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Na^+ AND Ca^{2+} PUMPS IN THE GILLS, EPIPODITES AND BRANCHIOSTEGITES OF THE EUROPEAN LOBSTER *HOMARUS GAMMARUS*: EFFECTS OF DILUTE SEA WATER

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Summary

Crude homogenates and plasma-membrane-enriched fractions were prepared from the epithelium of the gills, epipodites and branchiostegites of intermoult European lobsters *Homarus gammarus*, and Na^+/K^+-ATPase, Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange activities were quantified in these tissues. Lobsters were kept in sea water (salinity 35‰) or were adapted to dilute sea water (22.1‰). The lobster hyperregulates haemolymph osmolarity and Ca^{2+} levels in both media. Homogenates of the podobranchs, arthrobranchs and pleurobranchs had comparable Na^+/K^+-ATPase specific activities, and mean activities increased significantly for all three types of gills when the animals were kept in dilute sea water. In the epipodites and branchiostegites, Na^+/K^+-ATPase specific activities exceeded those in the gills, and exposure to dilute sea water greatly enhanced these activities. In sea water, 80 % of the total Na^+/K^+-ATPase activity is associated with the gills and epipodites (each tissue containing 40 %) and 20 % with the branchiostegites; in dilute sea water, the gills contained approximately 25 %, the epipodites 40 % and the branchiostegites approximately 35 % of the total activity, indicating the relative importance of the epipodites and branchiostegites for ionic hyperregulation in dilute media. In plasma membrane vesicles isolated from the gills, epipodites and branchiostegites, Ca^{2+} transport driven by ATP and by a Na^+ gradient was demonstrated. Exposure to dilute sea water enhanced Na^+/Ca^{2+} exchange and Ca^{2+}-ATPase activities in the epipodites and branchiostegites; in the gills, however, Ca^{2+} transport activities decreased. The role of these tissues and enzymes in Na^+ and Ca^{2+} handling by the lobster is discussed.

Key words: Crustacea, Decapoda, Na^+ pump, Ca^{2+} transport, branchial cavity, hyperregulation, lobster, *Homarus gammarus*.

Introduction

The European lobster *Homarus gammarus* is sometimes described as a stenohaline, non-regulating crustacean (Gilles, 1975; Péqueux, 1995). Indeed, this lobster may be considered to be an osmoconformer when living in sea water; however, when confronted with dilute sea water, the lobster hyperregulates its haemolymph osmolarity and by doing so it is able to survive salinities as low as 17 ‰ (Charmantier et al., 1984a,b). Because these animals may experience significant salinity changes in their natural habitat, such hyperregulatory activity is apparently of physiological significance. Clearly, *Homarus gammarus* is not a strictly stenohaline species as previously suggested.

The branchial cavity of *Homarus gammarus* harbours three structures that could be involved in ion transport underlying hyperosmoregulatory activity (Haond et al., 1998): the (trichobranchiate) gills, the epipodites and the branchiostegites. All three structures, but in particular the epipodites and the inner side of the branchiostegites, bear epithelia with mitochondria-rich cells and elaborate plasma membrane invaginations that harbour abundant Na^+/K^+-ATPase activity (Towle, 1984). Thuet et al. (1988) demonstrated increased Na^+/K^+-ATPase activity in a mixed preparation of gills and epipodites of postlarvae of *H. gammarus* transferred to dilute sea water, and this increase correlated with an increase in osmoregulatory capacity; since the gills and epipodites were pooled in their study, no conclusion could be drawn about the exact origin (i.e. gills, epipodites or both) of the increased activity.

When we challenged *H. gammarus* with dilute sea water, hypertrophy of epithelia was observed in the epipodites and branchiostegite, but not in the gills. It was concluded, therefore, that the gills are not involved in the hyperosmoregulatory response (Haond et al., 1998), whereas the epipodites and branchiostegites could be. Lobsters, such as *H. gammarus* and

H. americanus, hyperregulate haemolymph Ca^{2+} levels, particularly when the ambient medium becomes more dilute (Cole, 1941; Charmantier et al., 1984a,b). Such hyperregulatory activity in all likelihood requires continuous uptake of Ca^{2+} from the environment (Neufeld and Cameron, 1993) and may therefore involve the operation of ion-transporting epithelia associated with the branchial cavity. For postmoult blue crabs (*Callinectes sapidus*), it has been demonstrated that Ca^{2+} uptake reaches a maximum rate in animals that have not yet resumed eating, which excludes the intestinal route from involvement in the uptake of environmental Ca^{2+} (Neufeld and Cameron, 1994) and leaves the gills as the most likely site for Ca^{2+} uptake. Indeed, for the shore crab *Carcinus maenas*, it has been demonstrated that the posterior gills contain Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger activity that may drive the uptake of Ca^{2+} from the water (Flik et al., 1994; Lucu and Flik, 1999).

We have extended our histological study (Haond et al., 1998) and report on biochemical analyses of lobster gills, epipodites and branchiostegites, with an emphasis on Na^+/K^+-ATPase, Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange activities in basolateral plasma membranes. Lobsters acclimated to sea water and dilute sea water were compared.

