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Active ammonia excretion across the gills of the green shore crab *Carcinus maenas*: participation of Na^+/K^+-ATPase, V-type H^+-ATPase and functional microtubules

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Summary

Although aquatic animals are generally believed to export nitrogenous waste by diffusion of NH_3 or NH_4^+ across external epithelia, evidence for active ammonia excretion has been found in a number of species. In the euryhaline green shore crab *Carcinus maenas*, active excretion of ammonia across isolated gills is reduced by inhibitors of the Na^+/K^+-ATPase and vacuolar-type H^+-ATPase. In addition, a functional dynamic microtubule network is necessary, since application of colchicine, taxol or thiabendazole leads to almost complete blockage of active and gradient-driven ammonia excretion. Actin filaments seem not to play a role in the excretory process. The NH_4^+-dependent short-circuit current and the conductance of the isolated cuticle were reduced in a dose-dependent manner by amiloride, a non-specific inhibitor of the Na^+/H^+ exchanger and Na^+ channels. Combined with an analysis of gill morphology, the strong intracellular but weak apical abundance of V-type H^+-ATPase and the fact that ammonia flux rates are equal under buffered and unbuffered experimental conditions, our observations suggest a hypothetical model of transepithelial ammonia movement that features active uptake across the basolateral membrane, sequestration in acidified vesicles, vesicle transport *via* microtubules and exocytosis at the apical membrane.

Key words: Ammonia excretion, amiloride, crab, *Carcinus maenas*, colchicine, cuticle, microtubule, V-type H^+-ATPase.

Introduction

Non-ionic ammonia (NH_3) and, to a lesser degree, ammonium ion (NH_4^+) are toxic in most animals. Elevated ammonia levels in low-salinity media disrupt ionoregulatory function in the lobster *Homarus americanus* (Young-Lai et al., 1991) and the crayfish *Pacifastacus leniusculus* (Harris et al., 2001). Exposure of the green shore crab *Carcinus maenas* to 1 mmol l^{-1} total ammonia leads to increased ion permeability and salt flux across the gill; higher concentrations reduce both variables (Spaargaren, 1990). In fish, branchial gas exchange and oxidative metabolism are disturbed by excess ammonia (Wilkie, 1997). In mammals, elevated ammonia levels cause mucosal cell damage (Lin and Visek, 1991) and inhibit cyclic-AMP-regulated Cl^- transport across the colon (Prasad et al., 1995). Hydrated ammonium and potassium ions have the same ionic radius and, because of their K^+-like behavior, ammonium ions affect the membrane potential and excitability of neurons (Cooper and Plum, 1987). An effective ammonia detoxification or excretion system is therefore essential to maintain cellular functions.

Cellular uptake or excretion of non-ionic NH_3 is generally thought to occur by diffusion across the lipid bilayer of cellular membranes, although permeability to NH_3 is much lower than to CO_2 (Knepper et al., 1989). Indeed, some plasma membranes of animal cells are relatively impermeable to NH_3 (Burckhardt and Frömter, 1992; Garvin et al., 1987). The entry of charged ammonium ions into animal cells may be mediated by several transport proteins: NH_4^+-permeable K^+ channels (Latorre and Miller, 1983), the $Na^+/K^+/2Cl^-$ cotransporter (Kinne et al., 1986), the Na^+/H^+ exchanger (Kinsella and Aronson, 1981) or a recently described ammonium transporter related to the rhesus protein (Marini et al., 2000). In addition, abundant evidence suggests that NH_4^+ may be transported actively, utilizing the ubiquitous Na^+/K^+-dependent ATPase (Skou, 1960; Towle and Hølleland, 1987; Wall, 1997).

Cellular excretion of NH_4^+ may be mediated by an apical Na^+/NH_4^+ exchanger, as suggested for mammalian renal proximal tubules (Hamm and Simon, 1990) and the gills of marine teleosts (Randall et al., 1999). In freshwater teleost gills (Wilson et al., 1994) and mammalian inner medullary collecting ducts (Knepper et al., 1989), acidification exterior to the outer apical membrane may induce passive diffusion of non-ionic ammonia by diffusion trapping. Experiments with intact blue crabs (*Callinectes sapidus*) suggest that ammonia is excreted across the gills by diffusion of non-ionic NH_3 in

animals acclimated to sea water (Kormanik and Cameron, 1981) but by Na^+/NH_4^+ exchange in animals acclimated to low salinities (Pressley et al., 1981). Very little ammonia is excreted in the urine in this species (Cameron and Batterton, 1978).

The mechanism by which ammonia crosses the epithelial layer of the excreting tissue is not generally considered, it being assumed that NH_3 and NH_4^+ diffuse through the cytoplasm in a free state. However, under such conditions, the toxic effects of ammonia could be felt by multiple intracellular targets. In this study, we present the experimental basis for a novel mechanism of active ammonia excretion across a moderately tight epithelium, the gills of the euryhaline shore crab *Carcinus maenas*. We have previously demonstrated that isolated perfused gills from this animal are capable of transporting ammonia against a concentration gradient under physiologically meaningful conditions (Weihrauch et al., 1998, 1999). Active ammonia excretion across these gills was inhibited by apical amiloride, basolateral Cs^+ or basolateral ouabain, implicating the participation of an apical Na^+/NH_4^+ exchanger, basolateral NH_4^+-permeable K^+ channels and basolateral Na^+/NH_4^+-dependent ATPase, respectively. We show here that an intracellular V-type H^+-ATPase and an intact microtubule system are also required for active excretion of ammonia in this system, leading to an experimental model that includes acidification and ammonia-loading of intracellular vesicles, transport of vesicles along microtubules and exocytosis. Ultrastructural and molecular evidence support the model.

Materials and methods

Animals

Shore crabs *Carcinus maenas* L. were obtained from a fisherman in Kiel Bay, Germany, or were collected from the intertidal region of the Mount Desert Island Biological Laboratory in Salsbury Cove, Maine, USA. They were maintained at 15°C in recirculating biologically filtered aquaria containing diluted seawater of 10‰ salinity and were fed cleaned squid or beef heart twice weekly.

Measurement of active ammonia flux across perfused gills

Gills were prepared and perfused according to previously described methods (Siebers et al., 1985; Weihrauch et al., 1998) at a flow rate of 0.135 ml min^{-1}. Transepithelial potential differences (PD_{te}) were monitored using a millivolt meter (Keithley, type 197) to evaluate the quality of the preparation. Only gills generating an initial and continuously negative PD_{te} (–7.1±1.0 mV, N=32; mean ± S.E.M.) were employed.

