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Characterization of ion transport in the isolated epipodite of the lobster *Homarus americanus*

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SUMMARY

Unfolded epipodite isolated from American lobsters (*Homarus americanus*) acclimated to dilute seawater was mounted in an Ussing-type chamber for ion transport studies. The split epipodite is an electrically polarized, one-cell-layer epithelium supported with cuticle. Under open-circuit conditions, the transepithelial potential was –4.2±1.0 mV (*N*=38). In the short-circuited epithelium, the current averaged over all of the preparations was –185.4±20.2 A cm^{-2} (*N*=38) with a high conductance of 55.2±11.4 mS cm^{-2} (*N*=38), typical for a leaky epithelium. The Na:Cl absorptive flux ratio was 1:1.6; ion substitution experiments indicated that the transport of Na^+ and Cl^- is coupled. Basolateral application of the Cl^- channel blockers 5-nitro-2-(3-phenylpropylamino) benzoate (NPPB) and niflumic acid (NFA) dose-dependently inhibited short-circuit current (I_{SC}). Secretory K^+ (Rb^+) fluxes exceeded influxes and were inhibited by the Na^+/K^+-ATPase inhibitor ouabain and the K^+ channel blocker cesium. Western blot analysis showed that Na^+/K^+-ATPase α-subunit protein was more highly expressed in the epipodite of lobsters acclimated to 20 p.p.t. compared with animals acclimated to seawater (34 p.p.t.). 3-Isobutyl-1-methyl-xanthine (IBMX) stimulated a negative I_{SC} and enhanced apical secretory K^+ flux. Basolateral application of NPPB inhibited $J^{Rb}_{B\rightarrow A}$ fluxes, suggesting the interaction of K^+ channels with NPPB-sensitive Cl^- channels. The results are summarized in a transport model, suggesting apical $Na^+/K^+/2Cl^-$ co-transport, a dominant apical K^+-secreting channel and basolaterally located Cl^- and K^+ channels. This study represents the first comprehensive characterization of ion transport processes across the lobster epipodite epithelium and indeed in any tissue within the branchial cavity of the American lobster.

Key words: crustacean, epithelial transport, chloride channel, potassium channel, Na^+/K^+-ATPase.

INTRODUCTION

American and European lobsters (*Homarus americanus* and *Homarus gammarus*) mostly live in full-strength seawater (SW) where osmolarity of the hemolymph is close to the ambient SW. However, during migrations lobsters may be found in brackish waters (Lawton and Lavalli, 1995). For example, in the Great Bay Estuary, NH, USA, they are found in summer when salinity varies between 22 and 28 p.p.t. and in springtime when salinity drops below 15 p.p.t. (Short, 1992; Watson et al., 1999). Under laboratory conditions lobsters can survive in salinities as low as 8 p.p.t., and the lethal salinity was detected between 8 and 14 p.p.t., depending on water temperature (McLeese, 1956). Although hemolymph osmoconcentration is only slightly hyperosmotic in SW (Dall, 1970), it drops in dilute seawater (DSW) but is maintained 120–150 mosmol kg^{-1} above the osmolality of DSW (Charmantier et al., 1984; Charmantier et al., 2001; Lucu and Devescovi, 1999; Flik and Haond, 2000). Moreover, specific activity of Na^+/K^+-ATPase in the tissues of the branchial cavity of lobsters (i.e. gills, branchiostegites and epipodite) is much higher in DSW than in SW. For example, when lobsters were transferred from SW to DSW, basolateral infoldings in the epipodite epithelium became more extensive (Haond et al., 1998) and the specific activity of Na^+/K^+-ATPase more than doubled (Lucu and Devescovi, 1999; Flik and Haond, 2000). Immunocytochemical observations with a monoclonal antibody to the catalytic α-subunit of Na^+/K^+-ATPase revealed that the enzyme in lobster epipodite is specifically located in the infoldings of basolateral membranes (Lignot et al., 1999). In some hyperosmoregulating Crustacea acclimated to DSW, consensus exists about a cyclic AMP-protein kinase A pathway of rapid activation of Na^+/K^+-ATPase (Lucu and Flik, 1999; Genovese et al., 2006). Over a longer term, in order to maintain enhanced enzyme activity, production of enzyme protein may increase *via* gene induction or inhibition of transcript degradation.

While the Na^+/K^+-ATPase may be considered to be the driving force for osmoregulatory ion uptake across the epipodite epithelium, other ion transporters and channels must also be involved in transepithelial ion movements. In ion substitution experiments with the gill epithelium of the shore crab *Carcinus maenas*, absorptive fluxes of Cl^- were shown to depend in part on the simultaneous presence of Na^+ and K^+, evidence supporting an apically located $Na^+/K^+/2Cl^-$ co-transporter (Riestenpatt et al., 1996). The existence of a basolateral Cl^- channel was proposed by the same authors. A similar model was suggested for the hyperosmoregulating semi-terrestrial crab *Neohelice* (*Chasmagnathus*) *granulata* with the addition of apical electroneutral Na^+/H^+ exchange mediating Na^+ influx that was also sensitive to inhibition by the carbonic anhydrase inhibitor acetazolamide (Onken et al., 2003). The main purpose of this study was an examination of ion transport processes in lobster epipodite under DSW conditions, specifically the nature of K^+ and Cl^- fluxes and their interaction with Na^+ transport, in order to gain further insight into the osmoregulatory mechanisms in this economically important species.

MATERIALS AND METHODS

Animal and tissue preparation

Lobsters *Homarus americanus* Milne-Edwards 1835 weighing 250±50 g fresh mass were purchased from a commercial supplier in Trenton, ME, USA, close to Mount Desert Island Biological Laboratory, Salisbury Cove, MA, USA. Lobsters were acclimated to 20 p.p.t. SW at 18±2°C in experimental tanks (200 l) with recirculating filtered SW for at least one week before experiments. Lobsters were fed twice a week with wild-caught mussels *Mytilus edulis* L. Observation of the pleiopods' epidermal and setal characteristics indicated that the lobsters were in the intermoult stage (Aiken, 1980).

To remove a single epipodite, the carapace was slightly lifted in a living lobster and an epipodite was carefully excised at the base of the coxopodite with fine scissors. Two or three epipodites were taken from a single lobster over a period of 24 h when experiments were being performed. We observed no changes in the lobsters' behavior, food consumption or hemolymph ionic composition 24 h after the removal of up to three epipodites from their total of 14 epipodites. Following excision, the envelope-like epipodite was transferred to ice-cooled physiological saline and split into two hemiepipodites by careful dissection. Each single-cell-thick epithelium supported by cuticle was then mounted in an Ussing-type chamber as described below. The viability of the isolated preparation was evidenced by a stable transepithelial potential and short-circuit current (I_{SC}) over 5 h, the maximum duration of the experiments. Furthermore, at the end of each experimental period, the addition of 1.5 mmol l^{-1} ouabain to the basolateral side of the tissue consistently and drastically reduced the I_{SC} without an effect on epithelial conductance, showing that the transport function of the tissue remained intact.