Materials and methods

Animals

Adult female lobsters, *Homarus gammarus* (L.), caught in Scottish waters and weighing approximately 300 g (carapace length 89±5 mm, mean ± S.D., *N*=11), were obtained from a shellfish retailer (Jerry's Lobster Pond, Huissen, the Netherlands) and kept in 100 l glass aquaria filled with artificial sea water (Instant Ocean). The water was aerated and recirculated through Eheim pumps and filters. The lobsters were fed beef heart or cooked mussels on alternate days. The sea water had a salinity of 35 ‰ (1030 mosmol kg^{-1}). Lobsters were adapted to dilute sea water (22.1 ‰, 650 mosmol kg^{-1}) by diluting the sea water gradually with dechlorinated tapwater over a 7 day period. The animals were used for biochemical analyses after they had been exposed to dilute sea water for at least 2 weeks. No mortality was observed. Haemolymph was collected by puncture of an infrabranchial sinus near the base of a walking leg. The haemolymph was centrifuged for 3 min at 9000 **g** to remove cells; osmolality was determined on fresh plasma; the remainder of the plasma was subsequently stored at −20 °C until further ion analysis. Haemolymph osmolality was 1044±7 mosmol kg^{-1} (*N*=9) for seawater lobsters and 724±11 mosmol kg^{-1} (*N*=7) for dilute seawater lobsters. All specimens used were in intermoult stage C (Drach and Tchernigovtzeff, 1967).

Animals were killed by a median cut through the cerebral ganglia. The branchial cavity was carefully opened with scissors to obtain the carapace with its adhering branchiostegite epithelium, exposing the gills and associated epipodites. The branchiostegite epithelium was freed from the carapace using watchmaker's tweezers. In one experiment, the gills (podo-, arthro- and pleurobranchs) and epipodites were analysed individually for Na^+/K^+-ATPase activity; because no differences in Na^+/K^+-ATPase activity were found among gills or among epipodites (see Results), subsequent analyses were carried out with an unselected subsample of the combined gills and the pooled epipodites. Tissues were transferred immediately after dissection to an ice-cooled glass plate and finely minced with a razor blade before further processing. From a 300 g lobster, approximately 1.5 g of epipodites, 1.5 g of branchiostegites and 10 g of gills were obtained; approximately 3 g of gill tissue was used for the isolation of plasma membranes.

Membrane isolation

The procedure for isolating the plasma membranes of lobster tissues was that published for the isolation of plasma membranes from shore crab gills (Flik et al., 1994) with some modifications. In short, finely minced tissue from an individual lobster was homogenised (10 ml g^{-1}) in a hypotonic buffer containing 12.5 mmol l^{-1} NaCl, 1 mmol l^{-1} Hepes/Tris (pH 8.0), 1 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} Na_2EDTA and the serine protease inhibitor aprotinin (280 i.u l^{-1}) using an Ultraturrax device (2 min at 50 % of maximum speed). The suspension obtained was filtered through 140 μm mesh nylon cloth to remove particulate material and cuticle fragments. This filtrate was designated the original homogenate (H_0); 0.5 ml was set aside for further assays, and the remainder was centrifuged at 550 ***g*** for 15 min to remove nuclei and cellular debris. The resulting supernatant was centrifuged at 50 000 ***g*** for 30 min to collect the membranes. For branchiostegites processed this way, the pellet obtained consisted of a layer of red pigment mixed with the mitochondrial membranes and a fluffy layer of plasma membranes on top; special care was taken to discard the pigment layer (which does not stick firmly to the tube wall). The pelleted material of the gills and epipodites consisted of a solid brownish component firmly fixed to the wall of the tube and containing 90–95 % of the mitochondrial membranes (as assessed by succinate dehydrogenase activity; no further data shown) and, on top, a whitish fluffy layer. This fluffy layer was collected by careful swirling in a quantity of buffer (10 ml g^{-1} starting material) containing 0.5 mmol l^{-1} Tris/HCl (pH 8.5), 20 μmol l^{-1} Na_2EDTA and 50 μmol l^{-1} β-mercaptoethanol (Sarkadi et al., 1980). The membranes were resuspended in this buffer using 30 strokes in a Dounce homogenisation device and left in a centrifuge tube first for 30 min on ice and subsequently for 15 min at 37 °C. Every 5 min during this 45 min incubation period, the suspension was vigorously vortex-agitated for 15 s. Next, the suspension was cooled to 0 °C and centrifuged at 9000 ***g*** for 10 min to remove remaining mitochondrial fragments; the resulting supernatant was centrifuged at 50 000 ***g*** for 30 min to collect the plasma membranes. The pellet (P_3) was resuspended in 0.5–1.0 ml of buffer containing 150 mmol l^{-1} NaCl or KCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris (pH 7.4). Vesiculation of the membranes through shearing was achieved using 15 passes through a 23 gauge needle fitted to a tuberculin syringe. Membranes in KCl buffer were used for ATP-dependent Ca^{2+} transport assays, membranes in NaCl buffer for Na^+ gradient driven Ca^{2+} transport assays and marker enzyme determinations. All assays were carried out on the day

of isolation, during which the membranes were kept on ice. The protein content of the P_3 suspension ranged from 1 to 2 mg ml^{-1} bovine serum albumin (BSA) equivalents, as assessed using Bio-Rad's protein assay kit (catalogue no. 500-0002) based on Bradford's (1976) procedure.

