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CYCLIC AMP STIMULATION OF ELECTROGENIC UPTAKE OF Na^+ AND Cl^- ACROSS THE GILL EPITHELIUM OF THE CHINESE CRAB *ERIOCHEIR SINENSIS*

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Summary

Split gill lamellae (epithelium plus cuticle) of hyperregulating Chinese crabs acclimated to fresh water were mounted in a modified Ussing chamber. Active and electrogenic absorption of sodium and chloride were measured as positive amiloride-sensitive and negative Cl^--dependent short-circuit currents (I_{Na}, I_{Cl}), respectively. Both currents were characterized before and after treatment of the tissue with theophylline or dibutyryl cyclic AMP. Both drugs increased I_{Na} and I_{Cl}. A simple circuit analysis showed that I_{Na} stimulation reflected a marked increase in the transcellular Na^+ conductance, whereas the respective electromotive force was unchanged. The Michaelis constant (K_{Na}) for Na^+ current saturation was decreased after I_{Na} stimulation, indicating an increased affinity of the transport mechanism for its substrate. Consequently, the affinity for the Na^+ channel blocker amiloride decreased as expected for a competitive interaction between substrate and inhibitor. Analysis of the amiloride-induced current-noise revealed a marked increase in the number of apical Na^+ channels after I_{Na} stimulation with theophylline, whereas there was little change in the single-channel current. Stimulation of Cl^- absorption was accompanied by a substantial increase in both transcellular conductance and electromotive force, indicating an activation of the apical H^+ pump that provides the driving force for active Cl^- uptake *via* apical Cl^-/HCO_3^- exchange and basolateral Cl^- channels.

Introduction

During their ontogenesis, Chinese crabs (*Eriocheir sinensis*) are confronted with changing external salinities. In fresh water, Chinese crabs compensate for the ensuing salt loss and maintain a haemolymph osmolarity of approximately 650 mosmol l^{-1} by active uptake of NaCl across the posterior gills (Mantel and Farmer, 1983; Péqueux *et al.* 1988).

In investigations of ion fluxes and transepithelial potential differences (PD_{te}) across isolated and perfused gills, the general transport properties of this organ have been fairly

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well characterized (for a review, see Péqueux *et al.* 1988). However, a convincing model for the reported active uptake mechanisms of both Na^+ and Cl^- across this tissue was not presented until Schwarz and Graszynski (1989) mounted split gill lamellae in a modified Ussing chamber under voltage-clamp conditions. This made it possible to apply a number of current-based techniques which had been successfully used in the past to uncover and characterise electrogenic ion transport mechanisms in various epithelia. Thus, Onken *et al.* (1991*a*) succeeded in showing the nature of active Cl^- uptake by establishing a negative Cl^--dependent short-circuit current (I_{Cl}) in external Na^+-free saline. These authors proposed a Na^+-independent uptake of Cl^- *via* an apical Cl^-/HCO_3^- exchange and basolateral Cl^- channels, the process being driven by an apical V-type H^+-ATPase, as indicated by recent immunofluorescence labelling studies and the bafilomycin-sensitivity of the Cl^- current (Putzenlechner *et al.* 1992). A positive Na^+-dependent short-circuit current (I_{Na}) measured in external Cl^--free saline was shown to reflect electrogenic Na^+ uptake *via* apical Na^+ channels and the basolateral Na^+/K^+-ATPase (Schwarz, 1990; Zeiske *et al.* 1992). Therefore, independent mechanisms for electrogenic Na^+ and Cl^- uptake underlie the observed uptake fluxes in the posterior gills of *Eriocheir sinensis*.

In recent decades, evidence has accumulated that neuroendocrine factors (peptides and bioamines) are involved in the regulation of salt and water balance in Crustacea (Kleinholz, 1976; Kamemoto, 1982; Mantel, 1985). Although only a little is known about the regulatory influence of neuroendocrine peptides on salt absorption across gills of hyperregulating crabs, bioamines have clearly been identified as regulators of these processes. Zatta (1987) demonstrated changes in bioamine concentrations in haemolymph and gill tissue of *Carcinus maenas* after transfer of the animals to a dilute medium. Investigations of the Chinese crab indicated the presence of serotonin and dopamine receptors in the posterior ion-absorbing gills (Trausch *et al.* 1989). Kamemoto and Oyama (1985) found that pericardial organ extracts, dopamine and octopamine increase branchial cyclic AMP and Na^+ influx across isolated gills of *Callinectes sapidus*. Membrane-permeant dibutyryl cyclic AMP (dbc-AMP) was shown to increase both Na^+ influx (Lohrmann and Kamemoto, 1987; Bianchini and Gilles, 1990) and Cl^- influx (Bianchini and Gilles, 1990) across isolated and perfused crab gills. Thus, for bioamines, the full sequence of events from their source (pericardial organ) to their physiological effect (increase in cyclic-AMP-stimulated NaCl absorption) has been uncovered. Protein kinase C (Asselbourg *et al.* 1991) and calmodulin (Péqueux and Gilles, 1992) have also been shown to be involved in regulation of NaCl absorption across crab gills. However, it is still an open question which transport mechanisms in the gills are triggered by these second messengers. For *Eriocheir sinensis* it has been suggested that the Na^+/K^+-ATPase is regulated by these systems (Trausch *et al.* 1989; Bianchini and Gilles, 1990; Asselbourg *et al.* 1991; Péqueux and Gilles, 1992). Only for the gills of *Carcinus maenas*, where the transport mechanisms differ from those found in the Chinese crab (Onken and Siebers, 1992), has it been shown that addition of dbc-AMP to the internal bathing solution causes direct stimulation of the activity of the Na^+/K^+-ATPase (Sommer and Mantel, 1988).

In the present investigation, we focus on modulation of the mechanisms for electrogenic uptake of Na^+ (I_{Na}) and Cl^- (I_{Cl}) across posterior gills of freshwater-acclimated *Eriocheir sinensis* by the phosphodiesterase blocker theophylline (Johnsen

and Nielsen, 1978) and membrane-permeant dbc-AMP. I_{Na} and I_{Cl} were analysed kinetically as well as by circuit analyses. Additionally, the I_{Na} noise induced by the Na^+ channel blocker amiloride was recorded and evaluated on the basis of a simple blocking model. Most interestingly, our results indicate completely different interactions of theophylline or dbc-AMP with Na^+ or Cl^- uptake.

