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A V-ATPase DRIVES ACTIVE, ELECTROGENIC AND Na^+-INDEPENDENT Cl^- ABSORPTION ACROSS THE GILLS OF *ERIOCHEIR SINENSIS*

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Summary

Using biochemical and electrophysiological techniques, we have examined the proposal that an H^+-ATPase is involved in Cl^- uptake across the gills of the Chinese crab *Eriocheir sinensis*. Bafilomycin A_1 (1 μmol l^{-1}), a specific inhibitor of V-ATPases, was used to investigate the importance of this H^+-translocating enzyme in Cl^- transport across the gill. In homogenates of ion-transporting posterior gills, we found the activity of a bafilomycin-sensitive V-ATPase to be markedly higher than in the anterior gills, which are not involved in ion transport. A similar distribution was found for the Na^+/K^+- and the mitochondrial F_1F_o-ATPase. After differential and density centrifugation, the specific activity of the V-ATPase was enriched by a factor of 5. Neither Na^+/K^+- and F_1F_o-ATPase activities nor acid phosphatase activity copurified with the bafilomycin-sensitive ATPase activity, indicating that at least the major portion of V-ATPase activity is not of basolateral, mitochondrial or lysosomal origin. In fluorescence studies, using Acridine Orange or Oxonol V as dyes, membrane vesicles displayed ATP-dependent proton transport and membrane potential generation, which were markedly reduced in the presence of bafilomycin. In addition to these biochemical studies, we mounted split lamellae of posterior gills in an Ussing-type chamber and measured the negative short-circuit current (I_{sc}), which was shown to reflect active, electrogenic, Na^+-independent and ouabain-insensitive Cl^- absorption. After the addition of 1 μmol l^{-1} bafilomycin to the external bath, this I_{sc} was reduced to about 50–60 % of its original value. Concomitantly, the conductance of the preparation decreased by about 13 %. From these results, we conclude that an apical V-ATPase drives electrogenic Cl^- uptake across the posterior gills of the Chinese crab.

Key words: crab, gill, *Eriocheir sinensis*, V-ATPase, Cl^- absorption, bafilomycin A_1, Acridine Orange, Oxonol V, short-circuit current.

Introduction

Chinese crabs (*Eriocheir sinensis*) acclimated to fresh water maintain an outwardly directed osmotic gradient of about 650 mosmol l^{-1} across their body surface by active absorption of NaCl *via* the posterior gills (Mantel and Farmer, 1983; Péqueux *et al.* 1988). Investigating whole crabs, Krogh (1938) demonstrated that Na^+ and Cl^- can be absorbed independently. This was confirmed in electrophysiological studies on isolated and perfused whole gill preparations (Péqueux *et al.* 1988; Onken and Graszynski, 1989), which also indicated that the underlying transport mechanisms are electrogenic. The mechanism of Na^+ uptake has been clearly identified in voltage-clamp measurements on split gill lamellae mounted in modified Ussing-type chambers. In the presence of external Cl^--free saline, a positive, Na^+-dependent short-circuit current (I_{sc}), sensitive to submicromolar concentrations of external amiloride and to internal ouabain (Schwarz, 1990; Zeiske *et al.* 1992), was demonstrated. Thus, Na^+ absorption proceeds as in frog skin preparations *via* apical Na^+ channels and the basolateral Na^+/K^+-ATPase. In contrast, the mechanism for Cl^- absorption is still uncertain. Onken *et al.* (1991) showed that, in the presence of external Na^+-free saline, a negative Cl^--dependent I_{sc} could be measured and that this was susceptible to block with external 4-acetamido-4′-isothiocyanato-stilbene-2,2-disulphonic acid (SITS) and with internal diphenylamine-2-carboxylate (DPC) or acetazolamide. These observations are compatible with the assumption that Cl^- uptake proceeds *via* apical Cl^-/HCO_3^- antiport and basolateral Cl^- channels and depends on a functioning carbonic anhydrase. Owing to the insensitivity of this negative I_{sc} to internal ouabain, the authors discussed the possibility that Cl^- absorption is driven by an apical H^+ pump. This hypothesis was supported by their experimental findings, but there is no direct experimental support for the presence of an H^+ pump in *Eriocheir sinensis* gills and for its involvement in Cl^- absorption.

Bafilomycin A_1 has been shown to be a highly specific inhibitor of H^+-transporting V-ATPases (Bowman *et al.* 1988). In the present investigation, we used bafilomycin A_1 in biochemical and electrophysiological experiments on the gills of Chinese crabs acclimated to fresh water. Our results support the proposal that active and electrogenic Cl^- absorption across this tissue is driven by an apical V-type H^+ pump.

Some of the present results were reported at the 1992 meeting of the Deutsche Zoologische Gesellschaft (Putzenlechner *et al.* 1992) and at an international symposium on the occasion of the centennial of the Biologische Anstalt Helgoland in 1992 (Onken *et al.* 1994).