The morphology of the phyllobranchiate gills provides sufficient surface area, roughly 247 cm^2 g^{-1} fresh gill mass (Riestenpatt, 1995), to measure transepithelial ammonia fluxes directly. Excretion rates and ammonia concentrations were measured according to methods described in detail in an earlier publication (Weihrauch et al., 1998). Briefly, unless mentioned otherwise, gills were perfused and bathed in saline ($S_{standard}$) initially containing symmetrical amounts of ammonia (100 μmol l^{-1} NH_4^+) at identical pH values. Consequently, all measured net fluxes must be based on active transport mechanisms. The external medium was stirred constantly during each treatment. The concentration of ammonia selected was within the range of hemolymph ammonia levels measured in *C. maenas* (Durand and Regnault, 1998; Weihrauch et al., 1999). When a constant PD_{te} had been established (within approximately 30 min), the external bath and the perfusion solution were replaced and measurements were made for 30 min (controls). To continue the experiment with the same gill, the procedure was repeated with modified salines. Following application of a modified saline for 30 min, fluxes of total ammonia over the following 30 min were measured again. 2 ml samples were taken from the bath and internal perfusate after each experimental step.

Total ammonia concentrations (T_{Amm}) were determined with a gas-sensitive NH_3 electrode (Ingold, type 152303000). The sensitivity of the electrode measurements was approximately ±1.5 μmol l^{-1} in the T_{Amm} concentration range 50–100 μmol l^{-1}. To calculate net excretion, only the ammonia removed from the internal perfusion saline was considered to avoid including metabolically produced ammonia (Weihrauch et al., 1998). An alternative approach would be to measure the appearance of total ammonia in the external bath. However, as we show below, such a measurement would lead to an overestimation of transport rates as a result of ammonia production by the gill itself.

Measurement of gill resistance and calculation of transepithelial conductance

After removing the gills, single gill lamellae were isolated and split into their two halves (Schwarz and Graszynski, 1989). In this way, a single epithelial layer covered by an apical cuticle was obtained. Isolated cuticle was prepared by carefully removing the epithelial cells using a smooth rounded metal wire. Split gill lamellae or isolated cuticle were mounted in a modified Ussing chamber, allowing area (0.02 cm^2)-specific short-circuit currents (I_{sc}) and transepithelial resistances (R_{te}) to be measured. The chamber compartments were continuously superfused with saline at a rate of 0.5 ml min^{-1} by means of a peristaltic pump.

To measure PD_{te}, Ag/AgCl electrodes were connected *via* agar bridges (3% agar in 3 mmol l^{-1} KCl) to the chamber compartments; the separation distance of the preparation was less than 0.1 cm. A second pair of Ag/AgCl electrodes, connected through agar bridges, served as current electrodes to short-circuit the PD_{te} with an automatic clamping device (VCC 600, Physiologic Instruments, San Diego, USA). I_{sc} and R_{te} were calculated according to Riestenpatt et al. (1996). The transepithelial and transcuticular conductances were calculated as $G_{te}=1/R_{te}$ and $G_{cut}=1/R_{cut}$, respectively, where R_{cut} is the transcuticular resistance.

To assess the effects of NH_4Cl solutions on cuticular I_{sc} and R_{cut}, the isolated cuticle was superfused (0.5 ml min^{-1}) on both sides with a non-physiological ammonia-containing saline

(S_{NH_4Cl}) consisting of 248 mmol l^{-1} NH_4Cl and 2.5 mmol l^{-1} Tris adjusted to pH 7.8 with HCl. A clamp voltage of 10 mV with reference to the apical side was maintained to force transcuticular ion fluxes. Resistances for $S_{standard}$ and S_{NH_4Cl} were 9.0±0.1 Ω cm^2 (N=6) and 7.1±0.4 Ω cm^{-2} (N=5) (means ± S.E.M.), respectively.

Statistical analyses

All data presented in this study were corrected by the resistances of the salines employed. All results are presented as means ± S.E.M. Differences between groups were tested using one-way analysis of variance (ANOVA) and the Newman–Keuls multiple-comparison test. Statistical significance was assumed for P<0.05.

Salines and chemicals

The ionic composition of the salines ($S_{standard}$) used to bathe and perfuse isolated gills and to measure transepithelial resistance/conductance in gill half-lamellae contained (mmol l^{-1}) 248 NaCl, 5 $CaCl_2$, 5 KCl, 4 $MgCl_2$, 2 $NaHCO_3$, 2.5 Tris and 0.1 NH_4Cl. Immediately before use, 2 mmol l^{-1} glucose was added to the basolateral salines in all experiments, and the pH of all salines was adjusted to 7.8 (HCl). Amiloride, bafilomycin A_1, colchicine, cytochalasin D, ouabain and taxol were purchased from Sigma (St Louis, USA). The ammonia standard (0.1 mol l^{-1}) was obtained from Orion Research Incorporated (Boston, USA). CsCl and all other salts were of analytical grade and were purchased from Merck (Darmstadt, Germany). Thiabendazole was kindly provided by Dr R. Gräf (Munich).

Electron microscopy

Gills from crabs acclimated to 10‰ salinity were shock-frozen in a high-pressure freezer (Wohlwend Engineering GMBH, Sennwald, Switzerland) (Studer et al., 1989), followed by freeze-substitution in 1% OsO_4 in acetone and embedding in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a Philips electron microscope at 80 kV.

Molecular identification of vesicle-associated membrane protein cDNA

Total RNA was extracted from gill tissue under RNAse-free conditions (Chomczynski and Sacchi, 1987). Reverse transcription of poly(A) mRNA was initiated with oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). Amplification of a putative vesicle-associated membrane protein (VAMP) cDNA sequence was achieved *via* polymerase chain reaction (PCR) using degenerate primers based on published VAMP sequences (DiAntonio et al., 1993; Mandic and Lowe, 1999). The forward primer had the following composition: 5′-CARCARACNCARGCNCARGTNGA-3′; the reverse primer had the following composition: 5′-ATDATCATCATYTTNARRTTYT-3′, designed to produce a 198-nucleotide PCR product. Following separation on agarose gels, the PCR product was extracted from gel slices (Qiagen QiaQuick) and sequenced at the Marine DNA Sequencing Center of Mount Desert Island Biological Laboratory. The partial sequence was submitted to a BLAST search of GenBank (Altschul et al., 1997) and analyzed for open reading frame using DNASIS. The partial VAMP cDNA sequence from *C. maenas* gill was submitted to GenBank (Accession number AY035549).

Results

In preliminary experiments, gills were perfused and bathed in saline from which Tris buffer was omitted, to measure possible pH changes in the apical and basolateral media. Under these conditions, the rate of ammonia removal from the internal perfusate was 14.5±1.4 μmol g^{-1} fresh mass h^{-1} (N=5) (Fig. 1A). However, the rate of appearance of ammonia in the apical bath was 27.6±2.6 μmol g^{-1} fresh mass h^{-1}. The difference between these two values (13.1±3.8 μmol g^{-1} fresh mass h^{-1}) was interpreted to represent the apical release of ammonia produced metabolically by the gill itself. Thus, to avoid overestimation of transepithelial ammonia excretion, only the rate of ammonia removal from the internal perfusion medium was used to represent net active ammonia excretion across the gill. During these initial experiments, no significant changes in the pH (ΔpH<0.02) of the apical saline (pH 7.8; volume 30 ml) were detected. However, the pH of the recirculated internal perfusion saline decreased slightly from an initial value of 7.8 to 7.71±0.02.