Solutions

Lobster saline had the following composition (mmol l^{-1}): NaCl, 240; KCl, 5; $MgCl_2$, 5; $CaCl_2$, 5; Hepes, 10 (pH was adjusted by Tris base to 7.5). For low Na^+ and low Cl^- salines, Na^+ was partially replaced with N-methyl-D-glucamine (titrated by HCl) and Cl^- by sodium gluconate to give a final concentration of 25 mmol l^{-1} Na^+ and 25 mmol l^{-1} Cl^- at pH 7.5.

Chemicals

3-Isobutyl-1-methylxanthine (IBMX) and 5-nitro-2-(3-phenylpropylamino) benzoate (NPPB) were purchased from Sigma (St Louis, MO USA) and niflumic acid (NFA) from Calbiochem (San Diego, CA, USA). Chemicals were used from stock solutions in dimethylsulphoxide (DMSO) at 1000-fold greater than the desired final concentrations. The addition of final concentrations of 0.05–0.10% DMSO (as used in the experiments) to the control saline did not result in any significant effect on current, conductance or radioactive fluxes. Radioactive ^{36}Cl and ^{86}Rb dissolved in aqueous solution were purchased from Amersham Biosciences (Piscataway, NJ, USA).

Electrophysiology

I_{SC} and conductance (G) were measured in the split epipodite using a micro-Ussing type chamber with an aperture of 0.071 cm^2 (3 mm diameter). The microchamber with mounted tissue was connected to an automatic voltage clamp instrument (558C-5; Bioengineering, University of Iowa, IA, USA) by four Ag-AgCl electrodes, i.e. two voltage electrodes and two current electrodes (Mettler-Toledo reference electrodes InLab 301, Bedford, MA, USA). The electrodes made contact with the bathing solution *via* 3% agar bridges filled with 3 mmol l^{-1} KCl solution, permitting continuous monitoring of current. The saline on both sides of the preparation was circulated by a two-channel Watson-Marlow peristaltic pump (Sci 4000, Falmouth, Cornwall, UK) at a flow rate of 0.8 ml min^{-1}.

A voltage pulse of 1.0 mV (duration 1 s; 1000 s interval between pulses) was applied by a pulse generator, producing a deflection of current from which transepithelial resistance was measured. The resulting current response represented the resistance of the physiological saline and tissue. Electrical resistance of the bathing saline in the Ussing's chamber without tissue ranged from 8 to 12 Ω cm^2. Chamber resistance was subtracted from measured total resistance with tissue mounted, to obtain the resistance (and thus conductance) of the epipodite preparation proper. The measured I_{SC} was corrected according to Ohm's law. The I_{SC} is defined as negative when current flows across the tissue from the apical side to the basolateral membrane side.

Radioactive fluxes

Fluxes of ^{22}Na, ^{36}Cl and ^{86}Rb across the epipodite preparation were measured in separate experiments, simultaneously with determination of the I_{SC}. Fluxes were assessed after 30 min equilibration of radioactivity in the experimental setup. To this end, 10 ml of saline containing ^{22}Na (2.0 kBq ml^{-1}), ^{36}Cl (2.8 kBq ml^{-1}) or ^{86}Rb (0.14 MBq ml^{-1}) were recirculated on one side of the hemichamber. On the other side, 10 ml of saline was recirculated at an equal rate (0.8 ml min^{-1}), and the levels of saline in the hemichambers were kept equal to avoid any pressure difference. The closed recirculating system had no influence on I_{SC} during the experimental period, in comparison with open circulation employed in other experiments. Samples were taken for three flux periods of 30 min each from the radioactive side (20 µl diluted to 1 ml with saline) and from the initially non-radioactive side (1 ml), mixed with 3 ml scintillation cocktail (HiSafe OptiPhase, Perkin Elmer, Waltham, MA, USA), and counted by liquid scintillation counting (Beckman, Turku, Finland). Sample volumes removed from the experimental setup were replaced by equal amounts of saline. Separate preparations were used for influx ($J^{Rb}_{A\rightarrow B}$) and efflux ($J_{B\rightarrow A}$) determinations (A=apical and B=basolateral side). Radioactivity passing the epipodite preparation from the perfusion saline at one side of the epithelium to the saline collecting radioactivity at the opposite side was used to measure fluxes, expressed in µmol cm^{-2} h^{-1}.

Statistics

All results represent means ± s.d. where N is the number of single experiments. Significant differences between treatments were assayed by analysis of variance (ANOVA) in combination with Student's t-test and Tukey's test (unpaired and paired data). The level of statistical significance was set at $P<0.05$. The inhibition concentration of drugs (IC_{50} values) for NPPB and NFA were determined using a logistic function to fit the data (Graph Pad Prism, Aberystwyth, UK).

RESULTS

The split epipodite preparation isolated from lobsters acclimated to DSW is an electrically polarized, one-cell-layer epithelium supported with cuticle. This epithelium was mounted in an Ussing-type chamber and perfused apically and basolaterally with identical 240 mmol l^{-1} NaCl saline. Under open-circuit conditions, a transepithelial potential of –4.2±1.0 mV (N=38) was registered, the basolateral side of the epithelium having negative polarity. The short-circuited epipodite epithelium with transepithelial potential held at 0 mV generated a negative I_{SC} from the apical to basolateral side

of –185.4±20.2 μA cm^{-2} (N=38), with a conductance of 55.2±11.4 mS cm^{-2} (N=38).

Unidirectional Na^+, Cl^- and Rb^+ (K^+) fluxes were measured under short-circuited conditions (Table 1). The isolated epipodite preparation showed net $J^{Na}_{A\rightarrow B}$ and $J^{Cl}_{A\rightarrow B}$ influxes of 6.0 and 9.4 μmol cm^{-2} h^{-1}, respectively. In the absence of any chemical gradient between apical and basolateral sides with identical salines at both sides of the epithelium, sodium and chloride absorption must be maintained actively by the epithelium layer of the epipodite preparation (Table 1). Net ^{86}Rb efflux ($J^{Rb}_{B\rightarrow A}$) was –0.7±0.1 μmol cm^{-2} h^{-1} and the flux ratio (J_{in}/J_{out}) was 0.4±0.1. Under short-circuit conditions, basolateral application of 1.5 mmol l^{-1} ouabain had no effect on $J^{Rb}_{A\rightarrow B}$ influx; however, $J^{Rb}_{B\rightarrow A}$ efflux was reduced about 50% (48.4±3.2%; N=5), achieving a balance with $J^{Rb}_{A\rightarrow B}$ influx (Table 1). These results support an essential role of Na^+/K^+-ATPase in mediating potassium flux across the epipodite epithelium. Under short-circuit conditions, negative current from the apical to basolateral side must have resulted predominantly from chloride influx and also potassium efflux.

The effects on I_{SC} of ion substitution on both sides of the epithelium were analyzed in separate experiments where Na^+ in the control saline was partially replaced with N-methyl-D-glucamine (titrated by HCl) and Cl^- was partially replaced with sodium gluconate to make final concentrations of 25 mmol l^{-1} Na^+ and 25 mmol l^{-1} Cl^-. I_{SC} in 25 mmol l^{-1} Cl^- saline steadily decreased by 83%, from –160 μA cm^{-2} (control) to –28 μA cm^{-2}. In 25 mmol l^{-1} Na^+ saline, I_{SC} decreased by 77%, from –176 μA cm^{-2} (control) to –41 μA cm^{-2} (Fig. 1). In the low Na^+ and Cl^- saline, conductances decreased from 58.2±13.2 mS cm^{-2} (N=8) to 25.2±5.1 mS cm^{-2} (N=8). The same polarity and magnitude of I_{SC} reduction indicate that these ions are functionally coupled.