Materials and methods

Crabs

Chinese crabs (*Eriocheir sinensis*; Milne-Edwards) were obtained from commercial fishermen from Schleswig-Holstein, Germany. In the laboratory, the crabs were kept at 10–12 °C in running tap water (in mmol l^{-1}): Na^+, 2.0; K^+, 0.08; Ca^{2+}, 3.0; Cl^-, 1.7. Twice a week the animals were fed with Ewos fish food (Bertels GmbH, Halstenbek, Germany) or frozen fish. For the experiments, the crabs were killed by destroying the ventral ganglia. After lifting the dorsal carapace, the posterior (pleurobranchiae and posterior arthrobranchia) and anterior (podobranchiae and anterior arthrobranchiae) gills were removed.

Biochemistry

Membrane preparation

For each membrane preparation, the respective gills (posterior or anterior) of five animals were washed in ice-cold homogenization buffer (HB; all concentrations are given as final concentrations) containing 250 mmol l^{-1} sucrose, 10 mmol l^{-1} Hepes–Tris, 5 mmol l^{-1} EDTA and 3 mmol l^{-1} mercaptoethanol (pH 7.25). Homogenization was performed in a glass tube with a tight-fitting Teflon pestle (Potter homogenizer; Braun Melsungen, Germany) at 1400 revs min^{-1} with 30 strokes in a 7 % (w/v) homogenate. The homogenate was filtered through gauze (pore diameter 90 μm). The retained material was discarded. The filtrate (fraction H) was then centrifuged for 10 min at 500 ***g*** (Sorvall RC 5 B, rotor SS 34). The resulting pellet was discarded and the supernatant was centrifuged at 5000 ***g*** (Sorvall RC 5 B, rotor SS 34) for 15 min. The pellet, consisting largely of the mitochondria present in the gill tissue (very high F_1F_o-ATPase activity), was discarded. To separate cytosolic proteins and most lysosomes from a crude membrane pellet, the supernatant was centrifuged for 1 h at 100 000 ***g*** (Kontron UZ, rotor TFT 70.38). The resulting pellet was suspended in 5 ml of HB and layered on top of a two-step sucrose gradient, containing 33 or 40 % sucrose in 10 mmol l^{-1} Hepes–Tris and 0.32 mmol l^{-1} EDTA (pH 7.25). After centrifugation (2.5 h at 160 000 ***g***, Kontron UZ, swinging bucket rotor TST 41.14), the resulting microsomal fractions on top of the 33 % (fraction M_1) and 40 % (fraction M_2) sucrose solutions were isolated separately. Both fractions were diluted 10-fold with HB and centrifuged for 1 h at 100 000 ***g*** (Kontron UZ, rotor TFT 70.38). The resulting pellets were suspended in appropriate buffers for either ATPase assays or fluorometric measurements (see below). All preparative steps were conducted at 4 °C. Samples for ATPase assays were stored at −18 °C. Probes for fluorometric measurements were kept on ice until use.

ATPase assays

ATPase activities were measured according to Onishi *et al.* (1976) as liberated inorganic phosphate in the homogenate (fraction H) and in the microsomal fractions (M_1 and M_2), which were suspended in a mixture of 5 mmol l^{-1} Mops–Tris, 3 mmol l^{-1} mercaptoethanol and 0.32 mmol l^{-1} EGTA (pH 7.6).

SDS activation and measurement of Na^+/K^+-ATPase activity were conducted according to Kosiol *et al.* (1988). In each assay, 10–20 μg of membrane protein was used. Na^+/K^+-ATPase activity was defined as the difference between SDS-activated activity obtained with and without 5 mmol l^{-1} ouabain.

F_1F_o-ATPase activity was determined in the same way. However, instead of ouabain, 1 mmol l^{-1} sodium azide was used to define F_1F_o-ATPase activity.

For measuring V-ATPase activity, 50–100 μg of protein (not SDS-treated, since SDS activation of the V-ATPase was not successful in a preliminary test) was preincubated in a mixture of 50 mmol l^{-1} Mops–Tris, 1 mmol l^{-1} sodium vanadate (to inhibit P-type ATPases), 1 mmol l^{-1} sodium azide (to inhibit F_1F_o-ATPase) and 3 mmol l^{-1} $MgCl_2$ (pH 8.0) at 30 °C for 10 min. The reaction was initiated with 3 mmol l^{-1} Tris–ATP. After 15 min, the amount of liberated phosphate was measured as above (Kosiol *et al.* 1988). V-ATPase activity was defined as the difference between the activity obtained with and without 1 μmol l^{-1} bafilomycin A_1 (Bowman *et al.* 1988). In preliminary experiments, the dependence of the V-ATPase activity on pH and on Mg^{2+} and ATP concentrations was tested. Optimal conditions were found at pH values between 7 and 8 and at 3 mmol l^{-1} Mg^{2+} and ATP. Mg^{2+} could not be replaced by Ca^{2+}. Additional KCl, NaCl or Na_2SO_4 (each at 15 mmol l^{-1}) had no effect. The effect of the inhibitors of the F_1F_o-ATPase or the P-type ATPases on the bafilomycin-sensitive ATPase activity never exceeded 10 % of the control. With respect to vanadate, this finding was of special importance, because Beltran and Nelson (1992) found that vanadate concentrations below 1 mmol l^{-1} caused a 50 % inhibition of the V-ATPase of chromaffin granules and yeast vacuoles.

