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Electrophysiology of posterior, NaCl-absorbing gills of *Chasmagnathus granulatus*: rapid responses to osmotic variations

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Summary

In the present study, the influence of short-term osmotic variations on some electrophysiological properties related to NaCl absorption across posterior gills of *Chasmagnathus granulatus* was investigated. The transepithelial potential difference (V_{te}) of isolated and perfused gills increased significantly when hyposmotic saline (699 mosmol l^{-1}) was used instead of isosmotic solution (1045 mosmol l^{-1}). A reduction of the concentration of Na^+ or Cl^- at constant osmolarity did not produce any change in V_{te}. Transepithelial short-circuit current (I_{sc}) and conductance (G_{te}), measured with split gill lamellae mounted in a modified Ussing chamber, also increased after changing to hyposmotic salines (I_{sc}: from –89.0±40.8 μA cm^{-2} to –179.3±37.0 μA cm^{-2}; G_{te}: from 40.5±16.9 mS cm^{-2} to 47.3±15.8 mS cm^{-2}). The observed effects of reduced osmolarity were fast, reversible and gradually dependent on the magnitude of the osmotic variation. The acitivity of the Na^+/K^+-ATPase increased significantly after perfusion with hyposmotic saline, from 18.73±6.35 μmol P_i h^{-1} mg^{-1} to 41.84±14.54 μmol P_i h^{-1} mg^{-1}. Theophylline maintained part of the elevated V_{te} induced by hyposmotic saline, suggesting that an increased cellular cyclic AMP level is involved in the response to reduced osmolarity. In summary, the results indicate that the hemolymph osmolarity regulates active transbranchial NaCl absorption by modulating the activity of the basolateral Na^+/K^+-ATPase and by changing a conductive pathway, probably at the apical membrane.

Key words: *Chasmagnathus granulatus*, crab, cyclic AMP, gills, hyperosmoregulation, perfused gills, Na^+/K^+-ATPase, short-circuit current, split gill lamellae, transepithelial conductance, transepithelial voltage.

Introduction

Chasmagnathus granulatus is an estuarine, semi-terrestrial crab that is able to hyper- and hypo-regulate its ionic and osmotic concentrations (Mougabure Cueto, 1998; Charmantier et al., 2002). As is widespread among euryhaline crabs (for a review, see Péqueux, 1995), the posterior gills of this species are responsible for compensating ionic diffusive losses derived from exposure to a low salinity medium (Luquet et al., 2002). So far, the mechanism of active NaCl absorption across the posterior gills of hyperosmoregulating *C. granulatus* is unknown. However, based on data presented by Luquet et al. (2002), similarities with the transport mechanism shown for the posterior gills of the green shore crab *Carcinus maenas* could be anticipated. For *C. maenas*, coupled NaCl absorption *via* apical $Na^+/K^+/2Cl^-$ co-transporters and basolateral Na^+/K^+-ATPases and Cl^- channels has been proposed (Riestenpatt et al., 1996; Onken and Riestenpatt, 1998). As in the thick ascending limb of Henle's loop in the mammalian nephron (Greger, 1985), apical K^+ channels are of importance to allow transapical K^+ recycling, to promote passive, basolateral Cl^- exit *via* Cl^- channels and to give NaCl absorption its electrogenic character.

With respect to active NaCl secretion in hypoosmoregulating *C. granulatus*, Luquet et al. (2002) demonstrated its presence in posterior gills and its dependence on a functioning Na^+/K^+-ATPase. However, as in other hypoosmoregulating crabs (Green et al., 1959; Baldwin and Kirschner, 1976a,b; Evans et al., 1976), the entire mechanism is still unknown.

C. granulatus has developed bimodal ventilation and spends long periods on land (Halperin et al., 2000). These semi-terrestrial habits force *C. granulatus* to deal with sudden salinity changes: the water available in supratidal areas can vary from tide pools concentrated by evaporation to rain pools with significantly diluted seawater. Besides, during land visits the water retained within the gill chambers might evaporate, exposing the gills to high salinity conditions (Schmidt and Santos, 1993). As a consequence, the gills of this species must be able to rapidly switch between absorption, secretion and no transport. One way of changing

between different transport states would be endocrine regulation, as observed in a couple of crab species (Sommer and Mantel, 1988; Kamemoto, 1991; Mo et al., 1998; Onken et al., 2000; Morris, 2001). An alternative would be hormone-independent transport regulation triggered by the osmolarity of the internal medium, as has been demonstrated in the amphibian skin (Ussing, 1965) and other vertebrate epithelia (for a review, see Macknight, 1991) but also in the gills of Chinese crabs *Eriocheir sinensis* (Onken, 1996). A decreasing internal osmolarity resulted in a rapid stimulation of NaCl absorption, whereas an increasing internal osmolarity resulted in decreased transport rates. Thus, the osmotic influence on transport rates stabilises the hemolymph/blood osmolarity and was, therefore, called autoregulation (cf. Onken, 1996). In the gills of *E. sinensis*, the osmotic variations were shown to modulate the apical transporters involved in NaCl absorption (V-type H^+-ATPase and Na^+ channels; Onken, 1996).

