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Ion transport across posterior gills of hyperosmoregulating shore crabs (*Carcinus maenas*): amiloride blocks the cuticular Na^+ conductance and induces current-noise

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Summary

Split gill lamellae and gill cuticles of shore crabs (*Carcinus maenas*) adapted to 10 ‰ salinity were mounted in a modified Ussing-type chamber. With NaCl saline on both sides, split gill lamellae generated a short-circuit current (I_{sc}) of –301±16 μA cm^{-2} at a conductance (G_{te}) of 40±2 mS cm^{-2}. The net influxes of Na^+ and Cl^- were 8.3±2.6 and 18.2±2.7 μmol cm^{-2} h^{-1}, respectively. External amiloride (100 μmol l^{-1}) reduced G_{te} to approximately 50 % of the original value at unchanged I_{sc}; Cl^- fluxes remained unaffected, whereas Na^+ fluxes were markedly reduced by 70–80 %. The I_{sc} in the presence of external amiloride was almost completely inhibited by internal ouabain. At a clamp voltage of 50 mV (outside-positive), a positive current was measured at unchanged G_{te}. Under these conditions, amiloride reduced the current and conductance at half-maximal concentrations of 3.6 and 2.0 μmol l^{-1}, respectively. At outside-positive voltages, but not under short-circuit conditions, external amiloride induced Lorentzian components in the power density spectra. The amiloride-dependent changes in the corner frequency (linear) and of the low-frequency plateau ('bell-shaped') were as expected for channel blockade by amiloride with pseudo-first-order kinetics. With an outside-positive clamp voltage of 50 mV across isolated cuticles, a positive cuticular current (I_{cut}) of 25 188±3791 μA cm^{-2} and a cuticular conductance (G_{cut}) of 547±76 mS cm^{-2} were measured. External amiloride reduced I_{cut} and G_{cut} at half-maximal concentrations of 0.7 and 0.6 μmol l^{-1}, respectively. Amiloride-induced current-noise analysis gave similar results to those observed with split gill lamellae. Ion-substitution experiments with isolated cuticles further support inhibition by external amiloride of the cuticular Na^+ conductance of shore crab gills and not amiloride-sensitive transporters (Na^+ channels or Na^+/H^+ antiports) in the apical membrane.

Key words: amiloride, shore crab, *Carcinus maenas*, conductance, gills, cuticle, ion flux, NaCl absorption, short-circuit current, current-noise analysis, Ussing chamber.

Introduction

The gills of the shore crab *Carcinus maenas* have been used in a variety of studies to investigate transbranchial NaCl absorption in hyperosmoregulating crabs (see Péqueux et al., 1989; Péqueux, 1995; Onken and Riestenpatt, 1998). When acclimated to diluted sea water, shore crabs compensate for passive salt losses across the body surface with an active, coupled and Na^+/K^+ ATPase-dependent absorption of Na^+ and Cl^- *via* the posterior gills (Siebers et al., 1987; Lucu, 1990). The most recent model for this active NaCl absorption is based on simultaneous measurements of tracer fluxes and of transepithelial short-circuit currents (I_{sc}) and conductances (G_{te}) across split gill lamellae mounted in a modified Ussing-type chamber (Riestenpatt et al., 1996). The results indicated a similar mode of NaCl absorption to that described for the thick ascending limb of Henle's loop (TAL) in the mammalian nephron (Molony et al., 1989; Greger and Kunzelmann, 1990).

The electrogenic and coupled absorption of Na^+ and Cl^- seems to proceed *via* apical $Na^+/K^+/2Cl^-$ cotransport and basolateral Cl^- channels and Na^+/K^+-ATPases. Transcellular current flow occurs *via* apical K^+ channels and basolateral Cl^- channels. The presence of additional transapical pathways for NaCl absorption, such as Na^+/H^+ and Cl^-/HCO_3^- antiports, could not be confirmed in this study. Active Cl^- absorption was found to be directly related to the negative I_{sc}, suggesting the absence of any electroneutral NaCl absorption *via* parallel cation and anion antiports. The latter interpretation is also consistent with the finding that blockers of carbonic anhydrase inhibit neither Cl^- absorption across isolated and perfused gills (Böttcher et al., 1991) nor the negative I_{sc} (Onken and Siebers, 1992).

Amiloride has long been used as a probe for epithelial Na^+ channels and Na^+/H^+ antiports in a variety of Na^+-absorbing epithelial tissues (Benos, 1982; Garty and Benos, 1988). In two studies, external amiloride was observed to affect the transport characteristics of the gills of shore crabs. Na^+ fluxes, but not Cl^- fluxes, across isolated perfused gills were inhibited by external amiloride in a dose-dependent manner (K_{Ami}=40–70 μmol l^{-1}), and the inward negative transbranchial potential difference (PD_{te}) became hyperpolarised (K_{Ami}=50 μmol l^{-1}) (Lucu and Siebers, 1986; Siebers et al., 1987). It was proposed that these results reflected Na^+ absorption *via* apical Na^+/H^+ antiport. It has also been suggested that a passive, conductive and amiloride-sensitive paracellular pathway is at least partly responsible for the observed effects of external amiloride (Siebers et al., 1987, 1989). In a study of split gill lamellae (Onken and Siebers, 1992), the negative I_{sc} was increased to more negative values by the addition of external amiloride (K_{Ami}=10 μmol l^{-1}), and the transepithelial resistance (R_{te}) increased simultaneously. These results led to the proposal of an electrogenic Na^+ uptake *via* apical Na^+ channels or electrogenic $2Na^+/1H^+$ antiports in addition to symporter-mediated NaCl absorption (see above). In fact, in gill membrane vesicles of the shore crab, an electrogenic, amiloride-sensitive (K_{Ami}=280 μmol l^{-1}) $2Na^+/1H^+$ antiporter was identified (Shetlar and Towle, 1989). In addition to these effects at the cellular level, the isolated gill cuticle was demonstrated to be amiloride-sensitive: at a concentration of 1 mmol l^{-1}, the diuretic reduced the conductance of the cuticle of *Carcinus maenas* (Lignon and Péqueux, 1990). Thus, the location of the amiloride-sensitive site in the gills of the shore crab remains unclear.