Materials and methods

Crabs

Chinese crabs (*Eriocheir sinensis*; Milne-Edwards, 1853) were caught by commercial fishermen in rivers flowing into the North Sea. The animals were kept in running tap water containing (in mmol l^{-1}): Na^+, 2.0; K^+, 0.08; Ca^{2+}, 3.0; Cl^-, 1.7, at 12–14 °C, for at least 1 month before use. They were fed with Evans carp food (Bertels GmbH, Halstenbek, Germany) or frozen fish twice a week.

Preparation

The animals were killed by destroying the ventral ganglia. After lifting the dorsal carapace, the three posterior gills were removed. Single gill lamellae were isolated and split according to the method described by Schwarz and Graszynski (1989). These split lamella preparations, each consisting of a single epithelial layer covered by an apical cuticle, were mounted under microscopic control in a modified Ussing chamber with an epithelial area of 0.0078 cm^2. To minimize edge damage, Glisseal grease (Borer Chemie, Solothurn, Switzerland) was used to seal the edges of the preparation. The chamber compartments (volume 1 ml) were continuously perfused by gravity feed with aerated salines at a rate of approximately 12.5 ml min^{-1}.

Solutions and chemicals

The basic haemolymph-like saline, which was always used as the basolateral bathing solution, was composed of (in mmol l^{-1}): NaCl, 300; KCl, 8; $NaHCO_3$, 2; Hepes, 5; calcium gluconate, 8; glucose, 2; pH 7.6 (adjusted with Tris). In Cl^--free external saline, the chlorides were replaced with gluconates. In Na^+-free external saline, NaCl was replaced by choline chloride and $NaHCO_3$ by $KHCO_3$. The NaCl-free saline was prepared with an equimolar mixture of tetramethylammonium hydroxide (TMA) and gluconolacton instead of NaCl; $NaHCO_3$ was replaced by $KHCO_3$.

Amiloride was a gift from Merck, Sharp and Dohme (Munich, Germany); theophylline and ouabain were obtained from Serva; diphenylamine-2-carboxylic acid (DPC) was purchased from Fluka; dbc-AMP and dimethylsulphoxide (DMSO) were obtained from Sigma. All reagents, with the exceptions of DPC (stock solution of 0.5 mol l^{-1} DPC in DMSO) and amiloride (stock solution of 10 mmol l^{-1} amiloride in H_2O), were directly dissolved in the salines.

Electrical measurements

Ohmic variables

For measurement of PD_{te}, calomel electrodes were connected by agar bridges (3 % agar in 3 mol l^{-1} KCl) to the chamber compartments (the distance to the preparation was less

than 0.1 cm). The reference electrode was in the basolateral bath. Silver wires coated with AgCl served as current electrodes to short-circuit the epithelium (for measurement of short-circuit current I_{sc}) by an automatic clamping device (Van Driessche and Gullentops, Leuven, Belgium). The area-specific conductance between the tips of the voltage electrodes was calculated from small imposed voltage pulses (ΔPD_{te}) and the resulting current deflections (ΔI_{sc}). According to Schwarz and Graszynski (1989), the conductance of the solutions is high compared with the transepithelial conductance (G_{te}), so no correction was made for G_{te} and I_{sc}.

Noise analysis

For the current fluctuation analysis experiments, we used a specially constructed low-noise voltage-clamp apparatus designed and modified after the original version by Van Driessche and Lindemann (1978). Fluctuations of I_{sc} induced by amiloride were recorded digitally after passing the clamp current through a set of (anti-aliasing) high- and low-pass filters and after appropriate amplification at each step. Lorentzian curves in the I_{sc} noise spectra (cf. Zeiske *et al.* 1992) were obtained by adding amiloride to the external saline. The analysis of the blocker noise was carried out according to the two-state model, as outlined previously (Zeiske *et al.* 1992), yielding the Lorentzian variables S_o (plateau) and f_c (corner frequency). The linear relationship $f_c=f([AMI])$ also holds after stimulation with theophylline (not shown here), so the amiloride-dependent Na^+-channel open-probability (P_o) in a given preparation may simply be expressed as $P_o=I_{Na}(AMI)/I_{Na}(CTR)$, where $I_{Na}(AMI)$ is the Na^+-specific I_{sc} in the presence of a defined amiloride concentration ([AMI]) and $I_{Na}(CTR)$ is that in its absence. Because of the relationship $P_o=K_{AMI}/(K_{AMI}+[AMI])$, where K_{AMI} is the channel amiloride dissociation constant, a change in P_o at constant [AMI] indicates a change in K_{AMI}. For more details and for the determination of the single-channel current (i) and the channel density (M), see Zeiske *et al.* (1992).

Statistics

All values in the Results section are given as means ± standard error of the mean (S.E.M.). Differences between groups were tested using the paired Student's *t*-test. Differences were considered significant when values of P were less than 0.05. The S.E.M. values of the variables determined from Hanes–Woolf plots (maximal I_{sc} blockade with amiloride, K_{AMI}, K_{Na} and K_{Cl}) were obtained by analysing the data from individual experiments. In contrast, Figs 2, 3 and 5 represent the Hanes–Woolf plots of the respective mean values.

Results

Na^+ transport

Macroscopic variables

Electrogenic Na^+ uptake (I_{Na}) across split lamella preparations of posterior gills of the Chinese crab was investigated with Cl^--free (gluconate) saline in the external bath and physiological haemolymph-like NaCl saline in the internal bath (see Zeiske *et al.* 1992).

To identify I_{Na}, we studied the dose-dependence of I_{Na} on external amiloride for four split lamella preparations. At concentrations below 10^{-6} mol l^{-1}, amiloride is a specific inhibitor of Na^+ channels in epithelia (Benos, 1982), and the drug was shown to block electrogenic Na^+ uptake competitively across the gill epithelium of the Chinese crab in an earlier study (Zeiske *et al.* 1992). The influence of different external amiloride concentrations on I_{sc} is shown in Fig. 1. High doses of the blocker inhibit a major fraction (95±12 μA cm^{-2}) of the control short-circuit current. A more detailed analysis of the blocker saturation kinetics is shown in Fig. 2. In this Hanes–Woolf plot, the ratio of the amiloride concentration to the respective I_{sc} decrease ([AMI]/ΔI_{sc}) is plotted against the amiloride concentration ([AMI]). From the reciprocal slopes of the lines obtained with the four individual preparations, we calculated a mean maximal amiloride-induced current decrease (here defined as I_{Na}) of 95±12 μA cm^{-2}, which is identical to the extent of the I_{sc} blockade by 10^{-4} mol l^{-1} amiloride (see above). Therefore, 10^{-4} mol l^{-1} amiloride is an excellent probe to identify I_{Na}. The average half-maximal effect of the drug (K_{AMI}=0.44±0.07 μmol l^{-1}) was determined from the intercepts of the lines for the individual experiments with the abscissa (see also Fig. 2). For I_l, the part of I_{sc} that is not sensitive to external amiloride, we obtained 25±4 μA cm^{-2}.

