Transepithelial potential differences and Na⁺ flux in isolated perfused gills of the crab *Chasmagnathus granulatus* (Grapsidae) acclimated to hyper- and hypo-salinity

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Summary

We studied the transepithelial potential difference (TEPD) and ²²Na flux across isolated perfused gills (anterior pair 5 and posterior pairs 6–8) of the crab *Chasmagnathus granulatus* acclimated to either hypo- or hyper-osmotic conditions.

The gills of crabs acclimated to low salinity, perfused and bathed with 10‰ saline solutions, produced the following TEPDs (hemolymph side with respect to bath side): 0.4±0.7, –10.2±1.6, –10.8±1.3 and –6.7±1.3 mV for gills 5, 6, 7 and 8, respectively. Gills 6, 7 and 8 did not differ significantly. Reducing the saline concentration of both bath and perfusate from 30‰ to 20‰ or 10‰ increased significantly the TEPDs of these gills. TEPDs of gill 6 (representative of posterior gills) were reduced by 69±5% and 60±5% after perfusion with ouabain or BaCl₂ (5 mmol l⁻¹ each), respectively. The same gill showed a net ouabain-sensitive Na⁺ influx of 1150±290 μequiv g⁻¹ h⁻¹.

Gill 6 of crabs acclimated to high salinity produced TEPDs of –1.5±0.1 and –1.3±0.09 mV after perfusion with 30‰ or 40‰ salines, respectively. Perfusion with ouabain or BaCl₂ reduced TEPDs by 76±7% and 86±4%, respectively. A net ouabain-sensitive Na⁺ efflux of 2282±337 μequiv g⁻¹ h⁻¹ was recorded in gill 6 perfused with 38‰ saline.

Key words: isolated perfused gill, transepithelial potential difference, Na⁺K⁺-ATPase, ion flux, hypo-regulation, hyper-regulation, ouabain, crab, *Chasmagnathus granulatus*.

Introduction

Semiterrestrial estuarine crabs are adapted to a wide range of conditions. Water salinity varies from full-strength seawater to almost freshwater in a geographical and tidal basis. Excursions to intertidal and supratidal areas include the chance of entering into rain pools or into tide pools concentrated by evaporation. Moreover, aerial ventilation may cause evaporative concentration of water retained within the branchial chambers, leading to hyper-saline exposure of the gills and the gill chamber epithelium (Schmitt and Santos, 1993). Terrestrial and semiterrestrial crabs of marine or estuarine origin possess hyper- and hypo-regulation capacities, showing little variation in their internal osmotic and ionic concentrations within a wide range of salinity (Gross, 1964; Mantel and Farmer, 1983; Schubart and Diesel, 1998, 1999).

The gills of euryhaline crabs are histologically and functionally differentiated. While anterior gills are lined with thin epithelium and have a mainly respiratory function, the posterior ones play a key role in compensatory active uptake of ions (Siebers et al., 1982; Gilles and Pécqueux, 1986; Towle and Kays, 1986; Compère et al., 1989). The predominant cells in posterior gills are thick and possess large basolateral membrane interdigitations together with mitochondria (Copeland and Fitzjarrell, 1968; Compère et al., 1989; Luquet et al., 1997). There is much evidence from electrophysiological and ion flux studies on the ion-uptake capacity of posterior gills of different crab species (Gilles and Pécqueux, 1981, 1985; Pécqueux et al., 1988; Siebers et al., 1985; Lucu and Siebers, 1986; Burnett and Towle, 1990) (for a review, see Pécqueux, 1995).

Two models for the mechanisms involved in such active ion transport through the gills, have been reviewed recently (Onken and Riestenpatt, 1998). (1) In the gills of species such as *Carcinus maenas*, which possess limited capacity to invade low-salinity environments (weak hyper-regulators), it is proposed that the ions enter the cell by crossing the apical membrane through Na⁺/2Cl⁻/K⁺ symports coupled to K⁺ channels. (2) In strong hyper-regulators, such as *Eriocheir sinensis*, Onken and Riestenpatt suggest that Na⁺ crosses the apical membrane through epithelial Na⁺ channels while Cl⁻ is exchanged with HCO₃⁻, driven by an apical H⁺-V-ATPase. At
the basolateral side, Na\(^+\)K\(^+\)-ATPase and K\(^+\) and Cl\(^-\) channels are thought to drive ions into the hemolymph in both weak and strong hyper-regulating species.

