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Ammonia uptake in *Manduca sexta* midgut is mediated by an amiloride sensitive cation/proton exchanger: Transport studies and mRNA expression analysis of NHE7, 9, NHE8, and V-ATPase (subunit D)

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ABSTRACT

In this study participation of cation/proton exchangers (NHE) in ammonia uptake in the medial midgut of *Manduca sexta* larvae was investigated employing a modified Ussing chamber. There was a mean inward net ammonia ($NH_3 + NH_4^+$) flux of 194 ± 17 nmol cm^{-2} h^{-1} across the isolated epithelium under conditions of 0.1 mmol L^{-1} ammonia on both sides of the tissue and a 100-fold inwardly directed P_{NH3}-gradient (pH 8.5 luminal side, pH 6.5 hemolymphal side). Employing a 100-fold NH_4^+ gradient amiloride applied to the luminal side inhibited the influx in a dose-dependent manner, with a maximal inhibition of 75% at 20 mmol L^{-1} and an estimated $IC_{50} = 2$ mmol L^{-1}. Amiloride also caused a dose-dependent but smaller decrease in the short-circuit current (I_{sc}). No inhibition by apical or basal applied amiloride was noticed on cellular metabolic ammonia release, of which ca. 1/3 and 2/3 was secreted towards the apical and basal side, respectively. Using molecular methods full and partial sequence information of two putative cation/proton exchangers (MsNHE8, MsNHE7, 9) were obtained, both containing the characteristic amiloride binding motif. An mRNA expression analysis revealed ubiquitous expression patterns for both proteins, with similar expression levels for NHE8 in all tissues investigated and lower mRNA abundances for MsNHE7, 9 in the midgut sections of the caterpillar. In contrast, in this tissue high expression levels of the V-ATPase (D subunit) were detected, likely the sole pump responsible for energizing goblet cell K^+ excretion, but also involved in columnar cell ammonia uptake.

1. Introduction

Members of the cation/proton exchanger family (NHE, solute carrier family 9, SLC 9) are integral membrane proteins facilitating a monovalent cation/proton antiport and thereby playing an important role in diverse physiological processes. By moving predominately Na^+ or K^+ (Li^+ or NH_4^+ at slower velocities) in exchange for H^+, they are involved in the regulation of cellular and organellar pH in addition to the transport of salt and water across various epithelia and the restoration of the cell volume (Orlowski and Grinstein, 2004; Zachos et al., 2005).

NHE1 is regarded as the prototypical mammalian NHE with its structure being the best-understood of all members of the NHE family (Slepkov et al., 2005; Slepkov et al., 2007; Wakabayashi et al., 2000). The N-terminal membrane domain of NHE1 was shown to be necessary and sufficient for ion translocation (Murtazina et al., 2001; Touret et al., 2001; Wakabayashi et al., 1992). The C-terminus, on the other hand, is involved in regulatory functions, as indicated by numerous phosphorylation sites for different protein kinases and other sites responsible for interaction with accessory proteins. (Orlowski and Grinstein, 2004; Slepkov et al., 2007). The fourth transmembrane domain (TM4) is part of the ion conduction pathway (Slepkov et al., 2005) and also contains the amiloride binding motif "$F_1 \cdot F_2 \cdot X_3 \cdot X_4 \cdot X_5 \cdot L_6 \cdot P_7 \cdot P_8 \cdot I_9$", a pocket of nine amino acid residues which is well conserved among all members of the NHE family (Counillon et al., 1993; Counillon et al., 1997).

