluorescence quenching, they inferred that the K^+ transport of insects is driven by a proton gradient, generated by a V-type H^+-ATPase, *via* a distinct, electrophoretic nH^+/K^+ antiport (Wieczorek *et al.* 1989; Wieczorek, 1992).

This paper examines the K^+ transport at the apical membrane of the accessory cells of mechanoreceptor sensilla of the cockroach *Periplaneta americana* in an *in situ* preparation that preserves an unequivocal orientation of the transporting membrane. Both the input and the output compartments of the transport are accessible to microelectrodes. This allows quantitative measurements of the voltage, the intra- and extracellular pH and the impedance of the transporting membrane. The dependence of these variables on energy metabolism and the effect of putative inhibitors of a H^+/K^+ exchanger are reported. Results are supplemented by previous measurements of the K^+ activities in the same system (Thurm and Küppers, 1980). Some of the results have been published in a brief form (Küppers and Thurm, 1994).

Properties of the thread hair preparation

This investigation was centred on the apical membrane of the accessory cells of epidermal thread hair sensilla located on the ventral side of the cerci of *Periplaneta americana*. The accessory cells, the tormogene, trichogene and thecogene cells, represent a specialized part of the functionally monolayered epidermis. They envelop the dendrite of the sensory cell and confine a compartment beneath the cuticle: the receptor lymph space. This cavity is separated from the haemolymph and from the subcuticular space of the surrounding integument (Keil, 1984). The isolation prevents any net flux of solutes and confines the electrical circuit to the epithelial cells of the sensillum.

The apical membrane of the accessory cells constitutes at least 95 % of the resistance measured between the receptor lymph and the haemolymph (J. Küppers, unpublished observation). Poisoning energy metabolism using CN^- or NaN_3 raises this resistance from approximately 20 to 40–60 MΩ. The impedance of the apical membrane in the active state and during ATP depletion has been determined using current-clamp (Küppers, 1984). The conductance of this membrane is almost completely governed by an ATP-dependent pathway, as revealed by fitting a minimalist electrical circuit diagram to the vector plots of magnitudes and phase shifts recorded between 0.1 and 100 Hz. The ATP-dependent branch has a conductance of about 50 nS, while the sum of all conductances in parallel, including the intercellular clefts and the unstimulated dendrite, amounts to only approximately 5 nS.

The voltage across the apical membrane of the accessory cells is about 105 mV, outside positive, the K^+ activity (aK^+) within the receptor lymph is 140 mmol l^{-1} (corresponding to an aqueous KCl solution of 180 mmol l^{-1}) and aK^+ in the cytosol is about 105 mmol l^{-1} (Thurm and Küppers, 1980). Thus, the electrochemical potential difference for K^+, $\Delta\eta_K$, adds up to 110 meV (unit charge times ion-motive force, the energy referred to a single ion) or approximately 11 kJ mol^{-1}.

The only known function of the transport at the accessory cells of insect sensilla is the generation or enhancement of $\Delta\eta_K$, which drives the receptor current. Upon adequate mechanical stimulation, the receptor current enters the apex of the sensory dendrite and depolarises the spike-generating basal membrane of the sensory cell on the haemolymph side (Thurm and Küppers, 1980; Küppers and Thurm, 1979). The transport at sensilla is not concerned with excretion or resorption, unlike that of the gut, the rectum, the Malpighian tubules and the salivary glands.

Materials and methods

The cerci of adult *Periplaneta americana* L. were isolated. Their distal end was cut and they were glued to a glass capillary to be perfused by gravity (4 kPa). The flow rate was 150–200 μl min^{-1}. The standard perfusate used throughout these experiments was a high-K^+ saline which contained (in mmol l^{-1}): 140 KCl, 5 $KHCO_3$, 1 KH_2PO_4, 5 $MgCl_2$, 5 Tris, 5 EGTA, 30 glucose and 120 sucrose. pH was adjusted to 7.2 and pCa to approximately 9. 5 mmol l^{-1} NaN_3, 0.2 mmol l^{-1} harmaline or 1 mmol l^{-1} amiloride was added to this solution. The high K^+ concentration of this 'cytosol substitute' depolarizes the basolateral membrane to approximately 0 mV, indicating a dominating K^+ conductance. The values of the physiological parameters observed remained constant in successful preparations for several hours.

A substitute for haemolymph differed from the high-K^+ saline in the concentrations of the main cations, which were (in mmol l^{-1}): 125 Na^+, 20 K^+ and 5 Ca^{2+} (0 EGTA), according to an analysis of the *Periplaneta americana* haemolymph by Pichon (1970). This solution, used by Thurm and Küppers (1980), polarizes the basal membrane to approximately −30 mV without a significant effect on the ATP-dependent voltage drop across the apical membrane. The voltage between the receptor lymph and the haemolymph is about +70 mV in that case.