Assays

For each membrane vesicle preparation, the membrane configuration and Na^+/K^+-ATPase enrichment and recovery in P_3 were assessed following procedures published for shore crab gills (Flik et al., 1994). In short, Na^+/K^+-ATPase specific activity was determined for H_0 and P_3 using saponin (0.2 mg mg^{-1} membrane BSA equivalents) to open up resealed membranes and to optimise substrate accessibility and obtain values for maximum activity. The total activity (in μmol h^{-1}) in a preparation was then calculated as the product of the specific activity (μmol h^{-1} mg^{-1} protein) and the total amount of protein (protein content × volume; in mg). The recovery of Na^+/K^+-ATPase was calculated as the ratio of total activities in H_0 and P_3, and the enrichment in Na^+/K^+-ATPase was calculated as the ratio of the specific activities in H_0 and P_3.

Membrane configuration was assessed on the basis of the sensitivity to trypsin of the cytosolic part of the Na^+/K^+-ATPase and the permeabilisation of membranes by saponin. In short, for each preparation, the percentage of resealed membranes was determined by comparing the Na^+/K^+-ATPase activity in untreated (giving the activity of leaky sheets only) and saponin-treated (the activity of leaky sheets plus that of resealed membranes) membranes. Trypsin (Sigma, T0134) treatment of membranes (4500 BAEE trypsin units per milligram of membrane protein for 30 min at 25 °C) inactivates Na^+/K^+-ATPase in leaky membranes sheets and inside-out vesicles (IOVs), allowing the calculation of the percentage of right-side out vesicles (ROVs) by assessing the Na^+/K^+-ATPase activity after the trypsin had been quenched with trypsin inhibitor (Sigma T9253; 25 mg ml^{-1}) and opening up of the ROVs with saponin. Controls were run by adding trypsin inhibitor before the addition of trypsin. The percentage of IOVs was subsequently calculated as the difference between the percentages of resealed membranes and of ROVs (Flik et al., 1994).

Ca^{2+} transport was determined as described in detail for crab gills (Flik et al., 1994) using a rapid filtration technique to assess ^{45}Ca uptake into vesicles. ATP-driven Ca^{2+}-transport was determined on vesicles equilibrated in 150 mmol l^{-1} KCl in medium containing (in mmol l^{-1}): 150 KCl, 5×10^{-5} to 5×10^{-3} Ca^{2+}, 0.8 Mg^{2+}, 20 Hepes/Tris (pH 7.4), 0.5 nitrilo-triacetic acid (NTA), 0.5 EGTA, 0.5 *N*-(2-hydroxyethyl)-ethylenediamine-*N*,*N*′,*N*′-triacetic acid (HEEDTA), 0.005 thapsigargin, 5 μg ml^{-1} oligomycin B, 1 NaN_3 and 0 or 3 mmol l^{-1} TrisATP; the ^{45}Ca activity was 1–1.6 MBq ml^{-1}. Immediately before assay, the vesicles were K^+-clamped by addition of 2 μmol l^{-1} valinomycin B. The assay temperature was 37 °C, and the reaction time was 30 s. ATP-driven Ca^{2+} transport was calculated as the difference between Ca^{2+} transported into the vesicles in the presence and in the absence of ATP. Free Ca^{2+} concentrations were calculated using the program Chelator (Schoenmakers et al., 1992). The reaction was quenched by addition of 1 ml of ice-cold isotonic stop buffer (150 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes/Tris, pH 7.4, and 1 mmol l^{-1} EDTA). Filters (Schleicher and Schüll, ME 25) with retained vesicles containing ^{45}Ca were washed three times with 2 ml of stop buffer, transferred to a minivial, dissolved (30 min) in 4 ml of Aqualuma (Lumac) scintillation fluid and counted in a Wallac 1410 scintillation counter.

Na^+-gradient driven Ca^{2+} transport was calculated from the difference in ^{45}Ca taken up when vesicles equilibrated in 150 mmol l^{-1} NaCl (K^+-clamped by addition of 2.5 mmol l^{-1} KCl and 2 μmol l^{-1} valinomycin B) were transferred to a medium containing either 150 mmol l^{-1} NaCl (blank) or 150 mmol l^{-1} KCl. In preparing the media, a 25-fold dilution of the vesicle preparation was taken into account to obtain the following composition (in mmol l^{-1}): 150 NaCl or KCl, 20 Hepes/Tris (pH 7.4), 0.5 EGTA, 0.5 NTA, 0.5 HEEDTA, 0.8 Mg^{2+}, 5×10^{-4} to 2.5×10^{-2} Ca^{2+}. Free Ca^{2+} and Mg^{2+} concentrations were calculated as described above. The ^{45}Ca activity was 1 MBq ml^{-1}. Prewarmed (37 °C) medium (120 μl) and vesicle preparation (5 μl) were mixed and incubated for 5 s. The reaction was quenched by addition of 1 ml of ice-cold isotonic stop buffer; filters with collected and washed vesicles were processed for counting as described above.