From the differences between the tissue conductances before and after addition of 10^{-4} mol l^{-1} amiloride, we obtained a mean cellular Na^+ conductance (G_{Na}) of 1.18± 0.10 mS cm^{-2}. The mean remaining conductance (0.67±0.03 mS cm^{-2}) represents a leak conductance (G_l), which is not related to active Na^+ transport and probably represents the paracellular pathway.

The mean electromotive force of the cellular Na^+ uptake mechanism (E_{Na}) was

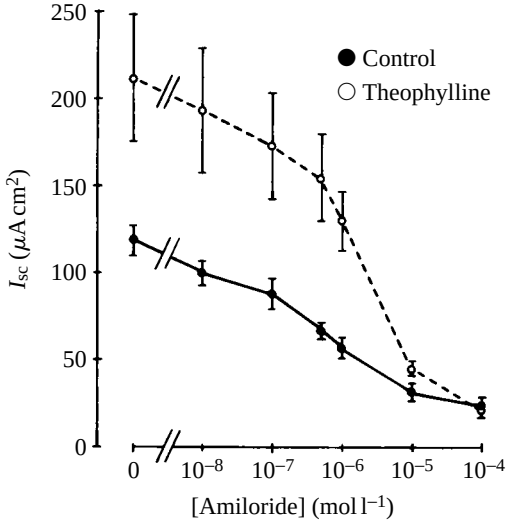

Fig. 1. Dose-dependence of I_{sc} inhibition on amiloride. In four experiments on different split lamella preparations, amiloride was added at increasing concentrations to the external Cl^--free saline (the internal solution was standard NaCl saline) before (filled circles, solid line) and after (open circles, broken line) addition of 2.5 mmol l^{-1} theophylline to the internal solution. The mean I_{sc} (± S.E.M.) is plotted against the amiloride concentration in the external saline.

calculated from the slopes of I_{Na}/G_{Na} plots for the different amiloride concentrations (which are linear; not shown) or from the individual I_{Na}:G_{Na} ratios of the four investigated preparations. We obtained, for the control case, a value of 80±5 mV.

To study the substrate kinetics of I_{Na}, we measured I_{sc} under different external Na^+ concentrations, identifying I_{Na} with 10^{-4} mol l^{-1} amiloride as described above. The averaged results of five experiments are shown in Fig. 3 in a Hanes–Woolf plot of the relationship between I_{Na} and [Na^+]. For high external [Na^+] (greater than 150 mmol l^{-1}) we observed a deviation from simple Michaelis–Menten kinetics. This abnormal behaviour, which seems to reflect a time- and concentration-dependent change in the maximal current (Onken *et al.* 1991*b*), has already been described (Zeiske *et al.* 1992). We therefore restricted our analysis to Na^+ concentrations of up to 150 mmol l^{-1}. For unstimulated tissues, the mean external Na^+ concentration at half-maximal Na^+ current (K_{Na}) was 60±11 mmol l^{-1} (mean ± S.E.M. of intercepts with the abscissa of the lines of the five individual preparations; see also Fig. 3).

Having characterized electrogenic Na^+ uptake under control conditions, we investigated the influence of internal theophylline, which is known to increase cellular cyclic AMP concentrations by blockade of the phosphodiesterase (Johnsen and Nielsen,

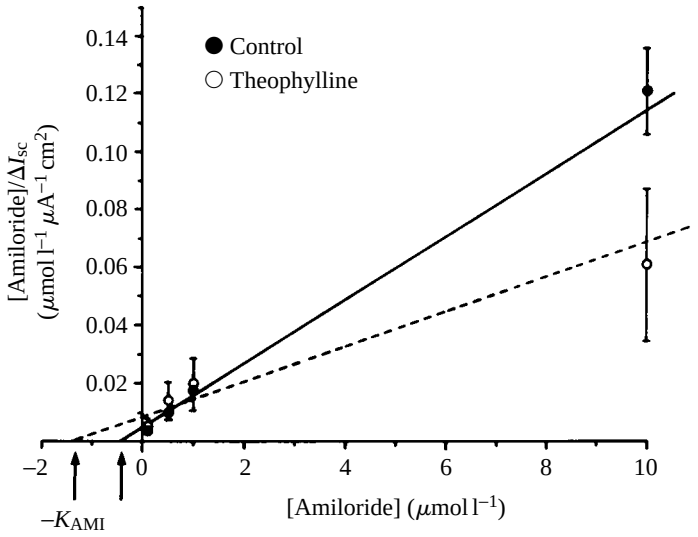

Fig. 2. A Hanes–Woolf plot of the dose-dependence of I_{sc} inhibition on amiloride before (filled circles, solid line) and after (open circles, broken line) addition of theophylline to the internal solution, as shown in Fig. 1. The ratios of the amiloride concentrations (in μmol l^{-1}) to the respective mean I_{sc} decreases ([amiloride]/ΔI_{sc}; N=4, ± S.E.M.) are plotted against the amiloride concentration (in μmol l^{-1}). The lines represent linear regressions for amiloride concentrations in the range 0.01–100 μmol l^{-1} (data points for 0.01 and 100 μmol l^{-1} are not shown). The mean maximal amiloride-induced I_{sc} decreases (I_{Na}) can be estimated from the reciprocal slopes of the lines, whereas the mean amiloride concentrations at half-maximal I_{sc} decrease (K_{AMI}) are reflected in the intercepts with the abscissa. The mean values of I_{Na} and K_{AMI}, which were obtained from plots for the individual experiments, are given in the text.

