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CALCIUM TRANSPORT IN GILL PLASMA MEMBRANES OF THE CRAB *CARCINUS MAENAS*: EVIDENCE FOR CARRIERS DRIVEN BY ATP AND A Na^+ GRADIENT

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Summary

A procedure was developed for the preparation of inside-out vesicles from plasma membranes isolated from the branchial epithelium of the green shore crab *Carcinus maenas* (L.). Procedures normally applied to fish branchial epithelium required the introduction of an additional hypotonic shock to obtain a preparation containing 22 % inside-out vesicles, 33 % right-side-out vesicles and 45 % leaky membrane fragments. In such membrane preparations, the first direct evidence for uphill (against a $[Ca^{2+}]$ gradient) ATP-dependent and Na^+-gradient-dependent Ca^{2+} transport in crustacean gills was found. The affinity for Ca^{2+} of the ATP-driven Ca^{2+} transporter was 149 $nmol\,l^{-1}$ and that of the Na^+/Ca^{2+} exchanger was 1.78 $\mu mol\,l^{-1}$; the V_{max} values were 1.73 and 9.88 $nmol\,min^{-1}\,mg^{-1}$ protein respectively. The relative importance of these carriers for Ca^{2+} transport in the branchial epithelium of the crab is evaluated on the basis of their calcium kinetics.

Introduction

The branchial epithelium of crustacean gills forms a delicate barrier between the external and internal media and serves an important role in respiration, acid–base balance, volume regulation and the transport of ions. Intensive research on Na^+ and Cl^- handling by crustaceans living in marine and brackish waters has been carried out (Mantel and Farmer, 1983; Lucu, 1990, 1993; Towle, 1993), but only a few reports on branchial Ca^{2+} handling are available at the moment (Greenaway, 1985; Cameron, 1989, 1990).

Studies on Ca^{2+} metabolism in crustaceans have focused on the processes of calcification in the carapace. Roer (1980) has shown that, during the late premoult stage, Ca^{2+} is resorbed from the carapace and subsequently transported across the hypodermis into the haemolymph. In aquatic crustaceans, most of the body Ca^{2+} is lost during this

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period *via* the gills and with the exuvia (Greenaway, 1974*a*, 1985), softening the exoskeleton and allowing ecdysis. In the postmoult stage, calcium remaining in the haemolymph is transported to the carapace across the hypodermis (Greenaway, 1974*b*, 1985). Moreover, crustaceans mobilize large amounts of calcium from the surrounding water during the postmoult period to calcify the enlarged cuticle and the carapace. This implies that the gills must be involved in inwardly directed (from water to haemolymph) and regulated Ca^{2+} transport.

In the hypodermal epithelium of postmoult blue crab *Callinectes sapidus*, Ca^{2+}-ATPase activity increases compared with that measured in the intermoult stages (Cameron, 1989). However, the kinetic properties of this ATPase (an affinity for Ca^{2+} too low to play a role in intracellular calcium homeostasis) suggest that this activity is that of a non-specific Ca^{2+}- and ATP-consuming phosphatase (with unknown function), similar to the one described in fish gills, and not that of a high-affinity Ca^{2+}-ATPase involved in the extrusion of Ca^{2+} from the cell (Flik *et al.* 1984). The inward transport of calcium across the hypodermis of *Carcinus maenas* was postulated to occur through the action of an ATPase or of a Na^{+}/Ca^{2+} exchanger for extrusion of Ca^{2+} from the cells (Roer, 1980). However, no direct biochemical evidence for Ca^{2+} transport across the plasma membrane, and thus for the existence of such carriers, has yet been reported. Morris and Greenaway (1992) have found evidence for high-affinity Ca^{2+}-ATPase activity ($K_{0.5}$ for Ca^{2+} in the nanomolar range) in the gills of the crab *Leptograpsus variegatus*. The kinetics of this enzymatic activity indeed favour a role of branchial epithelial cells in Ca^{2+} transport and homeostasis. However, Morris and Greenaway (1992) measured ATPase activity and this was assumed to reflect the transport activity. As shown in a study on ‘pumping and non-pumping’ ATPase activity in rat liver plasma membranes (Lin and Russel, 1988), one has to be cautious when making this assumption.

The hypodermis plays a pivotal role in the recycling of Ca^{2+} from the carapace during moulting and in calcification of the carapace; however, Ca^{2+} uptake from the environment is required to *augment* the body calcium pool after moulting and to recalcify the carapace rapidly (Greenaway, 1985; Neufeld and Cameron, 1993). Uptake of calcium from the environment could take place *via* the gills and the gut. Ca^{2+} uptake may reach a maximum rate in postmoult blue crabs (*Callinectes sapidus*) that have not yet resumed eating (Neufeld and Cameron, 1993) and, in this case, must occur *via* the gills. It seems reasonable then, to postulate an active Ca^{2+} transport mechanism for uptake *via* the gills. In this study, we concentrated on Ca^{2+} transport by the gills at the membrane level.