Acid phosphatase levels

For measuring levels of acid phosphatase, an enzyme which is often used as a marker enzyme for lysosome membranes (Trouet, 1974; Maunsbach, 1974), 10–50 μg of protein was preincubated at 37 °C in 100 mmol l^{-1} acetate buffer (adjusted to pH 5 using citric acid). After 10 min, the artificial substrate *p*-nitrophenylphosphate (5 mmol l^{-1}) was added to start the reaction in a volume of 900 μl. After a further 10 min, the reaction was stopped by the addition of 100 μl of NaOH (0.5 mol l^{-1}). The extinction of the product of the reaction, *p*-nitrophenol, was measured at 420 nm, and the enzyme activity

was calculated using an extinction coefficient of 18 600 l mol^{-1} cm^{-1} (Richterich and Colombo, 1978).

Protein content

The protein content was measured according to the method of Bradford (1976), modified according to Sedmark and Grossberg (1977), with bovine serum albumin as the standard.

Fluorometric measurements

The fluorometric measurements were conducted according to Wieczorek *et al.* (1991) and are described briefly here. For these experiments, we used only the microsomal fraction on top of the 40 % sucrose (M_2). This fraction was suspended in a mixture of 5 mmol l^{-1} Tris, 250 mmol l^{-1} sucrose and 3 mmol l^{-1} mercaptoethanol (adjusted to pH 8.0 using either HCl or gluconolactone). All assays were conducted at 30 °C in a Perkin Elmer LS3B fluorescence spectrometer.

Proton transport was measured with the fluorescent dye Acridine Orange. Standard assay mixtures with a final volume of 1.5 ml consisted of 75 mmol l^{-1} sucrose, 4 mmol l^{-1} Tris–HCl, 3 mmol l^{-1} Tris–ATP, 6 mmol l^{-1} $MgCl_2$ and 0.9 μmol l^{-1} Acridine Orange (pH 8.0). The reaction was started by the addition of 20 μl of vesicle suspension (containing 100–150 μg of protein), resulting in final fluorescence quenches of about 30 %. At the end of the assay, the pH gradients responsible for the Acridine Orange quenches were dissipated by adding 3 mmol l^{-1} NH_4Cl.

Membrane potential generation was measured with the fluorescent dye Oxonol V. The assay mixture, in a volume of 1.5 ml, contained 75 mmol l^{-1} sucrose, 4 mmol l^{-1} Tris–gluconate, 6 mmol l^{-1} $MgSO_4$, 0.6 μmol l^{-1} Oxonol V and 100–150 μg of protein (pH 8.0). The reaction was started by the addition of 1 mmol l^{-1} Tris–ATP. The membrane potentials responsible for the resulting fluorescence quenches were dissipated with 2 μg ml^{-1} gramicidin D.

1 μmol l^{-1} bafilomycin A_1 was used to inhibit the effects of V-ATPases on both proton transport and membrane potential generation (Bowman *et al.* 1988).

Electrophysiology

Split lamellae of posterior gills were obtained according to Schwarz and Graszynski (1989). The resulting flat epithelial sheets were mounted in a modified Ussing-type chamber (with an epithelial area of 0.0079 cm^2 exposed to the bathing solutions). Permanent perfusion of the external and internal chamber compartments (each 0.1 cm^3) with aerated salines was achieved by gravity flow (maximal flow rate: 125 bath volumes per minute). The basic, haemolymph-like NaCl saline consisted of (in mmol l^{-1}): 300, NaCl; 8, KCl; 2, $NaHCO_3$; 5, Hepes; 8, calcium gluconate; and 2, glucose (pH 7.6; adjusted with Tris). Measurement of transepithelial short-circuit current (I_{sc}) and conductance (G_{te}) was carried out according to Onken *et al.* (1991).

Chemical reagents

All reagents were of analytical grade. All substances not mentioned below were purchased from Merck. Acridine Orange, D-gluconolactone, EDTA, EGTA, gramicidin D, sodium vanadate and Tris–ATP were obtained from Sigma. Mercaptoethanol and ouabain were purchased from Serva. Oxonol V was from Molecular Probes. Bafilomycin A_1 was obtained from Dr K. Altendorf (University of Osnabrück, Germany). The substance was presolved in dimethylsulphoxide (DMSO; 100 mmol l^{-1}). At the final working concentration, DMSO alone had no effects on any measured variable. The concentration of bafilomycin A_1 was determined by spectrophotometry according to Bowman *et al.* (1988).

Statistics

All results are given as mean values ± standard error of the mean (S.E.M.). Differences between groups were tested with the paired Student's *t*-test. Statistical significance was assumed for $P<0.05$.

Results

ATPase activities in the gills of the Chinese crab

In eight experiments, we measured protein content and the activities of Na^+/K^+-ATPase, F_1F_o-ATPase and V-ATPase in the posterior gills of Chinese crabs, which are involved in active ion absorption (Péqueux *et al.* 1988). In three experiments, we determined the same variables in the anterior gills, which are not involved in ion transport (Péqueux *et al.* 1988).

The results, which are given as the specific activity per milligram protein and the quantity of ATPase activity per gram fresh mass, are summarized in Table 1.

