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ACTIVE AND ELECTROGENIC ABSORPTION OF Na^+ AND Cl^- ACROSS POSTERIOR GILLS OF *ERIOCHEIR SINENSIS*: INFLUENCE OF SHORT-TERM OSMOTIC VARIATIONS

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Summary

Split lamellae of posterior gills of Chinese crabs (*Eriocheir sinensis*) acclimated to fresh water were mounted in a modified Ussing-type chamber, and the transepithelial short-circuit current and conductance were measured. The epithelium shows independent active and electrogenic absorption mechanisms for Na^+ and Cl^- that can be measured as positive and negative short-circuit currents, respectively, in the absence of the counter ion. Increasing the osmolarity of the haemolymph-side saline by addition of sucrose resulted in a marked decrease in active uptake of both Na^+ and Cl^-. In contrast, increasing the internal osmolarity by addition of urea or moderately decreasing the haemolymph-side osmolarity resulted in a marked increase in Na^+ as well as Cl^- transport. Circuit analysis revealed that Na^+ current changes are mostly due to alterations in the apical amiloride-sensitive Na^+ conductance, while Cl^- current changes are caused not only by alterations in the transcellular conductance but also by changes in the electromotive force for Cl^- absorption. Osmotic perturbations in the external bath induced current changes in the same directions, but the magnitudes of the effects were smaller than those after internal osmotic variations, indicating that the external barrier has a lower water permeability than the internal barrier. Short-term osmotic perturbations did not significantly affect the leak conductance, which is not associated with active transport and which may mostly reflect the paracellular conductance.

Key words: crab gill, Chinese crab, *Eriocheir sinensis*, NaCl absorption, osmotic variations, sucrose, urea, Ussing-type chamber, short-circuit current.

Introduction

Chinese crabs (*Eriocheir sinensis*) spend the greatest proportion of their life in fresh water, where they maintain an outwardly directed osmotic gradient of about 550–650 mosmol kg^{-1} by active uptake of NaCl across the posterior gills (Mantel and Farmer, 1983; Péqueux *et al.* 1988). Krogh (1938) demonstrated that *E. sinensis* acclimated to fresh water are able to absorb Na^+ and Cl^- independently from each other. Through the use of split gill lamellae in Ussing-type chambers, the respective mechanisms have been elucidated. Active, electrogenic Na^+ absorption proceeds as in frog skin *via* apical Na^+ channels and the basolateral Na^+/K^+ pump and can be measured as positive, amiloride-sensitive short-circuit current (I_{Na}) using Cl^--free Na^+ saline in the external bath (Zeiske *et al.* 1992*a*). Active, electrogenic Cl^- absorption, which is reflected in a negative short-circuit current (I_{Cl}) in the presence of external Cl^- saline, proceeds *via* apical Cl^-/HCO_3^- antiport and basolateral Cl^- channels (Onken *et al.* 1991) and is driven by an apical V-type H^+ pump (Putzenlechner *et al.* 1992; Onken and Putzenlechner, 1995).

E. sinensis pass the first and last portions of their life cycle in sea water or brackish water (Peters and Panning, 1933). The haemolymph of seawater-adapted *E. sinensis* is approximately iso-osmotic with the ambient medium (900–1000 mosmol kg^{-1}; Mantel and Farmer, 1983). Thus, there is no requirement for NaCl absorption. In fact, the gills of seawater crabs display no net fluxes of Na^+ (Péqueux and Gilles, 1981) and only low transbranchial potential differences (Onken and Graszynski, 1989), indicating the absence of active and electrogenic salt absorption. In addition to the salinity of the ambient medium, factors that affect the haemolymph osmolarity and/or the permeability of the body surface (such as composition of food, moulting, land visits, etc.) also influence the demand for NaCl uptake. Thus, the freshwater-adapted animals must be able to regulate the magnitude of NaCl absorption. Dopamine has been shown to hyperpolarize the transbranchial potential difference measured in isolated perfused gills of freshwater-adapted crabs with NaCl saline on both sides of the epithelium (Detaille *et al.* 1992). Moreover, it has been reported that a transport-stimulating factor of unknown chemical nature is present in eyestalk extracts of *E. sinensis* acclimated to fresh water (Schöbel *et al.* 1994). The modulation of NaCl uptake across *E. sinensis* gills by

manipulating second messenger systems has been clearly demonstrated (Bianchini and Gilles, 1990; Asselbourg *et al.* 1991; Péqueux and Gilles, 1992; Riestenpatt *et al.* 1994). The absence of any net Na^+ influx when whole posterior gills of freshwater crabs were treated with high-NaCl salines (composed according to the haemolymph of seawater animals) was interpreted as a regulation of Na^+ absorption by the Na^+ level of the haemolymph (Péqueux and Gilles, 1981). However, when the haemolymph composition changes, its osmolarity also varies. For several vertebrate epithelia, transport modulation by alteration in cell volume induced by changing osmolarity of the body fluid has been demonstrated (for a review, see Macknight, 1991). Thus, it is conceivable that the haemolymph osmolarity itself also directly influences transport magnitude in *E. sinensis* gills.

In the present study, short-term osmotic perturbations on either side of split gill lamella preparations of crabs acclimated to fresh water were monitored. Their influence on the magnitudes of active and electrogenic absorption of Na^+ and Cl^- was also investigated.

Some of these results were reported at the 1993 annual meeting of the Deutsche Zoologische Gesellschaft in Salzburg (Onken *et al.* 1993).

Materials and methods

Crabs

Chinese crabs (*Eriocheir sinensis* Milne-Edwards) were obtained from commercial fishermen. The animals were caught in the rivers Eider (Schleswig-Holstein, Germany) or Havel (Brandenburg, Germany). In the laboratory, the animals were kept at 10–12 °C in running tap water (containing, in $mmol\,l^{-1}$: Na^+, 2.0; K^+, 0.08; Ca^{2+}, 3.0; Cl^-, 1.7) for at least 1 month before use. Twice a week, the crabs were fed with frozen fish or carp food (Bertels GmbH, Halstenbek, Germany).