1978). After addition of 2.5 mmol l^{-1} theophylline to the internal bath of the same preparations, the data analyses (Figs 1, 2 and 3) showed a substantially increased I_{Na} (189±37 μA cm^{-2}, see Fig. 1) and G_{Na} (2.95±0.75 mS cm^{-2}). Although maximal I_{sc} depression (193±38 μA cm^{-2}, see Fig. 2) was achieved with 10^{-4} mol l^{-1} external amiloride, the mean half-maximal effect of amiloride (K_{AMI}) surprisingly more than doubled to 1.11±0.27 μmol l^{-1}. However, I_l (21±3 μA cm^{-2}), G_l (0.81±0.07 mS cm^{-2}) and E_{Na} (68±8 mV) were not significantly affected by internal addition of theophylline. Therefore 10^{-4} mol l^{-1} amiloride reduced the theophylline-generated current increase by 100 %. The partly nonlinear behaviour of the $I_{Na}/[Na^+]$ kinetics was also observed in the presence of internal theophylline (see Fig. 3). Conspicuously, the drug caused a significant 50 % reduction in K_{Na} to 31±9 mmol l^{-1} (Student's *t*-test; $P<0.05$).

Addition of the membrane-permeant substance dbc-AMP (0.1 mmol l^{-1}) caused stimulation of I_{Na} and G_{Na} similar to that obtained with internal theophylline (Fig. 4). I_{Na} increased from 58±12 to 151±14 μA cm^{-2} (N=5) and G_{Na} increased from 0.73±0.07 to 2.04±0.16 mS cm^{-2} (N=5), whereas E_{Na}, G_l and I_l remained constant (82±20 mV, 1.14±0.1 mS cm^{-2} and 27±9 μA cm^{-2} respectively) before and (75±8 mV, 1.59±0.3 mS cm^{-2} and 27±5 μA cm^{-2} respectively) after stimulation with dbc-AMP. All currents were sensitive to internal ouabain, the 'classical' blocker of the Na^+/K^+-ATPase (Skou, 1965). A representative I_{sc} time course, demonstrating the stimulating effect of internal dbc-AMP and the blocking effect of external amiloride (10^{-4} mol l^{-1}) and internal ouabain (10^{-3} mol l^{-1}), is shown in Fig. 4.

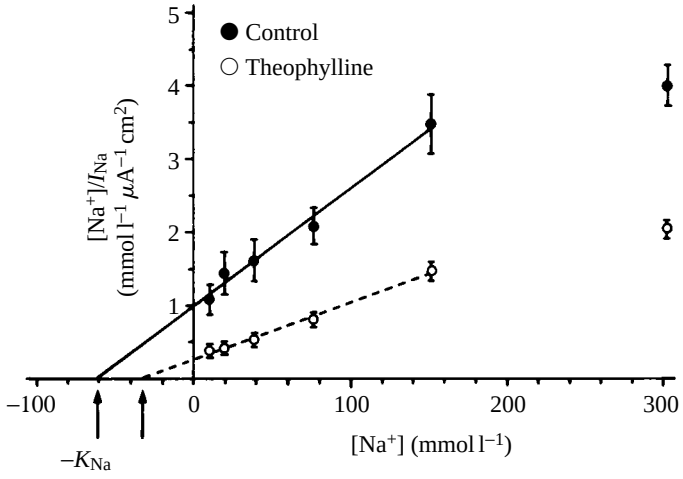

Fig. 3. Hanes–Woolf plot of the Na^+-dependence of I_{Na} before (filled circles, solid line) and after (open circles, broken line) addition of theophylline to the internal NaCl saline. I_{Na} was defined as the portion of I_{sc} that was sensitive to 10^{-4} mol l^{-1} external amiloride. The ratios of the external sodium concentrations (in mmol l^{-1}) to the respective mean (N=5, ± S.E.M.) sodium currents ($[Na^+]/I_{Na}$) are plotted against the external sodium concentration (in mmol l^{-1}). The lines represent linear regressions for the $[Na^+]$ range 0–150 mmol l^{-1}. The Na^+ concentrations at half-maximal I_{Na} (K_{Na}) are reflected in the intercepts with the abscissa. The mean values of K_{Na}, which were obtained from plots for the individual experiments, are given in the text.

Microscopic variables

In seven preparations, we analysed the amiloride-induced current-noise before and after treatment of the internal side with theophylline. Addition of amiloride to the external solution generated a Lorentzian current-noise characteristic of a Na^+ channel blockade (Zeiske *et al.* 1992). Evaluation of the spectra (Lorentzian variables S_o and f_c) and I_{sc} permits calculation of the single-channel current (i) and the channel density (M) according to our model (Zeiske *et al.* 1992). Table 1 summarises these values obtained from seven animals. In the absence of amiloride, theophylline induced a rise in I_{Na} in each case (average 2.2-fold). In the presence of amiloride, the Lorentzian plateaux (S_o) were substantially elevated by the phosphodiesterase blocker (average 4.3-fold). In three out of seven cases, f_c was significantly smaller in the theophylline-stimulated state. Table 1 also shows the variables i and M, as calculated using the two-state model of the amiloride blockade (Zeiske *et al.* 1992). No significant change in i was observed in four cases, but there was an increase of approximately 50 % in two preparations and a 25 % drop in one. Finally, we note that, without exception, there was a, sometimes dramatic, increase in channel density after theophylline treatment (average 1.9-fold).

Cl^- transport

Electrogenic Cl^- uptake across split lamella preparations of posterior gills of the Chinese crab was investigated with Na^+-free (choline) saline in the external bath and physiological haemolymph-like NaCl saline in the internal bath. The negative I_{sc} under

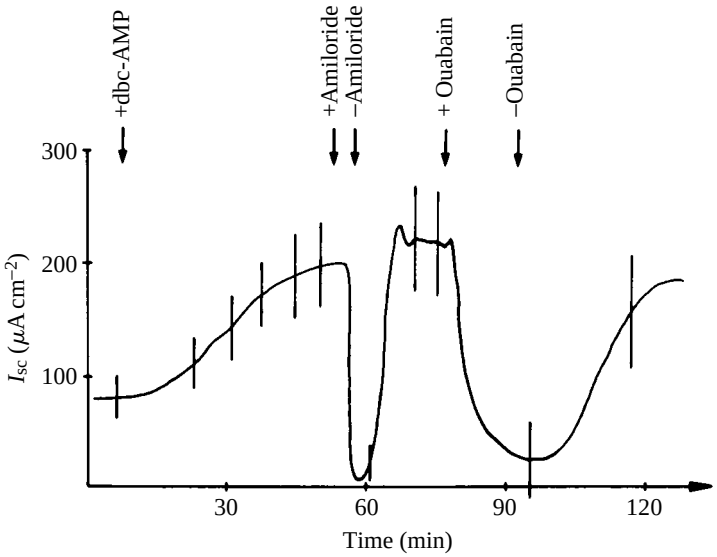

Fig. 4. Time course of the positive I_{sc} with Cl^--free saline in the external bath and NaCl saline in the internal bath, showing the stimulating effect of 10^{-4} mol l^{-1} internal dbc-AMP and the reversible inhibitions caused by external amiloride (10^{-4} mol l^{-1}) and internal ouabain (10^{-3} mol l^{-1}). The amplitudes of the current deflections, which are due to voltage pulses of ±10 mV, are proportional to the transepithelial conductance (G_{te}).