In the ensuing perfusion experiments, all solutions were buffered with 2.5 mmol l^{-1} Tris to ensure pH stability under all experimental conditions. Measuring active ammonia excretion over time, the initial net ammonia efflux was 14.7±2.5 μmol g^{-1} fresh mass h^{-1} (N=5) and was thus within the range measured under Tris-free conditions. Over a period of 3 h, the excretion rate decreased slightly but non-significantly by 4.7 μmol g^{-1} fresh mass h^{-1} (N=5) (Fig. 1B). Control rates of active ammonia excretion with symmetrical ammonia concentrations varied between experiments, ranging from approximately 12 to 26 μmol g^{-1} fresh mass h^{-1}, perhaps reflecting variability within natural populations.

Symmetrical addition of 1 μmol l^{-1} bafilomycin A_1, a specific inhibitor of V-type H^+-ATPase (Bowman et al., 1988), lowered the transbranchial active net ammonia efflux by 66% from 18.3±2.6 μmol g^{-1} fresh mass h^{-1} (control) to 6.3±1.7 μmol g^{-1} fresh mass h^{-1} (N=7; P<0.001). The concentration of 1 μmol l^{-1} bafilomycin A_1 employed is known to produce maximal inhibition of crustacean gill V-type H^+-ATPase without affecting the activity of Na^+/K^+-ATPase or F_1F_o-ATPase (Putzenlechner, 1994). After washout of the inhibitor, a minor but non-significant recovery of the efflux rate was detected (data not shown). A similar experiment was performed to verify whether the inhibitory effect of bafilomycin A_1 could be augmented by additional application of ouabain, a specific inhibitor of the Na^+/K^+-ATPase. The initial control efflux (19±3.8 μmol g^{-1} fresh mass h^{-1}) was reduced by application of 1 μmol l^{-1} bafilomycin A_1 to 6.3±2.1 μmol g^{-1} fresh mass h^{-1} (N=4; P<0.001) and was

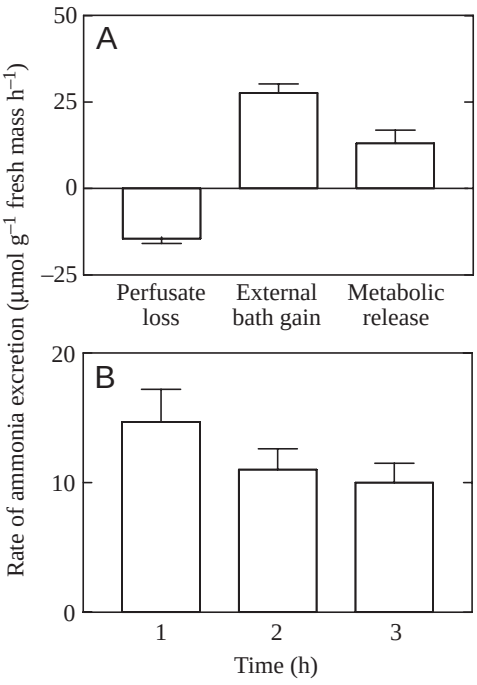

Fig. 1. Active ammonia excretion across the isolated perfused gill of the shore crab *Carcinus maenas*. At the beginning of all experiments, the internal perfusate and the external bath contained symmetrical concentrations of 100 μmol l^{-1} NH_4Cl. (A) Omission of Tris-HCl buffer in the saline. Rate of ammonia loss from the internal perfusate, rate of ammonia addition to the external bath and the calculated rate of metabolic ammonia release into the external bath are displayed (N=5). (B) Disappearance of total ammonia from the internal perfusion medium was measured as the rate of net active branchial ammonia excretion over an experimental period of 3 h (N=5). Data represent means + S.E.M.

further reduced to 0.9±1.4 μmol g^{-1} fresh mass h^{-1} by subsequent basolateral application of 5 mmol l^{-1} ouabain (N=4; P<0.05) (Fig. 2A). A partial but statistically insignificant recovery was measured after washout. It should be noted that the K_i for ouabain in *C. maenas* (K_i=2.9×10^{-4} mol l^{-1}) and other crustaceans is more than two orders of magnitude higher than the K_i in mammals (Postel et al., 1998; Towle, 1984).

In the next series of experiments, the effects of inhibitors of the cytoskeleton on active ammonia excretion were investigated. Basolateral application of 0.2 mmol l^{-1} colchicine, an inhibitor of the microtubule system (Wilson and Farrell, 1986), led to almost complete inhibition (to 2.3±1.3 μmol g^{-1} fresh mass h^{-1}) of the initial control efflux of 26.2±3.9 μmol g^{-1} fresh mass h^{-1} (N=6; P<0.001) (Fig. 2B). Following washout, the efflux recovered significantly to 15.0±5.4 μmol g^{-1} fresh mass h^{-1}. After establishing an outwardly directed ammonia gradient by adding 200 μmol l^{-1} NH_4^+ to the perfusing saline and none to the external bath, colchicine still reduced the initial efflux rate (37.1±3.9 μmol g^{-1} fresh mass h^{-1}) by 74% of the control value to 10.4±4.3 μmol g^{-1} fresh mass h^{-1} (N=5; P<0.001). After washout, efflux recovered to 72% of the control value (Fig. 2C).

In contrast to drugs affecting the microtubule system, no significant effects on active ammonia excretion were observed when 5 μmol l^{-1} cytochalasin D, a specific inhibitor of actin filaments (MacLean-Fletcher and Pollard, 1980), was added to the perfusion saline (N=5) (Fig. 2D). The slight and non-significant decrease in the excretion rate from 20.1±2.8 to 16.5±2.7 μmol g^{-1} fresh mass h^{-1} (P>0.05) resembled the control rates over the experimental period (Fig. 1B) (Weihrauch et al., 1998).