When ATP-driven and Na^+-gradient-driven Ca^{2+} transport were compared, the data were corrected for membrane configuration in a preparation. No differences were found in membrane configuration of tissues obtained from either seawater or dilute seawater animals. Approximately 50 % of the membranes were present as leaky sheets and 50 % as resealed membranes in all preparations. In addition, no differences were found in the percentage of IOVs (19±3.6 %, mean ± S.D., *N*=6; range 14±5 to 24±5 %, *N*=4–5) or in the percentage of ROVs (32±4.1 %, *N*=6; range 25±7 to 36±3 %, *N*=4–5) among the three tissues. The mean values for individual tissues are presented in Table 1; the values for membrane configuration have been used to correct the data on ATP-driven Ca^{2+} transport (which occurs in IOVs only) and Na^+/Ca^{2+} exchange (which occurs *in vitro* in all resealed membranes, i.e. IOVs plus ROVs)

Table 1. *Membrane configuration in vesicle preparations of lobster gills, epipodites and branchiostegites*

	Gill		Epipodites		Branchiostegite	
	SW	DSW	SW	DSW	SW	DSW
%L	50±5	48±3	50±5	51±6	59±14	54±9
%R	50±5	52±3	50±5	49±6	41±14	46±9
%IOV	14±5	18±3	20±6	16±4	24±16	21±5
%ROV	36±2	34±4	30±9	33±7	35±2	25±7

Lobsters from sea water (SW) and dilute sea water (DSW) were compared.

For details, see Materials and Methods.

L, leaky membrane fragments; R, resealed membranes; IOV, inside-out vesicles; ROV, right-side-out vesicles.

Values are means ± S.D., *N*=4–5.

for the sake of comparison of these Ca^{2+} transporter activities. All assays were carried out in triplicate.

Water and haemolymph Ca^{2+} content were estimated with an end-point colorimetric assay kit (Sigma, no. 595) using a combined calcium/phosphorus standard set (Sigma, no. 360-11) as reference. Water and haemolymph osmolality were assessed using a Vogel micro-osmometer using distilled water and a 300 mosmol kg^{-1} standard as reference.

Calculations and statistical analyses

The kinetic variables of the transport activities were obtained by linear regression analysis of Eadie–Hofstee-transformed data. Linear regression was based on the least squares method. Data are presented as means ± standard deviation, unless indicated otherwise. The significance of differences between mean values was assessed using Student's *t*-test or the Mann–Whitney *U*-test where appropriate, and significance was accepted when $P<0.05$.

Results

In female intermoult lobster kept in sea water (Ca^{2+} content 10.5±0.3 mmol l^{-1}; $N=6$), the mean haemolymph total Ca content was 14.8±1.0 mmol l^{-1} ($N=7$); for those kept in dilute sea water (Ca^{2+} content 6.9±0.4 mmol l^{-1}; $N=5$), the mean haemolymph total Ca content was 13.4±0.5 mmol l^{-1} ($N=6$).

No differences were found in Na^+/K^+-ATPase specific activity among individual podobranchs (associated with maxilliped 3 and pereiopods 1–4; the rudimentary podobranch associated with maxilliped 2 was not analysed), among the anterior and posterior arthrobranchs (associated with maxilliped 3 and pereiopods 1–4) or among the pleurobranchs (associated with pereiopods 2–5) (Fig. 1). However, in all instances, the specific activities were higher in gills from lobster exposed to dilute sea water: values are 3.2±0.3 and 5.6±0.3 μmol P_i h^{-1} mg^{-1} protein for the podobranchs, 3.2±0.2 and 5.0±0.5 μmol P_i h^{-1} mg^{-1} protein for the anterior arthrobranchs, 3.1±0.6 and 5.2±0.5 μmol P_i h^{-1} mg^{-1} protein for the posterior arthrobranchs and 3.9±0.1 and 6.2±0.5 μmol P_i h^{-1} mg^{-1} protein for the pleurobranchs in seawater and dilute seawater animals, respectively ($N=5$). No differences were found in Na^+/K^+-ATPase specific activity in homogenates of individual epipodites associated with maxillipeds and pereiopods, either in seawater or dilute seawater lobster. However, exposure to dilute sea water led to a significant 5.55-fold enhancement of the specific activity (mean values for the pooled data on epipodites 6.5±1.2 and 36.1±6.6 μmol P_i h^{-1} mg^{-1} protein for seawater and dilute seawater lobsters, respectively; $N=6$). For the branchiostegites, the mean Na^+/K^+-ATPase activities were 12.1±0.4 and 37.2±6.6 μmol P_i h^{-1} mg^{-1} protein for seawater and dilute seawater lobsters, respectively ($N=6$; Fig. 1). There being no individual differences among gills and epipodites within an animal, in all subsequent studies membranes were prepared from an unselected subsample of the pooled gills, from the pooled epipodites and from the pooled