Table 1. *Noise parameters obtained from seven split gill lamellae*

	I_{Na} (no AMI) (μA cm^{-2})		$10^{20}\times S_o$ (A^2 s cm^{-2})		f_c (s^{-1})		i (pA)		$10^{-6}\times M$ (cm^{-2})	
Number	Control	Theo-phylline	Control	Theo-phylline	Control	Theo-phylline	Control	Theo-phylline	Control	Theo-phylline
1*	49.1	153.6	2.6	10.8	48.5	42.1	0.23	0.25	219	619
2†	179.2	409.6	9.3	105.0	44.7	11.2	0.16	0.18	1120	2276
3	87.0	298.0	4.5	17.6	61.9	42.8	0.20	0.31	436	961
4	297.0	448.0	48.4	132.0	46.3	40.7	0.55	0.75	540	600
5	531.2	624.6	78.5	72.3	46.3	50.3	0.60	0.45	886	1400
6	228.0	454.0	1.6	3.3	44.9	44.3	0.02	0.02	11500	20300
7	222.7	376.3	6.1	24.6	62.0	38.1	0.13	0.16	1720	2350

*2.5 mmol l^{-1} theophylline; all others 5.0 mmol l^{-1}.
†1.5 μmol l^{-1} amiloride; all others 5.0 μmol l^{-1}.

these conditions reflects almost completely an active transcellular Cl^- uptake (I_{Cl}, see Onken *et al.* 1991*a*).

In our experiments (N=8) we found a mean control I_{Cl} of $-47\pm14\,\mu A\,cm^{-2}$ at an external chloride concentration $[Cl^-]_o$ of 306 mmol l^{-1}. From the differences between the tissue conductances before and after external substitution of Cl^-, we obtained estimates

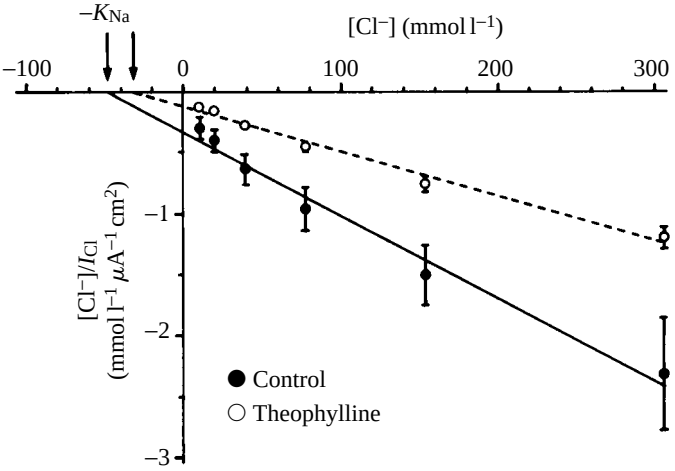

Fig. 5. Hanes–Woolf plot of the Cl^- dependence of I_{Cl} before (filled circles, solid line) and after addition of theophylline to the internal NaCl saline (open circles, broken line). I_{Cl} was defined as I_{sc} recorded in the presence of Na^+-free saline in the external bath and NaCl saline in the internal bath. For the correction of the increasing passive current with decreasing external $[Cl^-]$, see Onken *et al.* (1991*a*). The ratios of the external chloride concentration (in mmol l^{-1}) and the respective mean (N=4, ± S.E.M.) chloride currents ($[Cl^-]/I_{Cl}$) are plotted against the external chloride concentration (in mmol l^{-1}). The lines represent linear regressions for the $[Cl^-]$ range 0–306 mmol l^{-1}. The Cl^- concentrations at half-maximal I_{Cl} (K_{Cl}) are reflected in the intercepts with the abscissa. The mean values of K_{Cl}, which were obtained from plots for the individual experiments, are given in the text.

of the individual cellular conductance associated with active Cl^- uptake (G_{Cl}; cf. Onken *et al.* 1991*a*). The mean G_{Cl} was 1.36±0.10 mS cm^{-2}. The remaining conductance of 1.40±0.12 mS cm^{-2} and the positive current of 22±3 μA cm^{-2} with external NaCl-free saline are not associated with active Cl^- uptake. However, these values were ruled out as correct results for the passive paracellular leak variables (G_l and I_l). In five separate preparations, currents and conductances under these conditions were sensitive to 10^{-4} mol l^{-1} external amiloride (32 % current increase, 25 % conductance decrease), suggesting that cell-to-apical Na^+ movement contributed to G_l as defined here. From the individual I_{Cl}/G_{Cl} ratios, we calculated the individual electromotive forces for the cellular mechanism responsible for active Cl^- absorption (E_{Cl}). The mean E_{Cl} was −35±10 mV.

To investigate the substrate kinetics of I_{Cl}, we varied the external Cl^- concentration between 306 and 0 mmol l^{-1} and corrected for the respective passive currents (see Onken *et al.* 1991*a*). The pooled data from four experiments are presented in a Hanes–Woolf plot in Fig. 5. From the linear representations of Michaelis–Menten kinetics for the individual preparations, we obtained a mean control K_{Cl} of 54±9 mmol l^{-1}.

With 2.5 mmol l^{-1} theophylline in the internal bath, we repeated these analyses on the now-stimulated preparations and found substantially increased values for I_{Cl} (−261±46 μA cm^{-2}), E_{Cl} (−104±12 mV) and G_{Cl} (2.40±0.22 mS cm^{-2}). In three of the