The effects of two inhibitors of Cl^- channels, NPPB and NFA, were studied on I_{SC} in order to gain insight into the possible participation of Cl^- channels in the epipodite preparation. Basolaterally added NPPB and NFA inhibited I_{SC} dose-dependently with respective half-maximum inhibitory concentrations (IC_{50}) of 27.6 μmol l^{-1} (N=4) and 52.8 μmol l^{-1} (N=4) (Fig. 2). Mean conductance measured in control saline (55.2±15.9 mS cm^{-2}) did not significantly differ in NFA- and NPPB-treated groups (53.0±5.2 mS cm^{-2} and 56.1±19.7 mS cm^{-2}, respectively).

The potassium channel blocker cesium applied apically or basolaterally at a concentration of 12 mmol l^{-1} CsCl inhibited I_{SC} after a 90-minute incubation period by 61.9 and 42.6%, respectively (Fig. 3) but had no effect on $J^{Rb}_{A\rightarrow B}$. However, CsCl reduced $J^{Rb}_{B\rightarrow A}$ by 44.3±5.3% (P<0.001; apical side) and 30.4±7.7% (P<0.01; basolateral side). CsCl added apically reduced net $J^{Rb}_{B\rightarrow A}$ by 58.2±13.7% (P<0.01) and by 44.7±19.2% (P<0.05) when added to the basolateral saline (Fig. 3). Conductance of 49.0±18.2 mS cm^{-2} was not changed during the experiments.

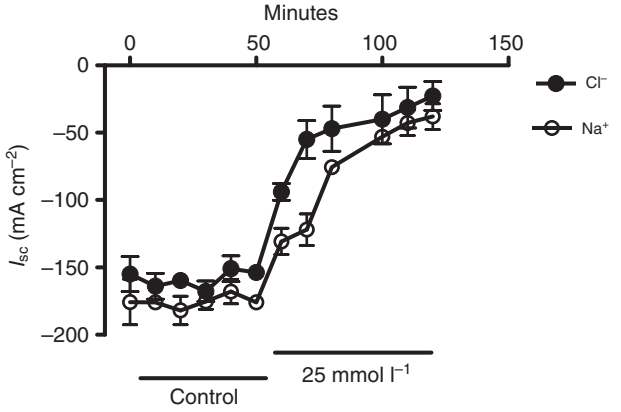

Fig. 1. A representative experiment on the time course of decrease in short-circuit current (I_{SC}) across the split epipodite in saline with 25 mmol l^{-1} Na^+ and 25 mmol l^{-1} Cl^- where the balance of sodium and chloride ions (to constitute the equivalent of 240 mmol l^{-1} NaCl saline) was substituted by N-methyl-D-glucamine chloride and sodium gluconate. Values are means ± s.d. of four separate experiments.

When the Na^+/K^+-ATPase inhibitor ouabain was added to the basolateral side of the tissue under I_{SC} conditions, current was drastically reduced to about 5% of control values. The remaining current was unaffected by 12 mmol l^{-1} CsCl added to either the apical or basolateral side (Fig. 4). The epithelial conductance of 58.2±5 ms cm^{-2} was not changed after ouabain and CsCl application. Moreover, the ouabain-insensitive portion of $J^{Rb}_{B\rightarrow A}$ effluxes (Table 1) was not affected by CsCl (0.65 μmol cm^{-2} h^{-1}).

Further evidence linking the Na^+/K^+-ATPase to transport processes in the epipodite was suggested by western blot analysis of the Na^+/K^+-ATPase α-subunit protein in partially purified membranes from the epipodite. The α-subunit showed an apparent molecular weight of 96±6 kDa (Fig. 5), a value in agreement with the 90 kDa phosphorylated α-subunit of the epipodite of *Homarus gammarus* (Lignot and Charmantier, 2001). By applying the same total amount of protein in each lane, it is apparent that the α-subunit

Table 1. Sodium, chloride and potassium (^{86}Rb) fluxes in hemiepipodite preparations isolated from the lobster *Homarus americanus*

	Influx		Efflux	Net	
	(μmol cm^{-2} h^{-1})				J_{in}/J_{out}
Na^+	21.2±2.4 (5)		15.2±0.9 (5)	6.0±1.7 (5)	1.4±0.1 (5)
		P<0.002			
Cl^-	27.2±5.4 (6)		17.9±5.3 (6)	9.4±1.0 (6)	1.6±0.2 (6)
		P<0.0001			
Rb^+	0.6±0.1 (8)		1.3±0.1 (8)	–0.7±0.1 (8)	0.4±0.1 (8)
		P<0.0001			
Rb^++1.5 mmol l^{-1} ouabain	0.7±0.1 (5)		0.7±0.1 (5)	–0.03 ±0.07 (5)	1.0±0.01 (5)
		NS			

Unidirectional ^{22}Na, ^{36}Cl and ^{86}Rb fluxes were measured under short-circuit conditions as described in the text. Rb^+ (K^+) fluxes were measured before and after inhibition of current by 1.5 mmol l^{-1} ouabain. J_{in}/J_{out} is the ratio of experimentally observed ion fluxes. The concentrations of Cl^- and K^+ (DSW) were identical on both sides of the epipodite preparation. The values are given as means±s.d. with number of observations in parentheses. The statistical significance of differences between measured fluxes and calculated and measured flux ratios with the level of statistical significance (t-test) is presented. NS=not significantly different.

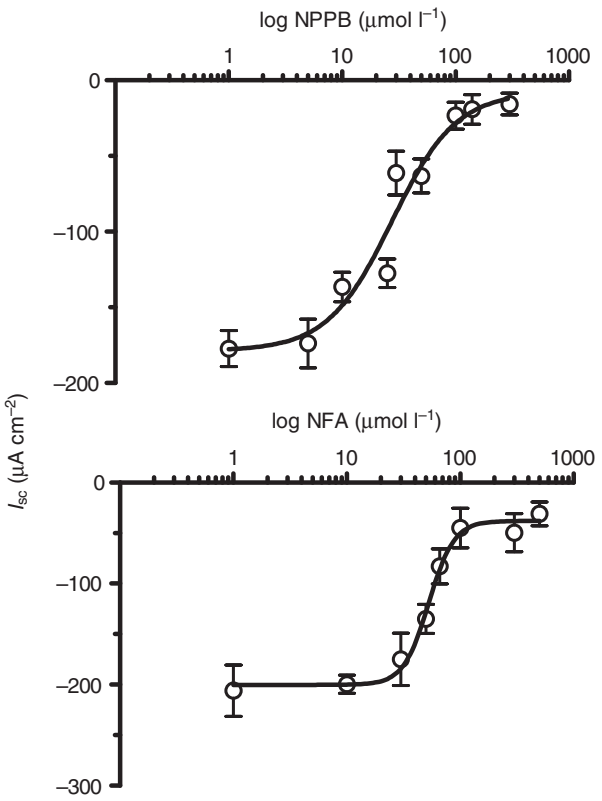

Fig. 2. Dose-dependent inhibition of short-circuit current (I_{SC}) after basolateral exposure of the epipodite preparation to the Cl^- channel blockers 5-nitro-2-(3-phenylpropylamino) benzoate (NPPB) and niflumic acid (NFA). Data are reported as means ± s.d. of four separate experiments.