Preparation

After killing the animals by destroying the ventral ganglia, the carapace was lifted and the three most posterior gills were removed. Under microscopic control, split lamellae, consisting of a single epithelial layer and the adherent cuticle (Schwarz, 1990), were prepared as described by Schwarz and Graszynski (1989). The preparations were carefully mounted in a modified Ussing-type chamber with an epithelial area of $0.01\,cm^2$. Continuous perfusion of both chamber compartments ($50\,\mu l$) with aerated salines was achieved by gravity flow. The rate of perfusion was adjusted to approximately $2\,ml\,min^{-1}$ and was not altered during an experiment.

Solutions and chemicals

The compositions of the principal salines are given in Table 1. The standard NaCl saline was made up according to the haemolymph of animals acclimated to fresh water (see Mantel and Farmer, 1983).

In contrast to former investigations (Onken *et al.* 1991; Zeiske *et al.* 1992*a*; Riestenpatt *et al.* 1994; Onken and Putzenlechner, 1995), in the present study nitrate was used instead of gluconate as a substitute for Cl^- (see Table 1). However, nitrate is an inhibitor of V-ATPases, acting on the cytosolic side (Nelson, 1987; Schirmanns and Zeiske, 1994) by reversibly dissociating the intracellularly attached V_1 sector from the transmembrane V_o sector (Bowman *et al.* 1992; Forgac, 1992). Because a V-ATPase is involved in active and electrogenic Cl^- absorption (I_{Cl}) across the gills of *E. sinensis* (Onken and Putzenlechner, 1995), Cl^- replacements with nitrate or gluconate were carried out in preliminary experiments. The effects (I_{Cl} decreased) were the same for both substitutions, indicating that the plasma membrane is impermeable to nitrate, as for gluconate. Similarly, using nitrate, no effect was observed on V-ATPase-mediated K^+ secretion across the midgut of *Manduca sexta* (Zeiske *et al.* 1992*b*). Nitrate only inhibited the V-ATPase in this tissue after permeabilization of the basolateral membrane with amphotericin B (Schirmanns and Zeiske, 1994).

In order to decrease osmolarity, the salines were mixed with 'hypo saline', which contained only the buffers, calcium gluconate, glucose and KNO_3. To increase the osmolarity, $350\,mmol\,l^{-1}$ sucrose or urea was added. The osmolalities of the salines were measured using a semimicro osmometer (Knaur, Berlin, Germany) and are given in Table 1 (mean ± S.E.M., N=3). Amiloride, kindly provided by Merck, Sharp and Dohme, was used at a concentration of $100\,\mu mol\,l^{-1}$. Diphenylamine-2-carboxylic acid (DPC), obtained from Fluka, was added from a stock solution in dimethylsulphoxide ($0.5\,mol\,l^{-1}$). The solvent alone has no significant effects on the measured parameters (see Onken *et al.* 1991). Theophylline (Serva), which stimulates I_{Na} and I_{Cl} across the *E. sinensis* gill epithelium (Riestenpatt *et al.* 1994), was employed at a concentration of $5\,mmol\,l^{-1}$ when the spontaneous currents were small.

Electrophysiology

The electrical equipment and measurement of the transepithelial short-circuit current (I_{sc}) and the tissue conductance (G_{te}) have been described in detail elsewhere (Onken *et al.* 1991).

The definitions of the cellular ion-specific currents (I_{Na}, I_{Cl}) and conductances (G_{Na}, G_{Cl}) as well as the definition of the leak conductance (G_l) are given in the Results. The electromotive forces for Na^+ and Cl^- absorption (E_{Na} and E_{Cl}) were calculated according to Ohm's law:

$$E_x = I_x/G_x\,,$$

where x is Na^+ or Cl^-.

The conductances of the solutions were high (approximately $150\,mS\,cm^{-2}$) compared with those of the tissues. Therefore no corrections to G_{te} and I_{sc} were necessary to compensate for possible errors arising from voltage-clamping.

Statistics

All data are given as mean ± S.E.M. Differences between groups were tested using paired Student's t-tests. Statistical significance was taken as $P<0.05$.

Results

Characteristics of the split gill lamellae

For 23 split lamellae of freshwater-adapted crabs studied using NaCl saline on both sides, the transepithelial conductance (G_{te}) was found to be 3.62±0.26 mS cm^{-2}. In parallel, a negative short-circuit current (I_{sc}) of −129±15 μA cm^{-2} was measured.

Following Zeiske *et al.* (1992*a*; see also Fig. 1), active electrogenic Na^+ absorption was defined as a positive, amiloride-sensitive short-circuit current (I_{Na}) using Cl^--free Na^+ saline as the external solution and NaCl saline internally. The transcellular Na^+ conductance (G_{Na}) was defined as an amiloride-induced decrease in G_{te}. After substituting external NaCl with $NaNO_3$, amiloride-sensitive Na^+ currents (I_{Na}) of 244±28 μA cm^{-2} and conductances (G_{Na}) of 3.00±0.35 mS cm^{-2} (N=21) were measured. The passive current driven by the outwardly directed [Cl^-] gradient (measured during application of amiloride) was very small (10–20 μA cm^{-2}) compared with I_{Na} (see Figs 1, 2 and 5; see also Zeiske *et al.* 1992*a*).

Active and electrogenic Cl^- absorption was defined according to Onken *et al.* (1991; see also Fig. 1) as a negative, Cl^--dependent short-circuit current (I_{Cl}) in the presence of external Na^+-free Cl^- saline and internal NaCl saline sensitive to internal diphenylamine-2-carboxylate (DPC). The cellular Cl^- conductance (G_{Cl}) was defined as the difference between G_{te} for externally Na^+-free Cl^- saline and the leak conductance (G_l, see below). In 21 split lamella preparations, Cl^--dependent currents (I_{Cl}) of −206±27 μA cm^{-2} and conductances (G_{Cl}) of 2.46±0.26 mS cm^{-2} were found, reflecting active and electrogenic Cl^- absorption. The passive currents driven by the outwardly directed [Na^+] gradient were very small: inhibition of Cl^- absorption with internal DPC (see Fig. 1 and Onken *et al.* 1991) resulted in a nearly complete reduction of the negative short-circuit current.