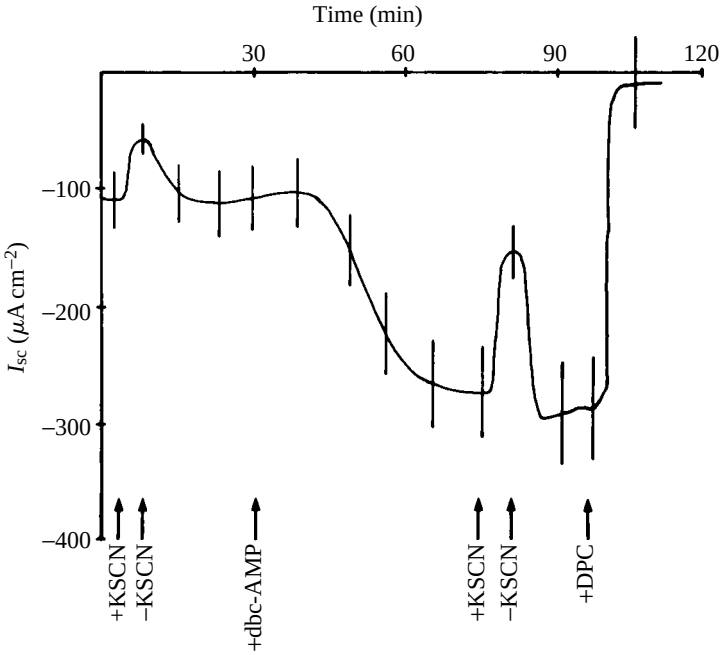

Fig. 6. Time course of the negative I_{sc} with Na^+-free saline in the external bath and NaCl saline in the internal bath, showing the stimulating effect of 10^{-4} mol l^{-1} internal dbc-AMP and the inhibition caused by external potassium thiocyanate (KSCN, 2×10^{-2} mol l^{-1}) and internal diphenylamine-2-carboxylate (DPC, 1.25×10^{-3} mol l^{-1}). The amplitudes of the current deflections, which are due to voltage pulses of ±10 mV, are proportional to the transepithelial conductance (G_{te}).

four experiments, K_{Cl} was less after treatment with theophylline, but in one case it increased. The mean value of 35±3 mmol l^{-1} was therefore not statistically different from the control (54±9 mmol l^{-1}) before I_{Cl} stimulation with theophylline (*t*-test for individual K_{Cl} values). I_{Cl} could be stimulated from 88±22 μA cm^{-2} to 228±21 μA cm^{-2} (N=3) by addition of 0.1 mmol l^{-1} dbc-AMP to the internal bath (Fig. 6). All negative currents before and after stimulation were equally sensitive to 20 mmol l^{-1} external KSCN and 1.25 mmol l^{-1} internal DPC. This is shown in a representative I_{sc} time course in Fig. 6.

Discussion

Endocrine control of ion transport has been demonstrated for various epithelial tissues, including crustacean gills (Foskett *et al.* 1982; Greger, 1985; Kamemoto and Oyama, 1985; Palmer, 1988; Reuss *et al.* 1991). Transduction of extracellular signals into intracellular signals is often mediated by receptor/G-protein mechanisms (Neer and Clapham, 1988). G-proteins may interact directly with transport proteins or determine the cellular concentration of second messengers, such as cyclic AMP, by activation or inactivation of their synthesis or degradation (Brown *et al.* 1990). Cyclic AMP is known to regulate the activity of transport proteins, usually by phosphorylation mediated by protein kinase A (Levitan, 1985). Various experimental procedures have been used to augment the intracellular cyclic AMP concentration for *in vitro* studies on ion-transporting epithelia. These include the procedures used in the present study, namely inhibition of the phosphodiesterase with theophylline (reduced degradation of cyclic AMP) and direct application of membrane-permeant cyclic AMP derivatives such as dbc-AMP.

Such manipulations increase the influxes of Na^+ and Cl^- across posterior gills of the Chinese crab and hyperpolarise the transepithelial potential differences under different conditions (Bianchini and Gilles, 1990). Our results also show increased magnitudes of both I_{Na} and I_{Cl}, reflecting a stimulated electrogenic uptake of Na^+ and Cl^- after internal application of theophylline or dbc-AMP. Moreover, the techniques used in the present study allow a much more detailed analysis of the changes that are induced by an increased intracellular cyclic AMP level than do flux and potential difference measurements on whole gills. Before discussing the effects of cyclic AMP, it should be noted that the principal mechanisms of the electrogenic uptake of Na^+ and Cl^- (see Onken *et al.* 1991*a*; Zeiske *et al.* 1992) seem to be unchanged after stimulation: before and after addition of theophylline or dbc-AMP, I_{Na} and I_{Cl} were equally sensitive to their specific inhibitors (see Figs 4, 6). Furthermore, the passive paracellular characteristics of the tissue seem not to be influenced by theophylline or dbc-AMP, for neither G_l nor I_l was significantly affected.

I_{Na} stimulation was due to an increase in G_{Na} at constant E_{Na}. E_{Na} was not affected by internal theophylline or dbc-AMP, so it is possible to exclude the Na^+/K^+ pump as a primary target for cyclic-AMP-dependent stimulation of I_{Na}. Apical Na^+ and basal K^+ diffusion potentials, if generated by the Na^+/K^+-ATPase, would therefore seem to be unaffected by cyclic AMP. This conclusion is in agreement with two reports on frog skin (Yonath and Civan, 1971; Nagel, 1978), but not with others (Aceves, 1977). The apical

fractional resistance of Chinese crab gills is very large (Onken *et al.* 1991*a*) so, as in other tight epithelia, G_{Na} (which is the principal determinant of I_{Na}) mainly represents the Na^+ conductance of the apical membrane. In principle, an increase in G_{Na} can be achieved by an enhanced single-channel conductance or by an increased number of open Na^+ channels. Our results obtained with I_{sc} noise-analysis strongly favour the latter possibility, because the single-channel currents (i) are hardly changed by I_{Na} stimulation, whereas the number of Na^+ channels (M) was considerably increased in each case, approximately in proportion to the observed effect on I_{Na} (Table 1). We cannot say whether the additional channels measured after I_{Na} stimulation were recruited from intracellular vesicles which fused with the apical membrane in response to an increase in intracellular cyclic AMP concentration (Van Driessche and Erlij, 1991), or whether they were recruited from a pool of formerly 'silent' channels already present in the apical membrane (Li *et al.* 1982). Another alternative would be a major increase in the open probability of spontaneously fluctuating channels, as has been suggested for the action of cyclic AMP on A6 cell Na^+ channels, studied with the patch-clamp method (Eaton and Marunaka, 1990). So far, only blocker-induced Na^+ channel-noise can be resolved by noise analysis of complex epithelia. However, there have been parallel noise analysis and patch-clamp studies of spontaneously fluctuating amiloride-sensitive Na^+ channels in A6 cells (Eaton and Marunaka, 1990). The 'new' Na^+ channels, if treated as a single population after I_{Na} stimulation in crab gill, seem nevertheless to differ from the channel ensemble which was active before current stimulation with theophylline or dbc-AMP. The affinity of the apical pathway for uptake of Na^+ increased significantly, because K_{Na} was found to be much lower after stimulation of I_{Na}. The concomitant decrease in the affinity of the channels for amiloride (increased K_{AMI}) after I_{Na} stimulation can simply be explained on the basis of the increased affinity for Na^+, because Na^+ and amiloride have been shown to be competitors (Zeiske *et al.* 1992). The observed 50 % drop in K_{Na} would then automatically result in an approximate doubling of K_{AMI}, as was observed.