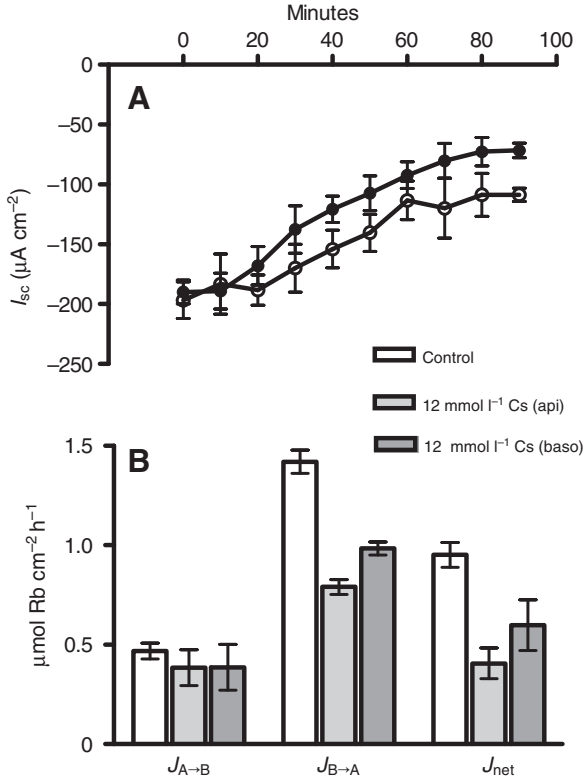

Fig. 3. (A) Inhibition of short-circuit current (I_{SC}) after apically (closed circles) and basolaterally (open circles) applied CsCl (12 mmol l^{-1}). Values are means ± s.d. of four separate experiments. (B) Unidirectional $J_{A\rightarrow B}K^+$ (Rb^+) influxes and $J_{B\rightarrow A}K^+$ (Rb^+) effluxes and J_{net} fluxes. Control saline: empty bars; saline with 12 mmol l^{-1} CsCl added apically (light colored bars); saline with 12 mmol l^{-1} CsCl added basolaterally (dark colored bars). Values are means ± s.d. of five separate experiments. The conductance remained unaffected by the presence of 12 mmol l^{-1} CsCl added either at the apical or basolateral side of the epipodite preparation (49.0±18.2 mS cm^{-2}). Analysis of variance (ANOVA) following Student's paired two-tailed *t*-test: $J_{A\rightarrow B}$ $P>0.05$; $J_{B\rightarrow A}$, control–Cs_{api} $P<0.001$; control–Cs_{baso} $P<0.01$; J_{net}, control–Cs_{api} $P<0.01$, control–Cs_{baso} $P<0.01$.

was more highly expressed in epipodites isolated from lobsters acclimated to DSW (lanes 4 and 5; Fig. 5) compared with lobsters acclimated to SW (lanes 1, 2 and 3; Fig. 5).

The phosphodiesterase inhibitor IBMX (1 mmol l^{-1}) added apically stimulated I_{SC} from –191.0±6.7 μA cm^{-2} (control; N=4) to –260.0±23.1 μA cm^{-2} (N=4; $P<0.001$), an increase of 36%. After stimulation of I_{SC} by IBMX, the apical addition of 12 mmol l^{-1} CsCl inhibited I_{SC} by 54.6% compared with the control value (N=4) (Fig. 6A). In accordance, $J^{Rb}_{B\rightarrow A}$ fluxes were stimulated by 1 mmol l^{-1} IBMX, from 1.52±0.19 μmol cm^{-2} h^{-1} to 1.88±0.21 μmol cm^{-2} h^{-1} (N=4; $P<0.05$) and inhibited by 12 mmol l^{-1} CsCl to 0.75±0.07 μmol cm^{-2} h^{-1} (N=4; $P<0.001$; Fig. 6B). Conductances before (51.9±6.0 mS cm^{-2}) and after IBMX and 12 mmol l^{-1} CsCl (55.2±15.9 mS cm^{-2}) were not changed.

Besides blocking chloride channels, basolateral application of NPPB at 50 μmol l^{-1} also inhibited $J^{Rb}_{B\rightarrow A}$ effluxes, suggesting a possible interaction of potassium channels with NPPB-sensitive chloride channels (Fig. 7). $J^{Rb}_{B\rightarrow A}$ fluxes were inhibited 35.5% by basolaterally applied 50 μmol l^{-1} NPPB, from 1.46±0.06 μmol cm^{-2} h^{-1} (N=4) to 0.94±0.29 μmol cm^{-2} h^{-1} (N=4; $P<0.05$) (Fig. 7).

DISCUSSION

Isolated gill lamella preparations from euryhaline hyperosmoregulating Crustacea and the split lobster epipodite have similar electrophysiological properties. Both consist of a cell monolayer with similar morphology, supported by cuticle (Lawson et al., 1994; Haond et al., 1998). The split epipodite preparation consists of a monolayer epithelium attached at the apical membrane domain to a 5 μm-thick chitinous cuticle (Haond et al., 1998). The cuticle of lobster gills is highly permeable to NaCl [10^{-3} cm s^{-1} (Avenet and Lignon, 1985)] and does not show selectivity for Na^+ over Cl^- as described for the less permeable cuticle of brackish water and freshwater Crustacea (Lignon, 1987). Cuticular conductivity in lobsters is 4.5-fold higher than that reported for the typical marine crustacean *Maia squinado* and 52-fold higher than the gill cuticle of *Carcinus maenas* (Pequeux and Lignon, 1992). The cuticle thus serves far less, or is even insignificant, as a barrier for monovalent ions in lobsters than it is in typical brackish water species.

Under short-circuited conditions, a large inward negative I_{SC} was generated from the apical side of preparation. The polarity and range of I_{SC} and conductance in isolated lobster epipodite were found to be similar to gill lamella preparations isolated from brackish water Crustacea (Onken and Siebers, 1992; Onken and Riestenpatt, 2002; Onken et al., 2000; Tresguerres et al., 2003). A large conductance (49.0±18.2 mS cm^{-2}), coupled with strong inhibition of I_{SC} by apically added CsCl (Fig. 4), indicate that overall conductance is dominated by paracellular pathways. Therefore, the lobster epipodite

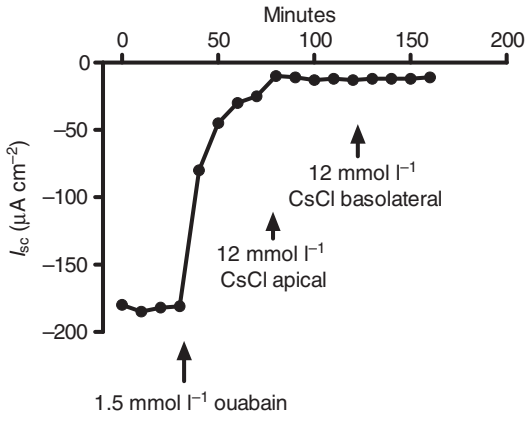

Fig. 4. Representative time course of short-circuit current (I_{SC}) in the lobster epipodite preparation before and after application of 1.5 mmol l^{-1} ouabain. Apically and basolaterally added 12 mmol l^{-1} CsCl had no effect on the ouabain-insensitive portion of I_{SC}. During the experimental procedure conductance remained unchanged (58.2±5.0 mS cm^{-2}).

preparation can be described as a leaky epithelium, in agreement with findings on isolated gill lamellae from other crustaceans (Riestenpatt et al., 1996; Onken et al., 2000; Tresguerres et al., 2003).

