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ACTIVE ABSORPTION OF Na^+ AND Cl^- ACROSS THE GILL EPITHELIUM OF THE SHORE CRAB *CARCINUS MAENAS*: VOLTAGE-CLAMP AND ION-FLUX STUDIES

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Summary

Mechanisms of active NaCl uptake across the posterior gills of the shore crab *Carcinus maenas* were examined using radiochemical and electrophysiological techniques. In order to measure short-circuit current (I_{sc}), transepithelial conductance (G_{te}) and area-related unidirectional fluxes of Na^+ and Cl^-, single split gill lamellae (epithelium plus cuticle) of hyperregulating shore crabs were mounted in a modified Ussing chamber. The negative short-circuit current measured with haemolymph-like NaCl saline on both sides of the epithelium could be inhibited by application of basolateral ouabain (ouabain inhibitor constant K_{Oua}=56±10 μmol l^{-1}), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB; K_{NPPB}=7.5±2.5 μmol l^{-1}) or Cs^+ (10 mmol l^{-1}). From the apical side, I_{sc} was nearly completely blocked by Cs^+ (10 mmol l^{-1}) or Ba^{2+} (15 mmol l^{-1}), whereas apical addition of furosemide (1 mmol l^{-1}) resulted in only a small current decrease. Cl^- influxes were linearly related to negative I_{sc}. The ratio between net influxes of Cl^- and Na^+ was found to be approximately 2:1. With a single membrane preparation, achieved by permeabilizing the basolateral membrane with amphotericin B, Cl^- influxes which were driven by a concentration gradient were shown to depend on the presence of apical Na^+ and K^+. On the basis of these observations, we propose that active and electrogenic absorption of NaCl across the gill epithelium of hyperregulating shore crabs proceeds as in the thick ascending limb of Henle's loop in the mammalian nephron. Accordingly, branchial NaCl transport is mediated by apical K^+ channels in cooperation with apical $Na^+/K^+/2Cl^-$ cotransporters and by the basolateral Na^+/K^+-ATPase and basolateral Cl^- channels.

Key words: crab, *Carcinus maenas*, gill epithelium, NaCl absorption, short-circuit current, ion fluxes, amphotericin B.

Introduction

The shore crab *Carcinus maenas* is a euryhaline species inhabiting coastal environments of the North Sea, western parts of the Baltic Sea and the east coast of North America. The animals tolerate a wide range of environmental salinities between approximately 10 ‰ and full-strength sea water. The body fluids of shore crabs adapted to sea water are iso-osmotic to the ambient medium. When acclimated to brackish water, the crabs hyperregulate their haemolymph osmolarity and counterbalance the ensuing salt losses by active uptake of NaCl across the posterior gills (Péqueux *et al.* 1988; Lucu, 1990; Towle, 1993).

Studies on isolated perfused gills (Lucu and Siebers, 1987) and on split gill lamellae in an Ussing chamber (Onken and Siebers, 1992) demonstrated an active and electrogenic (inward movement of negative charge) NaCl absorption across the low-resistance gill epithelium of hyperregulating shore crabs which proceeds in a coupled mode. The Na^+/K^+-ATPase energizes NaCl absorption and represents the basolateral pathway for Na^+ uptake (Lucu and Siebers, 1987; Onken and Siebers, 1992). Basolateral Cl^- channels allow the translocation of Cl^- from the cytosol to the haemolymph (Siebers *et al.* 1990). Thus, the basolateral transporters involved in transbranchial NaCl absorption seem to be identified.

With respect to transapical NaCl uptake, two different mechanisms have been proposed: parallel Na^+/H^+ and Cl^-/HCO_3^- antiports (Lucu and Siebers, 1986; Siebers *et al.* 1987; Lucu, 1989) or $Na^+/K^+/2Cl^-$ symport in parallel with K^+ channels (Onken and Siebers, 1992; Onken *et al.* 1995). An electrogenic $2Na^+/1H^+$ exchanger has been identified in gill membrane vesicles (Shetlar and Towle, 1989). However, its localization and its involvement in NaCl absorption are still uncertain. Moreover, taking into consideration the polarity of the electrophysiology of the epithelium with identical salines on both sides (outside-positive transepithelial potential difference, PD_{te}, and inward-negative short-circuit current, I_{sc}) as well as the independence of NaCl absorption and I_{sc} from a functioning carbonic anhydrase (Böttcher *et al.* 1991; Onken and Siebers,

1992), a major contribution of apical antiports to NaCl uptake seems unlikely. The alternative proposal of transapical NaCl absorption *via* $Na^+/K^+/2Cl^-$ symport, operating in parallel with K^+ channels, seems to be more consistent with the electrophysiological observations (Onken and Siebers, 1992; Onken *et al.* 1995). However, this hypothesis suffers from the lack of any direct experimental support.

Another feature of the *Carcinus maenas* gill epithelium is its interaction with the well-known diuretic amiloride, which reduces unidirectional Na^+ (but not Cl^-) fluxes (Lucu and Siebers, 1986), hyperpolarizes the outside-positive PD_{te} (Lucu and Siebers, 1986; Siebers *et al.* 1987) and increases inward-negative I_{sc} (Onken and Siebers, 1992) when applied to the apical bath. These effects were interpreted as evidence for the presence of either Na^+/H^+ antiports (Lucu and Siebers, 1986; Siebers *et al.* 1987) or Na^+ channels (Onken and Siebers, 1992) in the apical membrane. Recently, however, a cuticular, amiloride-sensitive Na^+ conductance has been reported (Riestenpatt and Siebers, 1995), suggesting that the effects of the diuretic amiloride do not reflect an interaction with the epithelium itself.

In summary, the apical mechanisms involved in electrogenic NaCl absorption across the gills of the shore crab are still unclear. Although it appears that active NaCl absorption is, at least in part, an electrogenic process, it is not known whether electroneutral, transcellular NaCl uptake participates in the overall absorption of NaCl.

In the present investigation, simultaneous measurements of area-related radioactive tracer fluxes and electrophysiological parameters in an Ussing chamber were conducted for the first time for crab gills. Our experiments focused on the mechanisms of active and coupled NaCl absorption across the gill epithelium of hyperregulating shore crabs, using several specific inhibitors of transport proteins. A single membrane preparation, achieved by permeabilization of the basolateral membrane with amphotericin B, was used to determine the K^+- and Na^+-dependence of the apical Cl^- transporter. Our findings support the view of completely electrogenic NaCl absorption *via* apical $Na^+/K^+/2Cl^-$ symport in parallel with apical K^+ exit through K^+ channels.

These results were reported in part at the annual meeting of the Deutsche Zoologische Gesellschaft (Riestenpatt and Siebers, 1994).

Material and methods

Crabs

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

Preparation

After killing the crabs by destroying the ventral ganglion (a needle was pressed through the ventral side of the body wall), the carapace was lifted and the three posterior gills were removed. Single gill lamellae were isolated and split according to the method described by Schwarz and Graszynski (1989). In this way, a single epithelial layer covered by an apical cuticle was obtained. The preparations were mounted in an Ussing chamber modified after De Wolf and Van Driessche (1986), allowing experimentation on epithelial areas of 0.02 or 0.01 cm^2. In order to minimize edge damage, silicone grease was used to seal the edges of the preparation. The chamber compartments were continuously perfused with salines at a rate of 0.5 $ml\,min^{-1}$ by means of a peristaltic pump.

Salines and chemicals

The haemolymph-like saline was composed of ($mmol\,l^{-1}$): 248 NaCl, 5 KCl, 2 $NaHCO_3$, 4 $MgCl_2$, 5 $CaCl_2$, 5 Hepes, 2 glucose. Immediately before use, the pH was adjusted to 7.7 with Tris.

Amphotericin B solution (0.27 $mmol\,l^{-1}$ amphotericin B in deionized water) was obtained from Sigma; ouabain was purchased from Fluka; CsCl, $BaCl_2$ and dimethylsulphoxide (DMSO) were obtained from Merck. Furosemide and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) were gifts from Farbwerke Hoechst (Frankfurt am Main, Germany). All reagents, with the exception of NPPB (stock solution of 10 $mmol\,l^{-1}$ NPPB in DMSO), were dissolved directly in the salines.