The leak conductance (G_l), which is not associated with active transport and which may mostly reflect the paracellular conductance, was considered to be the remaining G_{te} after amiloride treatment in either external Cl^--free Na^+ saline (when studying I_{Na}; see Figs 1, 2 and 5) or NaCl-free saline (when studying I_{Cl}; see Fig. 3). The diuretic was used in the latter case with external NaCl-free saline because an amiloride-sensitive conductance, which is absent in external Cl^--containing salines (see Zeiske *et al.* 1992*a*; Zeiske and Onken, 1993), was still present under these conditions (see Fig. 1 and Riestenpatt *et al.* 1994). The presence or absence of Na^+ in the external bath had no influence on G_l, indicating that the passive paracellular Na^+ conductance is very low. However, the true paracellular conductance should depend on external Cl^- concentration. Thus, the determination described above probably results in a small underestimation of G_l (and also an overestimation of G_{Cl} and an underestimation of E_{Cl}). However, this procedure for determining G_l and G_{Cl} is the best approximation available, because no specific reagent is currently available which quickly, completely and reversibly blocks I_{Cl} from the external side. The mean G_l of 21 split lamellae was found to be 1.16±0.07 mS cm^{-2}.

Influence of osmotic perturbations

In many epithelia, transport modulation by aniso-osmotic media has been demonstrated (for a review, see Macknight, 1991). For some tissues, it has been shown that this transport modulation occurs concomitantly with cell volume changes. Cell swelling, induced by hypo-osmotic media (Ussing, 1965) or by increasing the solution's osmolarity using a membranepermeant substance (Van Driessche *et al.* 1993), was reportedly accompanied by transport stimulation, whereas transport inhibition was observed (Ussing, 1965) simultaneously with cell shrinking induced by increasing the solution's osmolarity with a membrane-impermeant substance.

Table 1. *Composition of the principal salines*

Saline	NaCl	Na^+	Cl^-	NaCl-free	Hyper sucrose	Hyper urea	Hypo	3NaCl + 1hypo	$1Na^+$ + 3hypo	$1Cl^-$ + 3hypo
NaCl	300				300	300		225		
$NaNO_3$		300							75	
CC			300							75
NMG				300						
KCl	8		6		8	8		6		1.5
KNO_3		8		6			6	1.5	6.5	4.5
$NaHCO_3$	2	2			2	2		1.5	0.5	
$KHCO_3$			2	2			2	0.5	1.5	2
Sucrose					350					
Urea						350				
pH 7.6 with	Tris	Tris	Tris	HNO_3	Tris	Tris	Tris	Tris	Tris	Tris
Osmolality	587±4	566±2	564±3	383±9	1013±7	913±12	41±1	457±3	175±6	177±2

All salines contain in addition (in mmol l^{-1}): Hepes, 5; calcium gluconate, 8; glucose, 2.
All concentrations in mmol l^{-1}; osmolality in mosmol kg^{-1}.
Osmolality values are mean ± S.E.M. (N=3).
CC, choline chloride; NMG, *N*-methylglucamine.