In the present investigation, we focus on the effects of amiloride on NaCl absorption across split gill lamellae and on the amiloride-sensitivity of the cuticle. Our results indicate that the amiloride-induced changes in PD_{te}, G_{te}, I_{sc} and Na^+ fluxes are based on the inhibition of Na^+ movements across the cuticle, rather than the interaction between the drug and ion-transport proteins in the apical membrane.

Materials and methods

Crabs

Shore crabs (*Carcinus maenas* L.) were caught by commercial fishermen in Kiel Bay (Germany, Baltic Sea). Before experimental use, the crabs were kept at 16 °C for at least 1 month in diluted sea water (10 ‰ salinity) that was continuously aerated and filtered. The animals were fed three times a week with pieces of bovine heart.

Preparations

When the crabs had been killed by destroying their ventral ganglion by pressing a needle through the ventral side of the body wall and lifting the carapace, the three posterior gills were removed. Single gill lamellae, consisting of the gill epithelium and the adherent apical cuticle, were isolated and split according to the method described by Schwarz and Graszynski (1989). Isolated cuticles were obtained by mechanically peeling off the epithelium (Lignon, 1987). Separation of cuticle and epithelium could be easily controlled under the microscope since cuticle and epithelium differ in colour under the light of a halogen cold light. Split gill lamellae or isolated cuticles were mounted in an Ussing-type chamber modified after De Wolf and Van Driessche (1986) with an epithelial area of 0.02 cm^2 or 0.01 cm^2. To minimise edge damage, silicone grease was used. The chamber compartments (50 μl) were continuously perfused with salines by gravity flow (approximately 2 ml min^{-1}) or by means of a peristaltic pump (0.5 ml min^{-1}, to measure fluxes of radioactive tracers).

Salines and chemicals

The haemolymph-like saline used was composed of (mmol l^{-1}): 248 NaCl, 5 KCl, 2 $NaHCO_3$, 4 $MgCl_2$, 5 $CaCl_2$, 5 Hepes and 2 glucose. Immediately before use, the pH was adjusted to 7.7 with Tris. To prepare Cl^--free saline, the respective gluconates were used. In Na^+-free salines, NaCl was substituted with choline chloride, $KHCO_3$ was used instead of $NaHCO_3$ and KCl concentration was reduced to 3 mmol l^{-1}. Ouabain was purchased from Fluka. Amiloride was a gift from Merck, Sharp and Dohme (München, Germany).

Electrophysiological measurements

To measure the transepithelial potential difference (PD_{te}), calomel electrodes were connected *via* agar bridges (3 % agar in 3 mol l^{-1} KCl) with the chamber compartments (distance to the preparation <0.1 cm). The reference electrode was in the basolateral bath. Ag/AgCl electrodes served as current electrodes to short-circuit the preparation (measurement of short-circuit current, I^*_{sc}) using an automatic clamping device (VCC 600, Physiologic Instruments, San Diego, CA, USA). The area-specific resistance between the tips of the voltage electrodes (R_{tot}) was calculated from small imposed voltage pulses (ΔPD_{te}) and the resulting current deflections (ΔI). R_{tot} is the sum of the serial resistances of the solutions (R_s) and the tissue (R_{te} or R_{cut}). Because of the low values of R_{tot}, it was necessary to correct the R_{tot} and I^*_{sc} data to obtain values directly related to the preparations (R_{te} or R_{cut}, I_{sc} or I_{cut}). R_s was measured in the absence of a preparation separating the chamber compartments and was found to be 9 Ω cm^2 for NaCl saline (N=15) and Na^+-free saline (N=8) and 13 Ω cm^2 for Cl^--free saline (N=8). The corrected values of R_{te} and R_{cut}, respectively, result from subtracting R_s from R_{tot}, while the correction of I^*_{sc} followed Ohm's law (see Riestenpatt et al., 1996). In the results, only the corrected values of area-specific I_{sc}, I_{cut}, G_{te} (=1/R_{te}) and G_{cut} (=1/R_{cut}) are given.

For the current fluctuation ('noise') analysis experiments, we used a specially constructed low-noise voltage-clamp apparatus, designed and modified after the original version of Van Driessche and Lindemann (1978). Current fluctuations were digitally recorded after passing the clamp current through a set of (anti-aliasing) high-pass and low-pass filters and after appropriate amplification at each step. Fast Fourier analysis of

the current fluctuations yields the so-called power density spectrum, a double-logarithmic representation of the variance of I_{sc} over the frequency (see Fig. 3 for examples). Lorentzian components in the current noise spectra were obtained by adding amiloride to the external perfusion saline. To evaluate the spectra, the two-state model was used, producing the Lorentzian parameters S_o (low-frequency plateau):

$$S_o = \frac{4 \times I_{Na(Ami)} \times i \times (1 - P_o)}{2\pi f_c} \quad (1)$$

and f_c (corner frequency):

$$2\pi f_c = (k_{01} \times c_{Ami}) + k_{10}, \quad (2)$$

where $I_{Na(Ami)}$ is the amiloride-sensitive Na^+ current in the presence of submaximal concentration of amiloride, i is the single-channel current, P_o is the channel open probability, k_{01} and k_{10} are the association and dissociation rate constants, respectively, of the channel/amiloride interaction and c_{Ami} is the amiloride concentration. The channel open probability P_o:

$$P_o = \frac{k_{10}}{2\pi f_c} \quad (3)$$

was determined using values of k obtained from the linear $2\pi f_c/c_{Ami}$ plots or by using:

$$P_o = \frac{I_{Na(Ami)}}{I_{Na(Ctrl)}}, \quad (4)$$

where $I_{Na(Ctrl)}$ is the overall amiloride-blockable (100 μmol l^{-1}) current. The two procedures resulted in similar P_o values. To calculate the single-channel current, equation 1 was solved for i. To determine the number of channels per cm^2 (M), we used:

$$M = \frac{I_{Na(Ami)}}{i \times P_o}. \quad (5)$$

For further details, see Zeiske et al. (1992).

