Stoichiometry of K^+/H^+ antiport helps to explain extracellular pH 11 in a model epithelium

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Abstract The stoichiometry of K^+/H^+ antiport was measured fluorometrically by the static head method in highly purified vesicles from goblet cell apical membranes of larval lepidopteran midgut. The measured stoichiometry of 1 K^+/2 H^+ explains how the antiport results in electrophoretic exchange of extracellular H^+ for intracellular K^+, driven by the voltage component of the proton-motive force of an H^+ translocating V-ATPase that is located in the same membrane. In turn, the exchange of K^+ for H^+ helps to explain how the midgut contents are alkalinized to a pH of 11.

Key words: K^+/H^+ antiport; V-ATPase; K^+ transport; Alkalinization; *Manduca sexta*

1. Introduction

A remarkable property of lepidopteran insect midgut is the production of a luminal fluid that is the most alkaline in a biological system and can exceed pH 12 in some species [1]. The adaptive role of the high luminal pH is not known, although it may represent an evolutionary response to tannins that tend to reduce the digestibility of plant foods [2] by cross-linking proteins [3]. The mechanism by which the gut lumen is alkalinized is also unknown. It is clear that the overall mechanism of alkalinization has to involve the exchange of H^+ for cations such as K^+ or Na^+, or the exchange of anions such as Cl^- for anions such as OH^- or CO_3^{2-}. Obviously, the mechanisms that generate weakly alkaline fluids in vertebrates can not account for this strong alkalinization since they are based on HCO_3^- transport and thus could produce pH values no higher than 9.

Insect gastrointestinal and sensory epithelial possess a unique alkali metal ion transport system that resides in the apical plasma membrane and actively pumps ions out of cells [4,5]. In the larval midgut of the tobacco hornworm, *Manduca sexta*, it is situated in the apical membrane of goblet cells [4]. It transports K^+ electrogenically into the gut lumen, thereby creating a transmembrane voltage in excess of 250 mV, lumen positive [6]. One of the most appealing hypotheses of insect gut alkalinization was proposed by Dow [1], who suggested that alkalinization is, in part, due to H^+ distribution according to the voltage produced by the electrogenic K^+ transport. This thermodynamic explanation was supported by kinetic evidence linking alkalinization with electrogenic K^+ transport [7,8]; however, despite the attractiveness of the hypothesis, the intrinsic mechanism of alkalinization remained an enigma.

In recent years, the molecular architecture of the K^+ transport system in the *Manduca sexta* midgut has been deciphered; it was shown to consist of an electrogenic H^+ translocating V-ATPase energizing, by the voltage component of its proton motive force, electrophoretic K^+/nH^+-antiport [9–12]. Here we report experiments intended to deduce the stoichiometry of K^+/H^+ antiport. Using a vesicle preparation of highly purified goblet cell apical membranes from larval *Manduca sexta* midgut [13], we measured K^+-dependent vesicle acidification and alkalinization by the static head technique [14]. Based upon the resulting antiport stoichiometry ratio of K^+/2 H^+, we propose a broadly applicable, minimal model for strong extracellular alkalinization.

2. Materials and methods

2.1. Experimental insects, membrane purification, chemical reagents

Fifth instar larvae of *Manduca sexta* (Lepidoptera, Sphingidae), weighing 7–9 g, were reared under long-day conditions (16 h of light) at 27°C using a synthetic diet modified according to Bell and Joachim [15]. The preparation of vesicles from the posterior midgut epithelium followed published protocols [13,16]. Protein concentration was determined with Amido black [16]. All reagents were of analytical grade. Acridine orange, D-gluconolactone, TMA-chloride, TMA-hydroxide and Tris-ATP were purchased from Sigma. Gramicidin A was purchased from Fluka, and oxonol V from Molecular Probes.

2.2. ATP-dependent proton transport and K^+/H^+ antiport

Vesicle acidification and membrane potential generation were measured fluorometrically [11,12] using Acridine orange or oxonol V at excitation wavelengths of 493 and 620 nm, respectively, and at emission wavelengths of 530 and 645 nm, respectively. Fluorescence changes were monitored at an ambient temperature of 23–26°C by a Kontron SFM 23/B spectrofluorometer equipped with a MacLab (ADInstruments Ltd., UK). For ATP-dependent proton transport, partially purified goblet cell apical membranes were used [10]. Vesicles were resuspended in 10 mM Tris-MOPS (pH 7.0) containing either 30 mM TMA-chloride, 150 mM TMA-chloride or 150 mM TMA-gluconate. Assays in a final volume of 800 µl had a pH of 7.0 and consisted of 10 mM Tris-MOPS, 1 mM $MgCl_2$, 1 mM Tris-ATP, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, 0.5 mM sodium azide, 0.1 mM sodium orthovanadate, and either 30 mM TMA-chloride, 150 mM TMA-chloride or 150 mM TMA-gluconate. Assays measuring vesicle acidification contained 0.9 µM Acridine orange and had a protein content of 10–30 µg/ml. Assays measuring membrane potential generation contained 4 µM oxonol V and had a protein content of 50–60 µg/ml. For K^+/H^+ antiport, highly purified goblet cell apical membranes were used [12,13]. After incubation for at least 3 h at 4°C in a solution consisting of 10 mM Tris-MOPS (pH 6.5) and either 150 mM TMA-chloride or 150 mM KCl, 10 µl of vesicle suspension (approximately 10 µg of protein) was