Early work on salt and water regulation by intact fiddler crabs of the genus *Uca* (Green et al., 1959; Baldwin and Kirschner, 1976a; Evans et al., 1976) suggests that active ion excretion follows an extra-renal route. There is some physiological and histological evidence to suggest that the gills of hypo-regulating crabs are the organs involved in this function. Martinez et al. (1998), working with isolated perfused gills of *Ucides cordatus*, reported that gill 6 is capable of active ion excretion, while gill 5 is specialized in ion uptake. Ultrastructural studies also suggested an ion excretion capacity of the gills of crabs acclimated to hypersaline media; Martelo and Zanders (1986) and Luquet et al. (1997) described a cell architecture characteristic of an ion-excreting epithelium in the gills of grapsids and ocypodids. Some features of this cell architecture, such as the apparently low-resistance cell junctions, resemble those of the extensively studied vertebrate salt-secreting organs such as the avian salt gland (Riddle and Ernst, 1979), the teleost opercular epithelium (Ernst et al., 1980) and the rectal gland of elasmobranchs (Ernst et al., 1981).

*Chasmagnathus granulatus* Dana 1851 is a strong ion hyper- and hypo-regulating crab species that inhabits intertidal estuarine coasts of Brazil, Uruguay and Argentina (Boschi, 1964; Mougabure Cueto, 1998). The posterior gills of this species are believed to be involved in both ion uptake and excretion, since their epithelium thickness is increased to the same extent after transfer from full seawater to either dilute or concentrated seawater (Genovese et al., 2000).

The aim of this work was to establish, by electrophysiological and ion tracer flux experiments, the possible role of the different gill pairs of *C. granulatus* in ion-transport functions at low and high salinity. The participation of Na\(^+\)K\(^+\)-ATPase and K\(^+\) channels was also studied.

**Materials and methods**

**Animals**

Crabs were collected from Faro San Antonio beach (36°18'S 56°48'W), near the southern edge of the Rio de la Plata estuary, Argentina. Once in the laboratory, the animals were separated at random into two groups and acclimated in glass containers with aerated seawater of either 12 ‰ or 45 ‰ salinity for at least 2 weeks. Laboratory temperature was kept at 20±2°C. Stage C intermoult adult male crabs (Drach and Tchernigovtzeff, 1967) of 26±0.3 mm carapace widths were selected for the study.

**Gill perfusion**

Crabs were killed by destroying the ventral nervous ganglion with a spike. After removing the dorsal carapace, the gills were gently excised and placed in a Petri dish with saline solution. The afferent and efferent vessels of gills 5–8 were connected by fine polyethylene tubing of 0.4 mm diameter to a peristaltic pump (afferent) and to a glass tube (effluent). Perfusion rate was kept at 0.1 ml min\(^{-1}\). The tubing was held in position by an acrylic clamp and the preparation put into a glass beaker with the appropriate saline solution and constant aeration.

Gills 5–8 from crabs acclimated to low (12 %) and high (45 %) salinity were perfused and bathed with identical solutions, except that the perfusate contained 2 mmol l\(^{-1}\) glucose. Table 1 shows the composition of the saline solutions used in the different experiments. All solutions were adjusted with Tris-base to the physiological pH 7.75 for *C. granulatus* (Luquet and Ansaldo, 1997). The effects of the following drugs were tested in gills perfused with 10 % saline for low-salinity crabs and 38 % saline for high-salinity crabs: ouabain (a specific Na\(^+\)K\(^+\)-ATPase inhibitor) (Skou, 1965), 5 mmol l\(^{-1}\) applied basolaterally, and BaCl\(_2\) and CsCl (K\(^+\) channel blockers) (Zeiske, 1990; Draber and Hansen, 1994), 5–10 mmol l\(^{-1}\) applied at both apical and basolateral sides.