The greater activity of each ATPase in the posterior gills seems to reflect their role in ion transport. The ratios between ATPase activities per gram fresh mass in the homogenates of posterior and anterior gills are 7.5 for the Na^+/K^+-ATPase, 6.2 for the V-ATPase and 41.2 for the F_1F_o-ATPase. The ratios of specific activities are lower, 2.4 for Na^+/K^+-ATPase, 2.0 for V-ATPase and 13.3 for F_1F_o-ATPase, because the protein content in the posterior gill preparation was found to be markedly higher than in the anterior gill preparation.

Results from the differential, two-step sucrose gradient centrifugation showed that the protein content and the level of ATPase activity of the M_1 and M_2 fractions of the posterior gills exceeded those of the anterior gills. For example, the V-ATPase activity in fraction M_2 of posterior gills was found to be about 130 times higher than in the same fraction of anterior gills. A comparison of the specific activity of the Na^+/K^+-ATPase in the homogenate with that in the M_1 and M_2 fractions showed that the main enrichment was in the M_1 fraction, where the ratio of activity in the homogenate to activity in the M_1 fraction was 1:4.3 for posterior gills and 1:10.6 for anterior gills. In contrast, the specific activity of the V-ATPase was predominantly enriched in the M_2 fraction, where enrichment factors of 5.0 for posterior gills and 2.0 for anterior gills were obtained. No enrichment of the F_1F_o-ATPase activity was found in fractions M_1 or M_2 of posterior gills, although fraction

Table 1. *ATPase activities and protein content of homogenates (H) and fractions M_1 and M_2 of posterior and anterior gills of Chinese crabs acclimated to fresh water*

	Na^+/K^+-ATPase		V-ATPase		F_1F_o-ATPase		
Preparations	(U mg^{-1} protein)	(U g^{-1} fresh mass)	(U mg^{-1} protein)	(U g^{-1} fresh mass)	(U mg^{-1} protein)	(U g^{-1} fresh mass)	Protein (mg g^{-1} fresh mass)
Posterior gill							
H	1.2±0.1	82.0±4.0	0.02±0.004	1.36±0.18	0.40±0.07	27.20±1.80	68.0±4.9
M_1	5.1±0.7	11.7±0.3	0.01±0.004	0.02±0.01	0.20±0.07	0.46±0.11	2.3±0.3
M_2	2.4±0.4	9.6±0.4	0.10±0.014	0.40±0.03	0.45±0.03	1.80±0.28	4.0±0.7
Anterior gill							
H	0.5±0.1	11.0±3.5	0.01±0.005	0.22±0.12	0.03±0.01	0.66±0.12	22.0±5.8
M_1	5.3±0.4	3.7±0.6	–	–	0.03±0.01	0.02±0.01	0.7±0.1
M_2	3.4±0.2	0.4±0.1	0.02±0.005	0.003±0.001	0.06±0.01	0.008±0.001	0.13±0.01

U, μmol P_i min^{-1}; –, undetectable.
Values are mean ± S.E.M., N=8 for posterior gills and N=3 for anterior gills.

M_2 of anterior gills showed some enrichment (by a factor 2) of the F_1F_o-ATPase activity. However, in this fraction, the protein content and the quantity of F_1F_o-ATPase activity are extremely low.

Acid phosphatase in posterior gills of the Chinese crab

In five experiments, we measured the activity of acid phosphatase, which is often used as a marker enzyme for lysosomes (Trouet, 1974; Maunsbach, 1974), in the homogenate and in fractions M_1 and M_2 of posterior gills. The results are summarized as specific activity and as activity per gram fresh mass (Table 2).

About 15 % of the activity of the acid phosphatase in the homogenate was found after differential centrifugation in fractions M_1 and M_2. The specific activity was enriched in M_1 by a factor of 2.1, while in M_2 no significant enrichment was found.

ATP-dependent H^+ transport in membrane vesicles

We used fraction M_2 to measure ATP-dependent H^+ transport. When vesicles of posterior gills were added to a solution containing Mg–ATP, we observed a biphasic quenching of fluorescence (Fig. 1, line *a*). An instantaneous quenching was followed by an exponential one. The fluorescence quenching was maximal (approximately 30 %) after about 1 min. Some 75 % of the fluorescence quenching could be dissipated by addition of 3 mmol l^{-1} NH_4Cl, indicating that this portion reflects a fluorescence quenching of Acridine Orange caused by vesicle acidification. The remaining fluorescence quenching (25 %), which could not be dissipated by NH_4Cl, appears to be identical to the instantaneous fluorescence quenching that occurs immediately after the addition of vesicles, indicating that the presence of the vesicles was responsible for this element of the fluorescence quenching. When we repeated the same experiment in a solution containing 1 μmol l^{-1} bafilomycin A_1 (Fig. 1, line *b*), the quenching and dissipation with NH_4Cl were markedly smaller, indicating that a major portion of vesicle acidification has been inhibited. In five experiments, 1 μmol l^{-1} bafilomycin reduced the overall fluorescence quenching by 60±5 % and its dissipation with NH_4Cl by 84±3 %. Although ouabain was without effect on vesicle acidification, sodium azide reduced it by approximately 15 % (not shown). Thus, ATP-dependent H^+ transport in membrane vesicles obtained from posterior gills of the Chinese crab is due to the activity of a V-type ATPase. In three experiments, we used vesicles of the M_2 fraction of anterior gills and observed only a 10 % fluorescence quenching, which could not be dissipated with NH_4Cl. This result is consistent with our finding that the quantity of V-type ATPase in anterior gills is much lower than that in posterior gills (see above).