Electrical measurements

For measurements of the transepithelial potential difference (PD_{te}), Ag/AgCl electrodes were connected *via* agar bridges (3% agar in 3 $mol\,l^{-1}$ KCl) with the chamber compartments (distance to the preparation 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). The measured short-circuit current (I'_{sc}) represents the charge movement across both the salines and the preparation. The area-specific resistance between the tips of the voltage electrodes (R'_{te}) was calculated from small imposed voltage pulses (ΔPD_{te}) and the resulting current deflections (ΔI). R'_{te} is the sum of the serial resistances of the solutions (R_s) and the tissue (R_{te}). Owing to the low values of R'_{te}, it was necessary to correct the R'_{te} and I'_{sc} data in order to obtain values which are directly related to the preparation alone (R_{te}, I_{sc}). R_s was measured without a preparation separating the chamber compartments. For the haemolymph-like saline used, R_s was found to be 9.1±0.1 $\Omega\,cm^2$ (N=15). For NaCl solutions used in the amphotericin B experiments, R_s was 9.1±0.2 $\Omega\,cm^2$ (250 $mmol\,l^{-1}$) or 8.0±0.1 $\Omega\,cm^2$ (500 $mmol\,l^{-1}$) (N=5) and for choline chloride solutions used in the amphotericin B experiments R_s was 9.2±0.1 $\Omega\,cm^2$ (250 $mmol\,l^{-1}$) or 8.3±0.3 $\Omega\,cm^2$ (500 $mmol\,l^{-1}$) (N=4). The corrected R_{te} is obtained by subtracting R_s from R'_{te} according to:

$$R_{te} = R'_{te} - \left(\frac{R_{s,apical}}{2} + \frac{R_{s,basolateral}}{2} \right), \qquad (1)$$

where $R_{s,apical}$ is the resistance of the saline in the apical bath and $R_{s,basolateral}$ is the resistance of the saline in the basolateral bath. The correction of I'_{sc} followed Ohm's law. The transepithelial conductance G_{te} was calculated as $G_{te}=1/R_{te}$. In the Results section, only the corrected values are given.

Measurement of unidirectional Na^+ and Cl^- fluxes

The radioactive isotopes ^{36}Cl (NEN, Dupont) and ^{22}Na (Amersham) were used at a final activity of 37 MBq l^{-1} saline (Na^+ 148 MBq mol^{-1}; Cl^- 137 MBq mol^{-1}). Unidirectional influxes from the apical to the basolateral side ($J_{a\rightarrow b}$) or effluxes ($J_{b\rightarrow a}$) were measured over 60 min in a closed perfusion circuit (5 ml circulating through each chamber compartment at a pumping rate of 0.5 ml min^{-1}), allowing the accumulation of radioactivity in the superfusate. The closure of the perfusion circuit had no influence on I_{sc}. Only when the perfusion was stopped before and after flux measurements were transient I_{sc} inconstancies observed. ^{36}Cl radioactivity contained in 2 ml samples was determined with a PRIAS liquid scintillation counter (Packard, model PLD) after the addition of 4 ml of Insta Gel (Packard, no. 6013008). ^{22}Na radioactivity was either measured in 2 ml samples using the same procedure or estimated directly in 2 ml samples with a gamma-spectrometer (Fischer, Hamburg). Flux data were calculated from the respective counting rates, the volume of the perfusion compartment (5 ml) and time (1 h) and were expressed as $\mu mol\,h^{-1}\,cm^{-2}$. Influxes and effluxes of ^{22}Na and ^{36}Cl were measured in separate experiments. Owing to the high doses of radioactivity used, determination of influxes and effluxes in the same preparation was impossible since complete wash-out of the radioactivity required more than 3 h. Therefore, net influxes ($J_{net,a\rightarrow b}$) of the respective ions were calculated as the differences between the means ± S.E.M. of $J_{a\rightarrow b}$ and $J_{b\rightarrow a}$ according to:

$$J_{net,a\rightarrow b} = A - B \pm \sqrt{a^2 + b^2}\,, \qquad (2)$$

where A and B represent the mean values and a and b represent the standard errors of unidirectional influxes and effluxes, respectively.

Statistics

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

Results

Using haemolymph-like salines on both sides of the flat sheets, consisting of the cuticle and the underlying single layer of epithelial cells, a short-circuit current (I_{sc}) of $-375\pm14\,\mu A\,cm^{-2}$ (N=77) was measured, representing a negative charge flow from the apical to the basolateral side of the preparation. The corresponding transepithelial conductance (G_{te}) amounted to $45\pm1\,mS\,cm^{-2}$. Determinations of unidirectional Cl^- fluxes showed a mean Cl^- influx ($^{Cl}J_{a\rightarrow b}$) of $23.7\pm2.0\,\mu mol\,cm^{-2}\,h^{-1}$ (N=11) and a mean Cl^- efflux ($^{Cl}J_{b\rightarrow a}$) of $5.5\pm0.6\,\mu mol\,cm^{-2}\,h^{-1}$ (N=10). From these values, a net Cl^- influx ($^{Cl}J_{net,a\rightarrow b}$) of $18.8\pm2.1\,\mu mol\,cm^{-2}\,h^{-1}$ was calculated. Furthermore, $^{Cl}J_{a\rightarrow b}$ and the corresponding I_{sc} were linearly related (Fig. 1A), indicating the Cl^--dependence of I_{sc} and the electrogenicity of Cl^- uptake mechanisms. For Na^+, we measured a mean unidirectional influx ($^{Na}J_{a\rightarrow b}$) of $22.0\pm2.2\,\mu mol\,cm^{-2}\,h^{-1}$ (N=10) and a mean efflux ($^{Na}J_{b\rightarrow a}$) of $13.7\pm1.4\,\mu mol\,cm^{-2}\,h^{-1}$ (N=10). Thus, the mean net Na^+ influx ($^{Na}J_{net,a\rightarrow b}$) was calculated to be $8.3\pm2.6\,\mu mol\,cm^{-2}\,h^{-1}$. The net influx of Na^+ is markedly smaller than the net influx of Cl^-. A comparison of these flux values gives an approximately 1:2 relationship for the net transport of Na^+ and Cl^- across the gills.

Effect of ouabain

The involvement of the Na^+/K^+-ATPase in NaCl absorption by *Carcinus maenas* gill has been demonstrated previously (Lucu and Siebers, 1987; Onken and Siebers, 1992). However, dose-dependent blockage of NaCl uptake by ouabain, a specific inhibitor of the Na^+/K^+-ATPase (Skou, 1965), has not been reported so far. In five experiments, I_{sc} and G_{te} were measured when ouabain was added in stepwise increasing concentrations (0.001–10 mmol l^{-1}) to the basolateral superfusion saline. The effect of different concentrations of ouabain on the control current ($-398\pm37\,\mu A\,cm^{-2}$) is shown in Fig. 2. A more detailed analysis of blocker saturation kinetics may be obtained by the transformation of the data in a Hanes–Woolf plot (inset to Fig. 2). In this plot, the ratio of the ouabain concentration to the respective I_{sc} decrease ([ouabain]/ΔI_{sc}) is plotted against the ouabain concentration. From the reciprocal slope of the lines

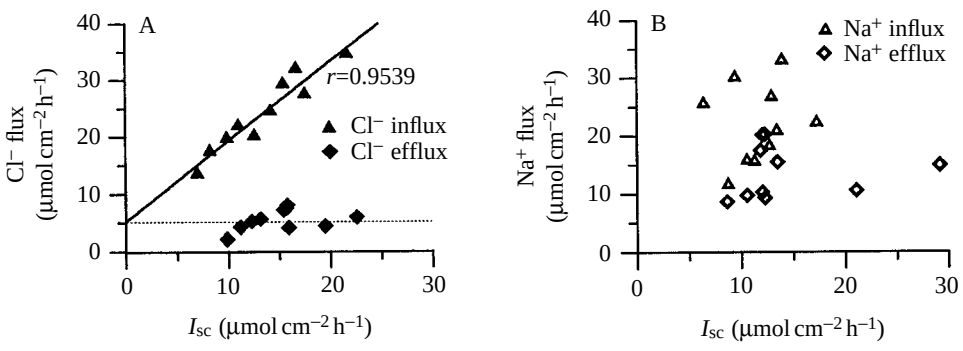

Fig. 1. Relationship between unidirectional Cl^- fluxes (A) and Na^+ fluxes (B) and the short-circuit current (I_{sc}) measured in split lamellae preparations of the shore crab *Carcinus maenas*. The straight line represents the linear fit of the estimated data. r is the coefficient of correlation (P=0.044). Regression lines where $r<0.4$ are not shown. The dotted line in A represents the calculated unidirectional Cl^- influx when I_{sc} equals zero.