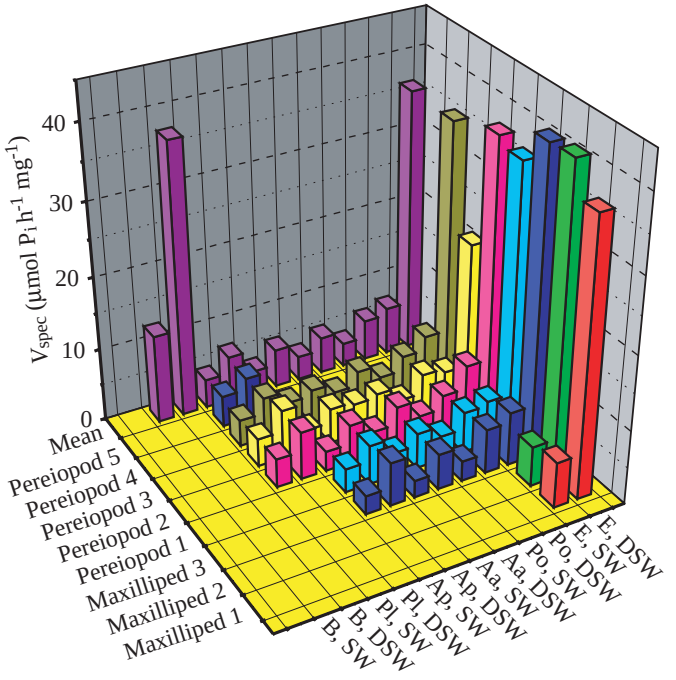

Fig. 1. Na^+/K^+-ATPase specific activities (V_{spec}) in the branchial chamber of the European lobster *Homarus gammarus* kept in sea water (SW) or dilute sea water (DSW). Mean values for individual gills ($N=5$), epipodites ($N=6$) and branchiostegites ($N=6$) as well as overall mean values for the gills, epipodites and branchiostegites are given (Mean). For clarity, no standard errors have been presented. In all cases, exposure to dilute sea water significantly enhanced Na^+/K^+-ATPase specific activity. Overall mean values are depicted in purple (Mean). E, epipodite; Po, podobranch; Aa, anterior arthrobranch; Ap, posterior arthrobranch; Pl, pleurobranch; B, branchiostegite.

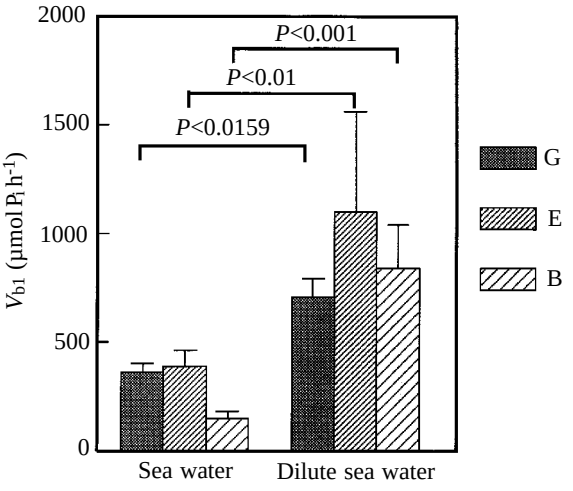

Fig. 2. The effects of dilute sea water on total Na^+/K^+-ATPase activity (V_{tot}, μmol P_i h^{-1}) in the gills ($N=5$) (G), epipodites ($N=6$) (E) and branchiostegites ($N=6$) (B) of the European lobster *Homarus gammarus*. Total activities were calculated as the product of the specific activity and the total amount of protein in a preparation. Values are means + S.D.

Table 2. *Kinetic parameters of Ca^{2+} transporters in the branchial cavity of lobster in sea water or dilute sea water*

		Gills		Epipodites		Branchiostegite	
		ATP	Na^+	ATP	Na^+	ATP	Na^+
Sea water	V_{max}	20±7	248±36	3±1	256±26	8±4	332±24
	$K_{0.5}$	0.150±0.045	2.74±0.65	0.240±0.023	4.26±1.26	0.206±0.034	4.34±1.48
Dilute sea water	V_{max}	6±3*	130±56*	8±3*	310±36*	13±2*	391±36*
	$K_{0.5}$	0.145±0.036	3.24±0.68	0.210±0.029	4.02±1.44	0.230±0.046	3.95±1.33

Ca^{2+} transport activities (V_{max}) are expressed in nmol min^{-1} mg^{-1} protein and were corrected for membrane configuration; the half-maximum activation concentrations for Ca^{2+} ($K_{0.5}$) are expressed in μmol l^{-1}.

ATP, ATP-driven Ca^{2+} transport; Na^+, Na^+-gradient driven Ca^{2+} transport (Na^+/Ca^{2+} exchange activity).

An asterisk indicates a value significantly different from the seawater value ($P<0.05$, $N=6$).

branchiostegites. Of the calculated total Na^+/K^+-ATPase activity in the three preparations of seawater lobsters, 40 % resides in the gills, 40 % in the epipodites and 20 % in the branchiostegites. After exposure to dilute sea water, total Na^+/K^+-ATPase activities in all three tissues had increased significantly, and the total activities associated with the three tissue compartment were now similar (25 % in the gills, 40 % in the epipodites and 35 % in the branchiostegites) (Fig. 2).