Table 2. *Activity of acid phosphatase of homogenate (H) and fractions M_1 and M_2 of posterior gills of Chinese crabs acclimated to fresh water*

	Acid phosphatase	
Preparation	(U mg^{-1} protein)	(U g^{-1} fresh mass)
H	0.04±0.0005	2.72±0.11
M_1	0.085±0.005	0.2±0.01
M_2	0.05±0.0005	0.2±0.01

U, μmol nitrophenol min^{-1}.
Values are mean ± S.E.M., N=5.

ATP-dependent membrane potential generation in membrane vesicles

The experiments described in this section were only carried out on fraction M_2 of the posterior gills. When we added ATP to a solution containing vesicles of this fraction in the appropriate assay buffer (see Materials and methods), we observed a fast fluorescence quenching of Oxonol V (Fig. 2, line *a*), indicating a net increase of positive charge in the vesicle interior. The maximal fluorescence quenching of about 25 % was reached within 40–50 s. The electrical potential across the vesicle membrane could be dissipated with the

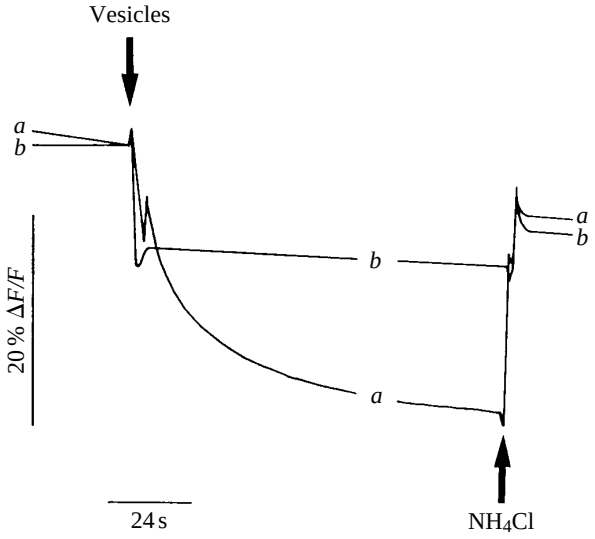

Fig. 1. A representative example showing the time course of ATP-dependent H^+ transport by membrane vesicles of posterior gills (fraction M_2) determined by the fluorescence quenching of Acridine Orange. Line *a*, standard assay in the absence of bafilomycin A_1. Line *b*, standard assay in the presence of 1 μmol l^{-1} bafilomycin A_1. The left-hand arrow indicates the time when vesicles were added (start of the reaction). The right-hand arrow indicates the addition of 3 mmol l^{-1} NH_4Cl (final concentration). $\Delta F/F$, fluorescence quenching.

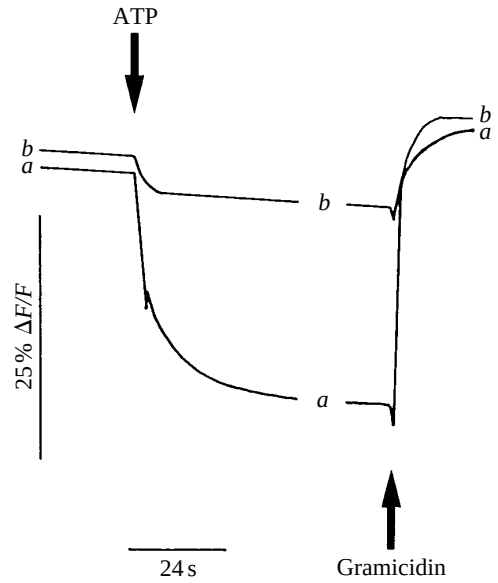

Fig. 2. A representative example showing the time course of ATP-dependent membrane potential generation by membrane vesicles of posterior gills (fraction M_2) determined by the fluorescence quenching of Oxonol V. Line *a*, standard assay in the absence of bafilomycin A_1. Line *b*, standard assay in the presence of 1 μmol l^{-1} bafilomycin A_1. The left-hand arrow indicates the time when ATP was added (start of the reaction). The right-hand arrow indicates the addition of 2 μg ml^{-1} gramicidin (final concentration).

ionophore gramicidin. When we conducted the same experiment in the presence of 1 μmol l^{-1} bafilomycin A_1, the fluorescence quenching and the dissipation were much smaller (Fig. 2, line *b*), indicating that membrane potential generation under control conditions was due to the activity of a V-type ATPase. In three experiments, 1 μmol l^{-1} bafilomycin reduced the fluorescence quenching by 50±6 % and its dissipation by 60±6 %.