In the absence of any chemical gradients between the apical and basolateral side of the epithelium and under short-circuited conditions, active sodium and chloride absorption and potassium secretion were clearly indicated (Table 1). More net Cl^- than Na^+ was absorbed by the epithelium (Table 1), and to maintain equilibrium the rest of the Na^+ may be transported *via* the paracellular pathway. Transcellular NaCl absorption across the epipodite epithelium may be considered to be electrogenic with a Na^+/Cl^- flux ratio of 1:1.6 (Table 1). Respecting the fact that fluxes and standard deviations were measured with different preparations it becomes evident that the observed Na^+/Cl^- ratio seems close to the 1:2 ratio expected for ion uptake *via* a $Na^+/K^+/2Cl^-$ co-transporter. In support of this suggestion is our observation that I_{SC}

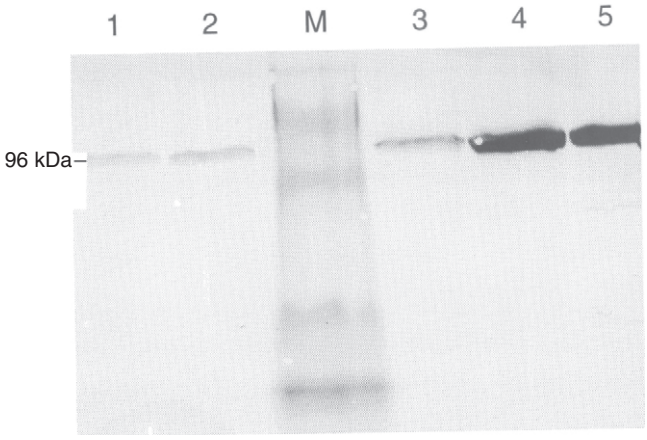

Fig. 5. Western blot of Na^+/K^+-ATPase α-subunit in partially purified membranes prepared from epipodites of lobster *Homarus americanus* from seawater (lanes 1, 2 and 3) and for 10 days in 18 p.p.t. dilute seawater (lanes 4 and 5). Blots were probed with a mouse monoclonal antibody against chicken Na^+/K^+-ATPase (cytosolic epitope of α-subunit), recognizing a 96 kDa protein as the prominent species.

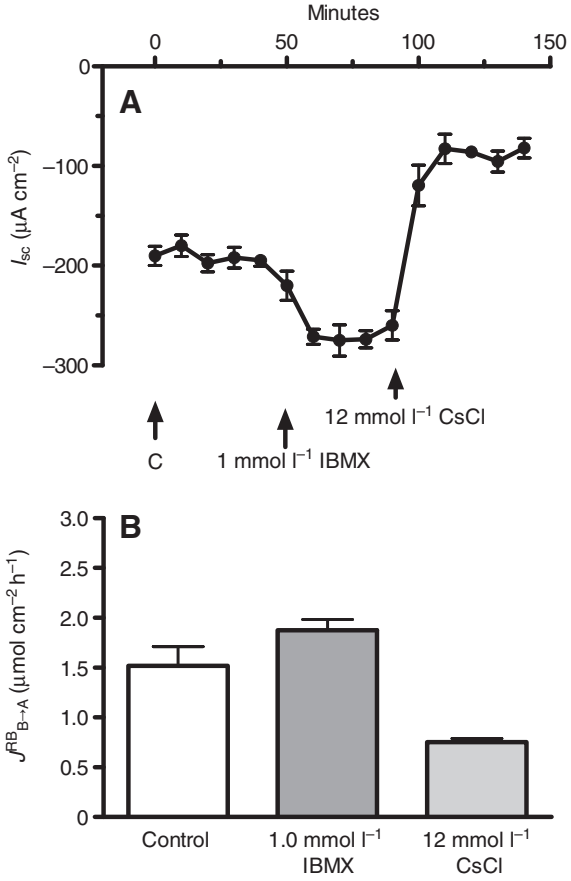

Fig. 6. Stimulation of short-circuit current (I_{SC}) (A) and $J^{Rb}_{B\rightarrow A}$ fluxes (B) in the lobster epipodite preparation by basolaterally added 1 mmol l^{-1} 3-isobutyl-1-methyl-xanthine (IBMX), and inhibition of I_{SC} and $J^{Rb}_{B\rightarrow A}$ after the successive addition of 12 mmol l^{-1} CsCl at the apical side. Values are means ± s.d. of four separate experiments.

and conductance across the epipodite were strongly reduced in either low (25 mmol l^{-1}) Na^+ or low (25 mmol l^{-1}) Cl^- saline, in which the osmotic equivalent of 240 mmol l^{-1} NaCl was provided by either N-methyl-D-glucamine chloride or sodium gluconate.

Further evidence for participation of the $Na^+/K^+/2Cl^-$ co-transporter in ion uptake across the epipodite was obtained using mercury as an experimental probe for co-transporter function (Jacoby et al., 1999; Kinne-Saffran and Kinne, 2001). In the epipodite preparation of the European lobster (*H. gammarus*), methyl mercury (Lucu et al., 2009) and mercury chloride (Č.L., unpublished) when applied in the nanomolar range at the apical side strongly inhibited both I_{SC} and Cl^- influxes. In this concentration range, Na^+/K^+-ATPase and I_{SC} were unaffected by basolateral application of the mercury compounds. These results support the coupling of ions *via* the $Na^+/K^+/2Cl^-$ co-transporter as suggested in earlier studies on crustaceans, including *Carcinus*, *Pachygrapsus* and *Chasmagnathus* (Lucu and Siebers, 1987; Pierrot et al., 1995; Riestenpatt et al., 1996; Onken et al., 2000; Onken et al., 2003; Tresguerres et al., 2003).