A double-barrelled, bevelled glass electrode (total tip diameter ⩽3 μm, see below) was guided by means of a motor-driven micromanipulator under stereomicroscopic control. The electrode was inserted through the thin cuticular cupola at the base of the hair into the receptor lymph space or was further advanced into a cell. One barrel of the microelectrode recorded the voltage, the other was used for either current injection or determination of pH (Fig. 1). The reference electrode made contact with the perfusate at the open end of the cercus. All three electrodes were filled with the standard perfusate. They were connected *via* AgCl-coated silver wires to a current-

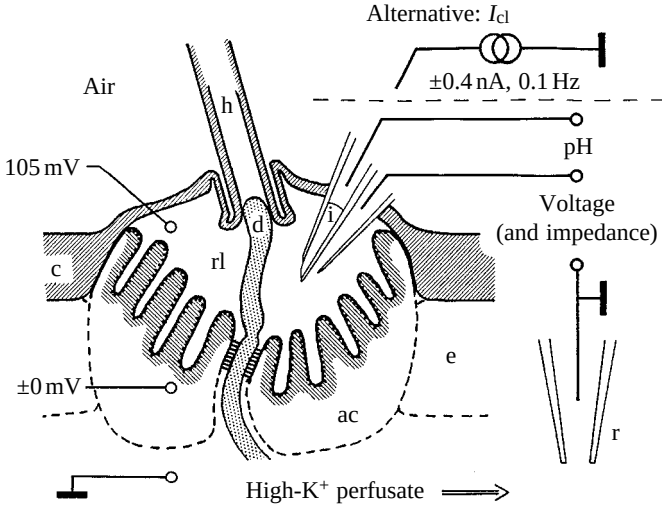

Fig. 1. A diagram of a cockroach hair sensillum showing the position of the electrodes and the principle of electrical recording. Broken lines symbolize membranes of low resistance. ac, accessory cells; c, cuticle; d, outer segment of the dendrite of the sensory cell; e, epidermis; h, hair; i, H^+ ionophore; alternative: I_{cl}, current to determine the resistance; r, reference electrode; rl, receptor lymph cavity. Voltages reflect the active state under experimental conditions. The diameter of the cuticular cupula is about 30 μm.

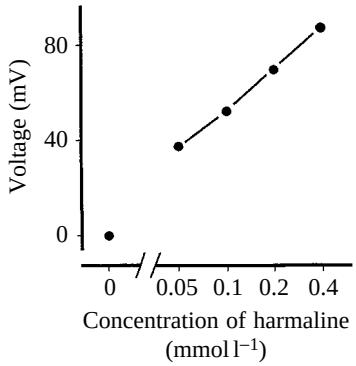

Fig. 2. The response of Ionophore II to different concentrations of harmaline in the standard perfusate at pH 7.2.

clamp device and two high-impedance amplifiers, respectively. The micromanipulator and electronic equipment were designed and constructed by our collaborators H. Meschede and J. Weil. Results were recorded by means of a two-channel chart recorder (SE 130, ABB Goerz, Wien) with the facility for pen synchronization.

The local transepithelial voltage (TEV) is a sensitive monitor of any artefactual shunt. A TEV of more than 100 mV (in the active state) ensures that a recording originates from the receptor lymph of an intact, unstimulated sensillum. Until the acute dependence of the low cytoplasmic pH on energy metabolism had been established (see Figs 3 and 7), the reversible polarization of the basal membrane by the haemolymph substitute was used to establish the intracellular position of the electrode. The very large tip of the electrode, which was required to penetrate the cuticle, resulted in a low percentage of successful cell impalements. The intracellular recordings are believed to originate from the outermost accessory cell, the tormogene cell, because this cell is by far the largest of the whole complex.

The resistance between the receptor lymph space and the haemolymph space was determined using a sinusoidal current, I_{cl}, of 0.1 Hz and an amplitude of ±0.4 nA. The resulting alternating voltage reflects the resistance and is superimposed on the actual transepithelial voltage.

For the pH microelectrodes, we used the liquid ion exchangers 'Hydrogen Ionophore I – Cocktail B' and 'Hydrogen Ionophore II – Cocktail A' from Fluka. One tip of the double-barrelled electrode was siliconized and the tip was filled with ionophore to a length of 300–500 μm. The electrodes were calibrated using commercial buffers of pH 6.0, 7.0 and 8.0 (Fluka) before and after each experiment. The response time was shorter than the time resolution of the recordings. Only those electrodes that responded with a change of more than 55 mV per pH unit were used. Measurements were discarded if the drift of the electrode during an experiment exceeded 0.1 pH unit h^{-1}.

We found that Ionophore II (but not Ionophore I) responded with approximately 60 mV per decade to harmaline (Fig. 2). We used this sensitivity of Ionophore II to verify that harmaline actually reached both faces of the apical membrane when added to the perfusate. The concentration of this agent in the receptor lymph was 90 % of that in the perfusate within approximately 5 min.

We found that electrodes containing Ionophore I were not suitable for intracellular recordings: a few minutes after impalement of a cell, the reading started to drift by several hundred millivolts, indicating a pH of 2 or even less. The electrodes slowly recovered in calibration buffer but showed little recovery in the perfusate. We were, therefore, unable to monitor the effect of harmaline on the intracellular pH.

Results

The ATP-dependent electrochemical proton potential across the apical membrane

The voltage across the apical membrane approached zero when energy metabolism was inhibited (Fig. 3). The pH of the receptor lymph was 6.8±0.1 (mean ± S.D., N=10) and that of the cytoplasm was 7.0±0.15 (N=13). This difference in pH was statistically significant ($P \leqslant 0.01$; Student's t-test), indicating that there is an anionic matrix within the receptor lymph cavity. We observed no significant changes in these values for periods of up to an hour, and so we define this state as the equilibrium state.