The presence of an autoregulatory mechanism in *C. granulatus* has been suggested in a previous study of the transepithelial voltage generated by isolated and perfused gills of *C. granulatus* adapted to diluted seawater (Luquet et al., 2002). To verify and to further characterise this regulation of NaCl absorption by osmotic changes, the present study investigated the influences of short-term osmotic variations on the transepithelial voltage generated by isolated and perfused gills, the short-circuit current across split gill lamellae mounted in a modified Ussing chamber, and the activity of the Na^+/K^+-ATPase.

Materials and methods

Animals

Chasmagnathus granulatus (Dana 1851) were collected at Faro San Antonio beach (36°18′ S 56°48′ W) near the southern edge of the Rio de la Plata estuary, Argentina during several campaigns conducted during 2000 and 2001. The animals were acclimated in plastic containers with aerated seawater of 2‰ salinity for at least two weeks. Water temperature was kept at 20±2°C. The animals were fed twice a week with commercially available pellets of rabbit food. Stage C intermolt adult male crabs (Drach and Tchernigovtzeff, 1967) of 30±3 mm carapace width were selected for the study.

Gill perfusion and measurement of the transepithelial potential difference (V_{te})

Crabs were sacrificed by destroying the ventral nervous ganglion with a spike. After removing the dorsal carapace, gill pair no. 6 (representative for posterior gills; Luquet et al., 2002) was removed and used for the experiments. The afferent and efferent vessels were connected by 0.4-mm diameter polyethylene tubing to a peristaltic pump (afferent) and to a glass tube (efferent). 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 and constant aeration. Under these conditions, isolated perfused gill preparations can remain viable for up to 15 h (Siebers et al., 1985).

For the measurements of transepithelial voltage, Ag/AgCl electrodes were connected by agar bridges to the external bath and to the glass tube collecting the perfusate. V_{te} was measured with a millivoltmeter (Metrix, Paris, France) and is given as the difference in electrical potential between the external and internal medium (reference electrode connected to the internal perfusate).

Split gill lamellae and measurement of short-circuit current (I_{sc}) and transepithelial conductance (G_{te})

Single gill lamellae were isolated and split under microscopic control according to Schwarz and Graszynski (1989). The split gill lamellae were mounted in a modified Ussing chamber (De Wolf and Van Driessche, 1986). An epithelial area of 0.002 cm^2 was exposed to the chamber compartments (volume approximately 50 µl), bathing the internal and external sides of the tissue. Continuous perfusion of both chamber compartments with aerated saline was achieved by gravity flow at a constant rate of approximately 2 ml min^{-1}.

To measure V_{te}, Ag/AgCl electrodes were connected *via* agar bridges (3% agar in 3 mol l^{-1} KCl) to both sides of the preparation (distance from the tissue, <1 mm). The reference electrode was in the internal bath. Silver wires coated with AgCl served as electrodes to short-circuit the transepithelial voltage by an automatic clamping device (VCC 600; Physiological Instruments, San Diego, USA). The transepithelial conductance (G_{te}) was calculated from imposed voltage pulses and the resulting current deflections (ΔI). As the resistance of the preparation was small, the values of G_{te} and I_{sc} were corrected for the influence of the resistance of the salines (for details, see Riestenpatt et al., 1996). In the Results, only the corrected values are shown.

Activity of the Na^+/K^+-ATPase

Posterior gill pair no. 6 was dissected and perfused as described above. The two gills from the same crab were perfused at the same time with $Iso_{sucrose}$ saline, and V_{te} was monitored. Once V_{te} became stable, one of the gills was perfused with Hyposmotic saline (treated), while the other continued as before (control). When the treated gill achieved a new stable V_{te} value, both gills were disconnected from the peristaltic pump, cut at the clamp level and immediately frozen at –40°C until the Na^+/K^+-ATPase activity assay was performed. In previous assays, freezing and thawing the gills did not produce any significant effect on the Na^+/K^+-ATPase activity, as the obtained values for frozen–thawed and freshly excised tissues were in the same range.

For the measurement of Na^+/K^+-ATPase activity, the gills were placed in 20 volumes of cold buffer [12.5 mmol l^{-1} NaCl, 1 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, 0.5 mmol l^{-1} PMFS (phenylmethylsulfonyl fluoride), adjusted to pH 7.6 with NaOH] and homogenized in a teflon–glass homogeniser (20 strokes). Homogenates were centrifuged at 11 000 ***g*** for

20 min at 4°C. Supernatants were discarded and pellets were resuspended in cold buffer [in *C. granulatus* gills, the greatest Na^+/K^+-ATPase activity is detected in this fraction (Rodriguez Moreno et al., 1998; G. Genovese, C. Luchetti and C. M. Luquet, unpublished data)].