However, in Crustacea, an apically located absorptive $Na^+/K^+/2Cl^-$ co-transporter has not been confirmed by the use of specific inhibitors. We have shown that rubidium influx in control epipodite preparations (0.5±0.1 µmol cm^{-2} h^{-1}; N=5) was not different from values obtained after incubation with 0.5 mmol l^{-1}

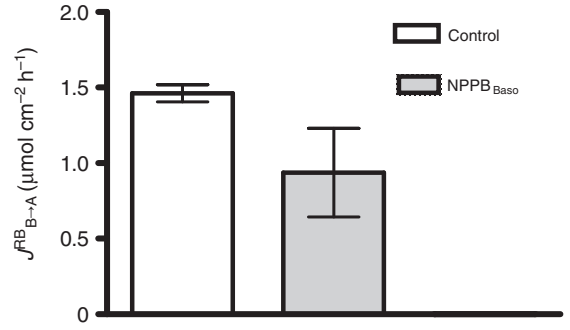

Fig. 7. Effect of basolaterally added 50 μmol l^{-1} 5-nitro-2-(3-phenylpropylamino) benzoate (NPPB) on $J^{Rb}_{B\rightarrow A}$ (N=4). Values are means ± s.d. of four experiments. P<0.05 for comparison between control and NPPB-treated group.

bumetanide (0.6±0.2 μmol cm^{-2} h^{-1}; N=5) or 1.0 mmol l^{-1} furosemide (0.5±0.2 μmol cm^{-2} h^{-1}; N=5). Furthermore, neither furosemide nor bumetanide have much effect on I_{SC} in *Carcinus* gills (Lucu, 1989; Riestenpatt et al., 1996). These observations may reflect the low permeability of the cuticle to organic compounds, interfering with access of inhibitors to the apical membrane (Onken and Riestenpatt, 2002). Nevertheless, at the molecular level, a $Na^+/K^+/2Cl^-$ co-transporter is clearly expressed in crustacean ion-transporting tissues (Luquet et al., 2005).

In epipodites of the European lobster *H. gammarus*, the Na^+/K^+-ATPase is a key generator of secondary active transport and Na^+/K^+-ATPase activity was found to increase following acclimation to DSW (Lucu and Devescovi, 1999). The catalytic α-subunit (Skou and Esmann, 1992) of the Na^+/K^+-ATPase was characterized in lobster epipodite *via* western blot analysis, using a monoclonal antibody against chicken (*Gallus gallus*) α-subunit. An α-subunit protein of 96±6 kDa was detected in epipodite from *H. americanus*, approximately the same size as in membrane preparations from epipodite of *H. gammarus* (Lignot and Charmantier, 2001) and in gills of *Carcinus maenas*, *Callinectes danae* and *Callinectes sapidus* (Towle and Kays, 1986; Lucu and Flik, 1999; Towle et al., 2001; Masui et al., 2002). Overall, the α-subunit of the Na^+/K^+-ATPase is highly conserved between species (Lucu and Towle, 2003). The amino acid sequence of the *Callinectes* α-subunit is 71–74% identical to the avian isoform and is 94% identical to the α-subunit sequence of *Homarus* (Towle et al., 2001). Furthermore, expression of the α-subunit protein of lobster epipodite is more apparent in partially purified membranes from animals acclimated to DSW than to SW, revealing upregulation of this protein during more than 10 days acclimation (Fig. 5), as also shown for *Carcinus* (Lucu and Flik, 1999).

In short-circuited lobster epipodite, the chloride channel inhibitor NPPB inhibited I_{SC} dose-dependently when applied basolaterally, with a half-inhibitory concentration of 27.6 μmol l^{-1}, a concentration about 4-fold higher than the concentration that inhibits I_{SC} in gill epithelium of the shore crab *Carcinus* (Riestenpatt et al., 1996). NFA inhibited I_{SC} dose-dependently with an IC_{50} about twice that for NPPB. Unfortunately, few specific potent anion channel blockers are available; most of them require high concentration and have undesirable non-specific side effects. The lack of high-affinity inhibitors of Cl^- channels has hindered the molecular identification of specific channel proteins. NPPB and NFA have been widely used in functional studies of understanding the role of Cl^- channels, particularly those activated by changes in intracellular Ca^{2+} (Jentsch et al., 2002; Fuller and Benos, 2000). Moreover, NFA is the most common blocker for the native Ca^{2+}-dependent chloride channel [CaCC (White and Aylwin, 1990)], showing inhibition of anion-driven current in *Xenopus* oocytes at concentrations in the 10 μmol l^{-1} range (Qu and Hartzell, 2001). A cDNA predicted to encode a Ca^{2+}-activated chloride channel has been identified in a normalized cDNA library prepared from multiple tissues of *H. americanus* (Towle and Smith, 2006); however, it is not known whether this particular channel is the active participant in the studies described here.

Pharmacological characterization of net effluxes of Rb^+ (substituting for K^+) was carried out with the potassium channel blocker Cs^+ (Zeiske et al., 1992). The secretory cesium-sensitive potassium channel in lobster epipodite has a physiological similarity to the high conductance open probability K^+ channel located in the apical membrane of the thick ascending limb of the loop of Henle (Wang, 1995; Giebisch, 1998). When 12 mmol l^{-1} CsCl was applied either apically or basolaterally, total $J^{Rb}_{B\rightarrow A}$ effluxes were significantly inhibited by 44.3% (apical side) and 30.4% (basolateral side). However, absorptive K^+ fluxes ($J^{Rb}_{A\rightarrow B}$) were not inhibited by CsCl beyond the level of the diffusible portion of ouabain-insensitive effluxes ($J^{Rb}_{B\rightarrow A}$). These results imply that K^+ channels significantly stimulate outward K^+ current, functioning as a Na^+/K^+-ATPase-modulated K^+ channel.

To estimate the contribution of K^+ secretion to I_{SC}, multiplication of the net K^+ efflux ($Rb_{B\rightarrow A}$) by the Faraday constant (1 μmol Rb cm^{-2} h^{-1}=26.8 μA cm^{-2}) allows the expression of K^+ flux in terms of current ($I_{SC}K^+$). Thus, the contribution of net K^+ efflux to total

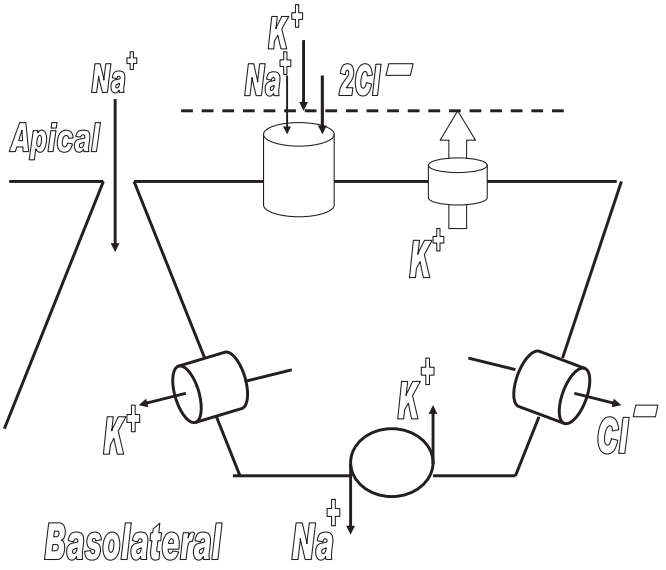

Fig. 8. A model showing the hypothetical mechanisms of electrolyte transport by the epipodite epithelium of the American lobster. The broken line represents the apically located cuticle. Absorption of NaCl occurs through the activity of electroneutral $Na^+/K^+/2Cl^-$ co-transport. Activity of basolateral Na^+/K^+-ATPase (Na^+/K^+) provides the driving force for the transport and also generates a high intracellular concentration of K^+, which exits through the apically located K^+ channels. This ensures production of an apically positive electrical potential which itself is the driving force for paracellular absorption of Na^+. Potassium and chloride transported into the cell by the co-transporter are hypothesized to exit the basolateral side by CsCl-sensitive K^+ channels and 5-nitro-2-(3-phenylpropylamino) benzoate (NPPB)- and niflumic acid (NFA)-sensitive Cl^- channels.