Procedures developed for the isolation of membranes from fish branchial epithelium (Flik *et al.* 1984, 1985*a*,*b*; Perry and Flik, 1988) were applied to crab branchial epithelium and yielded a plasma membrane vesicle preparation with a high proportion of resealed vesicles (more than 70 %), but too low a number of inside-out membrane vesicles (IOVs) to demonstrate Ca^{2+} transport. An additional hypotonic shock, as originally suggested for the preparation of red blood cell membrane IOVs (Sarkadi *et al.* 1980), yielded 22 % IOVs in the crab membrane preparation and allowed us to demonstrate ATP- and Na^{+}-gradient-driven Ca^{2+} transport.

We used intermoult stages of the euryhaline crab *Carcinus maenas* adapted for at least

2 weeks to 50 % sea water as Ca^{2+} transport was predicted to be enhanced under these conditions (Neufeld *et al.* 1980).

Materials and methods

Animals

Green shore crabs, *Carcinus maenas*, were collected early in January by netting in the Waddensea, The Netherlands. The animals were transferred to the laboratory and kept in running sea water at 6 °C, the temperature of the Waddensea at the time the crabs were caught. The mass of the animals ranged from 10 to 30 g. Only male specimens in intermoult stage were used. At least 14 days before experimentation, the sea water was diluted to 50 % with demineralized water over a 24 h period. The crabs were fed cod strips daily and kept at a photoperiod of 16 h:8 h (L:D). Less than 5 % 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*** and the plasma stored at −20 °C until assay. After the carapace had been opened, posterior gills 6–9 were dissected out and quickly cooled on ice. The posterior gills contain an abundance of mitochondria-rich cells (Compère *et al.* 1989), which are the equivalent of the ionocytes found in other ion-transporting epithelia. The Na^{+} contents of water and plasma were determined by flame photometry on 200-fold diluted samples using a Technikon model IV Autoanalyzer; $LiNO_3$ (5 mmol l^{-1}) was used as an internal standard. Water and plasma total calcium were determined with an endpoint colorimetric assay kit (Sigma, no. 587). A combined calcium–phosphorus standard set (Sigma, no. 360-11) was used as reference.

Isolation procedure

Gills from 2–4 animals were pooled to obtain 2–5 g of tissue. Tissue was disrupted by 20 strokes in a Douncer homogenization device equipped with a loosely fitting pestle in 10 ml g^{-1} of a hypotonic buffer containing 12.5 mmol l^{-1} NaCl, 1 mmol l^{-1} Hepes/Tris (pH 8), 1 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} EDTA and the serine protease inhibitor aprotinin (280 i.u l^{-1}). The resulting suspension was filtered over cheesecloth to remove particulate material and branchial septa. This filtrate was designated the original homogenate (H_0); 0.5 ml was set aside on ice for further assay and the remainder was centrifuged at 550 ***g*** for 15 min to remove nuclei and cellular debris. The resulting supernatant was centrifuged for 30 min at 50 000 ***g*** to collect the membranes. The pelletized material consisted of a brownish part fixed against the wall of the centrifugation tube (this part contains most of the mitochondrial fragments, indicated by around 90 % of the total branchial succinic acid dehydrogenase activity) and, above this, a whitish fluffy layer. The fluffy layer was carefully collected by mild 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} EDTA and 50 μmol l^{-1} β-mercaptoethanol (Sarkadi *et al.* 1980). The membranes were resuspended in the buffer by 20 strokes in the Douncer and left 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 agitated for 15 s. The suspension was

then cooled again to 0 °C and centrifuged for 10 min at 10 000 ***g*** (to remove remaining mitochondrial fragments); the resulting supernatant was centrifuged for 30 min at 50 000 ***g*** to sediment the plasma membranes (see below). The pellet (P_3) was resuspended in 400 μl of buffer containing 150 mmol l^{-1} KCl or NaCl, 0.8 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} Hepes/Tris (pH 7.4). Vesiculation was secured by 15 passes through a 23 gauge needle fitted to a tuberculin syringe. Membranes in potassium buffer were used for ATP-dependent Ca^{2+} transport assays, membranes in sodium buffer for Na^+-gradient-driven Ca^{2+} transport and marker enzyme determinations. Transport assays were carried out on the day of isolation. The membranes were kept on ice. When 0.5 ml of P_3 suspension was prepared, the protein content ranged from 1 to 1.5 mg ml^{-1} bovine serum albumin (BSA) equivalents (see below).

Assays and membrane characteristics

The protein content of the membrane preparation was determined according to Bradford's procedure (BioRad, catalogue no. 500-0002), using bovine serum albumin (BSA) as a reference. Two marker enzymes were used to characterize the membrane preparation: succinic acid dehydrogenase (SDH) for mitochondrial membrane fragments and ouabain-sensitive, sodium- and potassium-dependent ATPase (Na^+/K^+-ATPase) for basolateral plasma membranes (Flik *et al.* 1990). K^+-dependent, ouabain-sensitive *p*-nitrophenylphosphatase (K^+-pNPPase) activity (which reflects the dephosphorylation step of the Na^+/K^+-ATPase) was determined as the difference between the amounts of inorganic phosphate (P_i) released from pNPP in a medium containing 20 mmol l^{-1} Tris/Hepes (pH 7.4), 120 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl and 3 mmol l^{-1} Na_2pNPP and the amount of P_i released in the same medium but without KCl and supplemented with 1 mmol l^{-1} ouabain. The incubation was carried out at 37 °C for 30 min.