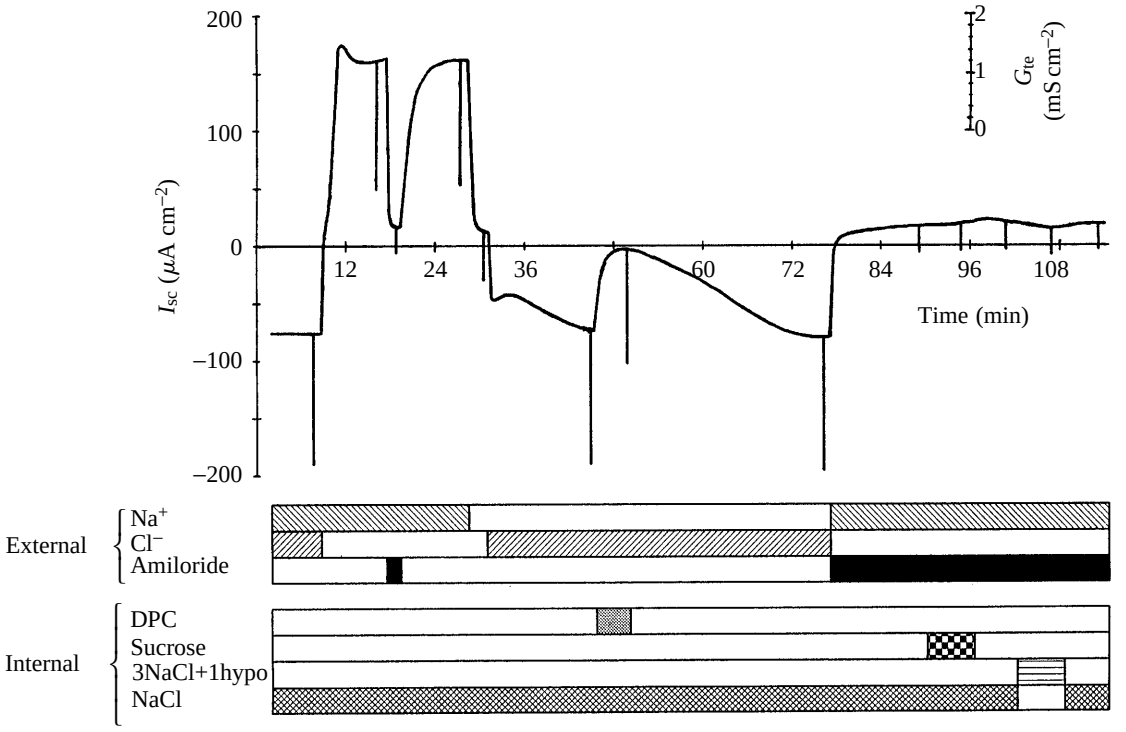

Fig. 1. Time course of the short-circuit current (I_{sc}) across a split gill lamella of a posterior gill of *Eriocheir sinensis* adapted to fresh water. The scale for the transepithelial conductance (G_{te}) corresponds to the vertical current deflections recorded in response to 50 mV voltage pulses. Applications of internal and external media are shown at the foot of the figure. Amiloride was applied at a concentration of 100 μmol l^{-1}. Diphenylamine-2-carboxylic acid (DPC) was applied at a concentration of 1 mmol l^{-1}. I_{Na} and G_{Na} were defined as the amiloride-sensitive parts of positive I_{sc} and of G_{te}, respectively, using Cl$^-$-free Na$^+$ saline as external solution and NaCl saline internally (t=10–30 min). The leak conductance (G_l) was defined as G_{te} in the presence of external amiloride with external Cl$^-$-free Na$^+$ saline (t=18–20 min and 78–114 min) or NaCl-free saline (see Fig. 3) and internal NaCl saline. When external NaCl-free saline without amiloride was used (t=30–32 min), G_{te} was significantly larger than G_l, indicating the presence of an amiloride-sensitive conductance under these conditions (see Riestenpatt *et al.* 1994). I_{Cl} was defined as the negative I_{sc} with external Na$^+$-free Cl$^-$ saline and internal NaCl saline which is sensitive to DPC (t=32–78 min). G_{Cl} is defined as the difference between G_{te} with external Na$^+$-free Cl$^-$ saline and G_l. For t=78–114 min, the effects of internal hyperosmolarity (addition of 350 mmol l^{-1} sucrose) and hypo-osmolarity (3 NaCl+1 hypo saline) on the leak conductance (G_l) are shown.

In the present study, the following manipulations were used to study the influence of osmotic perturbations on I_{Na} and I_{Cl} across short-circuited split gill lamellae of *E. sinensis*. The osmolarity of the salines was decreased by dilution with hypo-saline (see Table 1) or increased by addition of membrane-impermeant sucrose. In some experiments, the osmolarity of the internal saline was increased using urea, which is thought to be much more membrane-permeant than sucrose (see Stein, 1990). Of course, sucrose and urea are not significant osmolytes under physiological conditions. These substances simply served as experimental tools in the present investigation.

Internal osmotic variations

Addition of sucrose

Increasing the internal osmolarity by addition of 350 mmol l^{-1} sucrose resulted in a decrease of the Na$^+$- and Cl$^-$-specific currents and conductances. In seven experiments, I_{Na} was reduced significantly from 228±55 to 60±22 μA cm^{-2} (P<0.05) and G_{Na} was reduced significantly from 3.25±0.80 to 1.18±0.42 mS cm^{-2} (P<0.05). The electromotive force of Na$^+$ absorption (E_{Na}), which is I_{Na}/G_{Na}, decreased significantly from 72±9 to 49±4 mV (P<0.05). In six experiments, I_{Cl} was reduced significantly from −196±62 to −56±22 μA cm^{-2} (P<0.05) and G_{Cl} was reduced significantly from 2.58±0.54 to 1.77±0.38 mS cm^{-2} (P<0.05). The respective electromotive force (E_{Cl}) decreased significantly from −69±12 to −28±10 mV (P<0.05). Examples of such experiments are shown in Fig. 2 for I_{Na} and in Fig. 3 for I_{Cl}. In Fig. 4, the data are summarized as percentages of the control values before increasing the internal osmolarity. G_l was not affected by internal addition of sucrose (see Figs 1 and 3).

Addition of urea

In five experiments, urea was used instead of sucrose to increase the internal osmolarity. In all cases, whether urea alone was used to increase saline osmolarity (not shown) or whether it substituted for sucrose in already hyperosmotic conditions (see Figs 2, 3), Na$^+$ and Cl$^-$ currents and

conductances increased above the control value obtained with iso-osmotic salines on both sides of the epithelium. Thus, as expected for a membrane-permeant substance (see above), addition of urea mimicked the effect of internally diluted saline (see below). G_l was not affected by addition of urea to the internal saline (see Figs 2, 3). The transport-stimulating effect of urea was completely reversible (see Figs 2, 3), suggesting that impairment of the structure and function of cell proteins by urea at the concentrations and exposure times used was absent or of minor importance.

Reduction of internal osmolarity

Decreasing the internal osmolarity was achieved in two steps (see Fig. 3, t=72–126 min). In the first step, the internal Na^+ and Cl^- concentrations were reduced (to 226.5 and 229.5 mmol l^{-1}, respectively) at nearly constant osmolarity by substitution of NaCl with *N*-methylglucamine nitrate (3:1 mixture of NaCl saline and NaCl-free saline, see Table 1). In most cases, this manipulation resulted in a slowly reversible drop of the respective currents and conductances. These reduced magnitudes of the ion-specific electrical parameters after internal reduction of Na^+ and Cl^- levels served as control values for the subsequent osmotic change at constant internal NaCl, buffer and Ca^{2+} concentrations, when *N*-methylglucamine nitrate was omitted from the saline. This manipulation (3:1 mixture of NaCl saline and hypo saline) increased I_{Na}, I_{Cl}, G_{Na} and G_{Cl} (Fig. 4). In five experiments, I_{Na} increased significantly from 96±15 to 236±36 μA cm^{-2} (P<0.05) and G_{Na} increased significantly from 1.08±0.06 to 2.46±0.35 mS cm^{-2} (P<0.05), while E_{Na} was not significantly affected (87±9 mV *versus* 95±3 mV; P>0.05). In five experiments, I_{Cl} increased significantly from −128±26 to −231±34 μA cm^{-2} (P<0.05) and G_{Cl} increased significantly from 1.88±0.32 to 2.48±0.34 mS cm^{-2} (P<0.05). As with hyperosmolarity, E_{Cl} changed markedly, increasing significantly from −74±18 to −115±223 mV (P<0.05). An example of such an experiment for I_{Cl} is shown in Fig. 3 (t=72–126 min). In Fig. 4, the respective data are summarized as percentages of the control values before decreasing the internal osmolarity. The leak conductance (G_l) was not affected by reducing the osmolarity of the internal medium (see Figs 1, 3).