Na^+/K^+-ATPase activity was determined as described previously by Lucu and Flik (1999) by incubating 10 µl of sample with 500 µl assay solution, containing 100 mmol l^{-1} NaCl; 5 mmol l^{-1} $MgCl_2$; 0.1 mmol l^{-1} EDTA; 15 mmol l^{-1} imidazol; 3 mmol l^{-1} Na_2ATP and 12.5 mmol l^{-1} KCl with or without 1 mmol l^{-1} ouabain (pH 7.5; histidine-imidazol). Assay mixtures were then incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 ml 8.6% cold trichloroacetic acid. Liberated phosphate was quantified colorimetrically according to the modified method of Bonting and Cavaggio (1963) by adding 1 ml of a freshly made solution of 9.2% $Fe_2SO_4.7H_2O$ and 1.14% ammonium heptamolybdate in 3.63% H_2SO_4. After 30 min incubation at room temperature, the absorbance was recorded at 700 nm. The difference between phosphate released with and without ouabain was attributed to Na^+/K^+-ATPase activity. Protein concentration was determined in triplicate using the method of Lowry et al. (1951). Specific Na^+/K^+-ATPase activity was expressed in µmol P_i h^{-1} mg $protein^{-1}$.

Solutions and chemicals

Table 1 shows the composition of the salines used in the different experiments with perfused gills and with split gill lamellae. The osmolarity and ionic composition of Isosmotic saline is similar to the hemolymph of *C. granulatus* adapted to 30‰ salinity, where the crabs hardly maintain an osmotic gradient across their body surface (cf. Mougabure Cueto, 1998; Charmantier et al., 2002). Hyposmotic saline is composed according to the hemolymph of crabs adapted to a hyposmotic ambient medium of approximately 2‰ salinity (cf. Mougabure Cueto, 1998; Charmantier et al., 2002). The osmolarity of the salines Cl_{red} and Na_{red} is adjusted to the hemolymph of crabs adapted to 30‰ salinity. However, the concentrations of sodium or chloride are reduced to the level of Hyposmotic saline by partly substituting NaCl with choline chloride or $NaNO_3$, respectively. In the $Hypo_{sucrose}$ salines ($Hypo_{sucrose}1$–4), the osmolarity of Hyposmotic saline is stepwise increased by addition of sucrose until reaching the osmolarity of Isosmotic saline ($Iso_{sucrose}$). All solutions were adjusted with Tris base to the physiological pH of *C. granulatus* (7.75; Luquet and Ansaldo, 1997).

Theophylline was obtained from Serva, New York, USA. Forskolin and ouabain were purchased from Sigma, St Louis, USA. All other salts and reagents were purchased from Merck, Buenos Aires, Argentina.

Statistics

All data are given as means ± S.E.M. Differences between groups were tested using Student's *t*-test, paired Student's *t*-test or repeated-measures analysis of variance (RM-ANOVA) when appropriate. Differences were considered significant at $P<0.05$.

Results

Osmotic and ionic variations and their effect on V_{te} of isolated and perfused gills

In the first two series of experiments, the influence of osmotic and ionic changes on the transepithelial potential difference (V_{te}) of isolated and perfused posterior gills of *Chasmagnathus granulatus* was tested. For this purpose, the salines used as bath and perfusate were changed in the following sequence: from Isosmotic saline, we changed to salines with reduced concentrations of sodium or chloride (Cl_{red} or Na_{red}) at constant osmolarity. In a second step, the salines were changed to Hyposmotic saline, reducing the NaCl concentration and the osmolarity.

With Isosmotic saline as perfusate and bath, the transbranchial voltage stabilised at 2.14±0.32 mV and 1.36±0.13 mV in the Cl_{red} and Na_{red} experiments, respectively (external side positive; N=8 for each experiment); i.e. at very similar values to those observed in a previous study under similar conditions with *C. granulatus* adapted to 12‰ salinity (Luquet et al., 2002). No significant changes were observed after reducing the concentrations of chloride (2.16±0.41 mV;

Table 1. *Composition of the salines*

Saline	NaCl	KCl	$MgCl_2$	$CaCl_2$	$NaNO_3$	CC	Sucrose	Osmolarity
Hyposmotic	312	6.30	4.66	8.35	–	–	–	698.6
Isosmotic	468	9.46	7.50	12.53	–	–	–	1045.0
Cl_{red}	312	9.46	7.50	12.53	156	–	–	1045.0
Na_{red}	312	9.46	7.50	12.53	–	156	–	1045.0
$Hypo_{sucrose}1$	312	6.30	4.66	8.35	–	–	60	758.6
$Hypo_{sucrose}2$	312	6.30	4.66	8.35	–	–	140	838.6
$Hypo_{sucrose}3$	312	6.30	4.66	8.35	–	–	170	868.6
$Hypo_{sucrose}4$	312	6.30	4.66	8.35	–	–	285	983.6
$Iso_{sucrose}$	312	6.30	4.66	8.35	–	–	345.4	1045.0

All concentrations are expressed in mmol l^{-1}; osmolarity is expressed in mosmol l^{-1}. All salines also contained 5 mmol l^{-1} Hepes and 2.5 mmol l^{-1} $NaHCO_3$. Perfusates also contained 2 mmol l^{-1} glucose. CC, choline chloride.