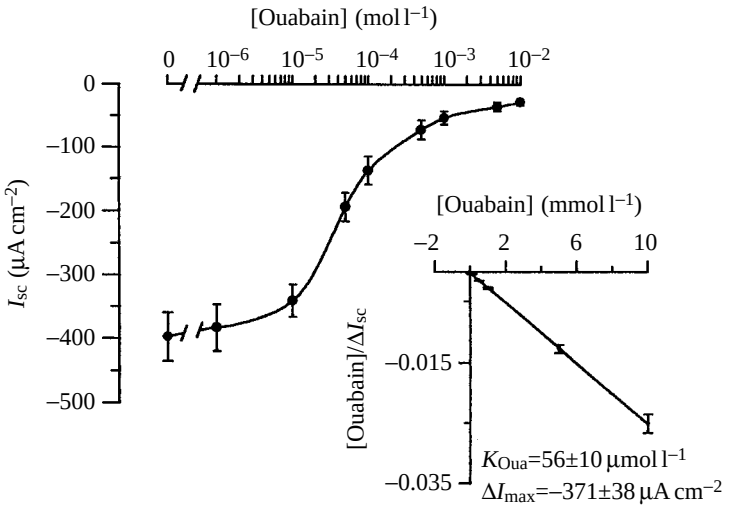

Fig. 2. Dose-dependence of I_{sc} inhibition by ouabain. In five experiments on different split lamellae preparations, ouabain was added at increasing concentrations to the internal solution. The mean I_{sc} (±S.E.M.) is plotted against the concentration of ouabain (in mol l^{-1}) in the internal saline. The inset shows the same data in a Hanes–Woolf plot. The ouabain concentration (in mmol l^{-1}) is plotted against the ratio of the ouabain concentration (in mmol l^{-1}) to the respective mean (±S.E.M.) I_{sc} decrease ([Ouabain]/ΔI_{sc}). The line represents the linear regression for concentrations of ouabain in the range 10^{-6} to 10^{-2} mol l^{-1} (coefficient of correlation r=1, P=0.004). The ouabain concentration at half-maximal I_{sc} decrease (K_{Oua}) is given by the intercept of the line with the abscissa, whereas the maximum ouabain-induced current decrease (ΔI_{max}) can be estimated from the reciprocal slope of the line. The mean values of ΔI_{max} and K_{Oua} were obtained from plots for the individual experiments.

obtained for the five individual preparations, we calculated the mean maximal ouabain-induced current decrease ΔI_{max} to be –371±38 µA cm^{-2}, indicating a strict dependence of I_{sc} on the activity of the Na^+/K^+-ATPase. The average half-maximal effect of the drug (K_{Oua}=55.5±10.2 µmol l^{-1}) was determined from the intercept of the lines with the abscissa. Ouabain also affected the conductance of the gill epithelium. Using high doses of the inhibitor (10 mmol l^{-1}), G_{te} was significantly reduced by 34±9 % to 32±7 mS cm^{-2} (N=5) (P<0.05).

Effect of NPPB

As shown previously for several epithelia, NPPB is a potent and specific inhibitor of Cl^- channels (Wangemann *et al.* 1986; Gögelein, 1988). In isolated perfused gills, basolateral Cl^- channels have been identified by the use of NPPB and other specific inhibitors of Cl^- channels in potentiometric and ion-flux studies (Siebers *et al.* 1990). Following the addition of NPPB (0.1 mmol l^{-1}) to the basolateral NaCl saline of the short-circuited split lamella preparation, the negative I_{sc} of –213±40 µA cm^{-2} decreased to –32±13 µA cm^{-2} (N=5). G_{te} remained unchanged. DMSO, which was used as primary solvent for the drug, was without any effect on the electrical parameters when added to the basolateral perfusion medium at a final concentration of 0.5 % (data not shown). The dose-dependence of the inhibition of I_{sc} by NPPB was studied in five experiments (Fig. 3). After transformation of the data into a Hanes–Woolf plot, a straight line was obtained, indicating simple Michaelis–Menten kinetics for the current blockage by NPPB. The half-maximal inhibitor concentration (K_{NPPB}) was 7.5±2.5 µmol l^{-1} NPPB in the basolateral bath. The maximum NPPB-induced current decrease (ΔI_{max}) was estimated to be –201±43 µA cm^{-2}.

Effect of K^+ channel inhibitors

In order to identify the electrogenic pathway of the apical membrane of the branchial epithelium, the specific K^+

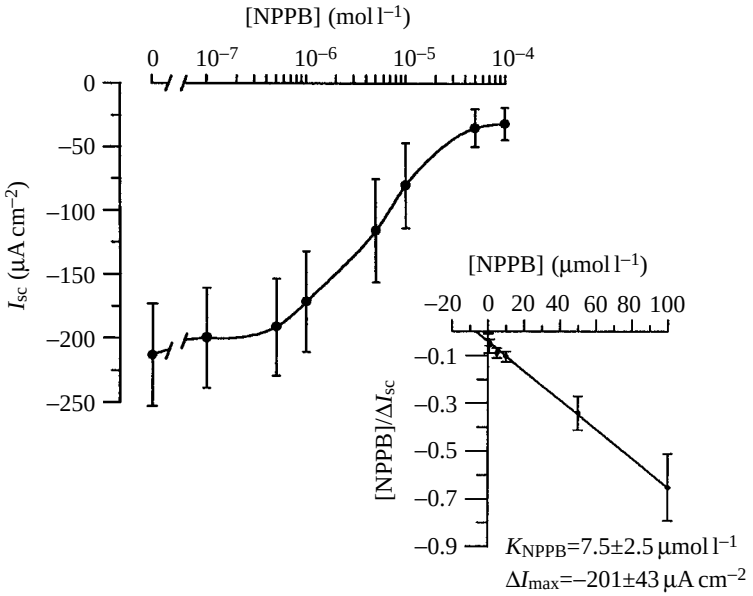

Fig. 3. Dose-dependence of I_{sc} inhibition by NPPB. In five experiments on different split lamellae preparations, NPPB was added to the internal solution and gradually washed out. The mean I_{sc} (±S.E.M.) is plotted against the concentration of NPPB (in mol l^{-1}) in the internal saline. The inset shows the same data in a Hanes–Woolf plot. The NPPB concentration in (µmol l^{-1}) is plotted against the ratio of the NPPB concentration (in µmol l^{-1}) to the respective mean (±S.E.M.) I_{sc} decrease ([NPPB]/ΔI_{sc}). The line represents the linear regression for concentrations of NPPB in the range 0.1–100 µmol l^{-1} (coefficient of correlation r=0.964, P=0.005). The NPPB concentration at half-maximal I_{sc} decrease (K_{NPPB}) is given by the intercept of the line with the abscissa, whereas the maximum NPPB-induced current decrease (ΔI_{max}) can be estimated from the reciprocal slope of the line. The mean values of ΔI_{max} and K_{NPPB} were obtained from plots for the individual experiments.