In all cases, the kinetics of Ca^{2+}-dependency (Ca^{2+} concentration ranges were 5×10^{-8} to 2.5×10^{-6} mol l^{-1} for ATPase activity and 5×10^{-7} to 25×10^{-6} mol l^{-1} for exchanger activity) obeyed single-Ca^{2+}-site kinetics, as indicated by the significance of the linearity of Eadie–Hofstee plots of the data (results not shown). Table 2 summarises the kinetic variables obtained for the three tissues in the two acclimation media. In all instances, Na^+/Ca^{2+} exchange activities greatly exceeded ATP-driven Ca^{2+} transport activities. Half-maximum activation concentrations of Ca^{2+} ($K_{0.5}$) for the ATP-driven transport were in the range 0.145–0.240 μmol l^{-1}, and $K_{0.5}$ values for the exchanger were in the range 2.74–4.34 μmol l^{-1}; no effects of dilute seawater exposure on these variables were observed. Interestingly, in dilute seawater lobsters, ATP-driven and Na^+-gradient-driven Ca^{2+} transport activity decreased in the gills but increased in the epipodites and branchiostegites. The effects of dilute sea water on Ca^{2+} transporter activities in the three tissues are presented in Fig. 3A–C, in which the activities of the transporters of the gills, epipodites and branchiostegites have been plotted as a function of the free Ca^{2+} levels that may occur in the cytosol of the transporting cells.

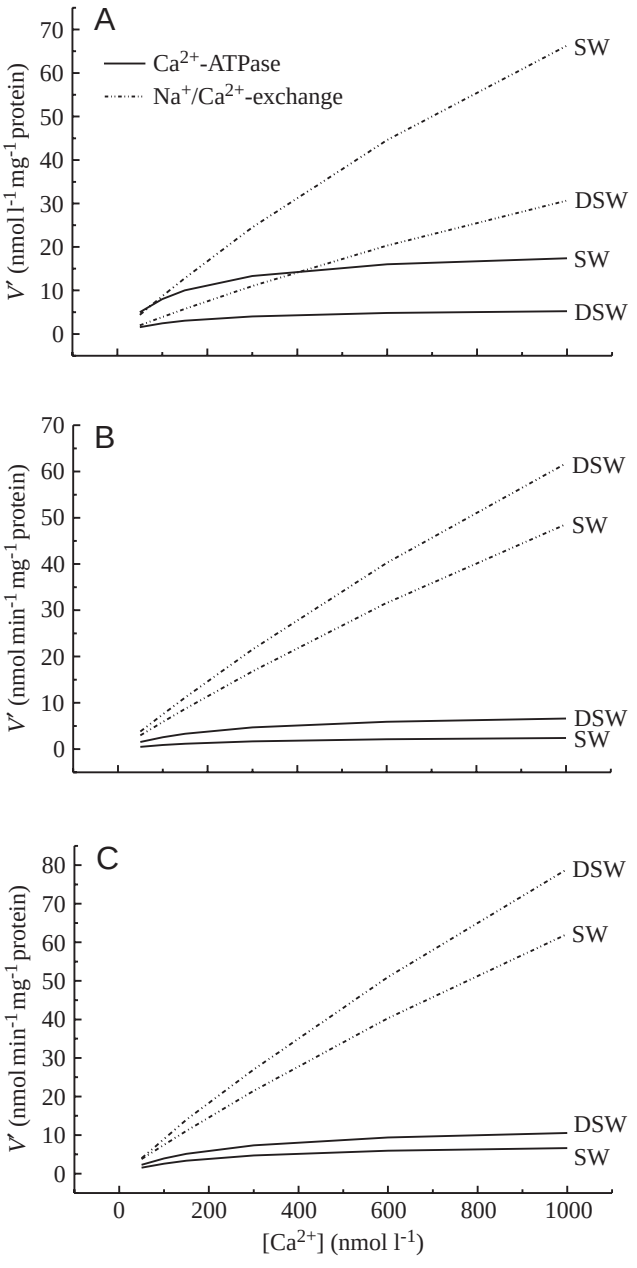

Fig. 3. Kinetics of Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange activities in gills (A), epipodites (B) and branchiostegites (C) of the European lobster *Homarus gammarus*. The curves were calculated on the basis of the kinetic variables presented in Table 2, according to the Michaelis–Menten equation: $V'=V_{max}[S]/(K_m+[S])$, where V' is the apparent velocity measured, V_{max} is the maximum velocity, $[S]$ is substrate concentration and K_m is the half-maximum activation concentration of substrate (Ca^{2+}). Values have been corrected for membrane configuration (see text). DSW, dilute sea water; SW, sea water.