Three detergents were tested for their suitability to unmask enzymatic activities that become latent as a result of membrane resealing. As shown in Fig. 1, digitonin and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate (Chaps) had no effect, or a slightly inhibitory effect, on Na^+/K^+-ATPase respectively, at concentrations where a clear stimulation was obtained with saponin. The optimum saponin concentration was 0.2 mg per milligram membrane BSA equivalents, and saponin at this concentration was used in all instances when determination of optimal activity was required.

Data on recovery (ratio of total activities in H_0 and P_3) and enrichment (ratio of specific activities in H_0 and P_3) of marker enzymes for the isolation procedure are given in Table 1. The protein recovery was 2.8±0.5 % BSA equivalents, the Na^+/K^+-ATPase activity recovery was 12.9±3.6 % and the K^+-pNPPase recovery 17.7±2.9 %. No purification occurred and low recovery was observed for SDH activity. We conclude from these results that the P_3 fraction obtained by this procedure reflects a plasma membrane preparation.

Membrane orientation was analyzed on the basis of K^+-pNPPase and Na^+/K^+-ATPase activity (Flik *et al.* 1990). Briefly, saponin was used to unmask enzymatic activities that are latent as a result of membrane resealing. The K^+-pNPPase activity was used to identify inside-out membrane vesicles (IOVs). The K^+-pNPPase assay replaced the assay of the exoenzyme acetyl cholinesterase that we have successfully applied to fish (Flik

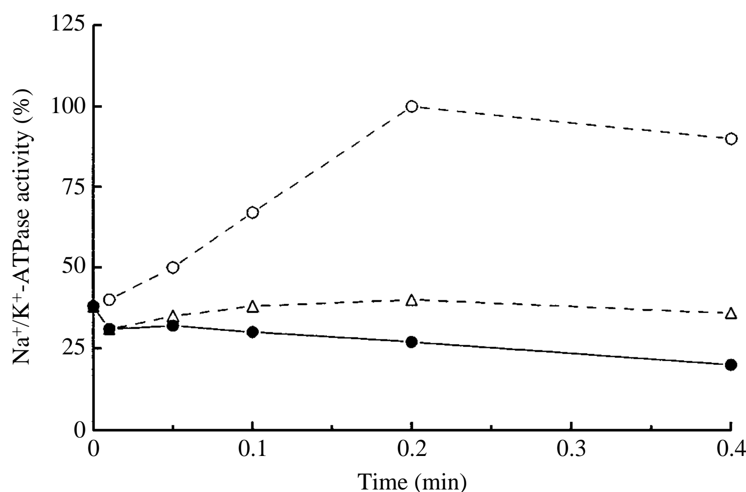

Fig. 1. Effects of detergents on Na^+/K^+-ATPase activity in a crab gill plasma membrane preparation. The highest Na^+/K^+-ATPase activity (designated 100 %) was observed at 0.2 mg saponin per milligram membrane protein (open circles). Chaps (filled circles) and digitonin (open triangles) had no stimulatory effects. Values are means for three different membrane preparations.

Table 1. *Purification and recovery of marker enzymes in membrane vesicles prepared from* Carcinus maenas *branchial epithelium*

	H_0	P_3	Enrichment factor	Percentage recovery
Total protein (mg BSA equivalents)	52.4±3.1	1.47±0.29	–	2.8±0.5
Specific activity of Na^+/K^+-ATPase (μmol P_i h^{-1} mg^{-1} protein)	17.2±8.8	79.3±11.7	5.8±2.2	12.9±3.6
Specific activity of K^+-pNPPase (μmol P_i h^{-1} mg^{-1} protein)	2.54±0.27	16.0±6.8	6.3±1.3	17.7±2.9
Specific activity of SDH (ΔA_{490} h^{-1} mg^{-1} protein)	110±28	111±28	0.9±0.3	1.1±0.2

The enrichment factor is the ratio of the specific activity in the plasma membrane fraction P_3 to that in the original homogenate H_0.

The percentage recovery is the ratio of the total activity (specific activity × total protein in the relevant fraction) in P_3 to that in H_0 multiplied by 100.

K^+-pNPPase, K^+-dependent, ouabain-sensitive pNPPase activity; SDH, succinate dehydrogenase.

Mean values ± S.D. are given for five preparations.

et al. 1990) and human red cell (Verbost *et al.* 1989) preparations; esterase activity proved to be too low to measure in our crab gill plasma membrane preparation. The trypsin-sensitivity of the cytosolic part of the Na^+/K^+-ATPase was the basis for the identification

of the right-side-out membrane vesicles (ROVs): after trypsin treatment, only ROVs are left with an intact Na^+/K^+-ATPase that may subsequently be unmasked by saponin. Inactivation of Na^+/K^+-ATPase activity in IOVs and leaky membrane fragments was optimal when they were treated with 4500 BAEE trypsin (Sigma, T0134) units per milligram membrane protein for 30 min at 25 °C. Trypsin activity was stopped by addition of 25 mg ml^{-1} soybean trypsin inhibitor (Sigma, T9253). In controls, the inhibitor was added before the addition of trypsin.