**Transepithelial potential difference (TEPD)**

Ag/AgCl electrodes were connected by agar bridges to the external bath and to the glass tube collecting the perfusate. Potential differences were measured in the internal perfusate with respect to the external bath by means of a millivoltmeter.

**Na\(^+\) flux**

Na\(^+\) outward and inward movements were measured in the same gill by applying \(^{22}\)Na first in the perfusate and afterwards in the bathing solution. Identical ionic concentrations (20 % or 38 %, for low- and high-salinity crabs, respectively) were used in the bath and perfusate. For Na\(^+\) efflux, \(^{22}\)Na was included in the perfusate at a final concentration of 0.25 μCi ml\(^{-1}\). After stabilization for 15 min, three samples (1 ml each) were collected from the perfusate and the bath at intervals of 15 min. Na\(^+\) efflux was calculated from the radioactivity that appeared in the bath. To avoid overestimated flux due to a possible leak, we also measured the radioactivity lost by the perfusate after passing through the gill. Gills showing large discrepancies between both methods of measurement were discarded. Results from measurements of radioactivity that appeared in the bath were chosen for the study.

<table>
<thead>
<tr>
<th>Salinity (%)</th>
<th>Component (mmol l(^{-1}))</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>KCl</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>10</td>
<td>156</td>
</tr>
<tr>
<td>20</td>
<td>312</td>
</tr>
<tr>
<td>30</td>
<td>468</td>
</tr>
<tr>
<td>32</td>
<td>500</td>
</tr>
<tr>
<td>34</td>
<td>531</td>
</tr>
<tr>
<td>36</td>
<td>562</td>
</tr>
<tr>
<td>38</td>
<td>593</td>
</tr>
<tr>
<td>40</td>
<td>625</td>
</tr>
</tbody>
</table>

Perfusate also included 2 mmol l\(^{-1}\) glucose.
After washing with non-radioactive solution, Na\textsuperscript{+} influx was measured by applying \(^{22}\text{Na}\) in the bath at a final concentration of 9 kBq (0.25 \(\mu\)Ci) ml\(^{-1}\). After stabilization for 15 min, radioactivity was measured in the collected perfusate at 10 min intervals during the subsequent 30 min.

Radioactivity was measured with a Canberra Series 35 plus gamma scintillation counter. Na\textsuperscript{+} efflux and influx were calculated according to the formula of Lucu and Siebers (1986):

\[
J = \frac{^{22}\text{Na}}{S \times \text{SRA} \times m},
\]

where \(J\) is the calculated unidirectional flux of Na\textsuperscript{+} in \(\mu\)equiv h\(^{-1}\) g\(^{-1}\); \(^{22}\text{Na}\) is the radioactivity (cts min\(^{-1}\)) collected during each interval; \(S\) is the number of samples collected during 1 h (6 and 4 for influx and efflux, respectively); \(\text{SRA}\) is the specific radioactivity (cts min\(^{-1}\) \(\mu\)equiv\(^{-1}\)) and \(m\) is the fresh mass of the gill (\(g\)).

### Chemicals

NaCl, KCl, MgCl\(_2\), BaCl\(_2\), KCN and glucose were obtained from Merck Argentina; NaHCO\(_3\) was obtained from Mallinckrodt USA; CaCl\(_2\) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were purchased from J. T. Baker, USA; ouabain was purchased from Sigma USA and \(^{22}\text{Na}\) was obtained from Amersham Pharmacia Biotech.

### Statistics

Data were analyzed by one- or two-way repeated measures analysis of variance (ANOVA) or paired \(t\)-test when appropriate (Sokal and Rohlf, 1981). All values are means ± S.E.M.

### Results

The preparations of isolated perfused gills remained viable for at least 3 h, since stable values of transepithelial potential difference (TEPDs) could be recorded along this period. The rate of perfusion was also constant during each experiment.