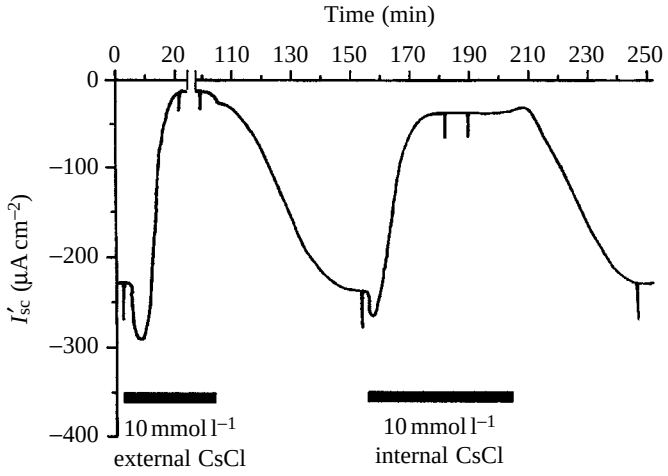

Fig. 4. Time course of the inward-negative measured I_{sc} (I'_{sc}) showing the inhibitory effect of external and internal addition of 10 mmol l^{-1} CsCl. The amplitudes of the current deflections, which are due to voltage pulses of 2 mV, are inversely proportional to the resistance between the tips of the voltage electrodes (R'_{te}; see Materials and methods).

channel inhibitor Ba^{2+} (Zeiske, 1990) was added to the apical bath. Following application of 15 mmol l^{-1} $BaCl_2$, the control I_{sc} (−519±90 μA cm^{-2}) was reduced by 73±9 % to −122±29 μA cm^{-2} and G_{te} (48±5 mS cm^{-2}) decreased by 56±3 % to 26±1 mS cm^{-2} (N=4) (P<0.05).

Using CsCl, another effective K^+ channel inhibitor (Zeiske, 1990; Draber and Hansen, 1994), we found an even more effective block of I_{sc} (Fig. 4; Table 1). Addition of 10 mmol l^{-1} CsCl to the apical bath decreased I_{sc} by 93±1 % and G_{te} by 62±2 % (P<0.05). The measurement of unidirectional Na^+ and Cl^- fluxes under these conditions revealed substantially decreased unidirectional influxes of Na^+ ($^{Na}J_{a\rightarrow b}$) from 23.2±2.4 μmol cm^{-2} h^{-1} to 14.2±1.6 μmol cm^{-2} h^{-1} (P<0.05) and of Cl^- ($^{Cl}J_{a\rightarrow b}$) from 27.1±2.5 μmol cm^{-2} h^{-1} to 5.3±0.5 μmol cm^{-2} h^{-1} (P<0.05). Therefore, the NaCl net influxes ($^{Cl}J_{net,a\rightarrow b}$=1.5 μmol cm^{-2} h^{-1} and $^{Na}J_{net,a\rightarrow b}$=−0.7 μmol cm^{-2} h^{-1}) were nearly abolished since the unidirectional NaCl effluxes ($^{Na}J_{b\rightarrow a}$ and $^{Cl}J_{b\rightarrow a}$) were not significantly affected by CsCl (Table 1).

When CsCl (10 mmol l^{-1}) was added to the basolateral bath, the negative I_{sc} (−567±132 μA cm^{-2}) decreased by 85±2 % to 79±12 μA cm^{-2} (N=3) (P<0.05). The simultaneously measured G_{te} (48±9 mS cm^{-2}) was reduced by 28±3 % to 34±5 mS cm^{-2} (P<0.05) (an example is shown in Fig. 4).

Effect of furosemide

The identification of apical K^+ channels and their involvement in transepithelial NaCl uptake (see above) suggested a mode of apical NaCl entry *via* a $Na^+/K^+/2Cl^-$ cotransport as described in the thick ascending limb of Henle's loop of the mammalian nephron. The diuretic furosemide has been shown to inhibit this $Na^+/K^+/2Cl^-$ cotransporter in various vertebrate tissues (Greger and Kunzelmann, 1990). In order to test the involvement of this enzyme in branchial NaCl absorption, we examined the effect of furosemide on I_{sc} and G_{te} in split lamellae preparations. Following apical addition of 1 mmol l^{-1} furosemide, negative I_{sc} (−343±63 μA cm^{-2}) was decreased by only 13±1 % to −296±51 μA cm^{-2} (N=5) (P<0.05). The transepithelial conductance (G_{te}) remained unchanged.

Table 1. *Short-circuit current (I_{sc}), transepithelial conductance (G_{te}) unidirectional influxes, unidirectional effluxes and mean net influxes ($J_{net,a\rightarrow b}$=mean $J_{a\rightarrow b}$ − mean $J_{b\rightarrow a}$) of Cl^- and Na^+ across single split lamellae of* Carcinus maenas *under control conditions and in the presence of 10 mmol l^{-1} CsCl in the external superfusate (apical bath)*

	Control	CsCl (10 mmol l^{-1})	P	N
Electrophysiological parameters				
I_{sc} (μA cm^{-2})	404±32	29±4	<0.010	24
G_{te} (mS cm^{-2})	40±1	26±1	<0.010	24
Cl^- fluxes (μmol cm^{-2} h^{-1})				
$^{Cl}J_{a\rightarrow b}$	27.1±2.5	5.3±0.5	<0.010	5
$^{Cl}J_{b\rightarrow a}$	4.8±1.7	3.8±2.2	0.083	5
$^{Cl}J_{net,a\rightarrow b}$	22.4±3.0	1.5±2.3		Calculated
Na^+ fluxes (μmol cm^{-2} h^{-1})				
$^{Na}J_{a\rightarrow b}$	23.2±2.4	14.2±1.6	<0.010	5
$^{Na}J_{b\rightarrow a}$	16.3±1.6	14.9±1.3	0.176	5
$^{Na}J_{net,a\rightarrow b}$	7.0±2.9	−0.7±2.1		Calculated

Data represent means ± S.E.M.

Statistical significance of differences were tested using the paired Student's t-test. Significant differences were assumed where P<0.05.

Permeabilization of the basolateral membrane

Since the above experiment did not clearly identify the pathway of apical NaCl entry, we investigated whether the apical Cl^- entry depends on the presence of other ions (e.g. K^+ and Na^+) in the apical bath. With this aim in mind, the basolateral membrane of the gill epithelium was electrically eliminated by the use of a relatively unspecific ionophore. The channel-forming polyene amphotericin B permeabilizes membranes for monovalent ions, water and small polar molecules (Holz and Finkelstein, 1970; Dawson, 1987). Single membrane preparations of epithelia were obtained by treating one side of the epithelium with amphotericin B (Dawson *et al.* 1990; Schirmanns and Zeiske, 1994). Basolateral addition of 14 μmol l^{-1} amphotericin B reduced the I_{sc} by 90±5 % to −39±23 μA cm^{-2} (N=10), indicating the permeabilization of the basolateral membrane for single-charged ions by the incorporation of polyene molecules (Fig. 5). Active NaCl absorption is assumed to have collapsed under these conditions since the depolarization of the cellular potential and the breakdown of the inwardly

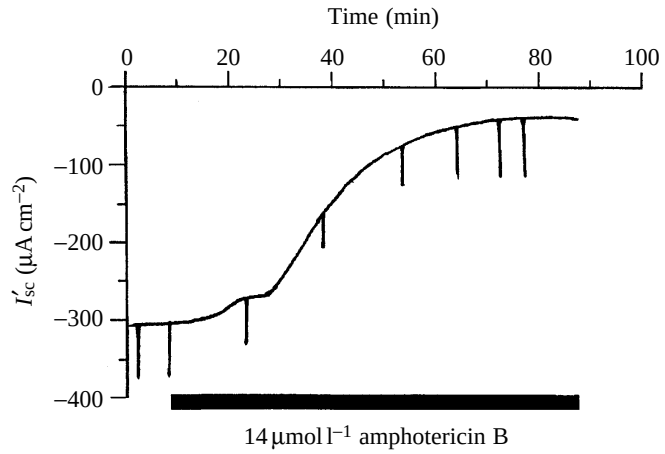

Fig. 5. Time course of the inward negative measured I_{sc} (I'_{sc}) showing the inhibitory effect of internal addition of 14 μmol l^{-1} amphotericin B. The amplitudes of the current deflections, which are due to voltage pulses of 1 mV, are inversely proportional to the resistance between the tips of the voltage electrodes (R'_{te}; see Materials and methods).