In all preparations saponin enhanced the K^+-pNPPase activity. Expressed as a percentage, the enhancement (mean values for specific activity V_{spec} were 13.6±5.9 and 16.0±6.8 μmol P_i h^{-1} mg^{-1} protein, in the absence and in the presence of saponin, repectively) was 18.6±2.6 % (N=5), indicating 19 % of IOVs in the preparation. The trypsin-*insensitive* Na^+/K^+-ATPase activity (V_{spec} 22.7±4.3 μmol P_i h^{-1} mg^{-1} protein) was 33±4 % (N=5) of the total activity (V_{spec} 69.6±12.9 μmol P_i h^{-1} mg^{-1} protein), indicating 33 % of ROVs in the preparation. In *untreated* membranes, the percentage resealing in P_3, indicated by the increase in Na^+/K^+-ATPase (from 32.2±6.1 to 79.3±11.7 μmol P_i h^{-1} mg^{-1} protein) upon saponin treatment was 59±12 %. This value did not differ (P>0.15) from the value calculated for *control-treated* membranes. In the latter, the percentage resealing indicated by the increase in Na^+/K^+-ATPase (from 31.8±5.5 to 69.6±12.9 μmol P_i h^{-1} mg^{-1} protein) upon saponin treatment was 55±10 %; from this value we calculated that there were 45±10 % leaky membrane fragments in the preparation and 22±8 % IOVs (percentage of resealed vesicles minus percentage of ROVs). The value for IOVs derived from Na^+/K^+-ATPase activity is in good agreement with the 18.6±2.6 % IOVs determined directly by the K^+-pNPPase activity assay (Table 2). The average configuration of the membrane preparation was therefore 22 % IOVs, 33 % ROVs and 45 % leaky membrane fragments.

Table 2. *Resealing and orientation of plasma membranes of* Carcinus maenas *branchial epithelium*

	− saponin	+ saponin	%R	%ROVs	%IOVs
Na^+/K^+-ATPase					
Untreated membranes	32.2±6.1	79.3±11.7	59	−	−
Control-treated membranes	31.8±5.5	69.6±12.9	55	−	−
Trypsin-treated membranes	−	22.7±4.3	−	33	22[a]
K^+-pNPPase					
Untreated membranes	13.6±5.9	16.0±6.8	−	−	19

Saponin was used at the optimum concentration of 0.2 mg mg^{-1} BSA equivalents. The specific activity is expressed in μmol P_i h^{-1} mg^{-1} protein. The percentage of resealing (%R) was calculated as the fractional increase in activity upon detergent treatment of the plasma membrane fraction.

[a]The percentage of inside-out vesicles (%IOVs) was calculated as the difference between the percentage of resealed membranes and the percentage of right-side-out (%ROVs). For further details on the procedure, see Materials and methods section.

K^+-pNPPase, K^+-dependent, ouabain-sensitive pNPPase activity.

Values are means ± S.D. for five preparations.

Applying the basolateral plasma membrane isolation method for fish gills to crab gills, we found a consistent, high degree of membrane resealing in the plasma membrane fraction, 73.5±4.2 %. However, the percentage of ROVs (71.1±4.3 %) did not differ significantly (P>0.15) from the percentage of resealed membranes. The calculated percentage of IOVs in these preparations was 3.8±2.2 % (N=5). In accordance with this low percentage of IOVs, we failed to show ATP-driven Ca^{2+} transport in these preparations (data not shown).

Ca^{2+} transport was determined as described in detail by Flik *et al.* (1990). In brief, a rapid filtration procedure was used to determine uptake of $^{45}Ca^{2+}$ into vesicles. The composition of the assay medium was (final concentrations, in mmol l^{-1}): Hepes/Tris, 20 at pH 7.4; NaCl, 150; Tris–ATP, 0 or 3; EGTA, 0.5; *N*-(2-hydroxyethyl)-ethylenediamine-*N*,*N′*,*N′*-triacetic acid (HEEDTA), 0.5; nitrilo-triacetic acid (NTA), 0.5; free Mg^{2+}, 0.8; free Ca^{2+}, 5×10^{-5} to 2.5×10^{-3}; NaN_3, 1.0; and oligomycin B (5 μg ml^{-1}); free Mg^{2+} and Ca^{2+} concentrations were varied by the addition of $MgCl_2$ and $CaCl_2$ and the actual free ion concentrations were calculated using the program *Chelator* (Schoenmakers *et al.* 1992*b*). The first and second protonations of the ligands in the calcium buffer (ATP, EGTA, HEEDTA and NTA) were taken into account and the stability constants were adjusted in accordance with the pH, temperature and ionic strength of the medium. The ^{45}Ca radioactive concentration was 1.0–1.6 MBq ml^{-1}. Incubation was carried out at the optimum *in vitro* temperature of 37 ˚C. ATP-driven Ca^{2+} transport was defined as the difference in Ca^{2+} uptake in the presence and in the absence of ATP. The incubation was terminated by quenching the reaction with 1 ml of ice-cold isotonic stop buffer (150 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes/Tris, at pH 7.4, and 1.0 mmol l^{-1} $LaCl_3$). The filters (Schleicher & Schüll, ME 25) with retained $^{45}Ca^{2+}$ containing vesicles were washed three times with 2 ml of stop buffer. After 30 min in scintillation fluid (Aqualuma, Lumac), the filters had dissolved and the radioactivity collected on them was subsequently determined by liquid scintillation counting.