In insects five NHE isoforms (NHE3, NHE7, 9, NHE8, NHE9 and NHE10) have been identified so far (Pullikuth et al., 2003). Employing Amiloride as an inhibitor, it was suggested that NHE proteins participate in fluid secretion by the Malpighian tubules of the fruit fly *Drosophila melanogaster* (Dow et al., 1994; Giannakou and Dow, 2001). Moreover, in studies on the yellow fever mosquito *Aedes aegypti* NHE3 and NHE8 (AeNHE3, AeNHE8) were characterized in terms of cellular localization and function (Piermarini et al., 2009; Pullikuth et al., 2006). AeNHE3 is predominantly expressed in the basolateral plasma membrane of the Malpighian tubules, the midgut

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and the ion transporting sector of gastric caecae (Pullikuth et al., 2006). For these tissues, it has been suggested that AeNHE3 is involved in transepithelial ion and fluid transport by moving Na^+ ions and K^+ ions in exchange for protons. Similar to mammalian NHE3, AeNHE3 was shown to be rather insensitive to amiloride and its derivate 5-*N*-ethyl-*N*-isopropyl amiloride (EIPA).

A thorough investigation of AeNHE8 by Piermarini et al. (2009) revealed that AeNHE8 is expressed primarily as an intracellular protein, which was confirmed by immunohistochemical localizations in Malpighian tubules (MT). Here it localizes to a subapical compartment (e.g., vesicles or endosomes), but not in the apical brush border. The lack of membrane insertion of that transporter after feeding suggested that AeNHE8 does not play a key role in the extrusion of excess Na^+ and K^+ after a blood meal to maintain a steady intracellular pH by moving Na^+ or K^+ in exchange for H^+. However, when expressed in *Xenopus laevis* oocytes, AeNHE8 mediates EIPA-sensitive Na^+/H^+ exchange, with clear preferences of Na^+ over Li^+ and K^+. The insect NHE isoforms NHE9 and NHE10 are only tentatively assigned to the NHE family (Pullikuth et al., 2003). They share only ca. 10% identity in amino acid sequence to the other insect and mammalian NHEs and exhibit no known amiloride binding pocket. The transport properties of both putative transporters are unknown to this date.

Cation/proton exchangers have also been suggested to play an important role in the digestive tract of the tobacco hornworm *Manduca sexta*. The intestine of *M. sexta* is a well characterized model system for an epithelium where nutrition uptake and water and ion homeostasis are not energized by a Na^+/K^+-ATPase but by a transepithelial inwardly directed K^+-gradient (Harvey et al., 1983; Jungreis and Vaughan, 1977; Weihrauch, 2006). The K^+-gradient is established by a V-type H^+-ATPase (V-ATPase), which energizes an electrogenic, amiloride sensitive $K^+/2H^+$ exchanger, both localized in the apical membrane of highly specialized cells, the goblet cells (Azuma et al., 1995; Lepier et al., 1994; Wieczorek et al., 1991). This K^+-gradient is used to enable nutrition uptake into the neighboring columnar cells by driving K^+-coupled transporters such as K^+/amino acid symporters (Hennigan et al., 1993; Xie et al., 1994).

Weihrauch (2006) showed that the midgut of *M. sexta* also exhibits an active ammonia uptake likely to maintain a steady state ammonia concentration in the hemolymph and to balance the low proteinous nitrogen intake from a herbal diet. Site of this ammonia uptake was suggested to be in the columnar cells since the entire luminal compartment of the *M. sexta* midgut is lined with microvilli from columnar cell brush border membranes which are also covering small “valve-like” apical openings of the neighboring goblet cells (Baldwin and Hakim, 1991). It was assumed that the active ammonia uptake is rather independent of goblet cell potassium excretion, represented by a massive positive short circuit current (I_{sc}) (Cioffi and Harvey, 1981; Harvey and Wolfersberger, 1979; Wood and Moreton, 1978). This assumption is supported by experiments employing the microtubule inhibitor colchicine, showing no effect on the I_{sc} but a substantial inhibition (ca. 50% of controls) of the active ammonia uptake (Weihrauch, 2006).

Earlier studies employing the non-specific NHE inhibitor amiloride suggested the existence of amiloride-sensitive NHE-like proteins to be present in the columnar cells of the medial midgut of the caterpillar, here being involved in active ammonia absorption (*note:* in this study, NH_3 refers to molecular ammonia, NH_4^+ to ammonium ions and ammonia to the sum of both) (Weihrauch, 2006).