Measurements of unidirectional NaCl fluxes

Radioactive isotopes, ^{36}Cl (ICN) and ^{22}Na (NEN, Dupont), were used at a final activity of 1 mCi l^{-1} (1 Ci=3×10^{10} Bq). Unidirectional influxes ($J_{a\rightarrow b}$) or effluxes ($J_{b\rightarrow a}$) were measured over a period of 60 min in a closed perfusion circuit (5 ml in each chamber compartment) allowing the accumulation of radioactivity in the superfusate. Net influxes of the respective ions were calculated as the differences between the means of $J_{a\rightarrow b}$ and $J_{b\rightarrow a}$. The radioactivity of ^{36}Cl contained in 2 ml samples was determined with a PRIAS liquid scintillation counter (Packard; model PLD) after addition of 4 ml of Insta Gel (Packard; no. 6013008). The radioactivity of ^{22}Na was measured in 2 ml samples using the same procedure or was determined directly in 2 ml samples using a gamma spectrometer (Fischer, Hamburg). Flux data were calculated from the respective specific activities, the volume of the perfusion compartment (5 ml) and time (1 h) and expressed as μmol h^{-1} cm^{-2}. Influxes and effluxes of ^{22}Na and ^{36}Cl were measured in separate experiments. It was impossible to measure influxes and effluxes in the same preparation since a complete wash-out of the radioactivity required an incubation of more than 3 h because of the high doses of radioactivity necessary.

Statistical analyses

All results represent means ± S.E.M. Differences between groups were tested with the paired Student's *t*-test. Significance was assumed for $P<0.05$.

Results

Split gill lamellae (gill epithelium and adherent apical cuticle)

Following the addition of amiloride (100 μmol l^{-1}) to the external NaCl saline, the mean negative I_{sc} of –301±16 μA cm^{-2} across split lamellae of posterior gills of *Carcinus maenas* was not significantly affected (–317±21 μA cm^{-2}; $P>0.05$; $N=20$). However, individual preparations responded with current increases (see Fig. 2), current decreases or unchanged currents under the influence of amiloride. G_{te} was significantly reduced in all cases (from 40±2 to 23±1 mS cm^{-2}; $P<0.05$; $N=20$). The unidirectional influxes (23.7±2.0 μmol h^{-1} cm^{-2}) and effluxes (5.5±1.8 μmol h^{-1} cm^{-2}) of Cl^- were not affected by external amiloride (21.3±2.0 and 6.8±1.0 μmol h^{-1} cm^{-2}, respectively; $P>0.05$, $N=5$; Fig. 1A). In contrast, the diuretic induced substantial decreases in the unidirectional Na^+ influxes (from 22.0±2.2 to 5.3±0.5 μmol h^{-1} cm^{-2}) and effluxes (from 13.7±1.4 to 3.7±0.5 μmol h^{-1} cm^{-2}; $P<0.05$, $N=5$; Fig. 1B). The calculated, mean Na^+ net influx decreased in the presence of amiloride from 8.3±2.6 to 1.6±0.7 μmol h^{-1} cm^{-2} ($N=5$).

When 5 mmol l^{-1} ouabain was added to the basolateral perfusion saline of the split gill lamella preparations following apical addition of amiloride (Fig. 2), I_{sc} (–294±42 μA cm^{-2}) and G_{te} (32±6 mS cm^{-2}) decreased to –44±12 μA cm^{-2} and 13±2 mS cm^{-2}, respectively ($N=5$; $P<0.05$), indicating the dependence of electrogenic ion uptake on the activity of the Na^+/K^+-ATPase also under these conditions.

Current-noise analysis was used to characterise the effect of amiloride in five split gill lamellae. Under short-circuit conditions, no Lorentzian components were observed in the presence of 5 μmol l^{-1} amiloride in the external bath. After increasing the driving force for inward movement of Na^+ by applying outside-positive clamp voltages (10–50 mV), positive currents were measured at unchanged conductances. Under these conditions, external amiloride caused a fast and reversible decrease in current and conductance, and Lorentzian components appeared in the power density spectra. Fig. 3A,B shows the dependence of the Lorentzian components in the power density spectra on the presence of external amiloride and on externally positive clamp voltages. At a clamp voltage of 50 mV, we analysed the dose-dependence of the effects of amiloride on macroscopic (current and conductance) and microscopic (corner frequency and low-frequency plateau) parameters. When applying different amiloride concentrations,

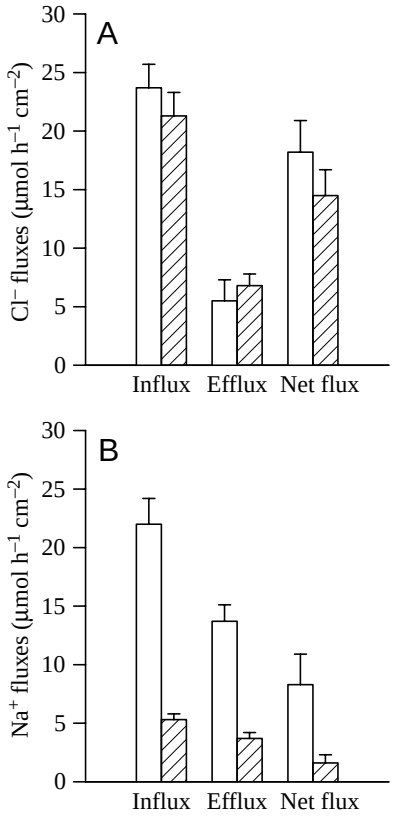

Fig. 1. The effects of external amiloride (100 μmol l^{-1}) on the fluxes of Cl^- (A) and Na^+ (B) across split lamellae of the posterior gills of shore crabs adapted to 10‰ salinity. Open columns, control values; hatched columns, after amiloride. Values are means + S.E.M. (N=5).