I_{SC} is about 6% and is equal to the ouabain-sensitive portion, indicating that active K^+ secretion is entirely inhibited by ouabain. This is also apparently the case for active NaCl absorption, because ouabain seems to almost completely abolish I_{SC} (Fig. 4).

When 1 mmol l^{-1} IBMX was added at the basolateral side of the epipodite preparation, I_{SC} and $J^{Rb}_{B\rightarrow A}$ (K^+) fluxes were stimulated. In posterior gills of the Chinese crab, IBMX treatment increased intracellular cAMP (Bianchini and Gilles, 1990) and also increased the transepithelial potential of perfused posterior gills of the hyperosmoregulating crab *Chasmagnathus* (Halperin et al., 2004). We suggest that the stimulation of K^+ noted in this study could be explained by a cAMP-dependent change in the phosphorylation state of the Na^+/K^+-ATPase and consequent activation of the enzyme (Kiroytcheva et al., 1999).

To summarize our findings on the split epipodite of lobster, a conventional model based on these and other transport studies in crustaceans is hypothesized (Fig. 8). Acclimation of lobsters to DSW is hypothesized to increase Na^+/K^+-ATPase activity through *de novo* synthesis or by activation of latent enzyme activity, enhancing the sodium motive force for hyperregulation. Inhibition of current by ouabain supports a pivotal role of the Na^+/K^+-ATPase enzyme in the epipodite, contributing to the IBMX-sensitive net K^+ (Rb^+) efflux *via* cesium-sensitive apical K^+ channels. By reducing cell Na^+, the Na^+/K^+-ATPase generates steep Na^+ gradients across the apical membrane which produces energy for the coupling of $Na^+/K^+/2Cl^-$ co-transport, supported in this study by the noted reduction of I_{SC} in low Na^+ and low Cl^- salines and by radioactive flux measurements showing a Na:Cl transport ratio close to 1:2. The net K^+ concentration gradient across the apical membrane favors flux *via* Cs-sensitive K^+ channels, supplying a driving force for co-transporter function as well as for movement of ions *via* the paracellular pathway. At the basolateral side, Cl^- is hypothesized to leave the cell *via* NPPB- and NFA-sensitive Cl^- channels (Fig. 8). The striking similarities in transport mechanisms between gills and epipodite in hyperosmoregulating Crustacea and the thick ascending limb in vertebrates suggests the possibility that during a long evolutionary history the vertebrate thick ascending limb preserves properties from SW ancestors.

LIST OF ABBREVIATIONS

DSW	dilute seawater
G	conductance
IBMX	3-isobutyl-1-methyl-xanthine
IC_{50}	half-maximum inhibitory concentration
I_{SC}	short-circuit current
NFA	niflumic acid
NKCC	$Na^+/K^+/2Cl^-$ co-transporter
NPPB	5-nitro-2-(3-phenylpropylamino) benzoate
SW	seawater