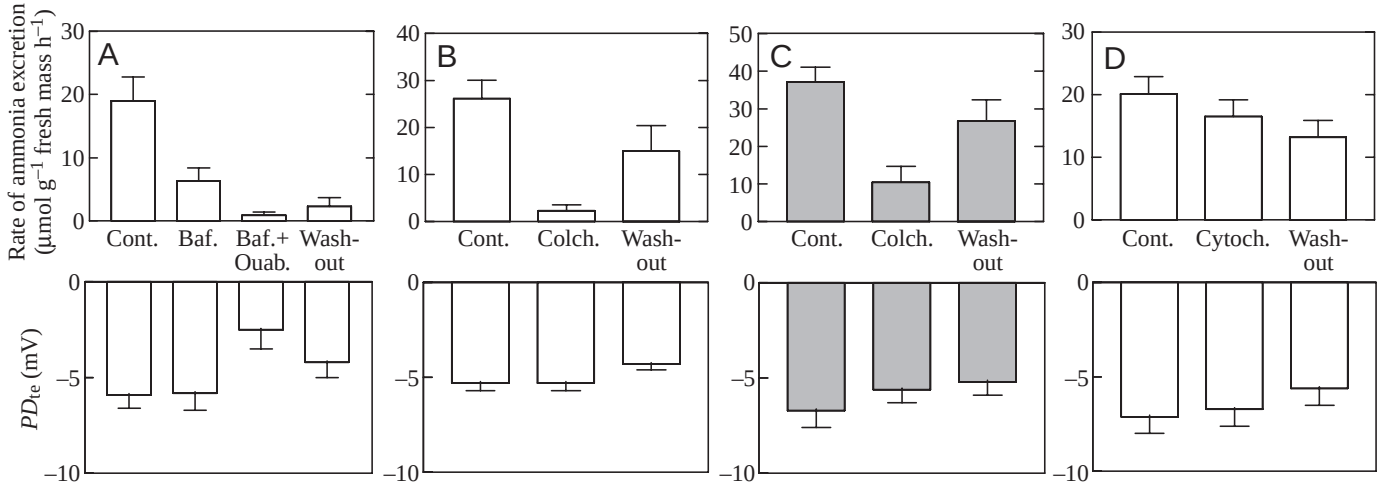

Fig. 2. Effects of inhibitors on active ammonia excretion and transepithelial potential difference (PD_{te}) across isolated perfused gills of the shore crab *Carcinus maenas*. The rate of disappearance of total ammonia from the internal perfusion medium was measured with symmetrical NH_4Cl concentrations (100 μmol l^{-1}) in the external and internal baths (A,B,D) or with 200 μmol l^{-1} NH_4Cl in the internal bath and none initially in the external bath (C). (A) Symmetrical application of bafilomycin A_1 (Baf.) (1 μmol l^{-1}) followed by basolateral addition of ouabain (Ouab.) (5 mmol l^{-1}) (N=4). (B) Basolateral addition of colchicine (Colch.) (0.2 mmol l^{-1}) (N=6). (C) Basolateral application of colchicine (0.2 mmol l^{-1}) with an initial outwardly directed NH_4^+ gradient of 200 μmol l^{-1} (N=5). (D) Basolateral addition of cytochalasin D (Cytoch.) (5 μmol l^{-1}) (N=5). Data represent means + S.E.M.

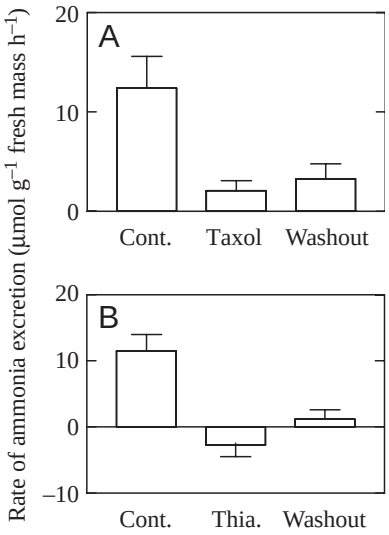

Fig. 3. Effects of microtubule inhibitors on the rate of active ammonia excretion across isolated perfused gills of the shore crab *Carcinus maenas* measured under initial conditions of symmetrical concentrations of NH_4Cl (100 μmol l^{-1}) in the external and internal baths. (A) Basolateral application of taxol (10 μmol l^{-1}) (N=6). (B) Basolateral application of thiabendazole (Thia.) (0.2 mmol l^{-1}) (N=6). Data represent means + S.E.M.

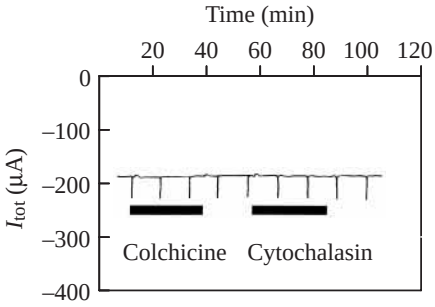

Fig. 4. Time course of the inward negative uncorrected short-circuit current (I_{tot}) measured over the split gill half-lamella of *Carcinus maenas* during application (bars) of basolateral colchicine (0.2 mmol l^{-1}) and cytochalasin D (5 μmol l^{-1}). The amplitudes of the current deflections, which are due to voltage pulses of 1 mV, are inversely proportional to the resistance between the tips of the voltage electrodes.

In all the above experiments, PD_{te} was monitored to detect changes in the electrophysiological variables of the gill (Fig. 2). With the exception of the addition of ouabain, which resulted in a reversible decrease in PD_{te} of 55% (Fig. 2A) due to a disruption of transepithelial Na^+ transport (Siebers et al., 1985), application of the various inhibitors had no significant effects on PD_{te}, indicating that Na^+ transport pathways were not affected by the remaining treatments.

For two other blockers of the microtubule system, microtubule hyper-stabilizing taxol (Nogales et al., 1995) and microtubule destabilizing thiabendazole (Davidse and Flach, 1978), a strong inhibitory effect on active ammonia excretion was observed (Fig. 3). Whereas basolateral application of 10 μmol l^{-1} taxol led to a decrease in the control rate (12.4±3.2 μmol g^{-1} fresh mass h^{-1}) of 77% to 2.1±1.0 μmol g^{-1} fresh mass h^{-1} (N=6; P<0.001), basolateral addition of 0.2 mmol l^{-1} thiabendazole altered the efflux rate of 11.5±2.5 μmol g^{-1} fresh mass h^{-1} to an apparent influx of ammonia (2.7±1.8 μmol g^{-1} fresh mass h^{-1}) (N=6; P<0.001), probably as a result of metabolic ammonia production and release across the basolateral membrane. A statistically significant recovery of ammonia efflux was observed after washout of thiabendazole but not of taxol.