Bafilomycin inhibits electrogenic chloride absorption

After mounting split lamella preparations in a modified Ussing-type chamber, we measured a negative short-circuit current (I_{sc}) when both sides of the epithelium were perfused with haemolymph-like NaCl saline. In agreement with former findings (Onken *et al.* 1991), this current became slightly more negative when we substituted Na^+ (using choline chloride and $KHCO_3$ instead of NaCl and $NaHCO_3$, respectively) in the external bath. The negative I_{sc} under these conditions was shown to reflect active, transcellular Cl^- uptake (Onken *et al.* 1991). In the present experiments, we confirmed that the negative I_{sc} was Cl^--dependent, was reduced by internal diphenylamine-2-carboxylate and was unaffected by internal ouabain (see Fig. 3A), before bafilomycin A_1 was added to the external bath. In five experiments with 1 μmol l^{-1} bafilomycin A_1, we observed a decrease of the negative I_{sc} by 43 % from -88 ± 16 to -50 ± 12 μA cm^{-2}. Simultaneously, the conductance of the preparation (G_{te}) decreased by 13 % from 3.64±0.59 to 3.18±0.56 mS cm^{-2}. The decreases in I_{sc} and G_{te} were both statistically significant ($P<0.05$). In Fig. 3B, two examples demonstrating the variation in the time course of the decrease in I_{sc} after external addition of bafilomycin are shown. In the left-hand example, 1 μmol l^{-1} bafilomycin caused a rapid reduction in I_{sc} of about 35 % which was, at least in part, reversible. In the right-hand example, 0.1 μmol l^{-1} bafilomycin was tested (causing a 34 % inhibition of I_{sc}) before the concentration was increased to 1 μmol l^{-1} (causing a 58 % inhibition of I_{sc}). In this experiment, the effects of the drug were slow, leading to stable reduced current levels after about 30 min. Although, in this example, the additional inhibitory effect of cyanide is shown instead of a washout, I_{sc} never recovered when the effect of bafilomycin was slow.

Discussion

V-ATPases are widely distributed and have diverse physiological functions (W. R. Harvey, 1992). Besides their role in pH regulation (transepithelial, contradirectional translocation of H^+ and base), which has been shown in a variety of tissues (e.g. Dow, 1992; Gluck and Nelson, 1992), V-ATPases of epithelial plasma membranes have been shown to energize a number of different processes related to the homoeostasis of ions and water in a variety of animals. In the midgut of plant-feeding (high K^+ load) insect larvae, an apical V-ATPase energizes K^+ secretion *via* basolateral K^+ channels and apical K^+/H^+ antiport (Wieczorek *et al.* 1991; Wieczorek, 1992; Zeiske, 1992; Schirmanns and Zeiske, 1994). Insects counterbalance a salt and water load by secretion across their Malpighian tubule epithelia. An apical V-ATPase plays a

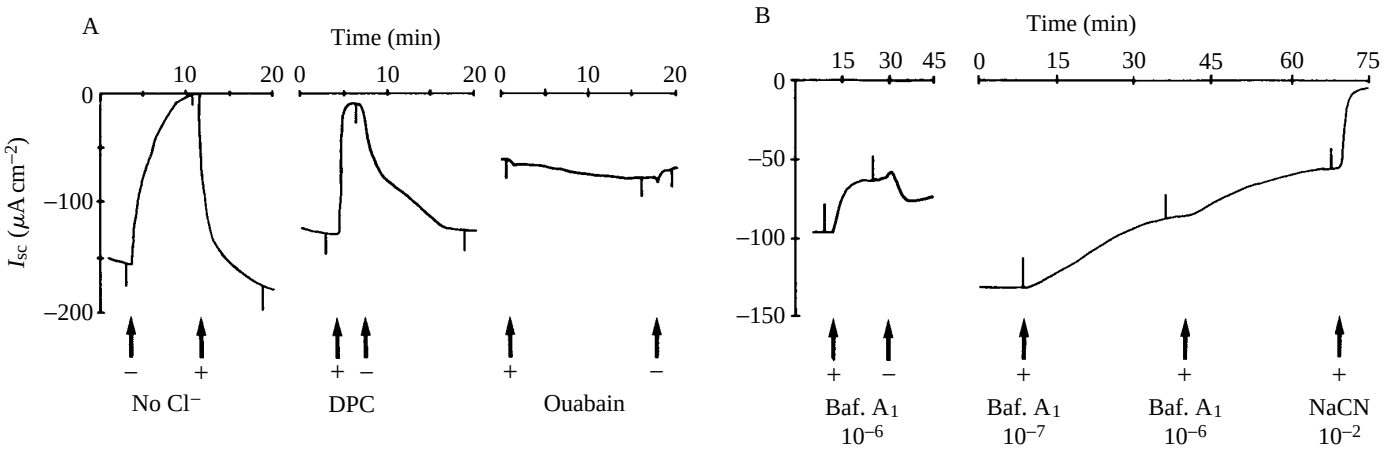

Fig. 3. Time courses of the negative short-circuit current (I_{sc}) across split lamella preparations with Na^+-free (choline substituted for Na^+) saline in the external bath (internal bath containing haemolymph-like NaCl saline). The vertical current deflections are induced by 5 mV voltage pulses and are proportional to the conductance of the preparation. (A) Typical influences of substitution of Cl^- (gluconate) in the external bath, internal addition of 1.25 mmol l^{-1} diphenylamine-2-carboxylic acid (DPC) and internal addition of 2 mmol l^{-1} ouabain. (B) Two examples showing the effects of bafilomycin A_1 (Baf. A_1, concentrations in mol l^{-1}) added to the external bath and the effect of sodium cyanide.