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REFERENCES

Aiken, D. E. (1980). Moulting and growth. In *The Biology and Management of Lobsters* (ed. J. S. Cobb and B. F. Phillips), pp. 91-163. New York: Academic Press.
Avenet, P. and Lignon, J. M. (1985). Ionic permeabilities of the gill lamina cuticle of the crayfish *Astacus leptodactylus* (E.). *J. Physiol. Lond.* **363**, 377-401.
Bianchini, A. and Gilles, R. (1990). Cyclic AMP as a modulator of NaCl transport in gills of euryhaline Chinese crab *Eriocheir sinensis*. *Mar. Biol.* **104**, 191-195.
Charmantier, G., Thuet, P. and Charmantier-Daures, M. (1984). La regulation osmotique et ionique ches *Homarus gammarus* (L) (Crustacea, Decapoda). *J. Exp. Mar. Biol. Ecol.* **76**, 191-199.
Charmantier, G., Haond, C., Lignot, J. H. and Charmantier-Daures, M. (2001). Ecophysiological adaptation to salinity throughout a life cycle: a review in homarid lobsters. *J. Exp. Biol.* **204**, 967-977.
Dall, W. (1970). Osmoregulation in the lobster *Homarus americanus*. *J. Fish. Res. Bd. Canada* **27**, 1123-1130.
Flik, G. and Haond, C. (2000). Na^+ and Ca^{2+} pumps in the gills, epipodites and branchiostegites of the European lobster *Homarus gammarus*: effects of dilute sea water. *J. Exp. Biol.* **203**, 213-220.
Fuller, C. M. and Benos, D. (2000). Ca^{2+}-activated Cl channels: a newly emerging anion transport family. *News Physiol.* **15**, 165-171.
Genovese, G., Senek, M., Ortiz, N., Regueira, M., Towle, D. W., Tresguerres, M. and Luquet, C. M. (2006). Dopaminergic regulation of ion transport in gills of the euryhaline semiterrestrial crab *Chasmagnathus granulatus*: interaction between D_1- and D_2-like receptors. *J. Exp. Biol.* **209**, 2785-2793.
Giebisch, G. (1998). Renal potassium transport mechanisms and regulation. *Am. J. Physiol.* **274**, F817-F833.
Halperin, J., Genovese, G., Tresguerres, M. and Luquet, C. M. (2004). Modulation of ion uptake across posterior gill of the crab *Chasmagnathus granulatus* by dopamine and cAMP. *Comp. Biochem. Physiol.* **139A**, 103-109.
Haond, C., Flik, G. and Charmantier, G. (1998). Confocal laser scanning and electron microscopical studies on osmoregulatory epithelia in the branchial cavity of the lobster *Homarus gammarus*. *J. Exp. Biol.* **201**, 1817-1833.
Jacoby, S. C., Gagnon, E., Caron, L., Chang, J. and Isenring, P. (1999). Inhibition of Na^+-K^+-$2Cl^-$ cotransport by mercury. *Am. J. Physiol.* **277**, C684-C692.
Jentsch, T. J., Stein, V., Weinreich, F. and Zdebik, A. A. (2002). Molecular structure and physiological function of chloride channels. *Physiol. Rev.* **82**, 503-668.
Kinne-Saffran, E. and Kinne, R. K. H. (2001). Inhibition by mercuric chloride of Na-K-2Cl cotransport acitivity in rectal gland plasma membrane vesicles isolated from *Squalus acanthias*. *Biochim. Biophys. Acta* **1510**, 442-451.
Kiroytcheva, M., Cheval, L., Luisa, M., Yves Martin, P., Favre, H., Doucet, A. and Feraille, E. (1999). Effect of cAMP on the activity and the phosphorylation of Na^+,K^+-ATPase in rat thick ascending limb of Henle. *Kidney Int.* **55**, 1819-1831.
Lawson, S. L., Jones, M. B. and Moate, R. M. (1994). Structural variability and distribution of cells in a posterior gill of *Carcinus maenas* (Decapoda, Brachyura). *J. Mar. Biol. Assoc. UK* **74**, 771-785.
Lawton, P. and Lavalli, K. (1995). Postlarval, juvenile, adolescent, and adult ecology. In *Biology Of The Lobster Homarus americanus* (ed. J. R. Factor), pp. 47-88. San Diego: Academic Press.
Lignon, J. M. (1987). Ionic permeabilities of the isolated gill cuticle of the shore crab *Carcinus maenas*. *J. Exp. Biol.* **131**, 159-174.
Lignot, J. H. and Charmantier, G. (2001). Immunolocalization of Na^+,K^+-ATPase in the branchial cavity during the early development of the European lobster *Homarus gammarus* (Crustacea, Decapoda). *J. Histochem. Cytochem.* **49**, 1013-1023.
Lignot, J. H., Charmantier-Daures, M. and Charmantier, G. (1999). Immunolocalization of Na^+,K^+-ATPase in the organs of the branchial cavity of the European lobster *Homarus gammarus* (Crustacea, Decapoda). *Cell Tissue Res.* **296**, 417-426.
Lucu, Č. (1989). Evidence for Cl^- exchangers in perfused *Carcinus* gills. *Comp. Biochem. Physiol.* **92A**, 415-420.
Lucu, Č. and Devescovi, M. (1999). Osmoregulation and branchial Na^+,K^+-ATPase in the lobster *Homarus gammarus* acclimated to dilute seawater. *J. Exp. Mar. Biol. Ecol.* **234**, 291-304.
Lucu, Č. and Flik, G. (1999). Na^+-K^+-ATPase and Na^+/Ca^{2+} exchange activities in gills of hyperregulating *Carcinus maenas*. *Am. J. Physiol.* **276**, R490-R499.
Lucu, Č. and Siebers, D. (1987). Linkage of Cl^- fluxes with ouabain sensitive Na/K exchange through *Carcinus* gill epithelia. *Comp. Biochem. Physiol.* **87A**, 807-811.
Lucu, Č. and Towle, D. W. (2003). $Na^+ + K^+$ ATPase in gills of aquatic crustaceans. *Comp. Biochem. Physiol.* **135A**, 195-214.
Lucu, Č., Dupčić-Radić, I. and Tomšić, S. (2009). Methyl mercury inhibits short-circuit current and Cl^- influx across isolated epipodite of European lobster *Homarus gammarus*. *Comp. Biochem. Physiol.* **149C**, 476-480.
Luquet, C. M., Weihrauch, D., Senek, M. and Towle, D. W. (2005). Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*. *J. Exp. Biol.* **208**, 3627-3636.
Masui, D., Furriel, R., McNamara, J., Mantellato, F. and Leone, F. (2002). Modulation by ammonium ions of gill microsomal (Na^+, K^+)-ATPase in the swimming crab *Callinectes danae*: a possible mechanism for regulation of ammonia excretion. *Comp. Biochem. Physiol.* **132C**, 471-482.
McLeese, D. W. (1956). Effects of temperature, salinity and oxygen on the survival of the American lobster. *J. Fish. Res. Board Can.* **13**, 247-272.
Onken, H. and Riestenpatt, S. (2002). Ion transport across posterior gills of hyperosmoregulating shore crabs (*Carcinus maenas*): Amiloride blocks the cuticular Na^+ conductance and induces current-noise. *J. Exp. Biol.* **205**, 523-531.
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.
Onken, H., Schoebel, A., Kraft, J. and Putzenlechner, M. (2000). Active NaCl absorption across split lamellae of posterior gills of the Chinese crab *Eriocheir sinensis*: stimulation by eyestalk extracts. *J. Exp. Biol.* **203**, 1372-1381.
Onken, H., Tresguerres, M. and Luquet, C. M. (2003). Active NaCl absorption across posterior gills of hyperosmoregulating *Chasmagnathus granulatus*. *J. Exp. Biol.* **206**, 1017-1023.
Pequeux, A. and Lignon, J. (1992). Permeabilite cuticulaire et ionoregulation chez les Crustaces Decapodes. *Cah. Biol. Mar.* **32**, 203-211.
Pierrot, C., Pequeux, A. and Thuet, P. (1995). Effects of ions substitutions and of inhibitors on transepithelial potential difference and sodium fluxes in perfused gills of the crab *Pachygrapsus marmoratus*. *Arch. Physiol. Biochem.* **103**, 466-475.

Qu, Z. and Hartzell, H. C. (2001). Functional geometry of the permeation of Ca^{2+} activated Cl^- channel inferred from analysis of voltage-dependent block. *J. Biol. Chem.* **296**, 18423-18429.
Riestenpatt, S., Onken, H. and Siebers, D. (1996). Active absorption of Na^+ and Cl^- across the gill epithelium of the shore crab *Carcinus maenas*: voltage-clamp and ion-flux studies. *J. Exp. Biol.* **199**, 1545-1554.
Short, F. T. (1992). *The Ecology of the Great Bay Estuary, New Hampshire and Maine: An Estuarine Profile and Bibliography. NOAA Coastal Ocean Program Publ.* 222 pp.
Skou, J. C. and Esmann, M. (1992). The Na^+, K^+ ATPase. *J. Bioenerg. Biomembr.* **24**, 249-261.
Towle, D. W. and Kays, W. T. (1986). Basolateral localization of $Na^+ + K^+$-ATPase in gill epithelium of two osmoregulating crabs *Callinectes sapidus* and *Carcinus maenas*. *J. Exp. Zool.* **239**, 311-318.
Towle, D. W. and Smith, C. M. (2006). Gene discovery in *Carcinus maenas* and *Homarus americanus via* expressed sequence tags. *Integr. Comp. Biol.* **46**, 912-918.
Towle, D. W., Paulsen, R. S., Weihrauch, D., Kordylewski, M., Salvador, C., Lignot, J. H. and Spanings-Pierrot, C. (2001). $Na^+ + K^+$-ATPase in gills of the blue crab *Callinectes sapidus*: cDNA sequencing and salinity-related expression of α-subunit mRNA and protein. *J. Exp. Biol.* **204**, 4005-4012.
Tresguerres, M., Onken, H., Perez, A. F. and Luquet, C. M. (2003). Electrophysiology of posterior, NaCl-absorbing gills of *Chasmagnathus granulatus*: rapid responses to osmotic variations. *J. Exp. Biol.* **206**, 619-626.
Wang, W. W. (1995). View of K^+ secretion through the apical K^+ channel of cortical collecting duct. *Kidney Int.* **48**, 1024-1030.
Watson, W. H., III., Vetrovs, A. and Howell, W. H. (1999). Lobster movements in an estuary. *Mar. Biol.* **134**, 65-75.
White, M. M. and Aylwin, M. (1990). Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+} activated Cl^- channels in *Xenopus* oocytes. *Mol. Pharmacol.* **37**, 720-724.
Zeiske, W., Onken, H., Schwarz, H. J. and Graszynski, K. (1992). Invertebrate epithelial Na^+ channels. Amiloride-induced current-noise in crab gill. *Biochem. Biophys. Acta* **1105**, 245-252.