Employing the medial midgut of *M. sexta*, in this study the effect of amiloride on the transepithelial ammonia flux and the K^+-carried short circuit current (I_{sc}) was further investigated.

Also, for the first time in a lepidopteran larva, tissue distribution and mRNA expression levels of two putatively amiloride sensitive NHE isoforms, namely MsNHE7, 9 and MsNHE8, as well as tissue expression levels of the V-Type H^+-ATPase, the dominant gut energizing cation pump, were demonstrated.

2. Materials and methods

2.1. Insects and tissue mounting

Larvae of *Manduca sexta* Linné 1763 (Lepidoptera, Sphingidae) were reared under long-day conditions (16 h of light) at 27 °C as described previously (Baldwin and Hakim, 1991) using a synthetic diet (Gypsy Moth Diet; MP Biomedicals, Solon, OH, USA). Larvae were used in the fifth instar of rapid growth with a live weight of 4–6 g (18–19 days after hatching).

For flux studies and electrical measurements, the medial midgut was dissected from cold-anaesthetized animals and adhering Malpighian tubules, tracheae and fatbody were carefully removed. The medial midgut was then opened along its length and the peritrophic membrane and luminal contents were removed. The midgut tissue was mounted as a flat sheet in a modified Ussing chamber with an aperture of 0.45 cm^2.

2.2. Electrical measurements

The transepithelial short circuit current (I_{sc}, $\mu A/cm^2$) is assumed to correspond mainly to the net secretion of K^+ ions (Cioffi and Harvey, 1981; Harvey and Wolfersberger, 1979; Wood and Moreton, 1978) and was measured in this study using an automatic voltage clamp apparatus (VCC-MC6, Physiologic Instruments, San Diego, CA) as described previously (Weihrauch, 2006). All experiments were conducted under short-circuited current conditions. The integrity of the preparation was controlled by monitoring the I_{sc} throughout the experiment. Only tissues exhibiting an initial $I_{sc} \geq 167\ \mu A/cm^2$ after equilibration (20–30 min after mounting) were employed. The dose-dependent inhibition of the I_{sc} is represented as percent of control (initial I_{sc}). Over a time period of 2 h the I_{sc} dropped in control experiments nearly linearly by $42.4 \pm 2.3\%$ ($n = 5$). All experiments employing inhibitors were corrected for the time-dependent decrease of I_{sc} measured under control conditions.

2.3. Ammonia flux measurements

2.3.1. Transport experiments employing a P_{NH3} gradient

In this experimental series both sides of the epithelium were exposed to solutions containing equal amounts of total ammonia (mmol L^{-1}): 30, KCl; 2.5, $KHCO_3$; 5, $CaCl_2$; 5, $MgCl_2$; 0.1 NH_4Cl, 5, Tris and 166, sucrose. The pH was adjusted by HCl to pH 8.5 (apical bath) and pH 6.5 (basal bath), respectively. Under these conditions a 100-fold inwardly directed partial pressure gradient for NH_3 (P_{NH3}) was established. The bathing solutions were stirred and aerated by airflow.

When a constant I_{sc} was established (within approximately 20–30 min after mounting) solutions on both sides of the epithelium were replaced, followed by a sampling period of 30 min. At the end of the sample period, two samples of 1.8 ml were taken from both sides of the epithelium (original chamber volume = 4 ml). If fluxes were measured under the effect of an inhibitor, the sample period started after full impact of the inhibitor indicated by a new constant I_{sc} (see also Fig. 2). All net fluxes were corrected for basal metabolic ammonia release, calculated as the difference of total ammonia added to and subtracted from the basal and apical solution at the end of the experiment, respectively.

2.3.2. Ammonia release experiments

In order to quantify the amount of ammonia originating from intestinal amino acid metabolism no NH_4^+ was added to the solutions (pH 7.4) on either side of the epithelium and the experimental period was prolonged to 60 min. If fluxes were measured under the effect