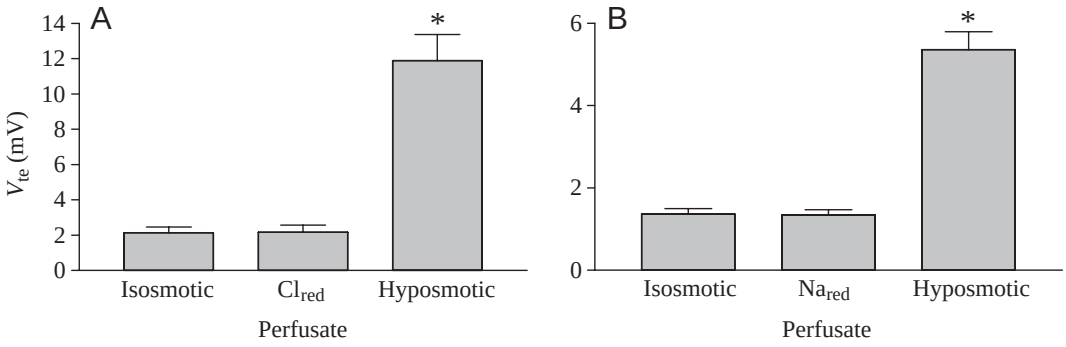

Fig. 1. Effects of reduced ion concentrations [(A) chloride, (B) sodium] and reduced osmolarity on the transepithelial potential difference (V_{te}) across isolated posterior gills of *Chasmagnathus granulatus* acclimated to 2‰ salinity. Mean + S.E.M. of eight values are given in each column. Asterisks indicate statistical differences (P<0.05). Isosmotic: 517.5 mmol l^{-1} Cl^- and 470.5 mmol l^{-1} Na^+ (1045 mosmol l^{-1}). Hyposmotic: 344.4 mmol l^{-1} Cl^- and 314.5 mmol l^{-1} Na^+ (698.6 mosmol l^{-1}). Cl_{red}: chloride concentration of Hyposmotic saline, but osmolarity of Isosmotic saline. Na_{red}: sodium concentration of Hyposmotic saline, but osmolarity of Isosmotic saline.

N=8) or sodium (1.34±0.13 mV; N=8) at constant osmolarity. However, when changing to Hyposmotic saline, V_{te} rapidly increased to significantly higher values (11.89±1.48 mV and 5.36±0.44 mV, respectively; N=8 in each case). These results are summarised in Fig. 1.

In another series of experiments, we changed between Isosmotic saline, Hyposmotic saline and salines of intermediate osmolarities prepared by adding sucrose to the basolateral Hyposmotic saline (salines $Hypo_{sucrose}$1–4 and $Iso_{sucrose}$). A representative time-course of one of these experiments is shown in Fig. 2, demonstrating the fast and reversible effects of osmotic changes. With Isosmotic saline and with $Iso_{sucrose}$ saline, the transepithelial voltage is low and not significantly different (1.14±0.30 mV and 1.88±0.25 mV, respectively; N=5). However, reduction of the saline's osmolarity significantly increased V_{te} (to 9.14±1.29 mV; N=5). In Fig. 3, the response of V_{te} to gradually reduced osmolarities is summarised, normalising the V_{te} increase with Hyposmotic saline to 100% response.

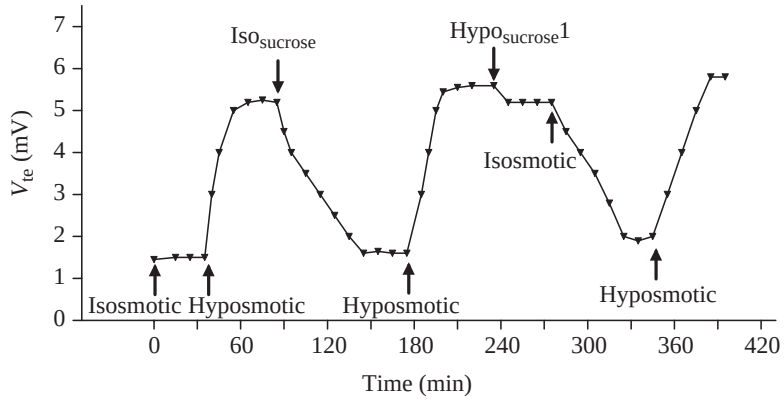

Fig. 2. Representative time-course of the transepithelial potential difference (V_{te}) across an isolated perfused posterior gill during a typical osmolarity change experiment. The arrows indicate application of the different treatments. Isosmotic: 1045 mosmol l^{-1}; Hyposmotic: 698.6 mosmol l^{-1}; $Hypo_{sucrose}$1: Hyposmotic saline plus 60 mmol l^{-1} sucrose (758.6 mosmol l^{-1}); $Iso_{sucrose}$: Hyposmotic saline plus 345.4 mmol l^{-1} sucrose (same osmolarity as Isosmotic saline).