Discussion

Five major conclusions can be drawn from this study. First, the podo-, arthro- and pleurobranchs contain similar Na^+/K^+-ATPase specific activities. Second, the total amounts of Na^+/K^+-ATPase activity in the gills and in epipodites are similar and make up 80 % of the activity associated with the branchial cavity; approximately 20 % is associated with the branchiostegites. Third, the epithelia of the gills, epipodites and branchiostegites harbour Na^+/K^+-ATPase, Na^+/Ca^{2+} exchange and Ca^{2+}-ATPase activities in their plasma membranes. Fourth, in all tissues, rates of Na^+-gradient-driven Ca^{2+} transport exceed rates of ATP-driven Ca^{2+} transport. Fifth, exposure to dilute sea water enhances the activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange in the epipodites and branchiostegites, enhances Na^+/K^+-ATPase activity in the gills slightly, but significantly, and decreases Ca^{2+} transporter activity.

Clearly, the tissues associated with the branchial cavity of the lobster provide us with a powerful model for future studies on the mechanisms of (up)regulation of Na^+- and Ca^{2+}-transporting enzymes. The hypertrophy observed in the epipodites and branchiostegites of lobsters exposed to dilute sea water (Haond et al., 1998) apparently comprises upregulating signals to genes coding for Na^+/K^+-ATPase, Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger molecules; in the gills, another suite of signals must occur because Na^+/K^+-ATPase activity was upregulated and Ca^{2+} transporter activity was downregulated.

Na^+/K^+-ATPase and osmoregulation

The osmoregulatory epithelia of crustaceans are characterised by a high Na^+/K^+-ATPase activity (Mantel and Farmer, 1983; Lucu, 1990; Pequeux, 1995), the enzymatic expression of the Na^+ pump (Towle and Kays, 1986; Lucu and Flik, 1999). In a shore crab (*Carcinus maenas*) posterior gill hemilamellar preparation, the short-circuit current is almost completely ouabain-inhibitable, showing that a Na^+/K^+-ATPase-mediated Na^+ current determines the electrical behaviour of the tissue; this current increases when the animal is challenged with dilute sea water, a medium that requires an upregulation of the osmoregulatory activity of the animal (Lucu and Flik, 1999). In *Homarus gammarus*, the epithelia of the epipodites and branchiostegites show all the characteristics of an ion-transporting epithelium, and the epithelia show a marked hypertrophy when the lobster is exposed to dilute sea water (Haond et al., 1998). Not unexpectedly, therefore, we observed a dramatic increase in Na^+/K^+-ATPase activity in these structures when the lobster was acclimated to dilute sea water. In seawater-adapted lobsters, the epipodites and the branchiostegites contain 40 % and 20 %, respectively, of the total Na^+/K^+-ATPase activity associated with the branchial cavity; in dilute sea water, the tissues harbour 30 % each. Thus, not only does the total amount of Na^+/K^+-ATPase activity associated with the branchial cavity increase, but the branchiostegites also become relatively more important in hyperregulating lobster. In osmoregulating crabs, the posterior gills harbour ionocytes, whereas the anterior gills display a typical gas-exchange epithelium made up of thin cells (Copeland and Fitzjarrell, 1968; Barra et al., 1983; Compère et al., 1989). As mentioned above, most of the Na^+/K^+-ATPase activity is associated with the posterior gills, and transfer to dilute sea water results in a considerable upregulation of the enzyme, whereas the anterior gills show little or no change in Na^+/K^+-ATPase activity (Harris and Bayliss, 1988; Welcomme and Devos, 1988; Corotto and Holliday, 1996). It has been demonstrated that only the posterior gills of osmoregulating crabs absorb Na^+ from the medium (Péqueux, 1995). If an analogy exists between the gills of the shore crab and the epipodites and branchiostegites of the lobster, it follows that Na^+ uptake must be enhanced in the epipodites and branchiostegites of lobsters in dilute sea water, and this would corroborate our prediction, based on histology, that these organs are important for osmoregulation in the lobster (Haond et al., 1998).

In the trichobranchiate gill of *H. gammarus*, the haemolymph space is bordered by a thin epithelial layer adjacent to the cuticle; two cell types make up the epithelial boundary, the thin cells and the ‘flange cells’. The latter cells send shallow cytoplasmic flanges to form a continuum with the thin cells in the periphery of the branchial tube; their perikaryon is associated with septa forming the lateral lacunae and afferent and efferent channels in the gills (Taylor and Taylor, 1992). The thinness of the epithelium is typical of an epithelium in which gas exchange occurs. However, when analysed biochemically, the gills collectively harbour a very significant proportion of the Na^+/K^+-ATPase activity in the branchial cavity. Indeed, at the ultrastructural level, it appears that the flange cells and thin cells contain basolateral plasma membrane invaginations (Haond et al., 1998) that are a likely site for this Na^+/K^+-ATPase activity. Moreover, mitochondria, a number of which are associated with the plasma membrane invaginations, are abundant in these gill cells, and such an organisation could warrant mitochondrial generation of ATP for Na^+/K^+-ATPase activity. However, in flange and thin cells, many mitochondria are also found distant from plasma membrane invaginations (Haond et al., 1998), and these may represent another pool of mitochondria energising general metabolism.