Na^{+}-dependent Ca^{2+} transport across plasma membranes was assayed as the difference in $^{45}Ca^{2+}$ accumulation upon transfer of membrane vesicles equilibrated in 150 mmol l^{-1} NaCl 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 suspension was taken into account to yield the following composition (final concentrations in mmol l^{-1}): NaCl or KCl, 150; Hepes/Tris, 20 at pH 7.4; EGTA, 0.5; HEEDTA, 0.5; NTA, 0.5; free Mg^{2+}, 0.8; and free Ca^{2+}, 5×10^{-4} to 2.5×10^{-2}. Free Ca^{2+} and Mg^{2+} concentrations were calculated as described above. The ^{45}Ca radioactive concentration was 0.5–0.8 MBq ml^{-1}. A 5 μl vesicle suspension was mixed with 120 μl of medium, both prewarmed to 37 ˚C. After 5 s of incubation, the reaction was stopped by addition of 1 ml of isotonic ice-cold stop buffer (150 mmol l^{-1} NaCl, 20 mmol l^{-1} Hepes/Tris at pH 7.4, 1.0 mmol l^{-1} EDTA). Membrane vesicles with retained $^{45}Ca^{2+}$ were collected by filtration as described above.

Calculations and statistics

Kinetic data were analyzed using Enzfitter, a non-linear regression data analysis program (Leatherbarrow, 1987). Significance of differences between mean values was

assessed by the Mann–Whitney *U*-test. Data are presented as mean values ±1 standard deviation (S.D.). Significance was accepted when $P<0.05$.

Results

Crabs kept for 2–4 weeks in 50 % sea water, containing 5.3 mmol l^{-1} Ca^{2+}, had mean haemolymph Ca^{2+} levels of 9.77±1.13 mmol l^{-1} (N=21). Apparently, these animals hyperregulated their internal Ca^{2+} levels. The low variation in plasma Ca^{2+} levels was taken as circumstantial evidence for the intermoult stage.

Membrane vesicles, characterized as the basolateral plasma membrane fraction, were isolated from posterior gills 6–9 of these intermoult crabs (see Materials and methods). As shown in Fig. 2, uphill (against a [Ca^{2+}] gradient) $^{45}Ca^{2+}$ accumulation in the vesicles occurred in the presence of ATP and was abolished by the ionophore A23187. Addition of the ionophore resulted in a loss of $^{45}Ca^{2+}$ from the loading vesicles. The apparently linear increase in $^{45}Ca^{2+}$ uptake observed during the first minute indicated that the uptake at 1 min reflected the initial velocity of the transporter.

Kinetic analysis of the ATP-driven Ca^{2+} transport (1 min determinations) obeyed Michaelis–Menten kinetics for a single enzyme (Fig. 3). The K_m for Ca^{2+} was 149 nmol l^{-1} and the maximum velocity was 1.73 nmol min^{-1} mg^{-1} protein.

As shown in Fig. 4, significantly more $^{45}Ca^{2+}$ was accumulated when Na^+-loaded vesicles were transferred to a medium containing an equimolar concentration of K^+ instead of Na^+, suggesting the presence of a Na^+/Ca^{2+} exchanger in these membranes.

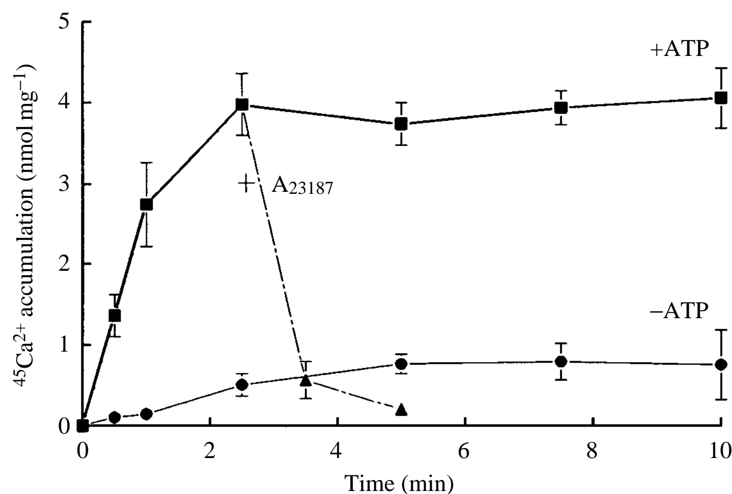

Fig. 2. ATP-driven Ca^{2+} transport in crab gill plasma membranes. Accumulation of $^{45}Ca^{2+}$ was stimulated by ATP (filled squares) and accumulated $^{45}Ca^{2+}$ was released by addition of the ionophore A23187 (filled triangles). Little $^{45}Ca^{2+}$ was associated with the membrane vesicle preparation in the absence of ATP (filled circles). The free Ca^{2+} concentration was 1 μmol l^{-1}. Note the linear increase in the rate of $^{45}Ca^{2+}$ uptake during the first minute of uptake. Values are means ± S.D. for five preparations.