dominant role in this process (Bertram *et al.* 1991; Maddrell and O'Donnell, 1992; Weltens *et al.* 1992). In the frog skin, ATP-dependent H^+ transport with the characteristics of a V-ATPase was found to support absorption of Na^+ under *in vivo* conditions (Ehrenfeld *et al.* 1990; B. J. Harvey, 1992). A similar mechanism has been proposed for salt absorbance in freshwater fish (Avella and Bornancin, 1990).

In the present investigation, we have demonstrated the presence of ATPase activity in the gills of Chinese crabs. The ATPase is sensitive to bafilomycin A_1 (see Table 1) which, at the concentration used (1 μmol l^{-1}), is a highly specific inhibitor of V-ATPases. A marked effect on other ATPases has only been found at far higher doses (Bowman *et al.* 1988). Therefore, we conclude that the bafilomycin-sensitive ATPase activity in the gills of the Chinese crab reflects the presence of a V-ATPase.

Our finding of a significantly higher activity of the Na^+/K^+-ATPase in posterior gills than in anterior gills (see Table 1) is in agreement with earlier results obtained on the same tissue (Péqueux *et al.* 1988). This distribution was interpreted as reflecting the physiological function of the respective gills. Posterior gills are involved in active salt absorption, which was thought to be driven solely by the Na^+/K^+-ATPase, while anterior gills are not involved in salt absorption but seem to be the main site of respiration (Péqueux *et al.* 1988). The high protein content of the posterior gills supports this picture of functional diversity. Moreover, the distribution of F_1F_o-ATPase activity also correlates with the results obtained from microscopic studies of *Eriocheir sinensis* gills. In the posterior, salt-absorbing gills there is an abundance of mitochondria, while the anterior gill epithelium is rather thin and the cells contain few mitochondria (Péqueux *et al.* 1988). V-ATPase activity shows the same distribution between posterior and anterior gills as the Na^+/K^+-ATPase and the mitochondrial F_1F_o-ATPase (see Table 1), which suggests that the V-ATPase may be involved in salt absorption.

Differential centrifugation on a two-step sucrose gradient resulted in only a moderate separation of the different enzymes (see Tables 1 and 2). However, the results allow some cautious conclusions to be drawn about the localization of the V-ATPase. The M_1 fraction seems to contain membranes of predominantly basolateral origin, with a marked enrichment of Na^+/K^+-ATPase activity. The activity of the V-ATPase was very low in fraction M_1, but was enriched in fraction M_2. Thus, the V-ATPase did not copurify with the Na^+/K^+-ATPase, suggesting that the major portion of V-ATPase is not a component of the basolateral membrane. The F_1F_o-ATPase was not significantly enriched in fractions M_1 or M_2 and the acidic phosphatase was slightly enriched in fraction M_1, suggesting that the V-ATPase activity of the M_2 fraction is not of mitochondrial or lysosomal origin. It seems unlikely that other organelles (e.g. the Golgi apparatus) with a general cellular function are a major source of the V-ATPase activity, because we found only low V-ATPase activities in the anterior gills. As discussed above, the V-ATPase activity seems to be related to transepithelial salt absorption. Thus, after ruling out a major basolateral or intracellular localization for the V-ATPase, it is reasonable to assume that at least a portion of the V-ATPase is located in the apical plasma membrane, as has been proposed for other salt-absorbing (Ehrenfeld *et al.* 1990; Avella and Bornancin, 1990) and salt-secreting (Wieczorek *et al.* 1991; Bertram *et al.* 1991) epithelia. However, more definitive results, for example a co-purification of the V-ATPase with an apical marker enzyme, are needed to support this hypothesis.

Besides their sensitivity to bafilomycin A_1, the dominant characteristic of V-ATPases is that they mediate electrogenic H^+ translocation. The results of our experiments with membrane vesicles using the fluorescent dyes Acridine Orange (vesicle acidification, see Fig. 1) and Oxonol V (membrane potential generation, see Fig. 2) demonstrate that these characteristics also apply to the bafilomycin-sensitive ATPase in the posterior gills of the Chinese crab.