Table 2. *Dependence of gradient-driven Cl⁻ influx ($^{Cl}J_{a\rightarrow b}$) on the presence of K⁺ and Na⁺ in the perfusion solutions*

	NaCl gradient +5 mmol l^{-1} KCl	NaCl gradient	*P*	*N*
K^+-dependence of the gradient-driven Cl^- influx $^{Cl}J_{a\rightarrow b}$ (μmol cm^{-2} h^{-1})	17.7±4.2	9.8±1.9	0.032	4
	NaCl gradient +5 mmol l^{-1} KCl	Choline chloride gradient +5 mmol l^{-1} KCl	*P*	*N*
Na^+-dependence of the gradient-driven Cl^- influx $^{Cl}J_{a\rightarrow b}$ (μmol cm^{-2} h^{-1})	9.8±2.3	6.4±1.4	0.025	5

Data represent means ± S.E.M.

Statistical significance of differences were tested using the paired Student's *t*-test. Significant differences were assumed for error probabilities $P<0.05$.

directed Na^+ gradient eliminate the driving force of secondary active transport processes across the apical membrane. G_{te} was not significantly affected by treatment with amphotericin B. As shown for other epithelia (Onken *et al.* 1991; Dijkstra *et al.* 1994), the transcellular conductance is mainly determined by the conductance of the apical membrane. Thus, with respect to the unchanged G_{te}, it could be anticipated that the polyenic membrane perforation is restricted to the treated membrane. Using a NaCl gradient across this preparation by applying 500 mmol l^{-1} NaCl + 5 mmol l^{-1} KCl in the apical bath and 250 mmol l^{-1} NaCl + 5 mmol l^{-1} KCl in the basolateral bath, an inward-positive I_{sc} of 1354±226 μA cm^{-2} and a G_{te} of 141±21 mS cm^{-2} (N=9) were measured. Following substitution of Na^+ by choline on both sides of the preparation, I_{sc} (−16±5 μA cm^{-2}; N=5) and G_{te} (10±1 mS cm^{-2}) decreased drastically. Moreover, the polarity of I_{sc} was changed. These results indicate that the single membrane preparation has a much higher conductance for Na^+ than for Cl^-. In the presence and in the absence of 5 mmol l^{-1} KCl and following substitution of Na^+ by choline, the unidirectional Cl^- influxes were measured in the presence of the Cl^- concentration gradient directed from the apical to the basolateral side. The Cl^- influx was significantly reduced when Na^+ or K^+ was absent from the superfusion solutions. The data from these experiments are summarized in Table 2.

Discussion

Methodological aspects

In the past, fluxes of radioactive tracers across crab gill epithelia have only been measured using whole perfused gills (Péqueux *et al.* 1988; Lucu and Siebers, 1986). Because of the complex morphology of the organs, the fluxes could not be related to the epithelial area and electrophysiological measurements were restricted to the determination of the transepithelial potential difference (PD_{te}), which does not measure transport quantities. Moreover, even with identical salines as perfusing and bathing media, the calculated net flux is not necessarily equal to the rate of active transport because the measurements were conducted under open-circuit conditions. Of course, a PD_{te} influences the unidirectional fluxes used to determine the net flux. Strictly, the most comprehensive conclusions that can be drawn from the results of such experiments are whether or not active transport and/or electrogenic mechanisms are involved. The effects of transport inhibitors on fluxes and PD_{te} may allow conclusions to be drawn about the mechanisms involved in transcellular ion translocation. However, the interpretation of these results is often ambiguous.

For some years, experiments have been performed with split lamellae of crab gills mounted into micro versions of an Ussing chamber (Schwarz and Graszynski, 1989). The validity of this preparation has been demonstrated and discussed extensively (Onken *et al.* 1991; Onken and Siebers, 1992). So far, split gill lamellae have been used to determine area-related transepithelial short-circuit currents (I_{sc}), reflecting the quantity of active charge transfer across the epithelium, and conductances (G_{te}). More advanced electrophysiological techniques, such as measurements of membrane potentials using microelectrodes (Onken *et al.* 1991) or current fluctuation analysis (Zeiske *et al.* 1992), have also been successfully employed on this preparation. These techniques allowed new insights into crab gill physiology (Onken *et al.* 1995). However, until the present study, the ionic nature of the currents has not been directly identified using flux measurements but was determined by substitution of the ion in question. Moreover, the restriction to electrophysiological

techniques did not allow putative electroneutral ion movements across the epithelium to be investigated.

All these problems can be solved when both influxes and effluxes of radioactive tracers and short-circuit currents across a single split gill lamella, mounted in an Ussing chamber, are measured simultaneously. In the present investigation, simultaneous measurements of influxes or effluxes and I_{sc} have been conducted for the first time on crab gills. Because of the small epithelial area in the Ussing chamber, it was necessary to use relatively high activities of ^{22}Na and ^{36}Cl. Unfortunately, a complete wash-out of the radioactive isotopes took too long for unidirectional influx and efflux, and thus the net flux, to be determined on the same preparation. Therefore, net fluxes had to be calculated from the mean influxes and effluxes determined on different preparations. Nevertheless, the validity of the respective results is shown by a more detailed analysis of the data (see Results and below). For example, when we compute the mean net inward movement of negative charge from the difference between the calculated net influxes of Na^+ and Cl^-, we obtain a value ($10.5\pm3.3\,\mu mol\,cm^{-2}\,h^{-1}$) which is not significantly different from the mean measured current ($13.5\pm0.7\,\mu mol\,cm^{-2}\,h^{-1}$). As in similar investigations on other epithelial tissues (Ussing and Zerahn, 1951; Diaz and Lorenzo, 1991), the simultaneous measurement of fluxes and currents across crab gill epithelia represents significant progress in methodology which may advance our understanding of their transport characteristics.

NaCl absorption across shore crab gills

Recent ion-flux measurements on isolated, perfused gills of hyperregulating shore crabs have demonstrated their ability to absorb Na^+ and Cl^- actively (Lucu and Siebers, 1986, 1987; Siebers *et al.* 1987, 1990). In the present study, the mean unidirectional influxes of Na^+ and Cl^- with identical salines on both sides of the short-circuited gill epithelium clearly exceeded the mean unidirectional effluxes (see Results). Thus, net influxes were obtained for both ions. Because of the different methodological approaches (Ussing chamber, short-circuit), these findings not only verify the presence of active NaCl absorption across shore crab gills but may also be used to measure active transport of Na^+ and Cl^- and to relate them to the epithelial area.