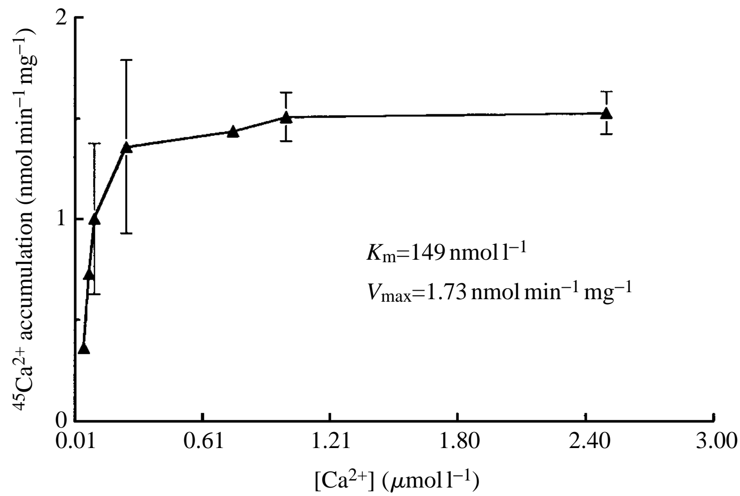

Fig. 3. Kinetics of ATP-driven (the difference between 1 min determinations of $^{45}Ca^{2+}$ accumulation in the presence and absence of ATP) Ca^{2+} transport in crab gill plasma membranes. Half-maximal activation by Ca^{2+} occurred at 149 nmol l^{-1} and a maximum velocity of 1.73 nmol min^{-1} mg^{-1} protein was calculated. Values are means ± S.D. for five preparations.

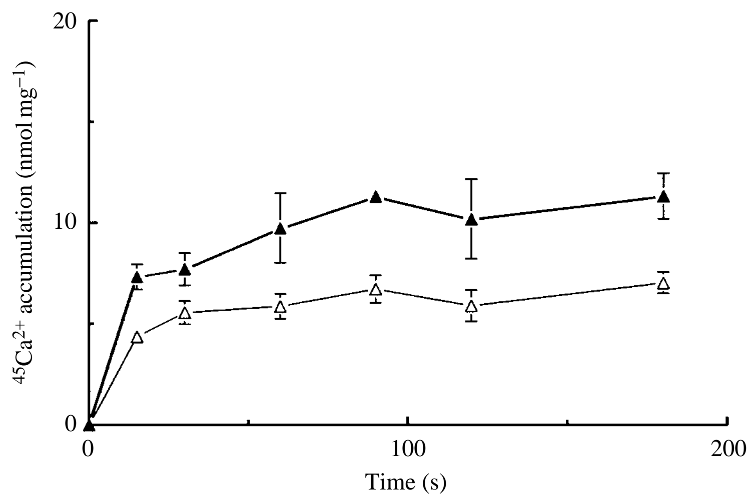

Fig. 4. Na^+-driven Ca^{2+} transport in plasma membranes of crab gills. $^{45}Ca^{2+}$ accumulation in vesicles loaded with 150 mmol l^{-1} Na^+ transferred to a medium containing 150 mmol l^{-1} K^+ (filled triangles) surpassed that in the same vesicles transferred to a medium containing 150 mmol l^{-1} Na^+ (open triangles). The free Ca^{2+} concentration was 1 μmol l^{-1}. Values are means ± S.D. for five preparations.