The usually clear decrease in f_c seen after addition of theophylline may be interpreted along these lines and may reflect a decreased blocking rate by amiloride because of an increased affinity of its receptor for the competing sodium ion. We cannot explain the origin of the decrease in K_{Na} after stimulation. Any alteration to the Na^+ channel resulting from cyclic AMP action could be 'translated' into a change in the single-channel kinetics of the Na^+/amiloride competition site.

The background and applicability of the amiloride noise analysis has been addressed earlier (Zeiske *et al.* 1992). The conserved linearity of the relationship between f_c and [AMI] (not shown here) in the presence of theophylline indicates the validity of the present analysis. As already mentioned, our conclusion that the cyclic-AMP-induced stimulation of I_{Na} is predominantly due to an increase in Na^+ channel density, which would be expressed as an increase in (apical) G_{Na}, relies on the extrapolation of the noise data (in the presence of amiloride) to the amiloride-free control case; this has already been discussed by Zeiske *et al.* (1992). The small change in single-channel current suggests that the variables determining i, i.e. the single-channel conductance and the net electrochemical driving force, are unlikely to be affected by cyclic AMP. At the comparably high doses of amiloride used for noise analysis, the intracellular voltage at

short circuit might be close to the basolateral membrane potential, because the apical fractional resistance will approach unity (Nagel, 1980; Nagel *et al.* 1981). If we accept constancy of E_{Na} under all conditions, then a constant single-channel current indicates that cyclic AMP has no influence on single-channel conductance. Amazingly, the increased channel affinity for Na^+ has no consequences for the conductance process at the single-channel level.

Our conclusions that changes in *M* but not in *i* are responsible for the augmented I_{Na} after cyclic AMP concentration has increased have also been reached by others who studied the effect of theophylline on frog skin (Katz and Van Driessche, 1987) or the effect of cyclic-AMP-stimulating antidiuretic hormone in different tight amphibian epithelia (Helman *et al.* 1983). These authors also observed an increased K_{AMI} after cyclic AMP treatments, but did not comment further on it.

Our results show that the cyclic-AMP-induced stimulation of I_{Cl} is accompanied by increases in both G_{Cl} and E_{Cl}. Cyclic-AMP-dependent activation of Cl^- channels is well known from various epithelial tissues (Greger *et al.* 1985; Schlatter and Greger, 1985; Katz and Van Driessche, 1987) and may also occur in crab gills. However, in view of their location in the basolateral membrane of crab gills, which is much more conductive than the apical barrier (Onken *et al.* 1991*a*), activation of Cl^- channels by intracellular cyclic AMP seems not to explain the marked G_{Cl} increase concomitant with I_{Cl} stimulation. Blockade of the channels with diphenylamine-2-carboxylate (DPC) resulted in a complete inhibition of I_{Cl}, but G_{te} was only slightly reduced (Onken *et al.* 1991*a*; see also Fig. 6). Therefore, the origin of the marked change in the cellular Cl^--dependent conductance should be located in the apical membrane. In our model for active and transcellular Cl^- uptake (see Introduction), the only apical conductance is associated with the electrogenic H^+ pump in parallel with the Cl^-/HCO_3^- antiport. A stimulation of this ATPase may result in an increase in (the apparent) G_{Cl}. Moreover, this assumption is also consistent with the observed increase in the electromotive force for active Cl^- uptake (E_{Cl}) after stimulation. An interaction between cyclic AMP and H^+-ATPase-driven transport has been investigated in several tissues. In insect epithelia, the interaction was positive, resulting in increased transport rates (Maddrell and O'Donnell, 1992). In other tissues, cyclic AMP produced ambiguous (Grinstein *et al.* 1992) or negative (Gluck and Nelson, 1992) effects on H^+ transport which were, at least in part, mediated by a V-type H^+-ATPase. The H^+-ATPase may be directly phosphorylated, as was suggested for the stimulating influence of protein kinase C on V-type H^+ pumps in human neutrophils (Nanda *et al.* 1992). An alternative is a (in the present case cyclic-AMP-dependent) modification of a cellular variable (i.e. $[H^+]$, $[Ca^{2+}]$), resulting either in the insertion of ATPase-containing vesicles into the apical membrane [as has been shown in other epithelial tissues (Stetson and Steinmetz, 1983; Schwartz and Al Awqati, 1986; Harvey, 1992)] or in the activation of existing ATPases. In this respect, the most straightforward hypothesis to explain the cyclic-AMP-dependent increase in I_{Cl} seems to be a primary stimulation of the apical Cl^-/HCO_3^- exchanger and a subsequent activation of H^+-ATPases by the resulting cellular acidification. A cyclic-AMP-dependent stimulation of Cl^-/HCO_3^- exchangers has been demonstrated in rat duodenal brush-border membranes (Dunk *et al.* 1989). However, because cyclic AMP is known to

interact with a variety of transporters, its detailed mode of stimulating the apical H^+ pump in the gill epithelium of the Chinese crab needs to be elucidated in further studies.

In summary, our results indicate that stimulation of Na^+ uptake across the posterior gills of the Chinese crab by an increased intracellular cyclic AMP concentration is achieved by an increase in the number of apical Na^+ channels, whereas stimulation of the basolateral Na^+/K^+ pump, which was suggested in former investigations with other techniques (Trausch *et al.* 1989; Bianchini and Gilles, 1990) seems to be unimportant or, at most, of minor importance. In contrast, the cyclic-AMP-induced increase in Cl^- uptake seems to be caused by a stimulation of apical H^+-ATPases, but an additional activation of basolateral Cl^- channels cannot be excluded.

In fresh water, cyclic-AMP-mediated hormonal stimulation of the transport pathways for Na^+ and Cl^- would seem to offer a number of advantages. (1) With regard to Na^+ transport, the increased density of Na^+ channels, with enhanced affinity for the substrate, will allow much more effective ion absorption from the highly dilute medium. (2) With respect to Cl^- transport, recruitment of H^+ pumps (through vesicle incorporation) will enhance transport capacity by increasing E_{Cl} and G_{Cl}, whereas a modulation of the Cl^- transporting molecule (see K_{Cl}) seems to be less important than in the case of Na^+ (see K_{Na}). (3) It could be envisaged that a fusion (supported by cyclic AMP) of transporter-containing sub-plasma-membrane vesicles supports the enhancement of I_{Na} and I_{Cl} at the same time.