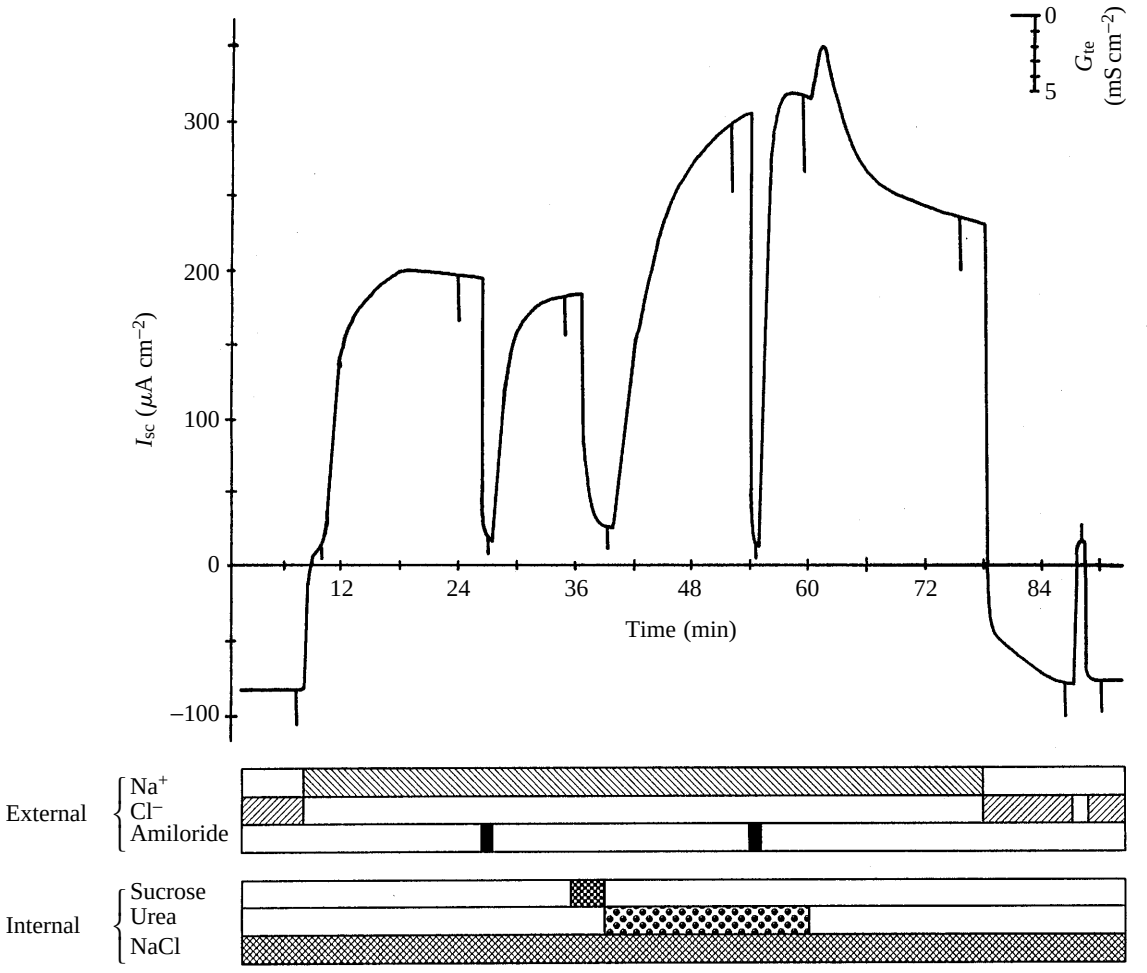

Fig. 2. I_{sc} time course showing the influence of internal addition of sucrose or urea (each 350 mmol l^{-1}) on the amiloride-sensitive (100 μmol l^{-1}) and Na^+-dependent current and conductance with external Cl^--free saline and internal NaCl saline. The scale for the transepithelial conductance (G_{te}) corresponds to the vertical current deflections recorded in response to 10 mV voltage pulses.

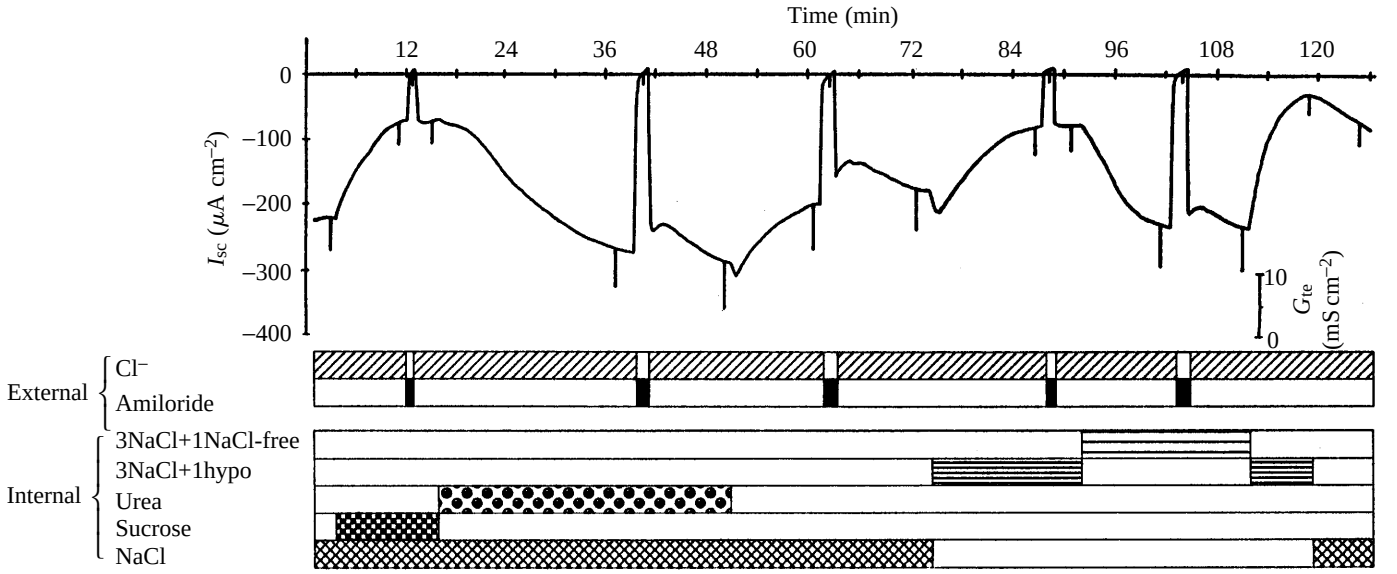

Fig. 3. I_{sc} time course showing the influence of internal addition of sucrose or urea (each 350 mmol l^{-1}) and internal dilution of NaCl saline with NaCl-free *N*-methylglucamine nitrate saline (3:1) or hypo saline (3:1) on the Cl^--dependent current and conductance with external Na^+-free saline. During all experiments, 5 mmol l^{-1} theophylline was present in the internal bath. The scale for the transepithelial conductance (G_{te}) corresponds to the vertical current deflections recorded in response to 10 mV voltage pulses.