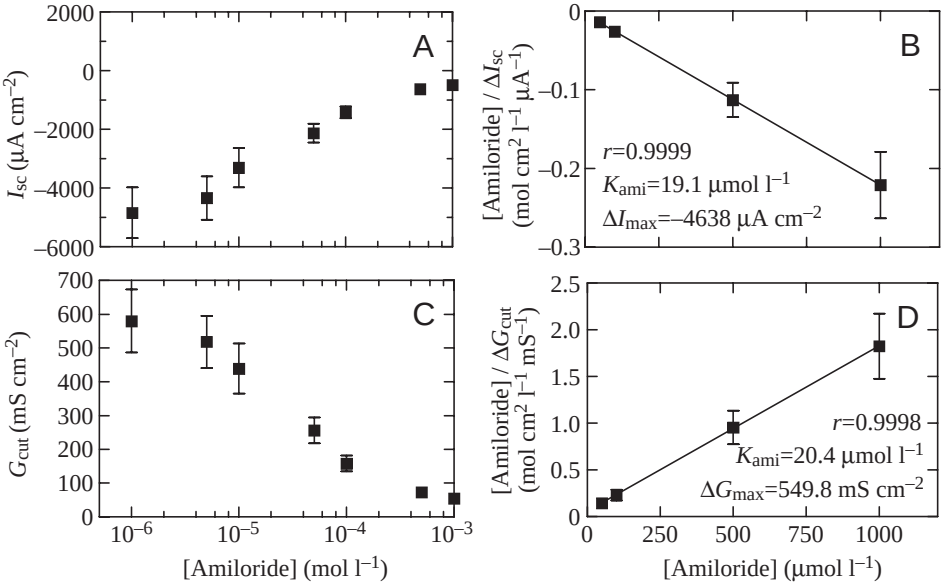

Fig. 5. Dose-dependence of cuticular short-circuit current (I_{sc}) and transcuticular conductance (G_{cut}) inhibition by amiloride. A clamp voltage of 10 mV with reference to the apical side was maintained to force transcuticular NH_4^+ fluxes. In four experiments on different isolated cuticles, amiloride was added at increasing concentrations to the external solution. (A) Mean I_{sc} plotted against the concentration of amiloride (in mol l^{-1}) in the external saline. (B) The I_{sc} data are shown in a Hanes–Woolf plot. The mean values of ΔI_{max} (maximum short-circuit current) and K_{ami} (inhibition constant for amiloride) were obtained from plots for the individual experiments. (C) Mean G_{cut} plotted against the concentration of amiloride (in mol l^{-1}) in the external saline. (D) The G_{cut} data are shown in a Hanes–Woolf plot. The mean values of ΔG_{max} (maximum transcuticular conductance) and K_{Ami} were obtained from plots for the individual experiments. Values are means ± S.E.M.

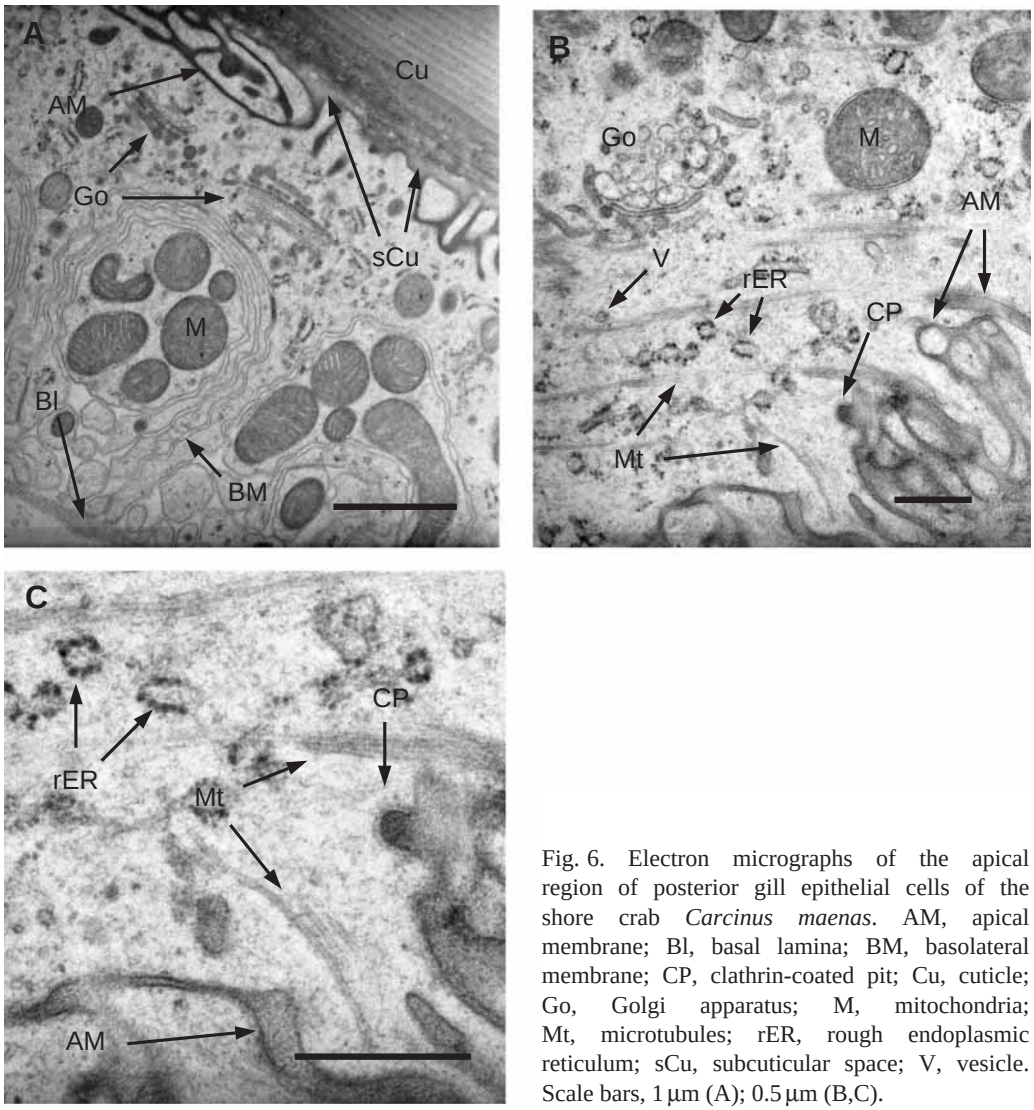

Fig. 6. Electron micrographs of the apical region of posterior gill epithelial cells of the shore crab *Carcinus maenas*. AM, apical membrane; Bl, basal lamina; BM, basolateral membrane; CP, clathrin-coated pit; Cu, cuticle; Go, Golgi apparatus; M, mitochondria; Mt, microtubules; rER, rough endoplasmic reticulum; sCu, subcuticular space; V, vesicle. Scale bars, 1 μm (A); 0.5 μm (B,C).

To evaluate changes in the electrical variables of the gill epithelium in response to application of the cytoskeleton inhibitors colchicine and cytochalasin D, the highly sensitive transepithelial short-circuit current (I_{sc}) and transepithelial resistance (R_{te}) were measured employing the preparation of the split gill lamella mounted in an Ussing-type chamber. Basolateral application of either 0.2 mmol l^{-1} colchicine or 5 μmol l^{-1} cytochalasin D had no significant effect on I_{sc} (colchicine, 318.8±51.6 μA cm^{-2}; control, 320.2±52.9 μA cm^{-2}, N=5; cytochalasin D, 353.4±61.5 μA cm^{-2}; control, 358.8±64.9 μA cm^{-2}, N=4) (Fig. 4). Control values of R_{te} measured in parallel were not altered following the addition of colchicine (24.1±1.7 Ω cm^2, N=5) or cytochalasin D (28.3±1.7 Ω cm^2, N=4) (data not shown).