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Abbreviations: MOPS, 3-morpholinopropanesulfonic acid; TMA, tetramethylammonium.

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added to 790 μl of a K^+-free mixture consisting of 10 mM Tris-MOPS (pH 7.0), 150 mM TMA-chloride, and 0.9 μM Acridine orange.

3. Results and discussion

3.1. Clamping of the membrane potential

The use of the static head method to determine the stoichiometry of K^+/H^+ antiport required the elimination of the voltage term in the free enthalpy equation for mK^+/nH^+ antiport,

$$\Delta G = m \cdot F \cdot \psi - R \cdot T \cdot m \cdot \ln\left[\frac{K^+_{in}}{K^+_{out}}\right] - n \cdot F \cdot \psi + R \cdot T \cdot n \cdot \ln\left[\frac{H^+_{in}}{H^+_{out}}\right]$$

to obtain defined static head conditions for $\Delta G = 0$, i.e.

$$m \cdot \ln\left[\frac{K^+_{in}}{K^+_{out}}\right] = n \cdot \ln\left[\frac{H^+_{in}}{H^+_{out}}\right]$$

Because valinomycin obviously could not be used for short circuiting of the membrane potential we took advantage of the anion channels in the goblet cell apical membrane which had been shown to be open at high Cl^- concentrations [11,17]. Evidence for the presence of anion channels is provided by Figs. 1 and 2: ATP-dependent vesicle acidification increased with increasing Cl^- concentration, and was much stronger in the presence of 150 mM Cl^- than in the presence of 150 mM gluconate which is believed not to cross anion channels (Fig. 1). Concordantly, a transmembrane voltage, produced by ATP-dependent proton transport in the presence of 150 mM gluconate, could not be established in the presence of 150 mM Cl^- (Fig. 2). Thus, in the presence of 150 mM Cl^-, the vesicle membrane was clamped at zero voltage.

3.2. Analysis of stoichiometry

For the measurement of K^+/H^+ antiport, contamination with inner mitochondrial membranes had to be excluded because these membranes contain a K^+/H^+ exchange mechanism (A. Lepier and H. Wieczorek, unpublished results). Therefore we used highly purified goblet cell apical membranes since they are essentially free of mitochondrial impurities [9,13]. Fig. 3 outlines the experimental design. Three groups of experimental vesicles were loaded with 150 mM KCl in pH 6.5 buffer, whereas control vesicles were loaded with 150 mM TMA-Cl in pH 6.5 buffer; thus in every case a pH gradient of 0.5 was established across the membrane when vesicles were added to a solution consisting of a pH 7.0 buffer. Assuming three different stoichiometric ratios (i) $K^+/3\ H^+$, (ii) $K^+/2\ H^+$, (iii) $2\ K^+/3\ H^+$, we calculated the $[K^+]_{out}$ necessary to reach the static head condition in which the driving forces of K^+ and of H^+ were balanced, preventing any net K^+/H^+ antiport.

Vesicles loaded with 150 mM KCl were acidified due to K^+/H^+ antiport (Fig. 4). After reaching a steady state (usually after 90 s), precise amounts of K^+ were added to the reaction mixtures. The addition of sufficient K^+ for a stoichiometry of $K^+/3\ H^+$ led to less than 50% dissipation of the fluorescence quench (mean ± S.D. = 48.6 ± 14.9; $n = 4$). This result indicated that the concentration of extravesicular K^+ was not high enough to meet static head conditions. The addition of sufficient K^+ for a stoichiometry of $K^+/2\ H^+$ led to 86% dissipation of the fluorescence quench (mean ± S.D. = 86.2 ± 2.4; $n = 4$); the remaining final dissipation of the fluorescence quench (14%) by the use of NH_4Cl was similar in size to that of control vesicles in which fluorescence quench was due only to the imposed pH gradient, but not to K^+/H^+ antiport. This result