#### Crabs acclimated to low salinity

**Transepithelial potential difference**

Posterior gills of crabs acclimated to low salinity, perfused and bathed with identical salines, showed hemolymph-side negative TEPDs, which increased significantly in absolute value as the concentration of saline solutions decreased; no significant TEPD was recorded in gill 5 (representative of anterior gills) (Fig. 1). Two-way repeated measures ANOVA comparing gills 6, 7 and 8 indicated that saline concentration was the only significant source of variation \((P<0.001, N=6\) for each gill). TEPDs for gill 6 ranged between \(-2.4±0.5\) mV \((N=6)\) in 30\% \(\circ\) saline and \(-10.2±1.6\) mV \((N=6)\) in 10\% \(\circ\) saline. Since the three posterior gills responded similarly to the different saline concentrations, gill 6 was chosen as representative for further experiments because it was bigger than gills 7 and 8 and therefore easier to handle.

Basolaterally applied ouabain (5 mmol l\(^{-1}\)) significantly reduced the TEPD of gill 6 by 69±5\% \((P<0.001, N=7)\). 5 mmol l\(^{-1}\) BaCl\(_2\) applied basolaterally caused a similar inhibitory effect of 60±5\% \((P<0.001, N=5)\). The same inhibitor was totally ineffective when applied at the apical (bath) side, even when the concentration was raised to 10 mmol l\(^{-1}\). In preliminary experiments CsCl (10 mmol l\(^{-1}\)) was also ineffective at the apical side (data not shown). The effects of ouabain and BaCl\(_2\) were totally reversed after washing out with normal saline solution (Table 2). Results from single experiments are shown in Fig. 2.

**Na\textsuperscript{+} influx**

A net Na\textsuperscript{+} influx of 1150±290 \(\mu\)equiv g\(^{-1}\) h\(^{-1}\) \((N=6)\) was calculated for isolated gills 6 of crabs acclimated to low salinity, perfused and bathed with 20\% \(\circ\) saline. Unidirectional flux of Na\textsuperscript{+} was 719±163 \(\mu\)equiv g\(^{-1}\) h\(^{-1}\) \((N=6)\) in the outward \((J_{\text{out}})\) direction and 1824±410 \(\mu\)equiv g\(^{-1}\) h\(^{-1}\) \((N=6)\) in the inward direction \((J_{\text{in}})\). The \(J_{\text{out}}/J_{\text{in}}\) ratio calculated from these results \((2.87±0.67)\) was significantly different \((P<0.05)\) from the ratio predicted by the Ussing equation (Ussing, 1949), which was 1.33±0.05. Ouabain \((5\text{ mmol l}^{-1})\) added to the perfusate caused a reduction of 35±5\% in \(J_{\text{in}}\) \((P<0.05, N=5)\); inhibition of the net Na\textsuperscript{+} influx calculated from this value was 70±20\%, \(N=5\) (Fig. 3).

### Table 2. Effect of different ion-transport inhibitors on the transepithelial potential difference (TEPD) of gill 6 of crabs acclimated to low salinity

<table>
<thead>
<tr>
<th>TEPD (mV)</th>
<th>Control</th>
<th>Inhibitor</th>
<th>Wash out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill 5</td>
<td>–6.6</td>
<td>–2.1</td>
<td>–6.8</td>
</tr>
<tr>
<td>Gill 6</td>
<td>–9</td>
<td>–3.8</td>
<td>–9</td>
</tr>
<tr>
<td>Gill 7</td>
<td>0.8</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Gill 8</td>
<td>1</td>
<td>0.8</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S.E.M.</th>
<th>0.8</th>
<th>0.5</th>
<th>0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Ouabain and BaCl\(_2\) (5 mmol l\(^{-1}\)) were each applied basolaterally.
Crabs acclimated to high salinity

Transepithelial potential difference

In a preliminary series of experiments, gills 6, 7 and 8 of crabs acclimated to high salinity, perfused with either 30% or 40% saline, produced TEPDs of the same polarity but substantially smaller than those shown by posterior gills of crabs acclimated to low salinity, i.e. −1.5±0.1 and −1.3±0.09 mV (N=13) for gill 6 perfused with 30% and 40% solutions, respectively. These values did not differ significantly. Gill 5 did not show any significant TEPD.