When metabolic activity was restored, the receptor lymph cavity became positive with respect to the cell by 105 mV and the pH of the receptor lymph increased by 0.35 to 7.15±0.1 (N=17) (Fig. 3). The increase in this transport-dependent pH is significant (P<0.001). At the same time, the pH of the

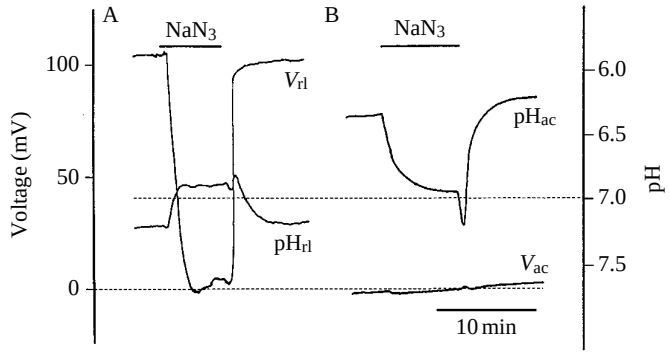

Fig. 3. (A) The upper trace (V_{rl}) represents the voltage between the receptor lymph cavity and the haemolymph space. It corresponds to the voltage across the apical membrane of the auxiliary cells, since the basal membrane was depolarized (see Materials and methods and compare with B and Fig. 7). In the presence of 5 mmol l^{-1} sodium azide, the apical voltage reversibly decayed to zero, while the pH of the receptor lymph (lower trace, pH_{rl}) reversibly decreased. (B) The effects of sodium azide on the voltage across the basal membrane (V_{ac}) and the intracellular pH (pH_{ac}).

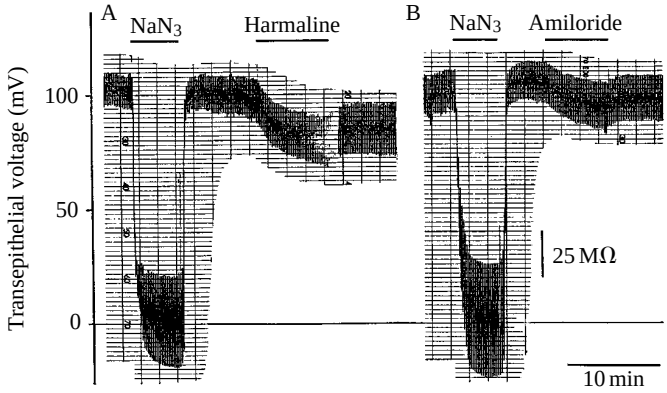

Fig. 4. The effects of 5 mmol l^{-1} NaN_3 on transepithelial voltage and resistance (monitored by the amplitude of the superimposed oscillating voltage) compared with the effects of (A) 0.2 mmol l^{-1} harmaline and (B) 1 mmol l^{-1} amiloride.

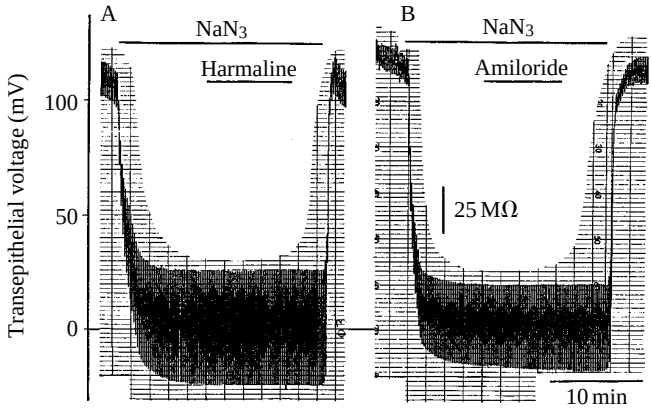

Fig. 5. During ATP depletion, 0.2 mmol l^{-1} harmaline (A) and 1 mmol l^{-1} amiloride (B) did not affect the membrane resistance.

cytoplasm decreased by 0.70 to 6.3±0.2 (N=18), presumably as a result of the presence of acid metabolic intermediates or products.

In conclusion, the active transport reduced the H^+ activity of the receptor lymph. The pH difference generated across the apical membrane amounted to 1.0 unit compared with the equilibrium distribution. Thus, the inwardly directed $\Delta\eta_H$ was approximately 45 meV (4.5 kJ mol^{-1}) at a voltage of 105 mV. Under these conditions, $\Delta\eta_K$ is 110 meV as previously determined (see above).

The effects of amiloride and harmaline

The influx of three protons would be required for the outward transport of one potassium ion *via* an antiport judging from the ratio $\Delta\eta_H/\Delta\eta_K$ only. Inhibiting such an electrophoretic antiport should markedly increase the H^+ activity of the receptor lymph and the resistance of the apical membrane. In addition, the membrane voltage will increase if the load to the primary transport is reduced (see Discussion). In contrast, inhibiting the ATP-dependent transport reduces the voltage and increases the resistance (Figs 4 and 5, see also Thurm, 1974).