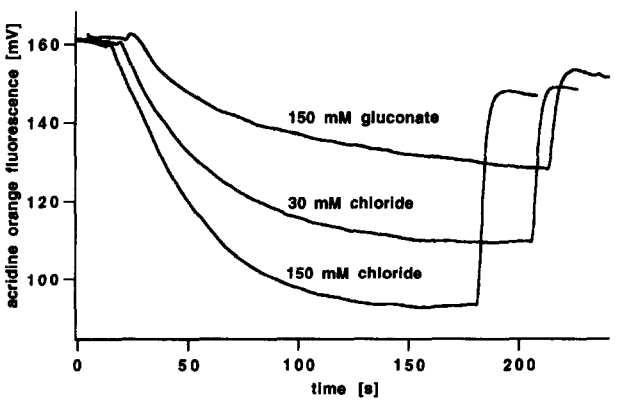

Fig. 1. Influence of anions on vesicle acidification as determined by the fluorescence quenching of Acridine orange. Assays of ATP-dependent proton transport in the presence of either 30 mM or 150 mM TMA-chloride or of 150 mM TMA-gluconate were started with $MgCl_2$. pH gradients were dissipated by the addition of 10 μl NH_4Cl (final concentration, 20 mM).

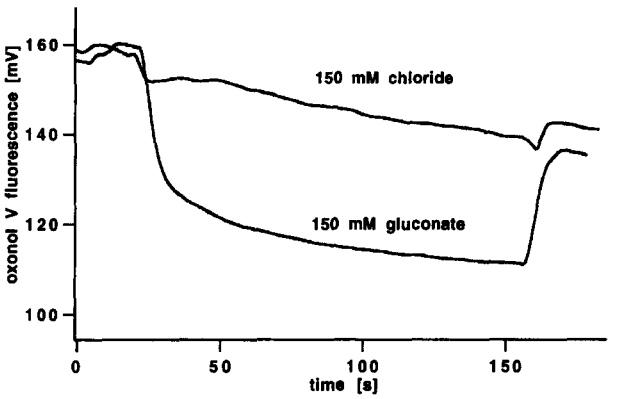

Fig. 2. Influence of anions on voltage generation across the vesicle membrane as determined by the fluorescence quenching of oxonol V. Assays of ATP-dependent proton transport in the presence of either 150 mM TMA-chloride or 150 mM TMA-gluconate were started with ATP. The membrane potential was dissipated by the addition of 10 μl gramicidin A dissolved in ethanol (final concentration, 5 μg/ml).

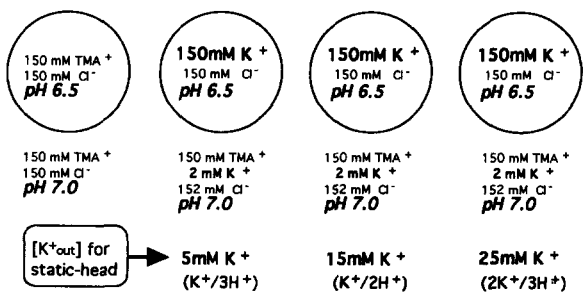

Fig. 3. Outline of the experimental design for analysis of K^+/H^+ antiport stoichiometry by the static head method. For explanation see text.

means that dissipation of the fluorescence quench by K^+ under $K^+/2\ H^+$ conditions most closely met static head conditions. The addition of sufficient K^+ for a stoichiometry of 2 $K^+/3\ H^+$ led to 94% dissipation of the fluorescence quench (mean ± S.D. = 94.0 ± 4.4; $n = 4$) which was clearly beyond the static head balance point. The interpretation of Acridine orange fluorescence as a measure of acidification and alkalinization in vesicles may be complicated by non-specific binding and quenching. However, in our analysis the final conditions after adding K^+ were otherwise identical to the control conditions except for the presence of intra- and extravesicular K^+. It is well known that Acridine orange fluorescence is not influenced by K^+. Since the static head point had already been reached under $K^+/2\ H^+$ conditions, we conclude that the transport stoichiometry of potassium/proton antiport in the goblet cell apical membrane is 1 $K^+/2\ H^+$, provided that coupling is 100%. That both processes are tightly coupled can be deduced from the following two arguments. First, ATP-dependent acidification of vesicles is possible only in the absence of extravesicular K^+; in the presence of extravesicular K^+, the development of a pH gradient is prevented by immediate K^+/H^+ exchange [11,12]. Second, the ion secreted across the epithelium of the isolated intact midgut is K^+ rather than H^+ [1,5].

3.3. Implications for cellular pH homeostasis

The stoichiometry of $K^+/2\ H^+$ antiport, together with the tight coupling of primary electrogenic H^+ transport and secondary electrophoretic antiport, necessarily leads to the withdrawal of H^+ from the bulk of the midgut lumen and its replacement there by secreted K^+ (Fig. 5). This exchange of cellular K^+ for H^+ would tend to acidify the cytoplasm and may thus maintain a constant intracellular pH in spite of an alkaline extracellular pH, provided that the antiport stoichiometry, as measured by us at neutral pH, is unchanged in the alkaline environment. This situation is reminiscent of that in alkaliphilic bacteria, where stoichiometrically equivalent electrophoretic $Na^+/2\ H^+$ antiport sustains a constant intracellular pH in an alkaline environment [18].