Electrogenic versus *electroneutral ion movements*

In recent investigations using perfused whole gills (Lucu and Siebers, 1986, 1987; Siebers *et al.* 1987, 1990) and split gill lamellae (Onken and Siebers, 1992), outside-positive transepithelial potential differences (PD_{te}) and inward-negative short-circuit currents (I_{sc}), respectively, have been measured using identical NaCl salines on both sides of the epithelium, indicating that electrogenic mechanisms are involved in NaCl absorption. The results of the present investigation allowed the relationship between the unidirectional NaCl fluxes and the simultaneously measured I_{sc} to be investigated. The effluxes of Na^+ and Cl^- showed no dependence on I_{sc} (Fig. 1A,B). In contrast, the Cl^- influx increased linearly with rising I_{sc} values (see Fig. 1A). It is interesting that the mean Cl^- influx at $I_{sc}=0$ ($5.3\,\mu mol\,cm^{-2}\,h^{-1}$), which is obtained from the intercept of the line with the ordinate, was found to be almost identical to the mean Cl^- efflux ($5.5\pm0.6\,\mu mol\,cm^{-2}\,h^{-1}$) determined on different preparations. Thus, it seems that *net* influx of Cl^- only occurs concomitantly with an inward-negative I_{sc}, indicating the absence of any active, cellular, electroneutral uptake of Cl^-. This strict Cl^--dependence of I_{sc} also excludes any active, electrogenic and Cl^--independent absorption of other ions (e.g. Na^+). For this reason, active Na^+ absorption across the gill tissue, which is evident from the observation of net influxes of Na^+ (Table 1), seems either to be directly coupled to electrogenic Cl^- uptake or to proceed *via* Cl^--independent, electroneutral mechanisms (Na^+/H^+ antiports). The inhibition of I_{sc} following substitution of Na^+ or Cl^- in the perfusion salines (Onken and Siebers, 1992) and after blockage of the Na^+/K^+-ATPase with ouabain (Fig. 2) suggests that Na^+ uptake is directly coupled to Cl^- absorption. In this case, a correlation between net Na^+ influx and negative I_{sc} would be expected. A correlation between unidirectional influxes of Na^+ and negative I_{sc} was not found (Fig. 1B). However, effluxes of Na^+ across the gill epithelium were found to be much higher than those of Cl^-. Moreover, effluxes and influxes varied greatly from preparation to preparation (see Fig. 1B). Therefore, it remains doubtful whether the unidirectional Na^+ influxes which represent transepithelial active *and* passive Na^+ movements are an appropriate measure of net Na^+ fluxes related to active transport. To determine whether transcellular, electroneutral Na^+ absorption occurs in the gill epithelium of *Carcinus maenas*, different isotopes (^{22}Na, ^{24}Na) could be used in the future to measure influxes and effluxes on the same preparation.

Transporters involved in electrogenic NaCl absorption

Basolateral Na^+/K^+-ATPase

In recent studies with whole gills (Lucu and Siebers, 1987) and with split gill lamellae (Onken and Siebers, 1992), the dependence of NaCl absorption across the gill epithelium on a functioning Na^+/K^+-ATPase in the basolateral membrane has been demonstrated. The results of the present investigation (see Fig. 2) confirm this finding. Ouabain, a specific blocker of the Na^+/K^+ pump (Skou, 1965), completely inhibited the negative I_{sc} which is, as discussed above, associated with the entire NaCl absorption. The half-maximal I_{sc} inhibition by basolateral ouabain (K_{Oua}, see inset to Fig. 2) agrees with ouabain inhibitor constants (K_{Oua}) obtained for gill tissues of other crustaceans such as *Eriocheir sinensis* (Graszynski and Bigalke, 1986; Schwarz and Graszynski, 1990), several species of fiddler crab (Wanson *et al.* 1984; D'Orazio and Holliday, 1985; Graszynski and Bigalke, 1986) and *Callinectes sapidus* (Neufeld *et al.* 1980). The conductance decrease, observed following ouabain addition, probably reflects a secondary influence on the apical membrane, as in the thick ascending limb of Henle's loop in the mammalian nephron (Greger *et al.* 1984).

Basolateral K^+ channels

Cs^+, which is known to block K^+ channels (Zeiske, 1990; Draber and Hansen, 1994), inhibited I_{sc} when applied to the basolateral bath (see Fig. 4). This finding indicates that, as in many other epithelia (Wills and Zweifach, 1987; Dawson *et al.* 1990) including crab gills (Onken *et al.* 1991), the basolateral membrane contains K^+ channels.

Basolateral Cl^- channels

In a variety of NaCl-absorbing epithelia (Gögelein, 1988), including crab gills (Drews and Graszynski, 1987; Onken *et al.* 1991), Cl^- channels have been shown to be the pathway for Cl^- to cross the basolateral membrane. Siebers *et al.* (1990) demonstrated that some blockers of Cl^- channels reduced PD_{te} as well as NaCl fluxes across isolated, perfused gills of hyperregulating shore crabs when applied to the basolateral saline, indicating the presence of a Cl^- conductance in the basolateral membrane. This finding is confirmed in the present study by the effect of NPPB on I_{sc} (see Fig. 3). As in experiments on whole gills, a K_{NPPB} of about $7\,\mu mol\,l^{-1}$ was obtained when the dose–response relationship was analysed (see inset to Fig. 3). In the thick ascending limb of Henle's loop, NPPB half-maximally inhibited the Cl^- conductance at a concentration of $8\times10^{-8}\,mol\,l^{-1}$ (Wangemann *et al.* 1986). However, as pointed out by Greger (1990), the blocking effect of NPPB and related substances varied considerably from tissue to tissue. The maximal NPPB-induced I_{sc} decrease (ΔI_{max}) is not significantly different from the control current, indicating that the whole transepithelial current flow proceeds *via* basolateral Cl^- channels.

Apical K^+ channels

Because of the limited knowledge of the transport mechanisms of the apical membrane, the question concerning which apical transport protein mediates the current flow occurring simultaneously with Cl^- absorption was unresolved. Since basolateral Cl^- channels have been demonstrated in *Carcinus maenas* gill epithelium (see above), the presence of these channels in the apical membrane can be excluded for thermodynamic reasons. Another candidate for the role of the apical electrogenic pathway was tested in this study by using two blockers of K^+ channels. The effects of apical addition of Cs^+ or Ba^{2+}, which are known as potent inhibitors of these channels (see above), on G_{te}, negative I_{sc} and NaCl influxes (see Results and Table 1) strongly indicate the presence of K^+ channels in the apical membrane of shore crab gills and their involvement in active NaCl absorption.

Apical Na^+/Cl^- symport

Influxes of Na^+ and Cl^- as well as the outside-positive PD_{te} and inward-negative I_{sc} across the gill epithelium of the shore crab depend on a functioning Na^+/K^+-ATPase (see above). Moreover, in a previous study, the current was almost completely abolished following substitution of Na^+ and Cl^- in the perfusion salines (Onken and Siebers, 1992). Finally, Cl^- influx across single membrane preparations, achieved by basolateral addition of the ionophore amphotericin B, depended on the presence of apical Na^+ (see Table 2). All these observations strongly indicate a coupled mode of transapical NaCl entry. In NaCl-absorbing epithelia, three different mechanisms of coupled NaCl absorption have been described so far: double ion exchange *via* Na^+/H^+ and Cl^-/HCO_3^- antiports (Murer and Burckhardt, 1983; Aronson and Seifter, 1983), Na^+/Cl^- symport (Velasquez, 1987; Cremaschi *et al.* 1992) and $Na^+/K^+/2Cl^-$ symport (Greger, 1985). Until now, attempts at pharmacological distinction between these mechanisms had not been successful in the gills of *Carcinus maenas*: SITS (Lucu, 1989), bumetanide (Lucu, 1989) and furosemide (see Results) showed only small effects on ion fluxes, PD_{te} and I_{sc} across shore crab gills. However, it is not yet clear whether apically applied inhibitors can cross the cuticle of crustacean gills and affect apically located transport proteins. In the isolated gill cuticle of several crustaceans, it has been shown that the permeability of the gill cuticle for large ions such as Tris, tetraethylammonium or thiocyanate is rather low. Moreover, blockers of epithelial ion transport such as amiloride or SCN^- also modified the transport properties of the cuticle (Lignon and Péqueux, 1990). When the gill cuticle of the shore crab was isolated and mounted in an Ussing-type chamber, it was shown that the conductance of the isolated cuticle was reduced by approximately 95 % following apical addition of amiloride. A half-maximal inhibitor concentration K_{Ami} of $1.2\,\mu mol\,l^{-1}$ was estimated for the amiloride-induced conductance decrease (Riestenpatt and Siebers, 1995). Thus, it seems that this diuretic at least cannot pass the gill cuticle of *Carcinus maenas*.

So far, the electrogenicity of NaCl absorption and its dependence on apical K^+ channels (see Fig. 4 and Table 1) suggests that transapical NaCl uptake proceeds *via* $Na^+/K^+/2Cl^-$ symport. This interpretation is supported by the finding of an approximately 1:2 relationship (1:2.27) between net Na^+ and Cl^- influxes under short-circuit conditions and by the K^+-dependence of Cl^- influxes across single membrane preparations (see Table 2).