To inhibit a putative nH^+/K^+ exchanger we used the drugs harmaline and amiloride, which have been reported to be effective blockers in vesicle preparations of the goblet cell apical membrane of *M. sexta* (Wieczorek *et al.* 1991). We raised the concentration of harmaline to 0.2 mmol l^{-1} and that of amiloride to 1 mmol l^{-1} (that is twice and 1.5 times, respectively, the concentrations used by Wieczorek and collaborators) until nonspecific effects clearly impeded the primary transport (see Fig. 4).

The effects of harmaline and amiloride on membrane voltage and impedance are compared with those of NaN_3 in Fig. 4A (a representative recording taken from seven experiments) and Fig. 4B (showing a representative recording from four experiments). Depletion of ATP caused the membrane resistance to increase by about 160 % (from 20 to 53 MΩ in Fig. 4A and from 24 to 65 MΩ in Fig. 4B). Both inhibitors produced a slight increase in the resistance (30 % for harmaline and 10 % for amiloride) and a slight decrease in the voltage, which were only partly reversible. This combination suggests some inhibition of the primary transport rather than an effect on an antiport. This inference is corroborated by the finding that harmaline or amiloride had no effect on the membrane resistance in the ATP-depleted state (compare Fig. 5A, which is representative of 13 recordings, and Fig. 5B, which is representative of four recordings, with Fig. 4).

Harmaline and amiloride did not significantly affect the pH of the receptor lymph (Fig. 6). Some of the slight decrease in the pH during the application of harmaline may be due to the reduced voltage and a corresponding increase in H^+ activity. The transient changes in pH at the beginning and end of amiloride application might reflect intracellular transients (Fig. 7).

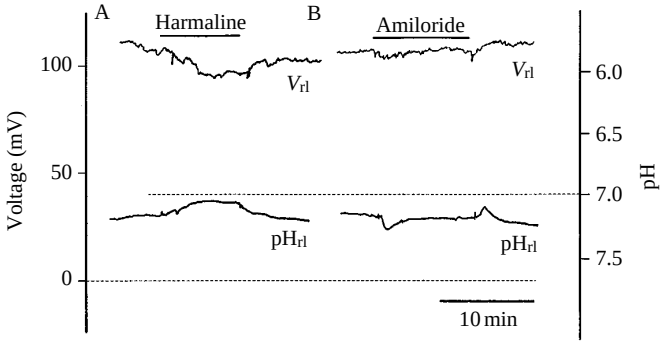

Fig. 6. Simultaneous recordings of voltage and pH in the receptor lymph. Changes induced by (A) 0.2 mmol l^{-1} harmaline and (B) 1 mmol l^{-1} amiloride are representative for 11 and 7 recordings, respectively.

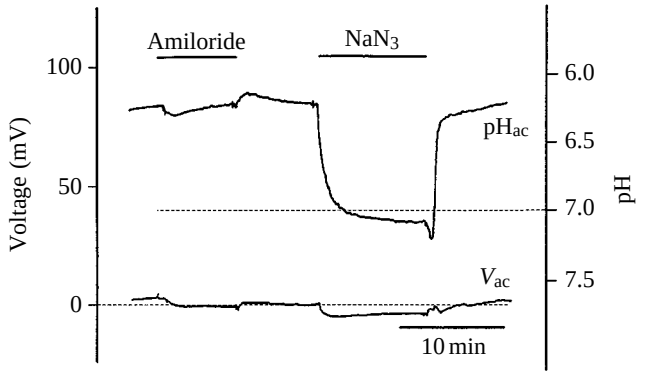

Fig. 7. Changes in the intracellular pH caused by 1 mmol l^{-1} amiloride and by blocking the energy metabolism with 5 mmol l^{-1} sodium azide. Representative recording of eight experiments.

Discussion

Observations of voltage changes and of shifts in the activities of a very restricted number of ion species in response to different experimental conditions cannot yield a convincing, positive identification of the ion specificity of a transport ATPase. However, a quantitative comparison of electrochemical potential differences, supplemented by measurements of resistance, may enable likely, possible and impossible mechanisms to be identified.

The thread hair sensillum of *P. americana* represents a very simple system in this context. The morphology prevents a significant net flux of solutes, and the low conductance in parallel to the transport provides nearly open-circuit conditions for the transport itself. Simultaneous recordings of voltage and pH or of voltage and resistance ensure that recordings originate from an uninjured sensillum and provide information about the position of the electrode tip, thus enhancing the reliability of the data and of their interpretation.

The conclusions drawn rely on reversible and reproducible ATP-dependent changes (Fig. 8) which were observed at the K^+ activity at which the transport actually works *in vivo*. They are inferred from two independent lines of evidence.