Previous studies have shown that amiloride, a blocker of epithelial Na^+ channels and the Na^+/H^+ exchanger (Kleyman and Cragoe, 1988), has a strong inhibitory effect on ammonia excretion by isolated crab gills when applied to the apical (cuticle) side of the epithelium (Lucu et al., 1989; Weihrauch et al., 1998). To investigate whether the amiloride-induced reduction in the rate of ammonia excretion may be based on a possible effect on the electrophysiological properties of isolated cuticle (Lignon, 1987) in addition to any effect on the epithelial cells themselves, NH_4^+-dependent I_{sc} and G_{cut} of the isolated gill cuticle of *C. maenas* were measured in a micro Ussing chamber. As expected in a cell-free system, a transcuticle potential difference (PD_{cut}) of 0 mV was measured (N=4). Following the imposition of a clamp voltage of 10 mV, a negative I_{sc} of –5800±1368 μA cm^{-2} and a G_{cut} of 683.0±165.2 mS cm^{-2} were measured. The detected current is probably the result of NH_4^+ effluxes, since cuticular permeability in *C. maenas* has been described to be 100- to 1000-fold smaller for monovalent anions than for monovalent cations (Lignon, 1987). After apical application of various amiloride concentrations (0.001–1 mmol l^{-1}), dose-dependent inhibition was observed for both I_{sc} and G_{cut} (Fig. 5). Linear regression in a Hanes–Woolf plot revealed simple Michaelis–Menten kinetics for I_{sc} and G_{cut}. For the NH_4^+-

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CARCINUS          : ----------------------------------------------VDEVVDIMRTNVEKVLERDQKL
DROSOPHILA        : MADAAPAGDAPPNAGAPAGEGGDGEIVGGPHNPQQIAAQKRLQQTQAQVDEVVDIMRTNVEKVLERDSKL
CAENORHABDITIS    : ------------------MDAQGDAGAQGGSQGGPRPSNKRLQQTQAQVDEVVGIMKVNVEKVLERDQKL
APLYSIA           : --------------------MSAGPGGPQGGMQPPREQSKRLQQTQAQVDEVVDIMRVNVEKVLDRDQKI
STRONGYLOCENTROTUS: ---------------------THNTTTHSSNYANRYTTNKRLQQTQAQVDEVVDIMRVNVDKVLERDQAL
XENOPUS           : ------------MSAPAAGPPAAAPGDGAPQGPPNLTSNRRLQQTQAQVDEVVDIMRVNVDKVLERDTKL

CARCINUS          : SELDARADALQQGASQFEQQAA------------------------------------------------
DROSOPHILA        : SELDDRADALQQGASQFEQQAGKLKRKFWLQNLKMMIIMGVIGLVVVGIIANKLGLIGGEQPPQYQYP-P
CAENORHABDITIS    : SQLDDRADALQEGASQFEKSAATLKRKYWWKNIKMMIIMCAIVVILIIIIVLWAGGK------------
APLYSIA           : SQLDDRAEALQAGASQFEASAGKLKRKYWWKNCKMMLILGAIIGVIVIIIIVWVVTSQDSGGDDSGSKTP
STRONGYLOCENTROTUS: SVLDDRADALQQGASQFETNAGKLKRKYWWKNCKMMIILAIIIVILIIIIVAIVQSQKK----------
XENOPUS           : SELDDRADALQAGASQFETSAAKLKRKYWWKNMKMMIIMGVICAIILIIIIVYFST-------------
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Fig. 7. Partial amino acid sequence of vesicle-associated membrane protein (VAMP) identified by PCR in gills of the shore crab *Carcinus maenas* aligned with VAMP sequences from fruit fly *Drosophila melanogaster* (GenBank Accession No. AAF47529), nematode *Caenorhabditis elegans* (GenBank Accession No. AAB61234), seahare *Aplysia californica* (GenBank Accession No. U00997), sea urchin *Strongylocentrotus purpuratus* (GenBank Accession No. AAB67799) and African frog *Xenopus laevis* (GenBank Accession No. P47193).

dependent I_{sc}, K_{ami} was 19.1 μmol l^{-1} and ΔI_{max} was –4638 μA cm^{-2}. K_{ami} for NH_4^+-dependent G_{cut} was 20.4 μmol l^{-1} and ΔG_{max} was 549.8 mS cm^{-2} (Fig. 5). Symmetrical application of 1 μmol l^{-1} bafilomycin A_1 had no significant effect on cuticular NH_4^+-dependent I_{sc} (control, –4294±206 μA cm^{-2}; bafilomycin, –3984±217 μA cm^{-2}) and G_{cut} (control, 454±45 mS cm^{-2}; bafilomycin, 400±80 mS cm^{-2}) (N=3).

Because our inhibitor studies indicated important roles for the V-type H^+-ATPase and microtubules in active ammonia excretion, we sought ultrastructural and molecular evidence that would help to support or refute such possibilities. A previous study showed that the B-subunit of the V-type H^+-ATPase was distributed throughout the cytoplasm of gill epithelial cells in *C. maenas* rather than being located specifically in the apical membrane, suggesting that the H^+-ATPase may be associated with cytoplasmic vesicles (Weihrauch et al., 2001b) as well as with the apical membrane. In the present study, transmission electron microscopy of sections obtained from posterior gills of *C. maenas* acclimated to 100 μmol l^{-1} external ammonia revealed an apparently dynamic system of membrane vesicles and Golgi bodies associated with the apical region of the epithelium (Fig. 6A). We also observed a well-developed microtubule assemblage associated with the apical membrane (Fig. 6B,C). In several sections, we were able to detect apparent interactions between membrane vesicles and the microtubules.

To ascertain whether any of the known exocytotic mechanisms are expressed in *C. maenas* gill, we attempted to identify one of the expected components, vesicle-associated membrane protein (VAMP, also called synaptobrevin) (Trimble et al., 1988). Using PCR with degenerate primers based on published VAMP sequences, we identified a VAMP-related sequence in a cDNA mixture prepared from *C. maenas* gill. Translation of the 132-nucleotide fragment revealed an amino acid sequence that is highly homologous to VAMP