Osmotic variations and their effects on I_{sc} and G_{te} across split gill lamellae

The transepithelial voltage is not a measure of transport rates and depends significantly on the resistance of the paracellular pathway. Thus, it may be that the above-demonstrated V_{te} changes are due to variations in the paracellular resistance at different osmolarities. To verify that osmotic changes influence the transport rates and not only the paracellular resistance, we conducted a series of experiments (N=3) with split gill lamellae mounted in a modified Ussing chamber, measuring the short-circuit current (I_{sc}; which depends only on transcellular parameters) and the transepithelial conductance (G_{te}).

The open-circuit voltage measured with split gill lamellae was in the same range as observed with isolated and perfused gills. Fig. 4 shows a representative time-course of an I_{sc} measurement where we changed between Isosmotic saline, $Iso_{sucrose}$ saline and Hyposmotic saline. The negative I_{sc} was hardly affected when NaCl was reduced at constant osmolarity (changing from Isosmotic saline to $Iso_{sucrose}$ saline). However, G_{te} was decreased after reduction of the NaCl concentration. When the osmolarity was reduced, the negative I_{sc} approximately doubled and G_{te} also increased again. The results of these experiments are shown in Table 2.

Osmotic variations and their effect on Na^+/K^+-ATPase activity

In order to investigate the possible involvement of a functioning Na^+/K^+-ATPase in the response to osmotic variations, we measured the specific activity of this enzyme in homogenates obtained from gills perfused with isosmotic ($Iso_{sucrose}$) or Hyposmotic saline. As shown in Fig. 5, the specific activity of the Na^+/K^+-ATPase in gills perfused and bathed with Hyposmotic saline (41.84±14.54 μmol P_i h^{-1} mg^{-1}) was significantly higher than in the gills perfused with $Iso_{sucrose}$ saline (18.73±6.35 μmol P_i h^{-1} mg^{-1}).

Osmotic variations and intracellular cyclic AMP

In the final series of experiments, we studied a

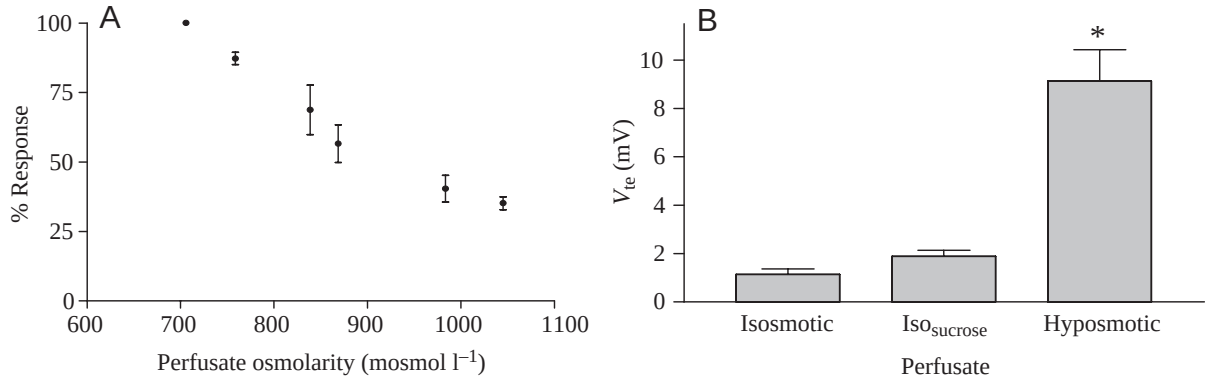

Fig. 3. Effect of osmotic manipulations on the hemolymph side of isolated posterior gills on the transepithelial potential difference (V_{te}). (A) Response of V_{te} to gradually reduced osmolarities. The data are given as percentages (± S.E.M.) of the V_{te} increase with Hyposmotic saline (100% response). N=20. (B) Response of V_{te} to ion concentration change at constant or reduced osmolarity. Mean ± S.E.M. of five values are given in each column. Asterisk indicates statistical difference (P<0.05). Isosmotic: 1045 mosmol l^{-1}; Hyposmotic: 698.6 mosmol l^{-1}; $Iso_{sucrose}$: Hyposmotic saline plus 345.4 mmol l^{-1} sucrose (same osmolarity as Isosmotic saline).

possible interaction between the effects of osmotic variations and the intracellular messenger cyclic AMP (cAMP). First, we measured V_{te} of isolated and perfused gills and analysed the effect of theophylline, a blocker of cAMP degradation by phosphodiesterases (Johnsen and Nielsen, 1978), before and after stimulating adenylate cyclase with forskolin (Seamon et al., 1981). As can be seen in Fig. 6A, a small stimulation of the outside positive V_{te} with Isosmotic saline can be observed after addition of theophylline (2.5 mmol l^{-1}) to the perfusate. Addition of forskolin (0.01 mmol l^{-1}) results in a much more pronounced V_{te} stimulation, and addition of theophylline after the forskolin treatment sustains the voltage elevated by forskolin. Thus, theophylline apparently keeps cAMP levels high. In a second experiment (Fig. 6B), we replaced the forskolin treatment with V_{te} stimulation by Hyposmotic saline. After the stimulation with Hyposmotic saline plus theophylline, the V_{te} level in the presence of Isosmotic saline plus theophylline was significantly higher than before treatment with Hyposmotic saline. These results (summarised in Fig. 7) suggest that Hyposmotic saline increased the cellular cAMP level.