In a second series of experiments, gill 6 was used as representative of posterior gills and the concentration of the bath and perfusate was increased stepwise from 30% to 40%, in an attempt to minimize the effects of a possible osmotic shock. A slight tendency to increased TEPD at higher saline concentrations was observed, with the highest values recorded at 36–38% (N=9). Nevertheless, these differences were not statistically significant (Fig. 4).

Effects of inhibitors are summarized in Table 3. Basolateral application of 5 mmol l⁻¹ ouabain significantly inhibited the TEPD by 76±7% (P<0.005, N=6). This effect was hardly reversed after washing out with normal saline. Similarly, 10 mmol l⁻¹ BaCl₂ applied at the basolateral side had an inhibitory effect of 86±4% (P<0.001, N=5) but did not cause consistent effects when applied at the apical side (N=9). Another series of experiments was performed to test whether the effects of BaCl₂ were due to diffusive potentials caused by the asymmetrical addition of Cl⁻ ions or by actual inhibition of K⁺ channels. Choline chloride (20 mmol l⁻¹) applied basolaterally caused a significant decrease in TEPD of 36±6% (P<0.01, N=5), whereas BaCl₂ under the same conditions produced a significantly higher effect of 84±5% (P<0.001, N=5). Fig. 5 shows the results of individual experiments with ouabain, BaCl₂ and choline chloride.

Na⁺ efflux

A mean net Na⁺ efflux of 2282±337 μequiv g⁻¹ h⁻¹ (N=9) was calculated for gill 6 of crabs acclimated to high salinity, perfused and bathed with 38% saline. Unidirectional Na⁺ efflux (J_{out}) was 2900±329 μequiv g⁻¹ h⁻¹ (N=9), while unidirectional influx (J_{in}) measured in the same preparations...
was 619±73 μequiv g⁻¹ h⁻¹ (N=9). The observed $J_{out}$/$J_{in}$ ratio (4.48±0.91) was significantly different ($P<0.05$) from the ratio predicted by the Ussing equation (0.95±0.01). $J_{out}$ was 58±9 % inhibited by basolaterally applied 5 mmol l⁻¹ ouabain ($P<0.001$, $N=4$); inhibition of net efflux calculated from this value was 81±6 %, $N=4$ (Fig. 3).

Discussion

Ion-uptake functions of crab gills have been extensively studied in the hyper-regulators Carcinus maenas, Eriocheir sinensis, Callinectes sapidus, (Burnett and Towle, 1990; Gilles and Péqueux, 1981; Onken and Siebers, 1992; Siebers et al., 1985) (for reviews, see Péqueux, 1995; Onken and Riestenpatt, 1998) and the hyper-hyporegulators Pachygrapsus marmoratus (Drews and Graszynski, 1987; Drews et al., 1989; Pierrot et al., 1995a,b). These TEPDs are sensitive to the dilution of the perfusion and bathing media, being the lowest absolute values measured at 30 ‰, which is near the physiological hemolymphatic concentrations of Na⁺ and Cl⁻ in this species (Mougabure Cueto, 1998). This change in potential difference seems to reflect an autoregulatory mechanism of gill tissue, which involves enhanced ion transport activity and/or increased paracellular resistance in response to hypo-osmotic stress. Onken (1996) and Onken and Riestenpatt (1998) reported that changes in short-circuit currents measured in split gill lamellae of Eriocheir sinensis respond to osmotic variations at the basolateral side. Our results indicate that these changes in TEPD take place in C. granulatus within a few minutes and are totally reversed when the gill is perfused again with iso-ionic saline. This rapid response should be important for a species that is often observed emerging from brackish or seawater and entering into rain pools for feeding on supratidal plants.

Radioactive tracer flux suggests that posterior gills of this species actively take up Na⁺ at low salinity. Both influx and efflux rates as well as net influx are somewhat high compared with data reported for other species in similar experimental conditions (Lucu and Siebers, 1986; Pierrrot et al., 1995a). This high rate of Na⁺ uptake is possibly a response to a high rate of ion loss by the animal. Gill and whole animal ionic permeability should be studied in order to test this hypothesis.