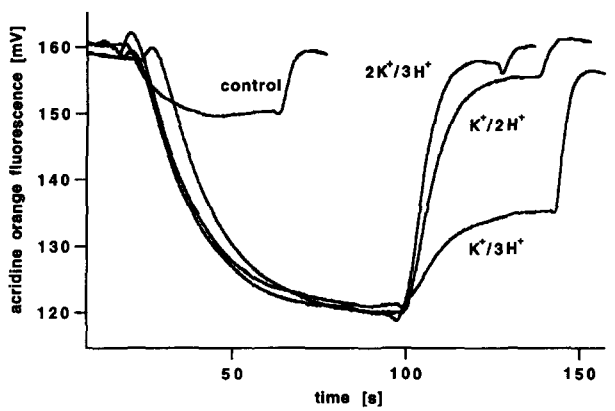

Fig. 4. Static head analysis of K^+/H^+ antiport stoichiometry based on the fluorescence quenching of Acridine orange. Assays of ATP-independent K^+/H^+ antiport were started by the addition of vesicles. Partial dissipation of the pH gradients was achieved by the addition of 5–15 μl KCl (final extravesicular concentrations see Fig. 3). Total dissipation of the partly collapsed pH gradients, as well as dissipation of the pH gradient in the control experiment, was achieved by the addition of 10 μl NH_4Cl (final concentration, 20 mM). For further explanations see Fig. 3 and text.

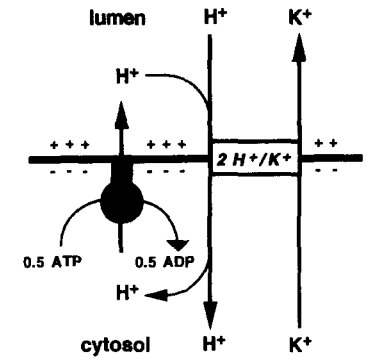

Fig. 5. Alkalinization driven by an electrogenic H^+ pumping V-ATPase. Electrogenic H^+ pumping V-ATPase and electrophoretic $K^+/2\ H^+$ antiport are the minimal elements required for extracellular alkalinization in the tobacco hornworm midgut. The H^+ V-ATPase couples the hydrolysis of 1 ATP to movement of 2 H^+ from cell to lumen. Driven by the voltage, the separate $K^+/2\ H^+$ antiporter exchanges luminal H^+ for cellular K^+, leading to luminal alkalinization. The H^+/ATP stoichiometry of the V-ATPase has not been measured directly so far, but the transmembrane voltage of more than 250 mV produced by the H^+ pump limits the value to not more than 2; this is in line with stoichiometry values determined for other V-ATPases [22,23]. The stoichiometry of the overall K^+ transport in the lepidopteran midgut has been investigated by comparison of rates of active K^+ transport with the P/O ratio calculated from oxygen consumption and estimated to be 2 K^+ transported per ATP hydrolyzed [24]. In the living midgut the free energy of ATP hydrolysis is sufficient to maintain a lumen pH of 11 with CO_3^{2-} as the gegenion [25].

3.4. Implications for extracellular alkalinization

The acidification argument can be reversed: due to the limited volume of the midgut lumen, the activity of voltage driven $K^+/2\ H^+$ antiport not only contributes to intracellular pH homeostasis, but may even be responsible for strong extracellular alkalinization (Fig. 5). Energetically, this alkalinizing mechanism can produce pH values of up to 11 while maintaining an intracellular pH of about 8.5 (assuming an extracellular K^+ activity of 0.06 M [19], an intracellular K^+ activity of 0.1 M [20], and a transmembrane voltage of 270 mV [6]); this situation is similar to that in alkaliphilic bacteria which maintain an intracellular pH of 8.5 facing an extracellular environment of pH 11 [18].

The model depicted in Fig. 5 shows the necessary and sufficient alkalinizing mechanism on the basis of identified elements. It is in accordance with kinetic evidence linking alkalinization with electrogenic K^+ transport [7,8]. Other ion transporting mechanisms, such as K^+ uniport, K^+-ATPase or Na^+/K^+-ATPase, which could also be involved in the alkalinization of the midgut, do not occur in the goblet cell apical membranes [9–12]; however, we can not exclude the possibility that further, so far unidentified, processes are also involved in midgut alkalinization. Taken together, our results support a new, broadly applicable mechanism for biological alkalinization. Since H^+ translocating electrogenic V-ATPases are widely distributed in endomembranes and plasma membranes (see refs. in [21]) this new concept may have broad implications for cell biology and related disciplines.

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