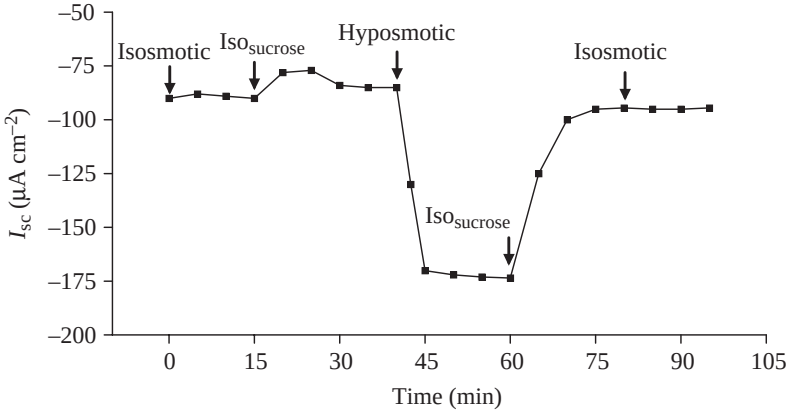

Fig. 4. Time-course of the short-circuit current (I_{sc}) across a split gill lamellae of a posterior gill (representative of three trials). The arrows indicate application of the different treatments. Isosmotic: 1045 mosmol l^{-1}; Hyposmotic: 698.6 mosmol l^{-1}; $Iso_{sucrose}$: Hyposmotic saline plus 345.4 mmol l^{-1} sucrose (same osmolarity as Isosmotic saline).

Discussion

For vertebrate absorptive epithelia, transport regulation by short-term osmotic variations has long been recognised (Ussing, 1965; for a review, see Macknight, 1991). In Crustacea, fast osmotic influences on transbranchial ion transport have been studied in the tight, NaCl-absorbing gill epithelium of the Chinese crab *Eriocheir sinensis*, adapted to freshwater (Onken, 1996). In a previous work with posterior gills of *Chasmagnathus granulatus* adapted to 12‰ salinity, Luquet et al. (2002) observed higher transepithelial voltages when salines of lower ionic and osmotic concentrations were used. As V_{te} was shown to reflect NaCl absorption, the results suggested that osmotic and/or ionic changes influence the transport characteristics of the gill epithelium of *C. granulatus*. In the present study, we investigated this phenomenon in greater detail.

When NaCl concentration and osmolarity of the salines were reduced, the outside positive transepithelial voltage (V_{te}) increased in the same manner as observed before (Luquet et al., 2002). The presented results with respect to reduction of the Na^+ or Cl^- concentration (Fig. 1) clearly show that the observed effects on V_{te} are due to the reduction in osmolarity and do not depend on the change in the concentrations of sodium or chloride. The possibility that the V_{te} increase in the presence of Hyposmotic saline may just reflect changes in paracellular resistance and not in transport rates can be excluded after measuring the short-circuit current (I_{sc}) across spilt gill lamellae (Fig. 4). The

Table 2. *Mean values of short-circuit current (I_{sc}) and transepithelial conductance (G_{te}) before and after osmotic manipulations*

	Isosmotic	$Iso_{sucrose}$	Hyposmotic	$Iso_{sucrose}$	Isosmotic
I_{sc} (μA cm^{-2})	–95.3±14.4	–89.0±40.6	–179.3±37.0[a]	–101.7±26.3	–101.3±14.2
G_{te} (mS cm^{-2})	58.7±18.9	40.5±16.9[b]	47.3±15.8[a]	42.3±15.3[b]	62.1±17.8

Mean ± S.E.M. of three values are given in each cell. Isosmotic: 1045 mosmol l^{-1}; Hyposmotic: 698.6 mosmol l^{-1}; $Iso_{sucrose}$: Hyposmotic saline plus 345.4 mmol l^{-1} sucrose (same osmolarity as Isosmotic saline). The different letters in each row indicate statistical differences among treatments (P<0.05).

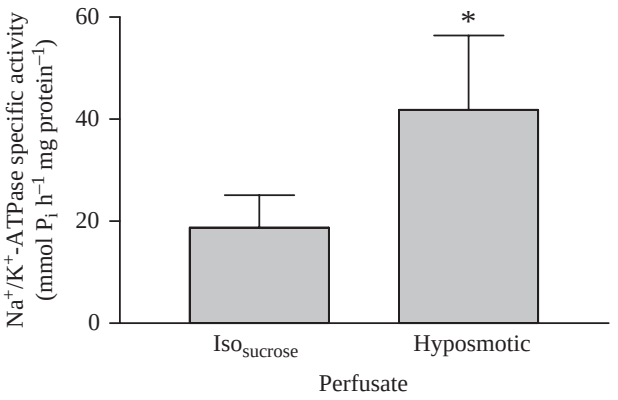

Fig. 5. Specific activity of the Na^+/K^+-ATPase in homogenates obtained from gills perfused with isosmotic ($Iso_{sucrose}$) or with Hyposmotic saline. $Iso_{sucrose}$: 1045 mosmol l^{-1}; Hyposmotic: 698.6 mosmol l^{-1}. Mean ± S.E.M. of five values are given in each column. Asterisk indicates statistical difference (P<0.05).