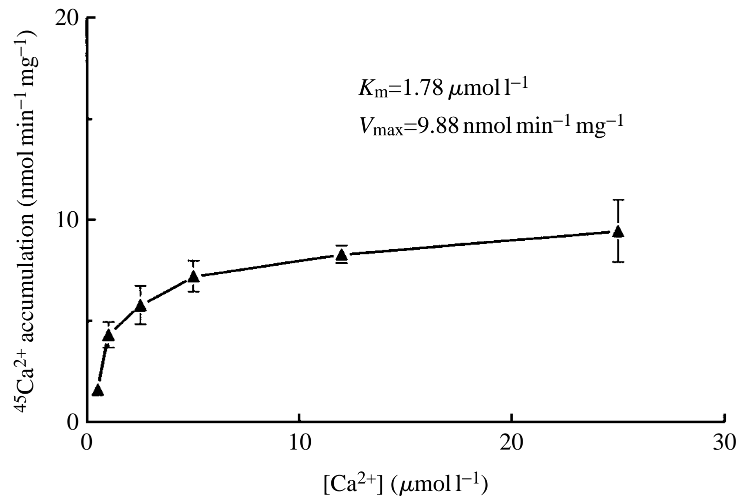

Fig. 5. Kinetics of Na^+ gradient-driven Ca^{2+} transport (the difference between 5 s determinations of Ca^{2+} accumulation upon transfer of vesicles loaded with 150 mmol l^{-1} Na^+ to a medium containing 150 mmol l^{-1} K^+ or Na^+). Half-maximal activation (K_m) occurred at 1.78 μmol l^{-1} Ca^{2+} and a maximum velocity of 9.88 nmol min^{-1} mg^{-1} protein was calculated. Values are means ± S.D. for five preparations.

Subsequently, we analyzed the Ca^{2+} kinetics of the exchanger (Fig. 5) to evaluate its activity relative to that of the ATP-driven Ca^{2+} pump. The Na^+/Ca^{2+} exchange activity obeyed Michaelis–Menten kinetics for a single enzyme; V_{max} was 9.88 nmol min^{-1} mg^{-1} protein and K_m was 1.78 μmol l^{-1}. The relative contributions of these two calcium carriers to Ca^{2+} extrusion in a branchial Ca^{2+}-transporting cell are presented in Fig. 6.

Discussion

We provide here the first direct evidence for active Ca^{2+} transport in basolateral plasma membranes of the gills of a crustacean, i.e. an ATP-driven Ca^{2+} uptake and a Na^+-gradient-driven Ca^{2+} extrusion mechanism. Both activities proved to be homogeneous (indicated by kinetics for a single enzyme), and their half-maximal activation by Ca^{2+} occurred at physiological cell Ca^{2+} concentrations. Two other important conclusions can be drawn from the data presented. First, the plasma membranes from green shore crab gills have a strong tendency to reseal predominantly as ROVs, which may hamper the direct demonstration of Ca^{2+} extrusion activity, which can only be measured in IOVs (Schoenmakers and Flik, 1992). However, when the membranes were subjected to a strong hypotonic shock (Sarkadi *et al.* 1980), sufficient IOVs were formed to measure such activities. Second, the activity of the Na^+/Ca^{2+} exchanger exceeds that of the Ca^{2+}-ATPase in the branchial plasma membrane and may, therefore, be predicted to play an important role in Ca^{2+} transport in this epithelium.

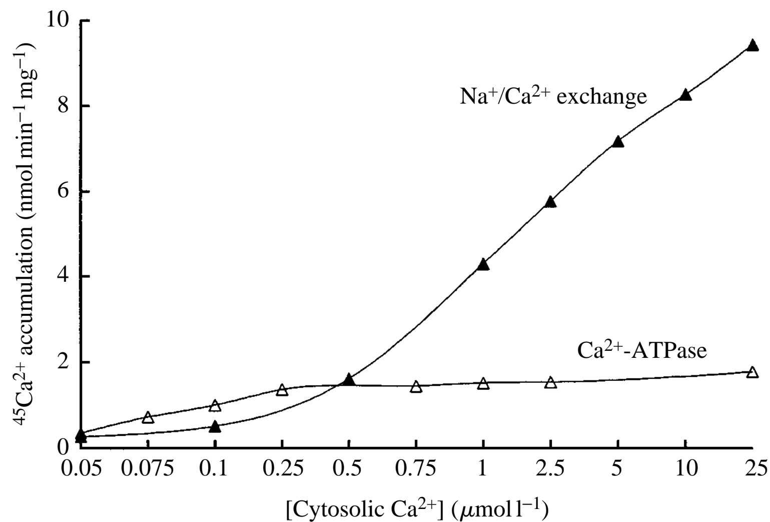

Fig. 6. The activities of the Ca^{2+}-ATPase (open triangles) and Na^+/Ca^{2+} exchanger (filled triangles) in plasma membranes of *Carcinus maenas* gills as functions of the Ca^{2+} concentration in the cell. The transport activities of both extrusion mechanisms were determined on samples from the same resealed vesicle preparations. Values are means for five preparations.

Haemolymph and water [Ca^{2+}]

Euryhaline crabs (e.g. the green shore crab *Carcinus maenas* and the blue crab *Callinectes sapidus*) maintain high plasma osmotic values when confronted with decreasing salinities (Siebers *et al.* 1982; Mantel and Farmer, 1983; Lucu and Siebers, 1986; Shetlar and Towle, 1989). Neufeld and Cameron (1993) have given a convincing electrochemical evaluation of branchial Ca^{2+} exchange in the blue crab and postulated that active Ca^{2+} transport mechanisms must exist in its branchial epithelium. There is evidence that haemolymph Ca^{2+} levels can be maintained hyperionic to the medium when crabs are acclimated to dilute sea water (*Carcinus maenas*, Greenaway, 1976; this study), indicating that net uptake of Ca^{2+} from the water must occur. Indeed, in intermoult *Carcinus mediterraneus*, which is closely related to the green shore crab used in this study, kept under identical water conditions to those described in this paper, whole-body (branchial) Ca^{2+} influx ($6.57\pm1.95\,\mu$mol h^{-1} g^{-1}) significantly exceeded the efflux ($3.15\pm2.63\,\mu$mol h^{-1} g^{-1}; means ± S.D., N=6; C. Lucu, unpublished data). The resulting net Ca^{2+} *influx via* the gills could explain the high Ca^{2+} concentration of the haemolymph. The transcellular movement of Ca^{2+} that must underlie this influx warranted further studies on Ca^{2+} transport mechanisms in the gills.