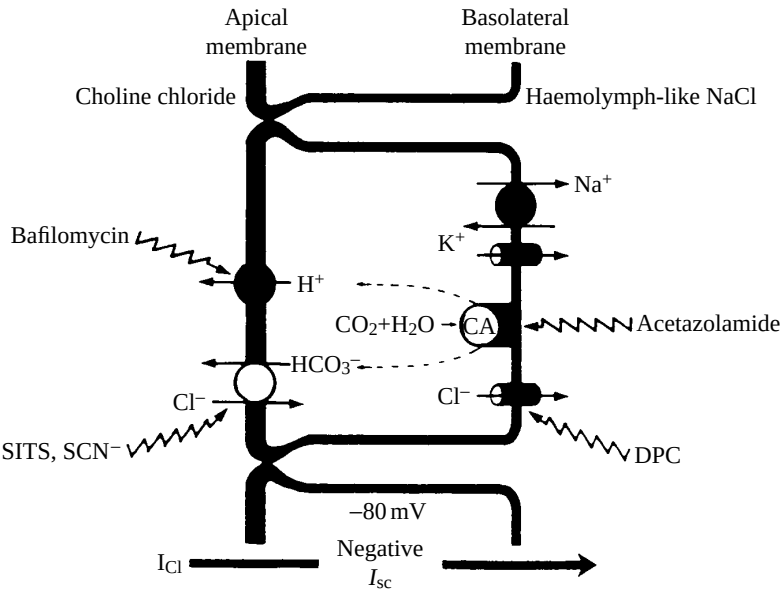

Fig. 4. Model of active, electrogenic and Na^+-independent Cl^- absorption across the posterior gills of freshwater-adapted Chinese crabs (see also Onken *et al.* 1991). Negative I_{sc} is carried *via* the apical, bafilomycin-sensitive H^+-ATPase and basolateral, DPC-sensitive Cl^- channels. Transapical Cl^- absorption proceeds *via* SITS- and SCN^--sensitive Cl^-/HCO_3^- antiport. The acetazolamide-sensitive carbonic anhydrase (CA) provides cellular H^+ and HCO_3^-. Although not directly dependent on a functioning Na^+/K^+-ATPase, this ion pump stimulates the chloride current, I_{Cl}, by its influence on the cellular electrical potential (*via* the generation and conservation of a K^+ concentration gradient across the basolateral membrane). DPC, diphenylamine-2-carboxylic acid; SITS, 4-acetamido-4′-isothiocyanato-stilbene-2,2-disulphonic acid; I_{sc}, short-circuit current.

The results of our biochemical experiments provide no information about the physiological role of the V-ATPase in the gills of the Chinese crab. However, the distribution of the bafilomycin-sensitive ATPase activity between the posterior and anterior gills suggests that this enzyme is involved in salt absorption across the posterior gills. Indeed, the results of our electrophysiological experiments confirm this suggestion and support the assumption of a predominantly apical location for the V-ATPase. External bafilomycin A_1 significantly reduced the negative I_{sc} (see Fig. 3B), which has been shown to reflect active and electrogenic absorption of Cl^- (Onken *et al.* 1991), and the transepithelial conductance. In fact, $1\,\mu mol\,l^{-1}$ bafilomycin did not abolish the negative I_{sc}, as might have been expected from the affinity of the inhibitor for the enzyme in biochemical tests (Figs 1, 2; see also Bowman *et al.* 1988). However, the effective dose of bafilomycin on V-ATPases in intact epithelia seems to be considerably higher (Bertram *et al.* 1991; Weltens *et al.* 1992; Schirmanns and Zeiske, 1994). With respect to the time course and reversibility of the I_{sc} inhibition by bafilomycin, our results show differences between preparations (see Fig. 3B), suggesting that a barrier with variable permeability to bafilomycin influences the access of the drug to its site of action. In this respect, it is worth noting that the epithelium is covered with cuticle on the apical side, and this might be responsible for these variations. The rapid onset of the I_{sc} reduction after external application of bafilomycin (see Fig. 3B, left-hand example) is compatible with the assumption that the V-ATPase, which drives Cl^- uptake, is located in the apical membrane. This interpretation is supported by the finding of a significant decrease in net influxes of Cl^- across isolated and perfused gills after external application of bafilomycin (Riestenpatt *et al.* 1993).

Considerable support has accumulated for a previous proposal that an apical H^+-ATPase drives active, electrogenic and Na^+-independent Cl^- absorption across the gill epithelium of Chinese crabs acclimated to fresh water. In the model shown in Fig. 4, the H^+ pump supports transcellular Cl^- absorption by maintaining a high, outwardly directed, transapical HCO_3^- gradient that drives Cl^- uptake *via* Cl^-/HCO_3^- antiport in the apical membrane and, by hyperpolarizing the cellular potential, drives Cl^- through channels across the basolateral membrane (see also Onken *et al.* 1991). It is only if the H^+-ATPase that pumps H^+ from the cytosol to the external bath is located apically that the finding of an outside-positive potential (reflected by the negative I_{sc}) for the epithelium makes sense. Moreover, an apical H^+ pump might, as in frog skin (Ehrenfeld *et al.* 1990; B. J. Harvey, 1992), support transapical Na^+ uptake *via* channels (Zeiske *et al.* 1992) under open-circuit conditions at low, *in-vivo*-like ambient ion concentrations. Lastly, this transepithelial electrical potential difference, which is generated under open-circuit conditions (see Onken and Graszynski, 1989), might be used to drive ion movements along the paracellular pathway. This transport model (Fig. 4) is not unique for the gill epithelium of freshwater-adapted Chinese crabs. Using different experimental approaches, the same mechanism was proposed for active Cl^- uptake by the so-called gamma-type mitochondria-rich cells of the amphibian skin (Larsen, 1991; Larsen *et al.* 1992).

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