As a first approach to understanding the mechanisms involved in gill ion uptake, it can be concluded that Na⁺/K⁺-ATPase located at the basolateral membrane is the major driving force, since both TEPD and Na⁺ influx are inhibited by ouabain in similar proportions. The reason why ouabain does not cause total inhibition could be incomplete access of the drug to the enzyme molecules, due to the complex basolateral membrane interdigitations of gill ionocytes, as suggested by Burnett and Towle (1990) to explain similar results obtained with Callinectes sapidus.

As our results imply, barium-sensitive K⁺ channels located in the basolateral membrane are also involved in generating the observed transepithelial potentials. The lack of effect of apical BaCl₂ and CsCl (preliminary data) suggests the absence of barium-sensitive K⁺ channels in this membrane. It has been reported that these channels are necessary for electrogenic uptake of Na⁺ and Cl⁻ across the apical membrane through Na⁺/2Cl⁻/K⁺ cotransporters in Uca tangeri and Carcinus maenas (Drews and Graszynski, 1987; Riestenpatt et al., 1996).

Onken and Riestenpatt (1998) have proposed that in strong hyper-regulators, such as Eriocheir sinensis, Na⁺ and Cl⁻ cross the apical membrane through Na⁺ channels and Cl⁻/HCO₃⁻ antiports, driven by an H⁺-V-ATPase. Although we have no direct evidence for apical transporters, expression of both Na⁺/H⁺ exchangers and H⁺-V-ATPase has been detected in gill 6 of C. granulatus in preliminary molecular biology experiments (D. Weihrauch and C. M. Luquet, unpublished
Acclimation to high salinity

The involvement of crab gills in hypo-regulatory ion excretion is still a matter of controversy. In the past two decades histological evidence has accumulated. There are reports of ultrastructural changes and increased posterior gill epithelium thickness after acclimation to hypersaline media (Martelo and Zanders, 1986; Luquet et al., 1997; Rosa et al., 1999; Genovese et al., 2000). Martínez et al. (1998) perfused gills 5 and 6 of *Ucides cordatus* acclimated in isosmotic medium, reporting net Na⁺ uptake by gill 5, even when the external saline was more concentrated than the perfusate. In contrast, they found net Na⁺ excretion by gill 6 at all concentrations tested. This experiment, however, is not comparable with the present results, since it was performed with asymmetrical perfusion. In addition, the authors acclimated the crabs to isosmotic medium. A clear difference does seem to exist between both species; however; whereas in *U. cordatus* different gills are specialized for transporting ions in opposite directions, the TEPDs measured on the three posterior gills of *C. granulatus* suggested that they have similar ion transport capacities. Thus gills 6, 7 and 8 of *C. granulatus* seem to be equally involved in both transport directions, after chronic acclimation to either low or high salinity.

At least two basolateral membrane proteins involved in ion uptake are also involved in ion excretion. These are Na⁺K⁺-ATPase, since both TEPD and Na⁺ flux are reduced by ouabain, and K⁺ channels, which are inhibited by basolateral application of BaCl₂. By contrast, our results provide no evidence for any apical ion-transport proteins.

Current models for salt excretion in vertebrates (gills, and opercular epithelia of teleost fish, rectal glands of elasmobranchs and avian salt glands) consider Na⁺ pumping into the paracellular space and a transcellular flux of Cl⁻, which in turn generates a positive transepithelial potential difference that drives Na⁺ efflux via low-resistance tight junctions (Ernst et al., 1980, 1981; Lowy et al., 1987). Our results on crabs acclimated to high salinity indicate that Na⁺K⁺-ATPase is the main driving force for ion extrusion. In addition, previous electron microscopic work shows shorter septate junctions in gills of *Uca uruguayensis* and *C. granulatus* acclimated to high salinity (Luquet et al., 1997; Rosa et al., 1999) compared with gills of the same species acclimated to low salinity. Thus, paracellular flux of ions through these junctions also seems possible. However, the vertebrate model predicts a positive transepithelial potential difference for driving paracellular Na⁺ efflux. This is not the case for the gills of *C. granulatus*, which produce a little negative potential difference. Therefore the routes followed by Na⁺ and Cl⁻ at the apical side seem to differ from known models for salt excretion and deserve further investigation.

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