Membrane isolation

Na^+/K^+-ATPase activity has been localized exclusively in the basolateral plasma membrane of blue as well as green shore crab gill cells (Towle and Kays, 1986; Morris

and Greenaway, 1992). Its enrichment in the membrane fraction used in this study, therefore, indicates that this fraction is enriched in basolateral plasma membranes. Moreover, SDH activity was not enriched in this fraction, indicating that mitochondrial membrane fragments had selectively been removed.

The values for enzyme enrichment and recovery of plasma membranes calculated on the basis of Na^+/K^+-ATPase and K^+-pNPPase were comparable, which strongly suggested that these activities indeed reflect the activity of the same enzymatic entity. This allowed a direct comparison of these activities, as used in the membrane orientation assays. The value of 22 % for IOVs, calculated indirectly on the basis of the trypsin treatment of membranes, was strongly supported by the comparable value of 19 % of IOVs determined directly on the basis of the K^+-pNPPase activity (Table 2). The configuration of the plasma membrane fragments in the preparation was therefore 22 % IOVs, 33 % ROVs and 45 % leaky fragments. These values compare very well with those obtained for plasma membrane vesicles prepared from fish tissues (Flik *et al.* 1985*a*, 1990), which have been shown to be suitable for the demonstration of membrane Ca^{2+} transport. It should be noted that others (Towle and Hølleland, 1987; Shetlar and Towle, 1989; Hølleland and Towle, 1990) have succeeded in isolating plasma membrane preparations from blue and green shore crab gills with sufficient IOVs to demonstrate energized Na^+ transport, applying procedures not very different from those normally applied to fish gill epithelium, but that yielded insufficient IOVs for measurements of Ca^{2+} transport. This suggests that species differences may exist with respect to membrane behaviour during isolation procedures and, therefore, that these procedures need careful evaluation.

Membrane Ca^{2+} transporters

Our kinetic studies indicated that both the ATP-driven and the Na^+-gradient-driven Ca^{2+} transport obeyed Michaelis–Menten kinetics and thus were the expression of a single class of carriers. This was taken as further evidence that the enzymatic activities in this membrane fraction reflect those present in the basolateral plasma membrane of the ion-transporting cells. The maximum velocities of the transporters are of the same order of magnitude as those found in, for instance, fish gills and intestine (Flik *et al.* 1990; Verbost *et al.* 1994) and suggest a Ca^{2+} transport capacity sufficient to explain the Ca^{2+} influx seen in these tissues.

Clearly, the Na^+/Ca^{2+} exchange activity in the crab gills may be an important mechanism for Ca^{2+} transport. Fig. 6 shows the activities of the two plasma membrane Ca^{2+} extrusion mechanisms as a function of cytosolic Ca^{2+} levels. For the exchange activity, it was assumed that the Na^+ gradient was not limiting its activity. At cytosolic Ca^{2+} concentrations up to $500\,nmol\,l^{-1}$, the rate of Ca^{2+}-ATPase-mediated transport exceeds that of exchanger-mediated transport. With cytosolic Ca^{2+} concentrations exceeding $500\,nmol\,l^{-1}$, the rate of export of Ca^{2+} from the cell depends predominantly on the activity of the exchanger. This situation is strongly reminiscent of that in the tilapia enterocyte, where Na^+/Ca^{2+} exchange determines the rate of extrusion of Ca^{2+} from the cell (Flik *et al.* 1990; Schoenmakers *et al.* 1992*a*, 1993). The crab gill may represent a second tissue in which Ca^{2+} transport is, to a large extent, mediated by a Na^+/Ca^{2+}

exchanger. The occurrence of these two Ca^{2+} transport mechanisms in a crustacean tissue lends support to the prediction from physiological experiments (Roer, 1980) that Ca^{2+} transport in the green shore crab hypodermis must be mediated by ATP-dependent as well as Na^+-dependent transport mechanisms. Further studies on the characterization of Ca^{2+} transport mechanisms in crustacean tissues seem warranted. In particular, the regulation of these mechanisms in gills and hypodermis during the moulting cycle may yield interesting new insights into the functioning of Ca^{2+} transporters. Euryhaline crabs may provide a particularly powerful model for the study of the Na^+-dependence of Ca^{2+} transport (when determined by the exchanger). The hyperregulation of plasma Na^+ in (these) crustaceans suggests that these animals may depend on a guaranteed inward Na^+ gradient for Ca^{2+} extrusion, irrespective of the ion composition of the ambient medium.

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