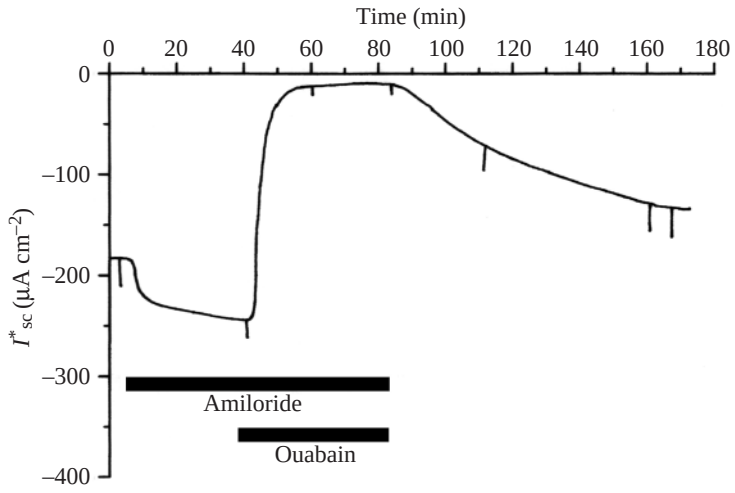

Fig. 2. Representative time course of the uncorrected short-circuit current (I^*_{sc}) across split gill lamellae of shore crabs adapted to 10‰ salinity showing that ouabain (5 mmol l^{-1}) completely inhibits the negative I^*_{sc} even in the presence of external amiloride (100 μmol l^{-1}). The vertical current deflections are due to voltage pulses (1 mV) and directly reflect the (uncorrected) conductance of the preparation in the chamber.

simple Michaelis–Menten kinetics was observed for the reductions in transepithelial current and conductance. The half-maximal decreases (K_{Ami}) in current and conductance induced by amiloride were analysed from Hanes–Woolf plots to be at 3.6±0.1 μmol l^{-1} (N=5; see Fig. 4) and 2.0±0.4 μmol l^{-1} (N=5; not shown), respectively. As theoretically predicted for a first-order rate process between blocker and ion channel (Van Driessche and Zeiske, 1980; Lindemann and Van Driessche, 1977), the plateau value (S_o) showed a 'bell-shaped' response to increasing c_{Ami} (maximum close to K_{Ami}; see Fig. 5), whereas the corner frequency increased linearly with the amiloride concentration (see Fig. 6). From the slope of the line (k_{01}=126.4±11.8 μmol^{-1} s^{-1} l) and from its intercept with the $2\pi f_c$ axis (k_{10}=70.9±2.1 s^{-1}), a K_{Ami} of 1.8±0.2 μmol l^{-1} (N=5) was calculated, which is close to the values obtained from the inhibition of macroscopic currents and conductances.

Isolated gill cuticles

Isolated cuticles of posterior gills of shore crabs were mounted in the Ussing-type chamber and perfused on both sides with haemolymph-like NaCl saline. As expected for a non-cellular system separating identical solutions, the electrical potential difference (open-circuit conditions) was 0 mV and the short-circuit current (short-circuit conditions) was 0 μA cm^{-2} (N=10). The transcuticular conductance (G_{cut}) was 583±71 mS cm^{-2} (N=10). To study ion movements, the transcuticular voltage was clamped to 50 mV (positive on the external side) to drive inward-directed flow of positive (and outward-directed flow of negative) ions. Under these conditions, a positive current I_{cut} of 25 188±3791 μA cm^{-2} (N=8) and a cuticular conductance (G_{cut}) of 547±76 mS cm^{-2} (N=8) were measured. Following apical addition of 100 μmol l^{-1} amiloride to the external bath, I_{cut} decreased to 1440±237 μA cm^{-2} and G_{cut} decreased to 27±4 mS cm^{-2} (N=8; P<0.05 for both). Both effects were fast and completely reversible when amiloride was washed out.

The dose-dependence of the inhibition of I_{cut} and G_{cut} by amiloride was studied in five experiments. Stepwise increases in concentration (0.5–100 μmol l^{-1}) of amiloride in the external perfusion saline resulted in stepwise decreases in current (control value 28 530±4373 μA cm^{-2}; N=5) and conductance (control value 600±100 mS cm^{-2}). After transformation of the data into Hanes–Woolf plots, straight lines were obtained, indicating simple Michaelis–Menten kinetics for the reduction in current (Fig. 4) and conductance (data not shown) elicited by amiloride. The average half-maximal effects of the drug on I_{cut} (K_{Ami}=0.7±0.1 μmol l^{-1}; N=5) and G_{cut} (K_{Ami}=0.6±0.1 μmol^{-1}; N=5) were determined from the intercepts of the lines with the abcissa. In another set of experiments (N=5), the dose/response curves of external amiloride were measured at a clamp voltage of +10 mV. The mean K_{Ami} values were at very similar amiloride concentrations (0.9±0.1 μmol l^{-1} in both cases) to those in the experiments with a clamp voltage of 50 mV (see Fig. 4).