External osmotic variations

Addition of sucrose

In three experiments, the external osmolarity was increased by addition of 350 mmol l^{-1} sucrose. The mean values of I_{Na} and I_{Cl} slightly but significantly decreased to 86±6 % (P<0.05) and 91±4 % (P<0.05) of the original control values, respectively. Thus, the effect of external addition of sucrose is much smaller than the same manipulation in the internal bath, which reduced I_{Na} and I_{Cl} to 27±7 % and 24±6 % of control values, respectively (see above and Fig. 4). An example of external addition of sucrose and its influence on I_{Na} is shown in Fig. 5.

Reduction of external osmolarity

To investigate the influence of a decrease in the external osmolarity, the external concentrations of Na^+ or Cl^- were first reduced to 75.5 or 76.5 mmol l^{-1}, respectively, at nearly constant osmolarity. This was achieved by substituting impermeant ions for both Na^+ and Cl^- (1:3 mixtures of Na^+ or Cl^- saline with NaCl-free saline, see Table 1). As expected, this manipulation decreased I_{Na} and I_{Cl} as well as G_{Na} and G_{Cl}. These reduced currents and conductances then served as new control values for the following reduction of the external osmolarity by omitting the Na^+ or Cl^- substitute from the saline (1:3 mixtures of Na^+ or Cl^- saline with hypo saline, see Table 1). Thus, in this second step, a reduction of the external osmolarity was achieved at constant substrate, buffer and Ca^{2+} concentrations. These hypo-osmotic salines caused I_{Na} (N=3) and I_{Cl} (N=3) to increase to 164±16 and 113±5 %, respectively, of the values in iso-osmotic salines. Although the osmotic gradient was approximately threefold higher than in the experiments with internal osmotic manipulations (see above), the percentage changes in the current were far lower. Thus, it appears that external hypo-osmolarity was much less effective than internal hypo-osmolarity. An example of a reduction of

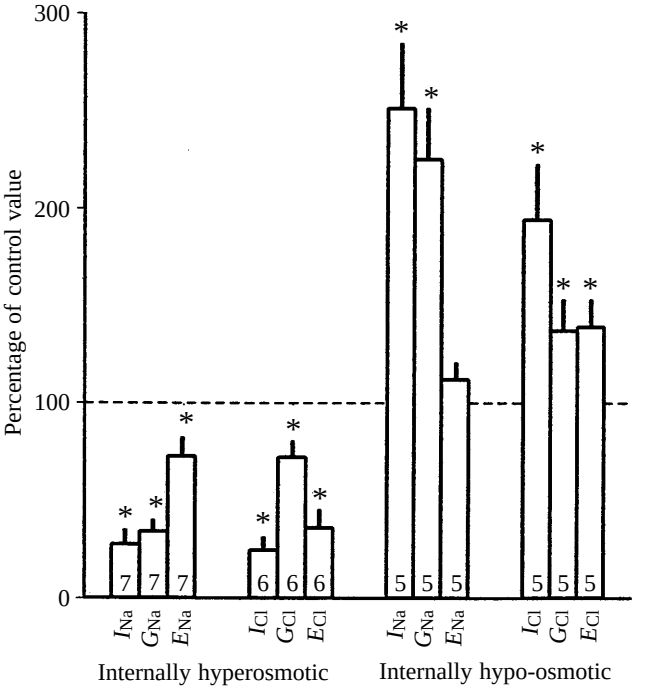

Fig. 4. Diagram summarizing the parameters that characterize active and electrogenic absorption of Na^+ and Cl^- after internal osmotic perturbations. The data are given as percentages (+ S.E.M.) of the control value before osmotic manipulation. *N* values are given in each column. Values marked with an asterisk are statistically different (P<0.05) from the control value before osmotic manipulation. For further details, see text.

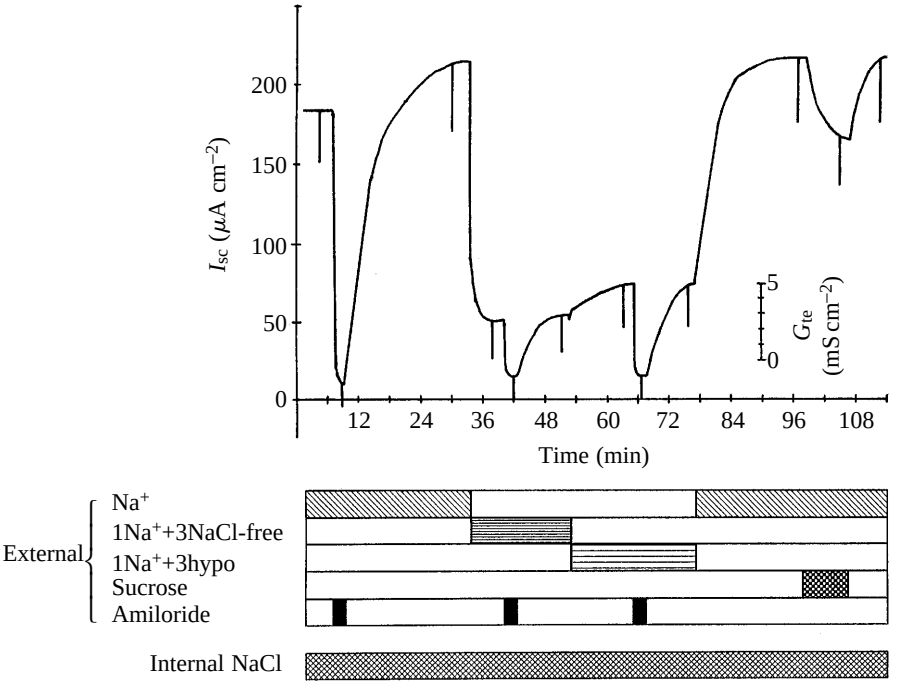

Fig. 5. Time course of the positive, amiloride-sensitive I_{sc} with external Cl^--free Na^+ saline (internal NaCl saline). Before addition and washout of sucrose (350 mmol l^{-1}) in the external bath, the external Cl^--free Na^+ saline was diluted 1:3 with NaCl-free *N*-methylglucamine nitrate saline or hypo saline. Amiloride was applied at a concentration of 100 μmol l^{-1}. The scale for the transepithelial conductance (G_{te}) corresponds to the vertical current deflections recorded in response to 10 mV voltage pulses.

the external osmolarity and its influence on I_{Na} is shown in Fig. 5.