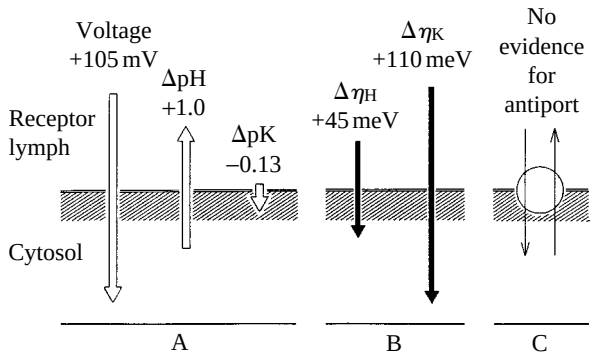

Fig. 8. A diagram summarizing the results. (A) The size and direction of the electrical and chemical driving forces for H^+ and K^+ as they are generated by the energy of ATP hydrolysis. (Arrows are drawn to scale using the Nernst relationship.) (B) This energy is reflected by electrochemical potential differences ($\Delta\eta$) for both H^+ and K^+. (C) We found no evidence for a proton/cation exchanger in the transporting membrane.

ATP-dependent shifts in pH

During ATP depletion, the following equilibrium state across the apical membrane was approached: the voltage was close to 0 mV, the pH of the receptor lymph was 6.8 and that of the cytosol 7.0. The transport generated a voltage of +105 mV and rendered the receptor lymph alkaline by 0.35 pH units (P<0.001). The pH of the cytosol was 6.3 in this active state, i.e. the cell became acidic by 0.7 pH units. The absolute, and even more the relative, increase of pH in the receptor lymph space by 1 unit was too large to be explained by experimental error.

The finding that the activity of the apical transport system renders the output compartment alkaline corresponds to observations on the midgut epithelium of lepidopteran larvae and on Malpighian tubules of *Drosophila melanogaster* (Chao *et al.* 1991; Wessing *et al.* 1993). The extremely high pH within the midgut lumen (>11) is consistently ascribed to the equilibration of protons with the high voltage across the open-circuited midgut epithelium (Dow, 1992).

To adapt the observations at the midgut to the H^+-ATPase hypothesis, a concept has been developed by Harvey (1992), Martin (1992) and Martin and Harvey (1994) that joins a primary, electrogenic proton transport with the alkalization of its output side by means of an electrophoretic nH^+/K^+ antiport which is driven by a proton gradient. This model of 'ion circuit analysis' has been accepted by others. On the basis of sophisticated assumptions, the concept tries to explain final states by their development. During this step-by-step analysis, the First Law of Thermodynamics is circumvented, because the system eventually arrives at a state that allows the driving energy of the proton potential difference to be ascribed only to the voltage component and the chemical potential difference to be disregarded. As a further explicit outcome, 'ion circuit analysis' allows the membrane voltage to exceed the reversal potential of the pump, which is limited by $\Delta G_{ATP}/m \times z_j F$, where ΔG_{ATP} is the free molar energy of ATP hydrolysis, m

is a stoichiometric factor, z_j is the valence of the ion species *j* and *F* is the Faraday constant.

An electrogenic ion pump converts the scalar energy of ATP hydrolysis into a vectorial electrochemical potential difference according to:

$$\Delta G_{ATP}/m = (\Psi^o - \Psi^i)z_jF + \boldsymbol{R}T\ln(a_j^o/a_j^i) + T\Delta S^*, \quad (1)$$

where ΔS^* denotes the fraction of entropy released during the hydrolysis of 1 mol of ATP: $\Delta S^*=\Delta S/\text{mol ATP}\times m$. Ψ is the electrical potential, i and o refer to the input and output compartments, respectively, $\boldsymbol{R}$ is the gas constant and *T* is the absolute temperature. Substituting the chemical potential difference: $\boldsymbol{R}T\ln(a_j^o/a_j^i)$ with $\Delta\mu_j$, then:

$$\Delta G_{ATP}/m = \Delta\Psi z_jF + \Delta\mu_j + T\Delta S^*$$
$$= \Delta\eta_j + T\Delta S^*. \quad (2)$$

The molar energy represented by $\Delta\eta_j$ may drive secondary transport processes by analogy to equation 2 and energize the flux or the accumulation of other solutes. The size of $T\Delta S^*$ and the relative contributions of $\Delta\Psi z_jF$ and $\Delta\mu_j$ to $\Delta\eta_j$ depend on the physiological surroundings that determine the working conditions of the transport process. For a given input energy ($\Delta G_{ATP}/m$ = constant), possible changes of the terms on the right-hand side of equation 2 are related by:

$$d(\Delta\Psi z_jF) + d(\Delta\mu_j) + d(T\Delta S^*) = 0. \quad (3)$$

The relative maximum entropy production is characterized by the state: $\Delta\Psi z_jF=\Delta\mu_j$ because then $d(\Delta\Psi z_jF)=-d(\Delta\mu_j)$ and $d(T\Delta S^*)=0$. If either $\Delta\Psi z_jF$ or $\Delta\mu_j$ reaches the input energy of the pump, ΔS^* declines to its minimum (equation 2). It follows from the Second Law of Thermodynamics that no term of $\Delta\eta_j$ will ever exceed the limit given by $\Delta G_{ATP}/m$, and, thus, invert the sign of the other term. This would require the input of entropy from an additional, independent energy source. This energy cannot be derived from a secondary process that is driven by the reaction under consideration.