Modelling active and electrogenic NaCl absorption across shore crab gills

Summarizing the results of the present study, the following model of NaCl uptake across the gill epithelium of *Carcinus maenas* is proposed (Fig. 6). Active NaCl absorption across the posterior gills of the shore crab is electrogenic and proceeds in a coupled mode. The Na^+/K^+-ATPase energizes NaCl uptake by establishing and maintaining Na^+ and K^+ concentration gradients across the plasma membranes. The Na^+ gradient (directed into the cells) drives transapical entry of Na^+, K^+ and Cl^- *via* $Na^+/K^+/2Cl^-$ symport. The K^+ gradient (directed out of the cells), in cooperation with the apical and basolateral K^+ conductances (K^+ channels), generates a negative cellular potential. This cellular negativity is responsible for driving the basolateral exit of Cl^- against the concentration gradient *via* Cl^- channels. Thus, the negative I_{sc} is carried *via* the apical membrane by K^+ (which is recycled *via* the apical symport)

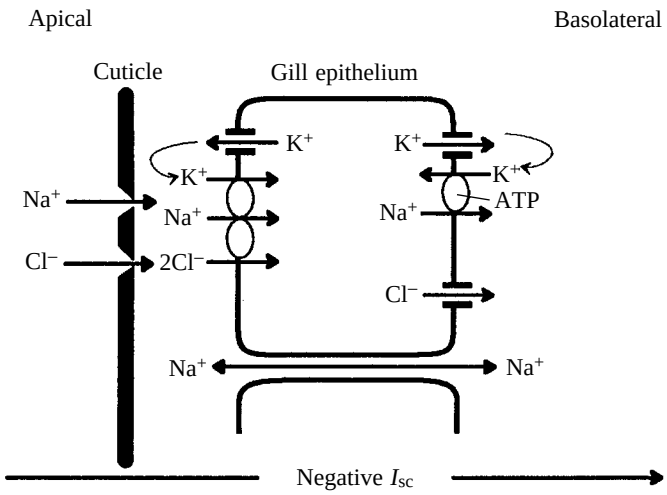

Fig. 6. Proposed functional model of active, electrogenic and coupled NaCl uptake across the posterior gills of the shore crab *Carcinus maenas*. Negative short-circuit current (I_{sc}) is carried *via* apical K^+ channels and basolateral Cl^- channels. Transapical NaCl transport proceeds *via* $Na^+/K^+/2Cl^-$ symport. This cotransporter is secondarily energized by the activity of the basolateral Na^+/K^+-ATPase, which generates a Na^+ gradient directed into the cell. Whereas K^+ leaves the cell *via* the conductive pathways in the apical and basolateral membrane, basolateral NaCl exit is effected *via* the Cl^- channels and the Na^+/K^+-ATPase.

through K^+ channels and *via* the basolateral membrane by Cl^- through Cl^- channels.

The Na^+ efflux under control conditions and the influx of Na^+ following inhibition of active NaCl absorption with Cs^+ exceed the respective fluxes of Cl^- by a factor of about 3 (see Table 1). Furthermore, ion substitution experiments showed that the conductance decrease after Na^+ substitution was much higher than that after Cl^- substitution (Onken and Siebers, 1992). Thus, the leak pathway of shore crab gills seems to be predominantly permeable to Na^+, allowing $1Na^+$:$2Cl^-$ absorption under open-circuit conditions to be balanced. Also, the gill cuticle of *Carcinus maenas* alone has been shown to be cation-selective, suggesting ion-selective channel-like pores in this cuticle (Lignon and Péqueux, 1990). However, owing to its manifold higher conductance (Lignon, 1987) when compared with that of the split gill lamella (see Results), the contribution of the cuticle to rate-limiting transport phenomena seems to be rather small.

In this transport model (Fig. 6), K^+ channels are the sole electrogenic pathway in the apical membrane. Thus, the Cs^+-induced conductance decrease ($14\pm1\,mS\,cm^{-2}$) reflects the cellular conductance, whereas the remaining conductance of $26\pm1\,mS\,cm^{-2}$ reflects the paracellular pathway (G_p). With a G_p/G_{te} ratio of 0.65, the gill epithelium of the shore crab belongs to the group of 'leaky' epithelia (see Schultz, 1979) which maintain only moderate osmotic gradients.

Interestingly, not only the transport mechanisms but also the characteristics of the paracellular pathway show significant similarities to the epithelium of the thick ascending limb of Henle's loop in the mammalian nephron (Greger, 1985; Molony *et al.* 1989).

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References

ARONSON, P. S. AND SEIFTER, J. (1983). Cl^- transport *via* anion exchange. *Fedn Proc. Fedn Am. Socs exp. Biol.* **43**, 2483–2484.

BÖTTCHER, K., SIEBERS, D., BECKER, W. AND PETRAUSCH, G. (1991). Physiological role of branchial carbonic anhydrase in the shore crab *Carcinus maenas*. *Mar. Biol.* **110**, 337–342.

CREMASCHI, D., PORTA, C., BOTTÀ, G. AND MEYER, G. (1992). Nature of the neutral Na^+–Cl^- coupled entry at the apical membrane of rabbit gallbladder epithelium. IV. Na^+/H^+, Cl^-/HCO_3^- double exchange, hydrochlorothiazide-sensitive Na^+–Cl^- symport and Na^+–$K^+/2Cl^-$ cotransport are all involved. *J. Membr. Biol.* **129**, 221–235.

DAWSON, D. C. (1987). Properties of epithelial potassium channels. *Curr. Top. Membr. Transp.* **28**, 41–71.

DAWSON, D. C., WILKINSON, D. J. AND RICHARDS, N. W. (1990). Basolateral K^+ channel noise; signals from the dark side. *Curr. Top. Membr. Transp.* **37**, 191–212.

DE WOLF, I. AND VAN DRIESSCHE, W. (1986). Voltage-dependent Ba^{2+} block of K^+ channels in apical membrane of frog skin. *Am. J. Physiol.* **251**, C696–C706.

DIAZ, M. AND LORENZO, A. (1991). Coexistence of absorptive and secretory NaCl processes in the isolated lizard colon: effects of cyclic AMP. *Zool. Sci.* **8**, 477–484.

DIJKSTRA, S., LOHRMANN, E., VAN KERKHOVE, E., STEELS, P. AND GREGER, R. (1994). Effects of dinitrophenol on active-transport processes and cell membranes in the Malpighian tubules of *Formica*. *Pflügers Arch.* **424**, 150–156.

D'ORAZIO, S. E. AND HOLLIDAY, C. W. (1985). Gill Na,K-ATPase and osmoregulation in the sand fiddler crab, *Uca pugilator*. *Physiol. Zool.* **58**, 364–373.

DRABER, S. AND HANSEN, U.-P. (1994). Fast single-channel measurements resolve the blocking effect of Cs^+ on the K^+ channel. *Biophys. J.* **67**, 120–129.

DREWS, G. AND GRASZYNSKI, K. (1987). The transepithelial potential difference in the gills of the fiddler crab, *Uca tangeri:* influence of some inhibitors. *J. comp. Physiol.* **157**B, 345–353.

GÖGELEIN, H. (1988). Chloride channels in epithelia. *Biochim. biophys. Acta* **947**, 521–547.

GRASZYNSKI, K. AND BIGALKE, T. (1986). Osmoregulation and ion transport in the extremely euryhaline fiddler crabs *Uca pugilator* and *Uca tangeri* (Ocypodidae). *Zool. Beitr. N.F.* **30**, 339–358.

GREGER, R. (1985). Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. *Physiol. Rev.* **65**, 760–797.

GREGER, R. (1990). Chloride channel blockers. In *Methods in Enzymology*, vol. 191 (ed. B. Fleischer and S. Fleischer), pp. 793–810. Orlando: Academic Press.

GREGER, R. AND KUNZELMANN, K. (1990). Chloride-transporting epithelia. In *Comparative Physiology*, vol. 3, *Basic Principles of Transport* (ed. R. K. H. Kinne), pp. 84–114. Basel: Karger.