As in the experiments with internal osmotic manipulations, no influence on the leak conductance (G_l) was observed during the experiments with external osmotic variations (see Fig. 5).

Discussion

Ionic variations and their influence on NaCl absorption

With isolated perfused posterior gills of *E. sinensis* acclimated to fresh water, Péqueux and Gilles (1981) observed that Na^+ influx disappeared when salines of high NaCl concentration (approximately 500 mmol l^{-1}) were used to bathe and perfuse the gills. The authors interpreted this result as regulation of active Na^+ absorption by the haemolymph Na^+ level. However, on the basis of the results from the present investigation, it is likely that transport inhibition by high NaCl levels is due to an osmotic effect.

In order to discriminate between effects induced by osmotic or by ionic changes to the bathing solutions, Na^+ and/or Cl^- concentrations in the present study were first reduced at a constant osmolarity before then decreasing the osmolarity. Decreases in currents (I_{Na}, I_{Cl}) and conductances (G_{Na}, G_{Cl}) after external reduction of Na^+ or Cl^- concentrations at constant osmolarity are obviously due to a reduction in the levels of transport substrate (see Onken *et al.* 1991; Zeiske *et al.* 1992*a*). In most cases, the reduction in internal NaCl levels at constant osmolarity was followed by a reduction in the currents and conductances (for I_{Cl} and G_{Cl}, see Fig. 3). The decrease in I_{Cl} was not due to inhibition of the V-ATPase by nitrate entering the cells. Substitution of NaCl with either *N*-methylglucamine nitrate or Tris-gluconate caused similar current decreases, indicating that the basolateral membrane is not permeable to nitrate (see Materials and methods). However, reduction of internal NaCl levels may decrease I_{Cl} and I_{Na} by causing cell shrinking (see below and Macknight, 1991), perhaps resulting from KCl loss from the cells (MacRobbie and Ussing, 1961). Moreover, an influence on basolateral (although unproven to date for crab gills) Na^+- and/or Cl^--dependent cotransporters may be of importance. Impairment of these cotransporters may result in changes in the cellular medium (pH, $[Ca^{2+}]$, etc.), hence affecting the activity of the transporters responsible for NaCl uptake.

Short-term osmotic variations and their influence on NaCl absorption

When the osmolarity of the internal bathing solution was decreased by dilution with hypo saline or increased by addition of membrane-permeant urea, I_{Na} and I_{Cl} increased markedly (Figs 3, 4). A reduction in these currents was observed after increasing the internal osmolarity by addition of membrane-impermeant sucrose (see Figs 2, 3, 4). Osmotic manipulation of the external bath induced current changes in the same directions (see Fig. 5), but the magnitudes of these effects were clearly smaller than those measured after internal osmotic variations. This result indicates that the external barrier (cuticle plus apical membrane) has, as expected for an epithelium facing a dilute external medium under *in vivo* conditions, a lower water permeability than the internal barrier (basolateral membrane). Similar changes in transepithelial transport induced by short-term osmotic perturbations, including the different sensitivities of the apical and basolateral epithelial surfaces, have been observed with other tight NaCl-absorbing epithelia (for a review, see Macknight, 1991). It is likely that

the changes in cellular volume that have been observed for other epithelial tissues (Ussing, 1965; Van Driessche *et al.* 1993) also occur in the gill epithelium of *E. sinensis*.

Osmotically induced transport modulation is due to modifications of apical transport capacity

I_{Na} can be completely inhibited by ouabain (Schwarz, 1990; Riestenpatt *et al.* 1994). Therefore, E_{Na} can be related to the functioning of the basolateral Na^+/K^+-ATPase and the ion gradients produced and maintained by this enzyme (for a discussion of the concept of E_{Na}, see Macknight *et al.* 1980). In the present study, changes in E_{Na} after internal osmotic manipulation were relatively small (see Fig. 4), excluding a major effect of these manipulations on the basolateral Na^+/K^+-ATPase. As with other epithelia, microelectrode impalements have demonstrated that the apical membrane of the gill epithelium of *E. sinensis* is the barrier along the transcellular pathway with the highest resistance (Onken *et al.* 1991, 1995). Therefore, the changes in transcellular conductance observed after internal osmotic manipulations (see Fig. 4) may be related to modification of the apical Na^+ channels.

With respect to the modulation of I_{Cl} by internal osmotic variation, circuit analysis shows that the alterations in I_{Cl} are clearly due to changes in both E_{Cl} and G_{Cl} (see Fig. 4). Bafilomycin A_1 is an effective inhibitor of I_{Cl} (Onken and Putzenlechner, 1995). The changes in E_{Cl} may therefore be related to the apical V-ATPase and the electrochemical gradients produced and maintained by this enzyme. By pumping H^+ from the cytosol to the apical medium, the V-ATPase produces a HCO_3^- concentration gradient ('used' for Cl^- absorption by apical Cl^-/HCO_3^- antiporters) and an electrical gradient (cell hyperpolarization, 'used' for driving Cl^- against a chemical gradient across basolateral Cl^- channels). Because the apical membrane is the barrier of highest resistance along the transcellular route (Onken *et al.* 1991, 1995), changes in G_{Cl} are also mainly determined by the conductance of the apical V-ATPase, which seems to be associated with the transmembrane V_o sector of this enzyme (Forgac, 1992). Because internal osmotic variations significantly affected E_{Cl} and G_{Cl} in the present study (see Fig. 4), it seems likely that they had a major influence on the apical V-ATPase.