Thus, an exclusive proton pump, such as a conventional V-ATPase, will render the output side both more positive and more acidic. This phenomenological analysis presumes nothing but causality between pump, voltage and ion distribution. It does not rely on coupled transporters, on details of the ion fluxes induced or on the physicochemical properties of the surroundings. It applies both to rather simple systems, such as vesicles or the closed receptor lymph cavity which do not allow the net flux of any matter, and to functionally sophisticated epithelia.

Because the active transport reduced the H^+ activity in the receptor lymph, we infer that the transport processes at the apical membrane are not governed by a primary proton transport.

The missing antiporter activity

The transport generated a $\Delta\eta_H$ of 45 meV and a $\Delta\eta_K$ of 110 meV. From the ratio $\Delta\eta_H/\Delta\eta_K$, it follows that a hypothetical nH^+/K^+ antiport might transfer K^+ if the value of *n* is greater than 2. Inhibiting such an electrophoretic antiport should perceptibly increase the membrane resistance. In this study, there is direct evidence (see Materials and methods) that harmaline, a putative antiport inhibitor, reached the receptor lymph space. From the effects on the primary transport process (Figs 4, 6), we infer that amiloride also reached the apical membrane. However, the pH, the voltage across the apical membrane and the resistance of this membrane did not reflect a specific target for these drugs (see Figs 4, 5, 6). In the ATP-depleted state, the conductance proved to be completely insensitive to both harmaline and amiloride (Fig. 5). This lack of effect might be because these drugs are not appropriate tools with which to inhibit an electrophoretic antiport. The result, however, is consistent with that obtained from the analysis of the electrical impedance, which showed no significant conductance in parallel to the ATP-dependent pathway (Küppers, 1984). We therefore conclude that the apical membrane of cockroach sensilla lacks an electrophoretic proton/cation exchanger.

The fact that the proton distribution did not equilibrate with the transport-dependent membrane voltage may be most simply ascribed to an appreciable H^+ affinity of the primary transport itself, as suggested by Wieczorek *et al.* (1986). Since the H^+ activity is 5–6 orders of magnitude lower than aK^+, it may be estimated that the proton specificity of the transport exceeds even that for K^+ by some orders of magnitude, thus leading to acidification of the output side under K^+-free experimental conditions, as observed by Wieczorek *et al.* (1991) and Wessing *et al.* (1993).

The electrochemical potential differences for K^+ and H^+ only add up to 155 meV. A lower limit for ΔG_{ATP} released by the pump may be estimated assuming the stoichiometric factor 2 (estimated by Harvey *et al.* 1981, for K^+/ATP). We calculate that ΔG_{ATP} is greater than 300 meV or 30 kJ mol^{-1}, which is consistent with general estimates for ΔG_{ATP}, which may be 40–50 kJ mol^{-1} in living animal cells.

In conclusion, both from the ATP-dependent shift in pH and from $\Delta\eta_H/\Delta\eta_K$ and the missing electrophoretic antiporter, we infer that the K^+ transport at the apical membrane of insect sensilla is not secondary to a proton transport. The transport phenomena observed match the performance of a primary, electrogenic cation pump of the kind that has been reviewed by Harvey *et al.* (1983).

This genuine concept of a primary K^+ transport through insect epithelia relies on analyses of the short-circuit current at the midgut epithelium of *Hyalophora cecropia* (Harvey and Nedergaard, 1964) and the midgut of *M. sexta*. In the latter case, the I_{sc} constitutes 100 % K^+ (Cioffi and Harvey, 1981; verified by Wolfersberger and Giangiacomo, 1983). Precise short-circuit conditions, defined by zero electrochemical gradients for the solvent and all solutes across the transporting membrane, are difficult to achieve in experimental situations. But even if short-circuit conditions are only approximated, we do not see how the flux of that ion species, which is driven by a metabolic reaction, can fail to contribute at least a detectable amount to the composition of the current. In these and related

experiments, however, the transport of K^+ always behaved as an indivisible functional entity.

In view of the heuristic value of short-circuit current analyses, and in view of the difficulties that arise in explaining established observations with the H^+-ATPase/antiport hypothesis (an example is discussed above), it is surprising that the well-founded K^+ transport concept has been so precipitately dismissed. A critical discussion of the antiport hypothesis has been avoided by the postulate that the hypothesized H^+ and K^+ fluxes are so intimately coupled to each other that the transport system as a whole behaves like an 'electrogenic K^+ pump'. The physicochemical nature of such tight coupling remains, however, in question. In considering these problems, Dow (1992) approached the outcome of our paper, when he suggested: 'Perhaps the interaction is so tight that alkali metal ions can actually substitute for protons in the ATPase'.

The common structural and biochemical features outlined in the Introduction, the functional analogies and the labelling by antibodies against the ATPase of *M. sexta* (Klein, 1992) justify the assumption that the K^+ pump at epidermal sensilla does not differ in essence from the catalytic element of the K^+-transporting system in the midgut or Malpighian tubules. In these epithelia, the ATPase has been reliably identified as a V-type ATPase (see references in Introduction). F-ATPases and the related V-ATPases are characterized by their specific molecular structure, showing homologies from the quaternary down to the primary level. Most of them seem to exhibit the extremely high proton specificity that is a prerequisite for the operation of H^+-translocating ATPases in the presence of abundant Na^+ and K^+.