negative I_{sc}, which is independent of the paracellular pathway and reflects active transcellular charge transport, increased when Hyposmotic saline was used but was unaffected by a reduction in the NaCl concentration at constant osmolarity (Fig. 4; Table 2). We always used solutions of identical ionic composition on both sides of the epithelium to avoid superposition of the signal reflecting active transport with transepithelial diffusive ion movements. However, when we studied the influence of gradually increased osmolarities (Figs 2, 3), sucrose was added only to the basolateral perfusion saline. The observed effects were almost identical to the results with bilateral osmolarity changes, indicating that this response is mainly caused by the hemolymph-side osmolarity change. The effects of reduced osmolarity on isolated gills and split gill lamellae are fast, reversible (Figs 2, 4) and gradually dependent on the magnitude of the osmotic variation (Fig. 3). Thus, the underlying mechanism perfectly accomplishes the demands for a hormone-independent regulation of hemolymph NaCl concentration and osmolarity by adjusting the rates of active NaCl absorption.

The results of the experiments with split gill lamellae allow a first insight into the mechanisms underlying the effects of osmotic variations. The G_{te} reduction at constant I_{sc} after reducing NaCl at constant osmolarity (see Table 2) probably reflects a decrease in paracellular conductance due to the reduction of the ionic strength of the solutions. On the other hand, the G_{te} increase at increased I_{sc} after also reducing the osmolarity (but at constant ionic strength) indicates a change in the transcellular conductance. In many epithelia, including crab gills (Onken et al., 1991, 1995), the apical membrane is, by far, the barrier of highest resistance along the transcellular pathway, and significant conductance changes are most likely due to modulations in this membrane. Thus, it seems likely that the G_{te} increase in the presence of Hyposmotic saline is related to the modulation of an apical, electrogenic transporter. If we

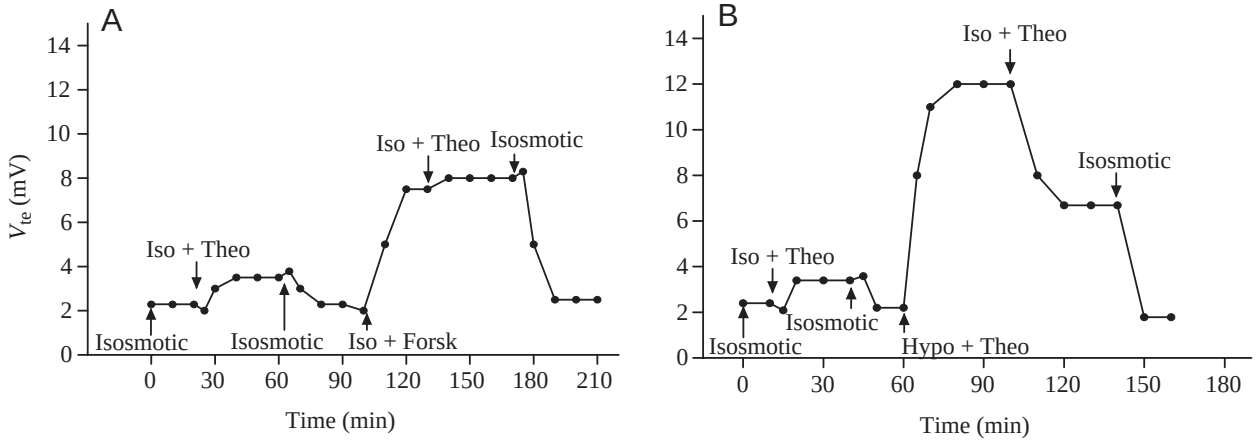

Fig. 6. Time-course of the transepithelial potential difference (V_{te}) across an isolated perfused posterior gill (representative of five trials). (A) Theophylline sustains the elevated V_{te} caused by forskolin. (B) Theophylline partially sustains the elevated V_{te} caused by Hyposmotic saline. Arrows indicate application of the different treatments. Iso + Theo: Isosmotic saline plus theophylline (2.5 mmol l^{-1}); Iso + Forsk: Isosmotic saline plus forskolin (0.01 mmol l^{-1}); Hypo + Theo: Hyposmotic saline plus theophylline (2.5 mmol l^{-1}).