Interestingly, modulation of active and electrogenic absorption of Na^+ and Cl^- by cellular cyclic AMP (Riestenpatt *et al.* 1994), by internal addition of eyestalk extract (Schöbel *et al.* 1994) or by a decrease in external pH (Onken *et al.* 1994), was mediated by similar changes to those observed in the present study after internal osmotic variations. In all cases, changes in I_{Na} were caused by changes in Na^+ conductance (at constant electromotive force), whereas alterations in I_{Cl} were caused by changes in electromotive force and in the conductance for Cl^-. Transport stimulation induced by increasing cellular cyclic AMP levels has in fact been demonstrated to be caused by increases in the number of open apical Na^+ channels (Riestenpatt *et al.* 1994) and in V-ATPase activity (Putzenlechner and Graszynski, 1995).

In frog skin (Zeiske and Van Driessche, 1984) and in cultured renal (A6) epithelium (Wills *et al.* 1991), osmotic perturbations were reportedly followed by alterations in the number of open apical Na^+ channels. In rabbit urinary bladder (Lewis and de Moura, 1984) and frog skin (Lacoste *et al.* 1993), a hypotonic shock induced fusion of transporter-containing vesicles with the apical membrane. However, in addition to vesicle insertion, two other cellular mechanisms might also be considered (see Benos *et al.* 1992). Aniso-osmotic media may induce modification of pre-existing transporters by membrane stretching/compression and/or by changing the concentrations of cellular transport modulators. More detailed investigations must be conducted in the future on crab gills to demonstrate cell volume changes induced by aniso-osmotic media and to characterize any link with transport modulation.

The leak conductance of E. sinensis *gills shows no osmotic sensitivity*

The paracellular shunt pathway of tight and leaky vertebrate epithelia shows an osmotic sensitivity (for a review, see Erlij and Martinez-Palomo, 1978). Osmotic gradients favouring water efflux induce local accumulations of fluid (so-called 'blisters') near the junctional regions of the intercellular space and increase the leak conductance (DiBona and Civan, 1973). The physiological relevance of the osmotic sensitivity of vertebrate tight junctions has received attention in several reports (e.g. Macknight *et al.* 1980). Lord and DiBona (1976) also observed 'blisters' induced by external hypertonic solutions in the surface epithelium of an invertebrate, the planarian *Dugesia tigrina*. The authors concluded that invertebrate septate junctions possess an osmotic sensitivity similar to that of vertebrate tight junctions. However, in the present investigation, modulation of the leak conductance by osmotic gradients was not observed (see Figs 1, 2, 3, 5). Thus, in the gill epithelium of *E. sinensis*, modulations induced by short-term osmotic variations seem to be restricted to the cellular transport pathway.

Physiological importance of transport modulation by osmotic changes

The magnitudes of the internal osmotic variations in the present investigation were chosen according to those encountered by the animals under natural conditions. In sea water, the haemolymph osmolality of *E. sinensis* (about 1000 mosmol kg^{-1}) is iso-osmotic with the external medium. When the animals migrate to fresh water, their haemolymph osmolality decreases to 550–650 mosmol kg^{-1}. In moulting or starving freshwater crabs, a further, reversible decrease to approximately 400 mosmol kg^{-1} has been observed (Schwabe, 1933). Of course, sudden osmotic variations of the internal medium, as applied in the present study, are never likely to be experienced by the animals. In the laboratory, the animals survive a direct transfer from sea water to fresh water or *vice versa*. However, the precise rate of the resulting haemolymph osmolarity changes is unknown.

The largest natural variation in external and internal osmolarity encountered by *E. sinensis* occurs when the juvenile animals migrate upstream from sea water to fresh water and when the adult crabs return to the sea to reproduce. With respect to a regulative influence of osmotic changes in the external and/or internal medium, it is of importance that, in the isolated epithelia of seawater-adapted crabs, NaCl absorption cannot be induced simply by diluting the salines. With salines made up according to the haemolymph of freshwater crabs, neither a net Na^+ influx (Péqueux and Gilles, 1981) nor any active short-circuit current (H. Onken, unpublished observation) can be measured across the gill epithelium of crabs acclimated to sea water. After transfer of seawater crabs to fresh water, gill Na^+/K^+-ATPase activity increases for about 2 days and major ultrastructural changes (increasing numbers of mitochondria, extension of apical infolding system, etc.) can be observed (Péqueux *et al.* 1988). On the basis of these observations, it can be assumed that transbranchial NaCl absorption in seawater-adapted crabs is the result of adaptive cellular responses. This more time-consuming adaptation may be initiated by a reduction in haemolymph osmolarity. However, the process is also likely to involve hormonal and/or neuronal control. Transfer of another crab species, the shore crab *Carcinus maenas*, from full-strength sea water to diluted sea water was followed by rapid changes in catecholamine concentrations in the haemolymph and gill tissue (Zatta, 1987).

After the generation of a transbranchial salt uptake by long-term adaptation (during periods of days) of the crabs to fresh water, the responses of NaCl absorption following short-term manipulations (over minutes) of the internal osmolarity are exactly those required to maintain NaCl homeostasis of the whole animal: an increased/decreased passive water gain and/or salt loss, resulting in dilution/concentration of the body fluid, can be compensated for by stimulation/inhibition of NaCl absorption. Because the results of the present investigation were obtained using isolated epithelia, the observed modulation of NaCl absorption obviously reflects a possible hormone-independent autoregulation. Thus, for *E. sinensis* living in fresh water, it may well be that the adaptive significance of autoregulation is that it allows the animal to compensate for moderate, irregular perturbations of NaCl homeostasis induced by food uptake (quantity and/or composition), by land visits or during moulting phases. Moreover, the mechanisms described may be important in inhibiting NaCl absorption when adult *E. sinensis* return to the sea for reproduction. During this phase, the animals pass through estuarine habitats, which are characterized by regular, fast and drastic changes in external osmolarity. These variations may effect changes in haemolymph osmolarity that can be buffered by subsequent reduction or stimulation of NaCl absorption.

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