The function ascribed to the V-type ATPase of *Enterococcus hirae* (*Streptococcus faecalis*) is essentially different from the electrogenic H^+ transport of most V-ATPases. It has been suggested that it drives an electroneutral exchange of Na^+ for K^+ (Kakinuma and Igarashi, 1989, 1994). The F_1F_o-ATPase of the anaerobic bacterium *Propionigenium modestum* allows Na^+ to substitute for protons (Laubinger and Dimroth, 1989; Dimroth, 1994), and it has been shown that Na^+ and H_3O^+ share the same binding site in the F_o moiety (Kluge and Dimroth, 1993). *Acetobacterium woodii* yields a further example of a Na^+-translocating F-ATPase (Reidlinger and Müller, 1994; Reidlinger *et al.* 1994). Site-directed mutagenesis of subunit c changed the binding specificity of F_o from *Escherichia coli* H^+-ATPase (Zhang and Fillingame, 1995). These examples show that minor modifications may affect the specificity of a proton-translocating ATPase.

From the structural evidence and the physiological evidence gained from the intact midgut epithelium of lepidopteran larvae, from Malpighian tubules and from epithelial sensilla, we infer that the K^+ transport at insect epithelia relies on a modified vacuolar ATPase. A V-ATPase with a reduced proton specificity, due to some modification at any structural level, will operate just like the electrogenic cation pump at insect epithelia.

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References

CHAO, A. C., MOFFETT, D. F. AND KOCH, A. (1991). Cytoplasmic pH and goblet cavity pH in the posterior midgut of the tobacco hornworm *Manduca sexta*. *J. exp. Biol.* **155**, 403–414.

CIOFFI, M. AND HARVEY, W. R. (1981). Comparison of potassium transport in three structurally distinct regions of the insect midgut. *J. exp. Biol.* **91**, 103–116.

DIMROTH, P. (1994). Bacterial sodium ion-coupled energetics. *Antonie van Leeuwenhoek* **65**, 381–395.

DOW, J. A. T. (1992). pH gradients in lepidopteran midgut. *J. exp. Biol.* **172**, 355–375.

GOGARTEN, P. G. AND TAIZ, L. (1992). Evolution of proton pumping ATPases: Rooting the tree of life. *Photosynth. Res.* **33**, 137–146.

HARVEY, W. R. (1980). Water and ions in the gut. In *Insect Biology in the Future* (ed. M. Locke and D. Smith), pp. 105–124. New York: Academic Press.

HARVEY, W. R. (1992). Physiology of V-ATPases. *J. exp. Biol.* **172**, 1–17.

HARVEY, W. R., CIOFFI, M., DOW, J. A. T. AND WOLFERSBERGER, M. G. (1983). Potassium ion transport ATPase in insect epithelia. *J. exp. Biol.* **106**, 91–117.

HARVEY, W. R., CIOFFI, M. AND WOLFERSBERGER, M. G. (1981). Portasomes as coupling factors in active ion transport and oxidative phosphorylation. *Am. Zool.* **21**, 775–791.

HARVEY, W. R. AND NEDERGAARD, S. (1964). Sodium independent active transport of potassium in the isolated midgut of the *Cecropia* silkworm. *Proc. natn. Acad. Sci. U.S.A.* **51**, 757–765.

KAKINUMA, Y. AND IGARASHI, K. (1989). Sodium-translocating adenosine triphosphatase in *Streptococcus faecalis*. *J. Bioenerg. Biomembr.* **21**, 679–692.

KAKINUMA, Y. AND IGARASHI, K. (1994). Purification and characterization of the catalytic moiety of vacuolar-type Na^+-ATPase from *Enterococcus hirae*. *J. Biochem., Tokyo* **116**, 1302–1308.

KEIL, T. A. (1984). Very tight contact of tormogene cell membrane and sensillum cuticle: ultrastructural basis for high electrical resistance between receptorlymph and subcuticular spaces in silkmoth olfactory hairs. *Tissue & Cell* **16**, 131–135.

KLEIN, U. (1992). The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: immunological evidence for the occurrence of a V-ATPase in insect ion-transporting epithelia. *J. exp. Biol.* **172**, 345–354.

KLUGE, C. AND DIMROTH, P. (1993). Kinetics of inactivation of the F_1F_o ATPase of *Propionigenium modestum* by dicyclohexylcarbodiimide in relationship to H^+ and Na^+ concentration: probing the binding site for the coupling ions. *Biochemistry, N.Y.* **32**, 10378–10386.

KÜPPERS, J. (1984). Stoffwechselabhängige Impedanzänderungen einer ionentransportierenden Membran. *Verh. dt. zool. Ges.* **77**, 302.

KÜPPERS, J. AND THURM, U. (1979) Active ion transport by a sensory epithelium. I. Transepithelial short circuit current, potential difference and their dependence on metabolism. *J. comp. Physiol.* **134**, 131–136.

KÜPPERS, J. AND THURM, U. (1982). On the functional significance of

ion circulation induced by electrogenic transport. In *Exogenous and Endogenous Influences on Metabolic and Neural Control* (ed. A. D. F. Addink and N. Spronk), pp. 313–327. Oxford, New York: Pergamon Press.

KÜPPERS, J. AND THURM, U. (1994). Ein electrogener, primärer K^+-Transport durch eine V-ATPase. *Verh. dt. zool. Ges.* **87**, 177.