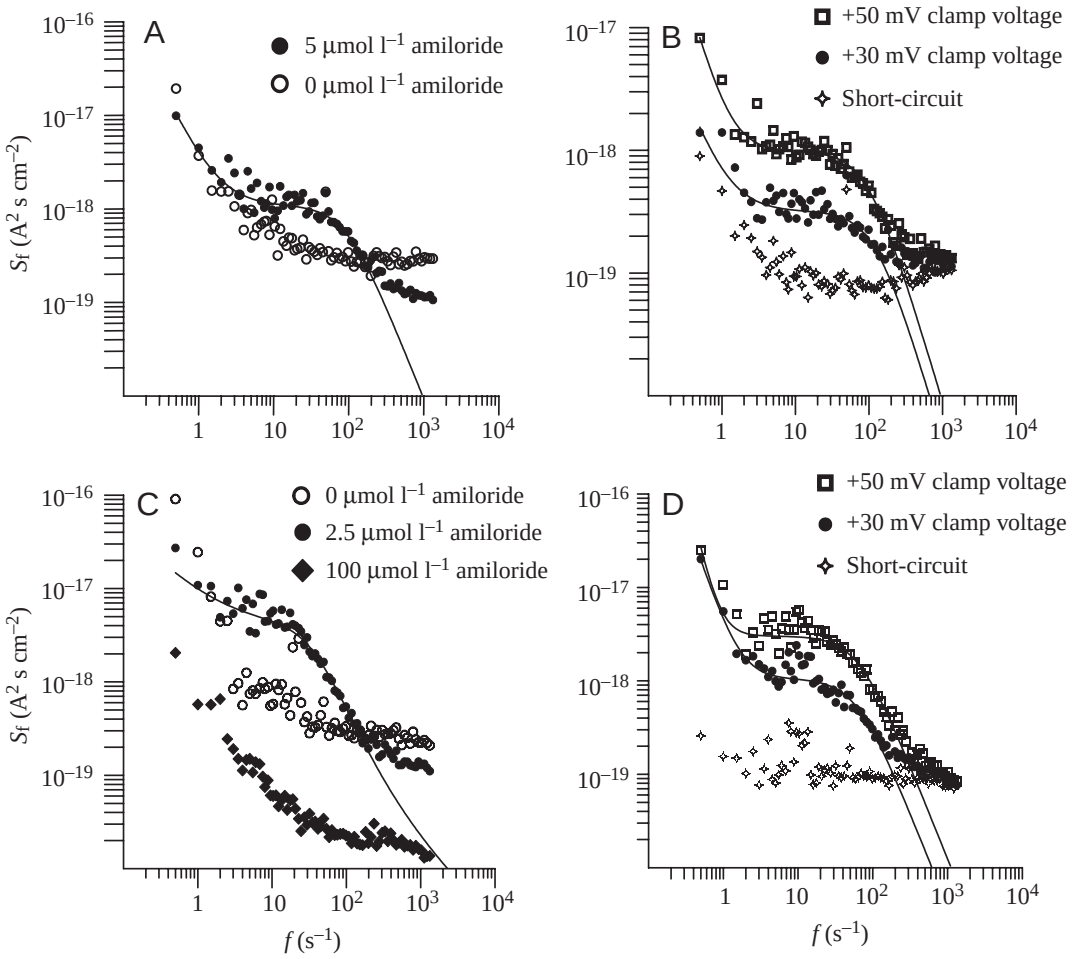

Fig. 3. Power density spectra obtained with split gill lamellae (A,B) or isolated gill cuticles (C,D) at different external amiloride concentrations (A,C; at a clamp voltage of 50 mV) and clamp voltages (B,D, at 5 μmol l^{-1} external amiloride) as indicated in the insets. S_f, power density; *f*, frequency.

Analyses of the current fluctuations (N=3) in the presence of amiloride revealed similar results to those obtained with split gill lamellae. Lorentzian components appeared in the power density spectra only when the clamp voltage was increased to 10–50 mV (outside-positive). The dependence of the Lorentzian component in the power density spectra on the presence of amiloride and the clamp voltage is shown in Fig. 3C,D. At a clamp voltage of 50 mV, changes in the Lorentzian variables S_o and $2\pi f_c$ with varying external c_{Ami} (1.25–10 μmol l^{-1}) were observed. As expected for a first-order rate process between blocker and ion channel (Van Driessche and Zeiske, 1980; Lindemann and Van Driessche, 1977), the plateau value (S_o) showed a 'bell-shaped' response to

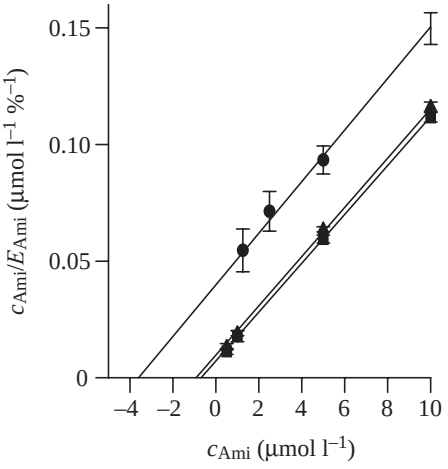

Fig. 4. Hanes–Woolf plots (concentration of amiloride, c_{Ami}, *versus* ratio of c_{Ami} to the induced change in I_{sc} as a percentage of the control current, E_{Ami}) of the influence of external amiloride on the currents across split gill lamellae at a clamp voltage of 50 mV (circles; mean 100 % control current=3635±475 μA cm^{-2}) and on the currents across isolated cuticles at clamp voltages of 50 mV (squares; mean 100 % I_{cut}=28 530±4889 μA cm^{-2}) or 10 mV (triangles; mean 100 % I_{cut}=4782±910 μA cm^{-2}). I_{cut}, cuticular current. The lines correspond to linear regressions (r^2>0.99 in all cases) over the entire amiloride concentration range used (1.25–200 μmol l^{-1} for split gill lamellae and 0.5–100 μmol l^{-1} for isolated cuticles). Values are means ± S.E.M. (N=5 in all cases).