GREGER, R., WITTNER, M., SCHLATTER, E. AND DI STEFANO, A. (1984). $Na^+2Cl^-K^+$ cotransport in the thick ascending limb of Henle's loop and mechanism of action of loop diuretics. In *Coupled Transport in Nephron* (ed. T. Hoshi), p. 96. Tokyo: Miura Foundation.

HOLZ, R. AND FINKELSTEIN, A. (1970). The water and nonelectrolyte permeability induced in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. *J. gen. Physiol.* **56**, 125–145.

LIGNON, J. M. (1987). Ionic permeabilities of the isolated gill cuticle of the shore crab *Carcinus maenas*. *J. exp. Biol.* **131**, 159–174.

LIGNON, J. M. AND PÉQUEUX, A. (1990). Permeability properties of the cuticle and gill ion exchanges in decapod crustacans. In *Comparative Physiology*, vol. 6, *Animal Nutrition and Transport Processes*, 2, *Transport, Respiration and Excretion: Comparative and Enviromental Aspects* (ed. J. Mellinger, J. P. Truchot and B. Lahlou), pp. 14–27. Basel: Karger.

LUCU, C. (1989). Evidence for Cl^- exchangers in perfused *Carcinus* gills. *Comp. Biochem. Physiol.* **92**A, 414–420.

LUCU, C. (1990). Ionic regulatory mechanisms in crustacean gill epithelia. *Comp. Biochem. Physiol.* **97**A, 297–306.

LUCU, C. AND SIEBERS, D. (1986). Amiloride-sensitive sodium flux and potentials in perfused *Carcinus* gill preparations. *J. exp. Biol.* **122**, 25–35.

LUCU, C. AND SIEBERS, D. (1987). Linkage of Cl^- fluxes with ouabain sensitive Na/K exchange through *Carcinus* gill epithelia. *Comp. Biochem. Physiol.* **87**A, 807–811.

MOLONY, D. A., REEVES, W. B. AND ANDREOLI, T. E. (1989). Na^+:K^+, $2Cl^-$ cotransport and the thick ascending limb. *Kidney Int.* **36**, 418–426.

MURER, H. AND BURCKHARDT, G. (1983). Membrane transport of anions across epithelia of mammalian small intestine and kidney proximal tubule. *Rev. Physiol. Pharmac.* **96**, 1–51.

NEUFELD, G. J., HOLLIDAY, C. W. AND PRITCHARD, J. B. (1980). Salinity adaptation of gill Na,K-ATPase in the blue crab *Callinectes sapidus*. *J. exp. Zool.* **211**, 215–224.

ONKEN, H., GRASZYNSKI, K., JOHANNSEN, A., PUTZENLECHNER, M., RIESTENPATT, S., SCHIRMER, C., SIEBERS, D. AND ZEISKE, W. (1995). How to overcome osmotic stress? Marine crabs conquer freshwater. New insights from modern electrophysiology. *Helgoländer Meeresunters.* **49**, 715–725.

ONKEN, H., GRASZYNSKI, K. AND ZEISKE, W. (1991). Na^+-independent, electrogenic Cl^- uptake across the posterior gills of the Chinese crab (*Eriocheir sinensis*): Voltage clamp and microelectrode studies. *J. comp. Physiol.* **161**B, 293–301.

ONKEN, H. AND SIEBERS, D. (1992). Voltage-clamp measurements on single split lamellae of posterior gills of the shore crab *Carcinus maenas*. *Mar. Biol.* **114**, 385–390.

PÉQUEUX, A., GILLES, R. AND MARSHALL, W. S. (1988). NaCl transport in gills and related structures. In *Advances in Comparative and Enviromental Physiology*, vol. 1 (ed. R. Greger), pp. 2–73. Berlin: Springer Verlag.

RIESTENPATT, S. AND SIEBERS, D. (1994). Active uptake of sodium and chloride across the posterior gills of the shore crab *Carcinus maenas*. Investigations on split gill lamellae using radioisotopes and the voltage-clamp technique. *Verh. dt. zool. Ges.* **87**, 192.

RIESTENPATT, S. AND SIEBERS, D. (1995). Der Einfluß von Amilorid auf die NaCl-Absorption durch die hinteren Kiemen der Strandkrabbe *Carcinus maenas*: Tracer-Flux-Studien und elektrophysiologische Untersuchungen an halbierten Kiemenlamellen und isolierten Cuticulae. *Verh. dt. zool. Ges.* **88**, 126.

SCHIRMANNS, K. AND ZEISKE, W. (1994). An investigation of the midgut K^+ pump of the tobacco hornworm (*Manduca sexta*) using specific inhibitors and amphotericin B. *J. exp. Biol.* **188**, 191–204.

SCHULTZ, S. G. (1979). Transport across small intestine. In *Membrane Transport in Biology*, vol. IVB (ed. G. Giebisch, D. C. Tosteson and H. H. Ussing), pp. 749–780. Berlin: Springer Verlag.

SCHWARZ, H.-J. AND GRASZYNSKI, K. (1989). Ion transport in crab gills: A new method using isolated half platelets of *Eriocheir* gills in an Ussing-type chamber. *Comp. Biochem. Physiol.* **92**A, 601–604.

SCHWARZ, H.-J. AND GRASZYNSKI, K. (1990). Characterization of Na^+-transport from posterior gills of the Chinese crab *Eriocheir sinensis* using voltage-clamp-technique. *Verh. dt. zool. Ges.* **83**, 555–556.

SHETLAR, R. E. AND TOWLE, D. W. (1989). Electrogenic sodium–proton exchange in membrane vesicles from crab (*Carcinus maenas*) gill. *Am. J. Physiol.* **257**, R924–R931.

SIEBERS, D., BÖTTCHER, K., PETRAUSCH, G. AND HAMANN, A. (1990). Effects of some chloride channel blockers on potential differences and ion fluxes in isolated perfused gills of shore crabs *Carcinus maenas*. *Comp. Biochem. Physiol.* **97**A, 9–15.

SIEBERS, D., WINKLER, C., LUCU, A., GRAMMERSDORF, U. AND WILLE, H. (1987). Effects of amiloride on sodium chloride transport across isolated perfused gills of shore crabs *Carcinus maenas* acclimated to brackish water. *Comp. Biochem. Physiol.* **87**A, 333–340.

SKOU, J. C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across the cell membrane. *Physiol. Rev.* **45**, 596–617.

TOWLE, D. W. (1993). Ion transport systems in membrane vesicles isolated from crustacean tissues. *J. exp. Zool.* **265**, 387–395.

USSING, H. H. AND ZERAHN, K. (1951). Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta physiol. scand.* **23**, 110–127.

VELASQUEZ, H. (1987). The azide diuretics. *Renal Physiol.* **10**, 184–197.

WANGEMANN, P., WITTNER, M., DI STEFANO, A., ENGLERT, H. C., LANG, H. J., SCHLATTER, E. AND GREGER, R. (1986). Cl^--channel blockers in the thick ascending limb of the loop of Henle. Structure activity relationship. *Pflügers Arch.* **407**, 128–141.

WANSON, S. A., PÉQUEUX, A. AND ROER, R. D. (1984). Na^+ regulation and (Na^++K^+)-ATPase activity in the euryhaline fiddler crab *Uca minax* (Le Conte). *Comp. Biochem. Physiol.* **79**A, 673–678.

WILLS, N. AND ZWEIFACH, A. (1987). Recent advances in the characterization of epithelial ionic channels. *Biochim. biophys. Acta* **906**, 1–31.

ZEISKE, W. (1990). Noise from apical potassium ion channels. *Curr. Top. Membr. Transp.* **37**, 157–190.

ZEISKE, W., ONKEN, H., SCHWARZ, H.-J. AND GRASZYNSKI, K. (1992). Invertebrate epithelial Na^+ channels: amiloride-induced current-noise in crab gill. *Biochim. biophys. Acta* **1105**, 245–252.