LAUBINGER, W. AND DIMROTH, P. (1989). The sodium ion translocating ATP-synthase of *Propionigenium modestum* pumps protons at low sodium ion concentrations. *Biochemistry, N.Y.* **28**, 7194–7198.

MADDRELL, S. H. P. (1980). The control of water relations in insects. In *Insect Biology in the Future* (ed. M. Locke and D. Smith), pp. 179–199. New York: Academic Press.

MARTIN, F. G. (1992). Circuit analysis of transmembrane voltage relationships in V-ATPase-coupled ion movements. *J. exp. Biol.* **172**, 387–402.

MARTIN, F. G. AND HARVEY, W. R. (1994). Ionic circuit analysis of K^+/H^+ antiport and amino acid/K^+ symport energized by a proton-motive force in *Manduca sexta* larval midgut vesicles. *J. exp. Biol.* **196**, 77–92.

MEAGHER, L., MCLEAN, P. AND FINBOW, M. E. (1990). Sequence of a cDNA from *Drosophila* coding for the 16-kDa proteolipid component of the vacuolar H^+-ATPase. *Nucleic Acids Res.* **18**, 6712.

NELSON, N. (1992). The vacuolar H^+-ATPase – one of the most fundamental ion pumps in nature. *J. exp. Biol.* **172**, 19–27.

NOVAK, F. J. S., GRÄF, R., WARING, R. B., WOLFERSBERGER, M. G., WIECZOREK, H. AND HARVEY, W. R. (1992). Primary structure of V-ATPase subunit B from *Manduca sexta* midgut. *Biochim. biophys. Acta* **1132**, 67–71.

PICHON, Y. (1970). Ionic content of haemolymph in the cockroach *Periplaneta americana. J. exp. Biol.* **53**, 195–209.

PIETRANTONIO, P. V. AND GILL, S. S. (1993). Sequence of a 17 kDa vacuolar H^+-ATPase proteolipid subunit from insect midgut and Malpighian tubules. *Insect Biochem. molec. Biol.* **23**, 675–680.

REIDLINGER, J., MAYER, F. AND MÜLLER, V. (1994). The molecular structure of the Na^+-translocating F_1F_o-ATPase of *Acetobacterium woodii*, as revealed by electron microscopy, resembles that of H^+-translocating ATPases. *FEBS Lett.* **356**, 17–20.

REIDLINGER, J. AND MÜLLER, V. (1994). Purification of ATP synthase from *Acetobacterium woodii* and identification as a Na^+-translocating F_1F_o-type enzyme. *Eur. J. Biochem.* **223**, 275–283.

SCHWEIKL, H., KLEIN, U., SCHINDLBECK, M. AND WIECZOREK, H. (1989). A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. *J. biol. Chem.* **264**, 11136–11142.

THURM, U. (1974). Basics of the generation of receptor potentials in epidermal mechanoreceptors of insects. In *Mechanorezeption* (ed J. Schwartzkoppf). *Abh. Rhein. Westf. Akad. Wiss., Opladen* **53**, 355–385.

THURM, U. AND KÜPPERS, J. (1980). Epithelial physiology of insect sensilla. In *Insect Biology in the Future* (ed. M. Locke and D. Smith), pp. 735–763. New York: Academic Press.

WESSING, A., BERTRAM, G. AND ZIEROLD, K. (1993). Effects of bafilomycin A_1 and amiloride on the apical potassium and proton gradients in *Drosophila* Malpighian tubules studied by X-ray microanalysis and microelectrode measurements. *J. comp. Physiol.* B **163**, 452–462.

WIECZOREK, H. (1992). The insect V-ATPase, a plasma membrane proton pump energizing secondary active transport: molecular analysis of electrogenic potassium transport in the tobacco hornworm midgut. *J. exp. Biol.* **172**, 335–343.

WIECZOREK, H., PUTZENLECHNER, M., ZEISKE, W. AND KLEIN, U. (1991). A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J. biol. Chem.* **266**, 15340–15347.

WIECZOREK, H., WEERTH, S., SCHINDLBECK, M. AND KLEIN, U. (1989). A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. biol. Chem.* **264**, 11143–11148.

WIECZOREK, H., WOLFERSBERGER, M. G., CIOFFI, M. AND HARVEY, W. R. (1986). Cation-stimulated ATPase activity in purified plasma membranes from tobacco hornworm midgut. *Biochim. biophys. Acta* **857**, 271–281.

WOLFERSBERGER, M. G. AND GIANGIACOMO, K. M. (1983). Active potassium transport by the isolated lepidopteran larval midgut: stimulation of net potassium flux and elimination of the slower phase decline of the short-circuit current. *J. exp. Biol.* **102**, 199–210.

ZERAHN, K. (1971). Active transport of the alkali metals by the isolated midgut of *Hyalophora cecropia*. *Phil. Trans. R. Soc. Lond. B* **262**, 315–321.

ZHANG, Y. AND FILLINGAME, R. F. (1995). Changing the ion binding specificity of the *Escherichia coli* H^+-ATP synthase by directed mutagenesis of subunit *c*. *J. biol. Chem.* **270**, 87–93.