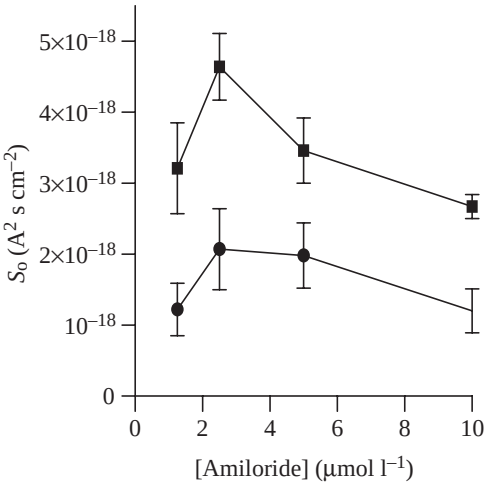

Fig. 5. The dependence of the mean Lorentzian plateau values (S_o) on increasing concentrations of external amiloride for split gill lamellae (circles; N=5) and isolated cuticles (squares; N=3) at a clamp voltage of 50 mV. Values are means ± S.E.M.

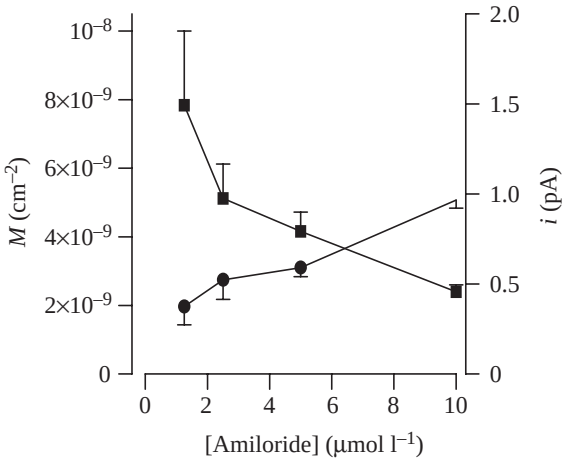

Fig. 7. The results of calculations of the 'channel' density (squares; N=3; mean + S.E.M.) and the single-'channel' current (circles; N=3; mean – S.E.M.) for isolated gill cuticles at a clamp voltage of 50 mV. Values are means ± S.E.M. i, single-'channel' current; M, the number of 'channels' per cm^2.

increasing c_{Ami} (maximum close to K_{Ami}; Fig. 5), whereas the corner frequency increased linearly with the amiloride concentration (Fig. 6). From the slope of the line (k_{01}=110.8±6.3 µmol^{-1} s^{-1} l) and from its intercept with the $2\pi f_c$ axis (k_{10}=57.8±1.1 s^{-1}), a K_{Ami} of 1.9±0.1 µmol l^{-1} (N=3) was calculated. Although true ion channels are unlikely to be present in the non-cellular cuticle, calculations of single-'channel' currents (i) and 'channel' densities (M) were conducted. An increase in i and a decrease in M were observed with increasing external amiloride concentration (Fig. 7).

Finally, experiments were conducted at a clamp voltage of 10 mV, and the effects of amiloride on the cuticular current and conductance were tested before and after substitution of Na^+ or Cl^- by choline and gluconate, respectively. The results are summarised in Table 1. In the presence of NaCl on both sides, 100 µmol l^{-1} external amiloride reduced both I_{cut} and G_{cut} by approximately 95 % (P<0.05), as was observed when dose–response curves were measured at clamp voltages of 10 and 50 mV (see above and Fig. 4). Substitution of Na^+ with choline on both sides of the isolated cuticle had an effect similar to that of external addition of amiloride, reducing cuticular current and conductance to approximately 5 % of the original values (P<0.05). Addition of amiloride in the absence of Na^+ slightly reduced G_{cut} (P<0.05), but did not significantly affect I_{cut} (P>0.05). When Cl^- was substituted with gluconate in the presence of Na^+, I_{cut} and G_{cut} were reduced by approximately 56 and 64 % (P<0.05), respectively. Subsequent addition of external amiloride reduced the cuticular current and conductance to values below 5 % of the control values in the presence of NaCl saline on both sides (P<0.05).

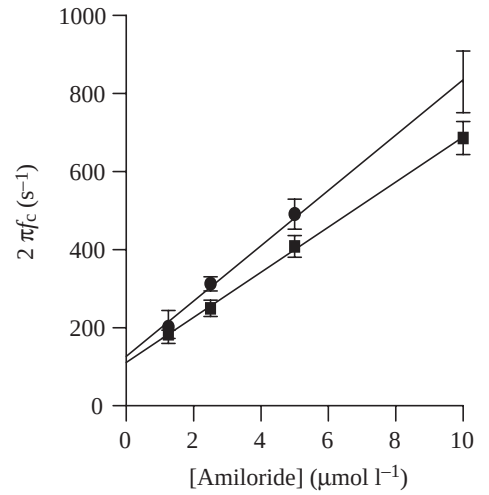

Fig. 6. The dependence of the mean values of $2\pi f_c$ on increasing concentrations of external amiloride for split gill lamellae (circles; N=5) and isolated cuticles (squares; N=3) at a clamp voltage of 50 mV. Values are means ± S.E.M. f_c, corner frequency.

Table 1. *Effects of external amiloride (100 µmol l^{-1}) before and after symmetrical ion substitutions on cuticular current and conductance in the presence of a transcuticular voltage of 10 mV (outside-positive)*

	I_{cut} (µA cm^{-2})	G_{cut} (mS cm^{-2})	N
Control	4507±604	583±71	10
Control + amiloride	268±35	29±4	10
$-Na^+_{i+o}$	191±34	28±3	5
$-Na^+_{i+o}$ + amiloride	161±35	19±4	5
$-Cl^-_{i+o}$	1965±289	212±24	5
$-Cl^-_{i+o}$ + amiloride	170±29	19±3	5

Values are means ± S.E.M.

I_{cut}, cuticular current; G_{cut}, cuticular conductance.

$-Na^+_{i+o}$, solutions lacking Na^+ on both sides (inside and outside); $-Cl^-_{i+o}$, solutions lacking Cl^- on both sides.