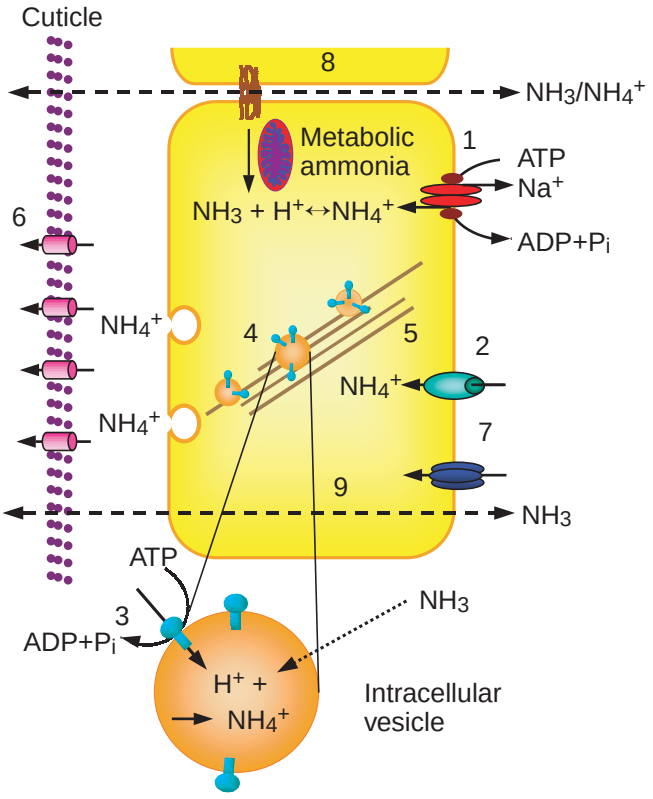

Fig. 8. Proposed hypothetical model of active ammonia excretion across the gills of the shore crab *Carcinus maenas*. According to this model, NH_4^+ is pumped across the basolateral membrane by the Na^+/K^+-ATPase (1) or traverses the membrane *via* Cs^+-sensitive channels (2). Dissociation of cytosolic NH_4^+ to H^+ and NH_3 is accompanied by diffusion of NH_3 into vesicles acidified by a V-type H^+-ATPase (3). The ammonia-loaded vesicles (4) are then moved *via* microtubules (5) to the apical membrane, where they fuse with the external membrane, releasing NH_4^+ into the subcuticular space. The NH_4^+ is then believed to diffuse across the cuticle *via* amiloride-sensitive structures (6). The possibility of an additional ammonium transporter, probably in the basolateral membrane (7), cannot be discounted. Rates of paracellular ammonia diffusion (8) and non-ionic transcellular diffusion of NH_3 (9) are considered to be low at physiologically meaningful transepithelial ammonia gradients. P_i, inorganic phosphate.

sequences determined for other invertebrate and vertebrate species (Fig. 7).

Discussion

Previous investigations on the ammonia excretion mechanism in the gills of *C. maenas* and other crab species suggested that ammonia from the hemolymph space is transported into the cytosol by basolateral Na^+/K^+-ATPase (Lucu et al., 1989; Towle and Hølleland, 1987) and Cs^+-sensitive channels, probably K^+ channels (Weihrauch et al., 1998). In isolated gills, active ammonia efflux was only partially (approximately 60%) inhibited by blocking the Na^+/K^+-ATPase or by omission of Na^+, but was almost completely inhibited (>90%) after addition of dinitrophenol (Weihrauch et al., 1998), suggesting that a second, Na^+-independent ATP-requiring component is involved in ammonia excretion. The present study shows that inhibition of the V-type H^+-ATPase, which has been identified at the molecular level in *C. maenas* gill (Weihrauch et al., 2001b), reduces active ammonia net efflux by 66%. The finding that simultaneous application of bafilomycin A_1 and ouabain almost completely blocked active ammonia excretion identified the V-type H^+-ATPase and the Na^+/K^+-ATPase as the two major ATP-requiring participants in the excretion mechanism.

Active ammonia transport across biological membranes could be mediated by a proton pump *via* two different mechanisms: (i) the proton gradient generated by the pump drives a parallel H^+/NH_4^+ exchanger, or (ii) the proton pump generates a partial pressure gradient for NH_3 over the membrane, inducing transmembrane diffusion of gaseous ammonia. The latter has been suggested for ammonia transport across the apical membrane in the gills of freshwater trout (Wilson et al., 1994) since in this tissue the H^+-ATPase is localized predominantly in the apical membrane (Sullivan et al., 1995). In addition, buffering the external medium with Hepes dramatically inhibited ammonia excretion across fish gill because, under this condition, the partial pressure gradient for NH_3 diffusion across the apical membrane was abolished.

However, in gill epithelial cells of *C. maenas*, the V-type H^+-ATPase is distributed throughout the cytoplasm and only faintly detectable in the apical region (Weihrauch et al., 2001b). In the present study, no significant changes in pH in the apical medium were detected during excretion nor was the active ammonia excretion rate altered when a buffer (2.5 mmol l^{-1} Tris-HCl) was applied in the apical saline (Fig. 1) (Weihrauch et al., 1998). These results indicate that in *C. maenas* acidification of the outer apical membrane is not responsible for driving a putative H^+/NH_4^+ exchanger nor does it generate a partial pressure gradient for diffusion of non-ionic NH_3 across the apical membrane. However, we cannot discount the possibility of unstirred layers between the gill lamellae and thus cannot exclude the possibility of pH gradients immediately external to the cuticle. Indeed, we suspect that the subcuticular space, between the cuticle and the apical membrane, represents a classic unstirred layer. Diffusion of NH_3 coupled with transport of H^+ to produce NH_4^+ could theoretically occur in these unstirred layers to which buffer might not penetrate.

However, we suggest that the proton pump of crab gills, rather than being restricted to apical membranes, may be inserted into the membranes of cytoplasmic vesicles, generating an inwardly directed partial pressure gradient for NH_3 and leading to the accumulation of NH_4^+ within the vesicles. It has been shown that radioactively labeled methylamine diffuses into acidified vesicles, where it is protonated into its membrane-impermeable ionic form methylammonium (Riejngoud and Tager, 1973). We suggest that a similar mechanism functions in crab gills, where cytoplasmic NH_3 diffuses into vesicles acidified by the V-type H^+-ATPase and is trapped in the vesicles as NH_4^+.

The almost complete inhibition of both active and gradient-driven net ammonia excretion by microtubule inhibitors (colchicine, thiabendazole and taxol) indicates a microtubule-dependent ammonia excretion mechanism. In contrast, blocking the actin filaments with cytochalasin D, which causes a small increase in exocytotic and endocytotic movements in frog nephron epithelia (Verrey et al., 1995), had no significant effect on active ammonia excretion across the isolated gill. We suggest that NH_4^+-loaded vesicles are transported along the microtubule network to the apical membrane, where ammonia is released by membrane fusion and exocytosis. This suggestion is supported by the abundance of Golgi bodies and vesicles in gill epithelial cells (Fig. 6A), by the presence of bundles of microtubules oriented towards the apical membrane and by the endo/exocytotic activities represented by apparent clathrin-coated pits (Fig. 6B,C).

Following application of bafilomycin A_1, colchicine, thiabendazole, taxol and cytochalasin D, PD_{te} remained unchanged, indicating that changes in ammonia excretion during treatment with these inhibitors were not caused by alterations to the osmoregulatory NaCl uptake machinery or by damage to the integrity of the preparation. The unaltered I_{sc} and G_{te} across the split half-lamella during exposure to colchicine also supported our conclusion that a functional microtubule network is necessary for the process of active ammonia excretion but that its inhibition does not affect the electrical variables of the gill epithelium, at least over the short term (Fig. 2).