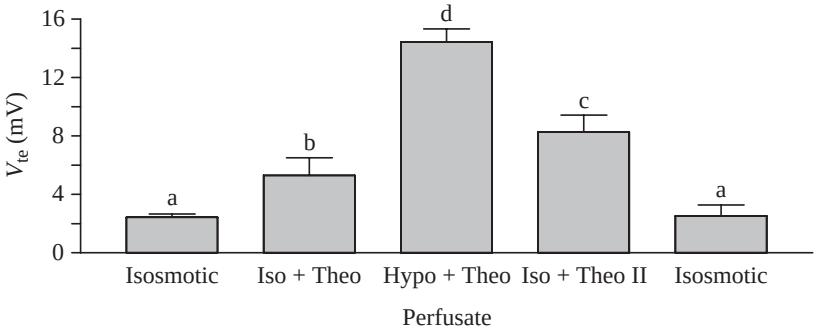

Fig. 7. Effects of the interaction between osmotic variations and theophylline on the transepithelial potential difference (V_{te}) in isolated posterior gills. Mean ± S.E.M. of five values are given in each column. The different letters indicate statistical differences among treatments (P<0.05). Iso + Theo: Isosmotic saline plus theophylline (2.5 mmol l^{-1}). Hypo + Theo: Hyposmotic saline plus theophylline (2.5 mmol l^{-1}). Iso + Theo II: after perfusing with Hyposmotic saline plus theophylline.

assume that active NaCl absorption across the gills of *C. granulatus* also follows the same mechanism as proposed for *C. maenas* (cf. Riestenpatt et al., 1996; see Introduction), it seems conclusive that the osmotic stimulation of NaCl absorption is at least partly based on the increase of an apical K^+ conductance. However, a detailed analysis of the mode of active NaCl absorption in *C. granulatus* is still missing and the above hypothesis needs to be readdressed after successful characterization of the transport mechanism.

The basolateral Na^+/K^+-ATPase was shown to energize NaCl absorption across *C. granulatus* posterior gills (Luquet et al., 2002). In the present study, the activity of this ATPase was approximately doubled when Hyposmotic saline was used instead of Isosmotic saline (see Fig. 5). This finding clearly demonstrates that, apart from a possible modulation of an apical conductive pathway (see above), the basolateral Na^+/K^+-ATPase is modulated by the osmotic variation and participates in the stimulation of NaCl absorption in the presence of hyposmotic salines. Fast activation of the Na^+/K^+-ATPase in hyposmotic media has been observed with myocytes (Venosa, 1991; Whalley et al., 1993) and also with epithelial cells (Coutry et al., 1994). A modulation of the *in situ* activity of the ATPase due to changes in transapical NaCl absorption and respective alterations in cellular sodium would not be detectable in activity measurements as conducted in the present study. Thus, it seems that osmotic variations modulate the activity of the ATPase by phosphorylation/dephosphorylation processes (cf. Cheng et al., 1999) or by rapid insertion of ATPase-containing vesicles into the basolateral membrane (cf. Venosa, 1991; Carranza et al., 1998). The activation of the Na^+/K^+-ATPase during hyposmotic conditions represents a major difference compared with the autoregulatory response of the gills of *E. sinensis*, in which only apical transporters (V-type H^+-ATPase and Na^+ channels) are involved (Onken, 1996). Some results obtained at our laboratory suggest that dopamine, an extensively studied bioamine likely to be involved in the adaptation to low salinity (for a review, see Morris, 2001), also modulates the electrical properties and Na^+/K^+-ATPase activity of the gills of *C. granulatus* (J. Halperin, M. Tresguerres, G. Genovese, A. Pozzi and C. M. Luquet, unpublished data).

Apart from the cell volume, a multitude of cellular parameters has been shown to change following exposure of cells to anisosmotic media (see Lang et al., 1998). One of these factors is an increase in cellular cAMP by activation of adenylate cyclase in hyposmotic media (Watson, 1989, 1990). As shown in Fig. 6A, an artificial increase in intracellular cAMP concentration in *C. granulatus* posterior gills by the addition of theophylline and/or forskolin in isosmotic salines results in an increase in V_{te}. So far, these findings are consistent with an increase in the activity of the Na^+/K^+-ATPase after increasing cellular cAMP (J. Halperin, M. Tresguerres, G. Genovese, A. Pozzi and C. M. Luquet, unpublished data). When theophylline was present in the Isosmotic saline after stimulation with Hyposmotic saline, V_{te} was maintained at a higher level than under the same conditions before the hyposmotic stimulation (see Figs 6B, 7), suggesting that the change to hyposmotic salines was accompanied by a rise in cellular cAMP. The same second messenger has already been shown to mediate the stimulatory effects of dopamine and serotonin in *E. sinensis* (Trausch et al., 1989; Mo et al., 1998; see Morris, 2001 for a review). However, since theophylline preserved only part of the hyposmotic activation, other second messengers might also be involved in the autoregulatory response observed in *C. granulatus* gills.

In summary, we propose that reduction of the hemolymph-side osmolarity results in an increase of cellular cAMP that, in turn, stimulates active NaCl absorption across the posterior gills of *C. granulatus* by activating the basolateral Na^+/K^+-ATPase. A conductive pathway, probably at the apical membrane, is also stimulated by signalling events that are still to be elucidated. Under physiological conditions, this autoregulation could be an important mechanism to rapidly stabilise the hemolymph osmolarity and NaCl concentration when the animals move between ambient media of different salinities.

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