Discussion

Amiloride has long been a widely used inhibitor of epithelial Na^+ transport (Benos, 1982). The diuretic has been shown to block Na^+ channels, Na^+/H^+ antiports, Na^+/Ca^{2+} antiports (for references, see Garty and Benos, 1988) and even paracellular pathways (Balaban et al., 1979). Amiloride has been used in a number of studies of NaCl absorption across the posterior gills of hyperosmoregulating shore crabs (*Carcinus maenas*). A variety of effects have been observed, and their interpretation has been equally variable, suggesting interactions between the diuretic and apical Na^+/H^+ antiports (Lucu and Siebers, 1986; Siebers et al., 1987), apical Na^+ channels (Onken and Siebers, 1992), the paracellular pathway (Siebers et al., 1987, 1989) and the gill cuticle (Lignon and Péqueux, 1990). In the light of later findings (Riestenpatt, 1995; Riestenpatt et al., 1996) indicating that active NaCl absorption across shore crab gills seems to proceed exclusively in a coupled mode, as in the thick ascending limb of the mammalian nephron *via* apical, K^+-dependent NaCl cotransport (see Introduction), the effects of amiloride on the posterior gills of shore crabs needed to be reinvestigated.

The electrophysiological parameters and the flux data under control conditions measured in the present study with split gill lamellae of posterior gills of hyperosmoregulating shore crabs are very similar to the values published in previous studies of the same preparation (Onken and Siebers, 1992; Riestenpatt et al., 1996). The polarity and magnitude of the short-circuit current (I_{sc}), the magnitude of the transepithelial conductance (G_{te}) and the magnitudes of the measured unidirectional and calculated net fluxes of Na^+ and Cl^- are in the same range as observed recently. The same applies to the experiments with isolated cuticles. Given that the cuticle is cation-selective (Lignon, 1987) (see below), the cuticular conductances measured in the present study are consistent with the molar area-specific Na^+ conductance of $2.05\pm0.53\,mS\,cm^{-2}/mmol\,l^{-1}$ determined in an earlier study of the isolated gill cuticles of *Carcinus maenas* (Lignon, 1987).

In the present study, external addition of amiloride did not significantly affect the I_{sc}, but decreased the conductance of the split gill lamellae. With respect to the increased I_{sc}/G_{te} ratio, these results are consistent with findings on isolated and perfused gills, in which amiloride caused a hyperpolarization of the outside-positive transbranchial potential difference (Lucu and Siebers, 1986; Siebers et al., 1987). When studying split gill lamellae of *Carcinus maenas*, Onken and Siebers (1992) also observed a decrease in the conductance of split gill lamellae after application of external amiloride. However, in these experiments, the negative I_{sc} increased to more negative values. On the basis of this observation, the authors proposed an active, electrogenic and transcellular Na^+ absorption *via* Na^+ channels or electrogenic Na^+/H^+ antiports independent of the coupled NaCl absorption *via* apical symporters.

Amiloride-induced increases in I_{sc} were observed in approximately one-third of the preparations in the present study. However, in the remaining two-thirds, I_{sc} did not change or even decreased after the addition of amiloride. The unidirectional fluxes and the net influx of Na^+ across split gill lamellae of shore crabs were markedly reduced by amiloride, whereas Cl^- fluxes remained unchanged (Fig. 1). Similar results have been obtained with isolated and perfused gills (Lucu and Siebers, 1986; Siebers et al., 1987). These findings may suggest that external amiloride induces very complex effects at the epithelial level: blockade of active and electrogenic Na^+ absorption *via* apical Na^+ channels or electrogenic antiports and a change in Cl^- absorption from a Na^+-coupled to a Na^+-independent mode.

Na^+-independent Cl^- absorption *via* apical Cl^-/HCO_3^- antiports and basolateral Cl^- channels driven by an apical H^+ pump has been observed to generate a negative I_{sc} across split gill lamellae of the Chinese crab *Eriocheir sinensis* adapted to fresh water (Onken and Putzenlechner, 1996; Onken and Riestenpatt, 1998). This negative, Cl^--dependent I_{sc} across the gill lamellae of the Chinese crab was independent of a functioning Na^+/K^+-ATPase. In the present study, however, the negative I_{sc} across shore crab gill lamellae was almost completely blocked by ouabain, even in the presence of external amiloride (see Fig. 2). Thus, a change in Na^+-coupled Cl^- absorption to Na^+-independent Cl^- absorption had not been induced by amiloride.

A more detailed analysis of the effects of amiloride on split gill lamellae and on isolated cuticles (present study) also indicated the absence of active and electrogenic Na^+ absorption *via* Na^+ channels or electrogenic $2Na^+/1H^+$ antiports in the apical membrane. Both split lamella preparations and isolated cuticles showed very similar responses to external amiloride. At a clamp voltage of +50 mV, and thus in the presence of an increased driving force for inward Na^+ movement, the addition of the diuretic resulted in fast and reversible reductions in currents and conductances across split gill lamellae and isolated cuticles. Even the values of K_{Ami} were similar for split gill lamellae and isolated cuticle (see Results and Fig. 4). These findings indicate that the effect of amiloride is due to an interaction between the drug and the external side of the cuticle and not with transporters in the apical membrane or with the paracellular junctions. Thus, our findings clearly support the data obtained on isolated cuticle in a previous study using high amiloride concentrations (Lignon and Péqueux, 1990).