The possibility of an apical amiloride-sensitive Na^+/NH_4^+ exchange across the apical membrane prompted our examination of the role of the cuticle. Amiloride has been shown to have an inhibitory effect on ammonia transport in renal proximal tubules (Knepper et al., 1989), colonic crypt cells (Ramirez et al., 1999), teleost gills (Randall et al., 1999) and crustacean gills (Lucu et al., 1989; Weihrauch et al., 1998). In contrast to vertebrate tissues, crustacean gills are covered with an ion-selective cuticle. Although the conductance of the isolated gill cuticle of *C. maenas* has been shown to be approximately 10 times higher than that of the combined epithelium plus cuticle (Riestenpatt, 1995), ion selectivity is

demonstrable, with the permeability for monovalent anions (Cl^-, HCO_3^-) being 100–1000 times lower than that for Na^+ (Lignon, 1987).

The present study employing the isolated cuticle showed that NH_4^+-dependent I_{sc} and G_{cut} were inhibited by amiloride in a dose-dependent manner. At an amiloride concentration of 100 μmol l^{-1}, a concentration commonly used in investigating Na^+ and NH_4^+ fluxes across the gill epithelia of *C. maenas* (Lucu et al., 1989; Lucu and Siebers, 1986; Onken and Siebers, 1992; Weihrauch et al., 1998), more than 70% of the cuticular NH_4^+-dependent I_{sc} and G_{cut} were blocked. In a recent electrophysiological study investigating the effects of amiloride on Na^+ influx across split gill lamellae and isolated cuticle of *C. maenas*, it was shown that apical amiloride inhibits both the Na^+-dependent transepithelial I_{sc} and G_{te} and also the transcuticular I_{sc} and G_{cut} with similar values for K_{ami} (Onken and Riestenpatt, 2002). These authors suggested that the effects of amiloride were due to an interaction between the diuretic and the outer cuticle and not with transporters in the apical cell membrane or paracellular junctions. They concluded, however, that amiloride may interact directly with cellular transporters in the gills of other crab species (Onken and Riestenpatt, 2002).

The values of K_{ami} for the cuticular NH_4^+-dependent I_{sc} and G_{cut} (approximately 20 μmol l^{-1}) obtained in our study are approximately 20-fold higher than the values calculated for the Na^+-dependent I_{sc} and G_{cut} (approximately 1 μmol l^{-1}) employing an identical experimental design. Comparison of the NH_4^+-dependent G_{cut} (683±165 mS cm^{-2}) and the Na^+-dependent G_{cut} (583±71 mS cm^{-2}) (Riestenpatt, 1995) showed a higher conductance of the cuticle for NH_4^+ ions than for Na^+ ions, confirming earlier measurements (Lignon, 1987). We only can speculate that because of the smaller hydrated ionic size of NH_4^+ (approximately 0.38 nm *versus* approximately 0.56 nm for Na^+), blockage of a cation-permeable structure in the cuticle by amiloride is less efficient. However, from these experiments, the presence of a Na^+/NH_4^+ exchanger in the apical membrane itself cannot be excluded.

On the basis of previous observations and insights gained from the present study, we have constructed a hypothetical model for transbranchial ammonia excretion in *C. maenas* functioning at physiological ammonia concentrations. In this model (Fig. 8), we suggest that hemolymph ammonia enters the epithelial cell across the basolateral membrane *via* NH_4^+-permeable Cs^+-sensitive channels and also *via* the Na^+/K^+-ATPase in exchange for Na^+ (Lucu et al., 1989; Towle and Hølleland, 1987). The nature of the NH_4^+-permeable channel is unknown, but it may be related to a recently described rhesus-like protein that appears to mediate transfer of NH_4^+ across cell membranes (Marini et al., 2000). Using reverse transcription and PCR, we have recently identified such a rhesus-like protein in the gills of *C. maenas* (Weihrauch et al., 2001a).

The pool of ammonia imported from the hemolymph and produced by gill metabolism occurs within the cytoplasm in a pH-dependent equilibrium between NH_4^+ and NH_3 ($pK_{Ammonia}$=9.48) (Cameron and Heisler, 1983). In our working model, we suggest that non-ionic NH_3 diffuses along its partial pressure gradient into intracellular vesicles acidified by a proton pump. Because of the low pH within the vesicles, NH_3 would be converted into its membrane-impermeable ionic form NH_4^+ and therefore trapped in this compartment. Our microtubule inhibitor studies suggest that the NH_4^+-loaded vesicles may be transported along the microtubule network to the apical membrane, where NH_4^+ would be released by exocytosis into the subcuticular space. Our demonstration of a vesicle-associated membrane protein (VAMP) sequence in cDNA prepared from *C. maenas* gill RNA (Fig. 7) shows that at least one component of the exocytotic machinery is expressed in this tissue, providing circumstantial evidence for branchial exocytotic activity.

From the subcuticular space, NH_4^+ would diffuse along a concentration gradient *via* amiloride-sensitive structures across the cuticle into the external medium of the gill chamber. At physiologically meaningful outwardly directed ammonia gradients (50–400 μmol l^{-1}), transepithelial diffusion of ammonia is considered to be low, comprising 12–21% of the total efflux (Weihrauch et al., 1998).

In the present report, the proposed mechanism of exocytotic ammonia excretion is supported only by indirect evidence. To investigate its validity more thoroughly, more direct experiments are necessary. Feasible future approaches include measurements of the capacitance as an indicator of the exocytotic activity of the apical membrane under control and high-ammonia conditions (Zeiske et al., 1998), the use of radioactively labeled methylamine/methyl-ammonium as a traceable competitive inhibitor for ammonia movements (Talor et al., 1987) and the use of laser confocal microscopy combined with video-image analysis to trace intracellular pH-labeled compartments (Miller et al., 1994).

The possibility of an exocytotic ammonia excretion mechanism should be considered since, in this situation, toxic ammonia is trapped in vesicles within the cell rather than diffusing through the entire cytoplasm, where it could cause major damage. In aquatic animals facing an inwardly directed ammonia gradient in the natural environment (Weihrauch et al., 1999), the active component of the mechanism would provide protection for the gill epithelial cells and, indeed, for the entire organism against passive NH_4^+ influxes. Microtubule-mediated transport of ammonia-loaded vesicles and exocytosis at the apical membrane would permit a potent ammonia detoxification mechanism in such organisms without compromising ionic permeability. Whether such a mechanism applies broadly across species is not known. However, some aquatic species, including the South American rainbow crab *Chasmagnathus granulatus* (Rebelo et al., 1999), the prawn *Nephrops norvegicus* (Schmitt and Uglow, 1997) and three fish species in the family Batrachoididae (Wang and Walsh, 2000), tolerate high environmental ammonia levels. Among the adaptive mechanisms in these species may be an active ammonia excretion process similar to that described here.

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