Na^+/K^+-ATPase specific activity was similar in homogenates of the three types of gills, and this observation provides biochemical evidence that no functional specialisation involving Na^+/K^+-ATPase among the gill areas occurs, a conclusion in line with the morphological homogeneity of these gills (Haond et al., 1998). Thin cell equivalents are also found in the anterior respiratory gills of crabs such as *Callinectes sapidus* (Copeland and Fitzjarrell, 1968), *Eriocheir sinensis* (Barra et al., 1983) and *Carcinus maenas* (Compère et al., 1989). Assuming that the primary function of the gills is respiration rather than ion uptake from the water, the Na^+/K^+-ATPase may function in acid–base regulation and waste excretion (Taylor and Taylor, 1992). Our observations that branchial Na^+/K^+-ATPase activity increases in dilute-seawater-

exposed lobsters and that such treatment does not influence the ultrastructure of the gills (Haond et al., 1998) suggest that the increased metabolic rate during increased hyperregulatory activity requires an adjustment of branchial Na^+/K^+-ATPase activity. A similar conclusion was reached for enhanced Na^+/K^+-ATPase activity in the anterior gills of shore crabs exposed to dilute sea water (Lucu and Flik, 1999).

The decrease in branchial Ca^{2+} transporter activity in lobsters exposed to dilute sea water and the concomitant increase in Na^+/K^+-ATPase activity may therefore reflect an adjustment in housekeeping enzyme activity more than in specific ion (Ca^{2+}) transport activity. An important conclusion is that the biochemical changes are not necessarily reflected by morphological changes: the ultrastructure of the gills of this lobster was not notably affected by exposure to dilute sea water (Haond et al., 1998). However, the membrane invaginations seen in the lobster branchial epithelium represent a specialisation considered typical of ion-transporting epithelia and are absent from respiratory cells in fish gills (Laurent and Dunel, 1980). Given the Na^+/K^+-ATPase activity present in the gills (40 % of the total activity associated with the branchial cavity in seawater lobster) and the ultrastructural arrangements, these organs could contribute potentially and significantly to ion transport. Corotto and Holliday (1996) provided evidence that the anterior gills (lacking typical ion-transporting cells) in hyperregulating *Hemigrapsus nudus* (and *Carcinus maenas*) may contribute significantly to ion transport when the animals are exposed to dilute sea water, a conclusion also reached by (Lucu and Flik, 1999) for *Carcinus maenas*. Clearly, studies are required that evaluate ion fluxes in isolated and perfused gills of lobster.

Ca^{2+} transporters in the branchial cavity

The basolateral plasma membranes of the epithelia of the gills, epipodites and branchiostegites of the lobster *H. gammarus* contain the machinery for Ca^{2+} extrusion that, in terms of its components, is comparable among these tissues and similar to that found in crab (Flik et al., 1994) and fish (Verbost et al., 1994; Van der Heijden et al., 1997) gills. Apparently, such a biochemical structure is of wider occurrence, but not universal, in epithelia rich in Na^+/K^+-ATPase: ferret and dog red blood cells lack a Na^+/K^+-ATPase, extrude Ca^{2+} from the cell by Ca^{2+}-ATPase activity and bring into play the inward Ca^{2+} gradient to extrude Na^+ *via* a Na^+/Ca^{2+} exhanger (Ortiz and Sjodin, 1984; Parker, 1987). Interestingly, the three tissues studied in the lobster exhibit a differential response to dilute seawater exposure. In dilute sea water, Ca^{2+} transporter activities in the epipodites and branchiostegite were upregulated, as was Na^+/K^+-ATPase activity; however, as discussed above, in the gills, only Na^+/K^+-ATPase activity increased, but Ca^{2+} transporter activity decreased. We conclude from this observation that the epithelia of the epipodites and branchiostegites are specialised for Ca^{2+} transport, whereas the epithelium of the gills is not. The increased Na^+/K^+-ATPase activity in the gills may therefore relate to an enhanced metabolic rate (discussed above), a challenged acid–base balance and enhanced waste excretion in the gills proper as well as in other tissues of hyperregulating lobsters (Henry and Wheatly, 1992; Piller et al., 1995).

The significant increase in Na^+/K^+-ATPase activity together with Na^+/Ca^{2+} exchange activity in lobsters exposed to dilute sea water strongly suggest a role for the exchange mechanism in Ca^{2+} uptake from the water, but do not resolve the role of the Ca^{2+}-ATPase. In a recent study on crab (*Carcinus maenas*) gills (Lucu and Flik, 1999), we have provided evidence, based on Ussing chamber experiments, that inward Ca^{2+} transport across the branchial epithelium is for the most part Na^+/K^+-ATPase-dependent (ouabain-inhibitable) and, therefore, in all likelihood mediated indirectly by the exchanger mechanism. The function of the Ca^{2+}-ATPase in Ca^{2+} transport remains unclear, but a housekeeping role for this enzyme in cellular Ca^{2+} homeostasis has been proposed (Flik et al., 1996). The presumedly enhanced Ca^{2+} turnover in Ca^{2+}-transporting epithelia of hyperregulating lobsters may, of course, be predicted to account for the enhanced housekeeping activity of Ca^{2+} transporting enzymes.

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