The similarities between the effects of amiloride on split gill lamellae and isolated cuticle were also observed with respect to the parameters obtained by amiloride-induced current-noise analysis. Noise analysis was used in the present study with the expectation that amiloride-induced Lorentzian components in the power density spectra would be visible only if the diuretic were to interfere with epithelial Na^+ channels. In fact, under short-circuit conditions, no Lorentzian components could be detected in the presence of amiloride. However, when the driving force for inward movement of Na^+ was increased by clamping to an outside-positive voltage, amiloride-induced Lorentzian components were clearly expressed in split gill lamellae and isolated cuticles (Fig. 3). Moreover, the amiloride-dependent shifts in the low-frequency plateau (S_o; bell-shaped) and the corner frequency

(f_c; linear) agreed perfectly with the theoretical two-state model of pseudo-first-order channel blockade (see Figs 5, 6) (cf. Lindemann and Van Driessche, 1977; Van Driessche and Zeiske, 1980). The clearly higher values of S_o for isolated cuticle (see Fig. 5) are probably due to the larger currents in these preparations in which the epithelium does not act as a series resistance. Of course, amiloride-sensitive, epithelial Na^+ channels cannot be expected to be present in a non-cellular layer such as the cuticle. Consequently, it is hardly surprising that the noise analysis data obtained in the present study also show clear differences from results obtained with Na^+ channels in epithelial tissues. The association (k_{01}) and dissociation (k_{10}) rate constants for the interaction between amiloride and its binding site, which can be determined from plots of $2\pi f_c$ *versus* c_{Ami}, seem to be considerably higher in shore crab cuticle than observed for Na^+ channels in epithelial tissues, including the gill epithelium of Chinese crabs (Helman and Kizer, 1990; Zeiske et al., 1992). Moreover, when calculating the single-'channel' current (i) and the 'channel' density (M), the changes observed with increasing blocker concentration (Fig. 7) are not consistent with the theoretical model, which assumes constancy of i and M. In this light, there appears to be little point in interpreting the measured single-channel currents and channel densities with respect to the gill cuticle. Nevertheless, to our knowledge, it is a new and important observation that a biological, but non-membraneous, barrier shows such a high degree of similarity with epithelial Na^+ channels.

Both substitution of Na^+ and addition of amiloride resulted in similar decreases in transcuticular current and conductance (Table 1), suggesting that the diuretic inhibited a Na^+ conductance. In the absence of Na^+, amiloride had only a minor effect, which may be due to the inhibition of a small permeability of the cuticle for choline (which served as substitute for Na^+) or to an effect on cuticular anion permeability. The permeability characteristics of the crustacean cuticle have been attributed to the lipoproteic, uncalcified, chitin-free epicuticle, which lacks the waterproofing wax layer of the insect cuticle (Lignon, 1987; Lignon and Péqueux, 1990). It has been proposed that the selective permeability of the epicuticle is due to specific pores discriminating between anions and cations and between particles of different size (Lignon and Péqueux, 1990). On the basis of this model, it is possible that amiloride inhibits the cation conductance of the shore crab gill cuticle in general. Nevertheless, further experiments with other cation species are needed to verify this hypothesis. The relatively large reductions in the transcuticular current and conductance after substitution of Cl^- with gluconate are puzzling and contrast with the results of a previous study of the shore crab gill cuticle (Lignon, 1987). The sum of the conductance decreases induced by wash-out of Na^+ and Cl^- ($555\pm71+371\pm75=926\pm103\,mS\,cm^{-2}$) is far larger than the conductance in the presence of NaCl ($583\pm71\,mS\,cm^{-2}$), suggesting that the presence of Cl^- has a positive influence on the permeability of the cuticle for Na^+.

How could Na^+-coupled Cl^- absorption continue after blockade of the cuticular Na^+ conductance by amiloride? First, it is important to realise that amiloride did not completely abolish the influx and efflux of Na^+ (Fig. 1). Even at the maximal dose of the diuretic, 20–30 % of the Na^+ fluxes were maintained. Moreover, the paracellular pathway of the shore crab gill epithelium seems to be cation-selective, with a high conductance ($26\pm1\,mS\,cm^{-2}$) (Riestenpatt et al., 1996). Thus, Na^+ actively absorbed from the subcuticular space might be replaced by recycling along this pathway. Such paracellular Na^+ recycling may also explain the increases in I_{sc} observed in individual preparations (Onken and Siebers, 1992) (see Results). Of course, under otherwise unchanged conditions, a decrease in cuticular conductance should result in a decrease in I_{sc}. However, this current-decreasing effect might be compensated, or even over-compensated, by the current-increasing effect of paracellular Na^+ recycling.

Apart from direct measurements of changes in cuticular conductance, the most significant 'fingerprints' of an amiloride-induced inhibition of the cuticular cation conductance of crustacean gills seem to be the hyperpolarization of the outside-positive PD_{te} and the reduction in transbranchial influxes and effluxes of Na^+ (see Fig. 1). The posterior gills of *Uca tangeri* and *Carcinus maenas* adapted to low salinities appear to be very similar with respect to the changes in PD_{te} due to ion substitutions and transport inhibitors (cf. Drews and Graszynski, 1987; Krippeit-Drews et al., 1989). External amiloride also induced a hyperpolarization of the outside-positive PD_{te} in the posterior gills of *Uca tangeri*. As in shore crab gills, amiloride reduced both the influxes and the effluxes of Na^+ across the gills of *Callinectes sapidus* (Cameron, 1979). Thus, as in *Carcinus maenas*, and also in *Uca tangeri* and *Callinectes sapidus*, the effects of amiloride might be due to an interaction with the cuticle and not with the external surface of the gill epithelium. In contrast, however, in whole *Procambarus* spp. (Kirschner et al., 1973) and *Astacus leptodactylus* (Ehrenfeld, 1974) and in posterior gills of *Eriocheir sinensis* (Riestenpatt, 1995), only the Na^+ influxes were affected by amiloride, suggesting that, in these animals, the diuretic acted at the level of the apical membrane and not on the cuticle. In the posterior gills of Chinese crabs, amiloride has been reported to increase the cuticular conductance (Péqueux and Lignon, 1989), and the presence of apical Na^+ channels has been convincingly demonstrated using amiloride-induced current-noise analysis (Zeiske et al., 1992). However, the cuticular conductance of the anterior gills of Chinese crabs has been reported to be reduced by the diuretic (Péqueux and Lignon, 1989). It seems that the observed effects of amiloride on the cuticle of *Carcinus maenas* cannot be generalised for all Crustacea.

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