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References

ACEVES, J. (1977). Sodium pump stimulation by oxytocin and cyclic AMP in the isolated epithelium of the frog skin. *Pflügers Arch.* **371**, 211–216.

ASSELBOURG, V., TRAUSCH, G., GILLES, R. AND DEVOS, P. (1991). Protein kinase C and ion transport in the posterior gills of the Chinese crab *Eriocheir sinensis*. *Mar. Biol.* **109**, 115–119.

BENOS, B. J. (1982). Amiloride: a molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* **242**, C131–C145.

BIANCHINI, A. AND GILLES, R. (1990). Cyclic AMP as a modulator of NaCl transport in gills of the euryhaline Chinese crab *Eriocheir sinensis*. *Mar. Biol.* **104**, 191–195.

BROWN, A. M., YATANI, A., VAN DONGEN, A. M. J., KIRSCH, G. E., CODINA, J. AND BIRNBAUMER, L. (1990). Networking ionic channels by G proteins. In *G Proteins and Signal Transduction* (ed. N. M. Nathanson and T. K. Harden), pp. 1–9. New York: Rockefeller University Press.

DUNK, C. R., BROWN, C. D. A. AND TURNBERG, L. A. (1989). Stimulation of Cl/HCO_3 exchange in rat duodenal brush border membrane vesicles by cyclic AMP. *Pflügers Arch.* **414**, 701–705.

EATON, D. C. AND MARUNAKA, Y. (1990). Ion channel fluctuations. 'Noise' and single-channel measurements. *Curr. Topics Membr. Transport* **37**, 61–114.

FOSKETT, J. K., HUBBARD, G. H., MACHEN, T. E. AND BERN, H. A. (1982). Effects of epinephrine, glucagon and vasoactive intestinal polypeptide on chloride secretion by teleost opercular membrane. *J. comp. Physiol.* **146**, 27–34.

GLUCK, S. AND NELSON, R. (1992). The role of the V-ATPase in renal epithelial H^+ transport. *J. exp. Biol.* **172**, 205–218.

GREGER, R. (1985). Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. *Physiol. Rev.* **65**, 760–797.

GREGER, R., SCHLATTER, E. AND GÖGELEIN, H. (1985). Cl^--channels in the apical cell membrane of the rectal gland 'induced' by cyclic AMP. *Pflügers Arch.* **403**, 446–448.

GRINSTEIN, S., NANDA, A., LUKACS, G. AND ROTHSTEIN, O. (1992). V-ATPases in phagocytic cells. *J. exp. Biol.* **172**, 179–192.

HARVEY, B. J. (1992). Energization of sodium absorption by the H^+-ATPase pump in mitochondria-rich cells of frog skin. *J. exp. Biol.* **172**, 289–309.

HELMAN, S. I., COX, T. C. AND VAN DRIESSCHE, W. (1983). Hormonal control of apical Na permeability in epithelia. Studies using fluctuation noise analysis. *J. gen. Physiol.* **82**, 201–220.

JOHNSEN, A. H. AND NIELSEN, R. (1978). Effects of antidiuretic hormone, arginine vasotocin, theophylline, fillipin and A 23187 on cyclic AMP in isolated frog skin epithelium (*R. temporaria*). *Acta physiol. scand.* **102**, 281–289.

KAMEMOTO, F. I. (1982). Crustacean neuropeptides and osmoregulation. In *Neurosecretion (Molecules, Cells, Systems)* (ed. D. S. Farner and K. Lederis), pp. 329–335. New York: Plenum Press.

KAMEMOTO, F. I. AND OYAMA, S. N. (1985). Neuroendocrine influence on effector tissues of hydromineral balance in crustaceans. In *Current Trends in Comparative Endocrinology* (ed. B. Lofts and W. N. Holmes), pp. 883–886. Hong Kong: Hong Kong University Press.

KATZ, U. AND VAN DRIESSCHE, W. (1987). Effect of theophylline on the apical sodium and chloride permeabilities of amphibian skin. *J. Physiol., Lond.* **397**, 223–236.

KLEINHOLZ, L. H. (1976). Crustacean neurosecretory hormones and physiological specifity. *Am. Zool.* **16**, 151–166.

LEVITAN, I. B. (1985). Phosphorylation of ion channels. *J. Membr. Biol.* **87**, 177–190.

LI, J. H.-Y., PALMER, L. G., EDELMAN, I. S. AND LINDEMANN, B. (1982). The role of sodium-channel density in the natriferic response of the toad urinary bladder to an antidiuretic hormone. *J. Membr. Biol.* **64**, 77–89.

LOHRMANN, D. M. AND KAMEMOTO, F. I. (1987). The effect of dibutyryl cyclic AMP on sodium uptake by isolated perfused gills of *Callinectes sapidus*. *Gen. comp. Endocr.* **65**, 300–305.

MADDRELL, S. H. P. AND O'DONNELL, M. J. (1992). Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. exp. Biol.* **172**, 417–429.

MANTEL, L. H. (1985). Neurohormonal integration of osmotic and ionic regulation. *Am. Zool.* **25**, 253–263.

MANTEL, L. H. AND FARMER, L. L. (1983). Osmotic and ionic regulation. In *The Biology of Crustacea*, vol. 5 (ed. D. E. Bliss and L. H. Mantel), pp. 54–126. London: Academic Press.

NAGEL, W. (1978). Effects of antidiuretic hormone upon electrical potential and resistance of apical and basolateral membranes of frog skin. *J. Membr. Biol.* **42**, 99–122.

NAGEL, W. (1980). Rheogenic sodium transport in a tight epithelium, the amphibian skin. *J. Physiol., Lond.* **302**, 281–295.

NAGEL, W., DURHAM, J. H. AND BRODSKY, W. A. (1981). Electrical characteristics of the apical and basal–lateral membranes in the turtle bladder epithelial cell layer. *Biochim. biophys. Acta* **646**, 77–87.

NANDA, A., GUKOVSKAYA, A., TSENG, J. AND GRINSTEIN, S. (1992). Activation of vacuolar-type proton pumps by protein kinase C. Role in neutrophil pH regulation. *J. biol. Chem.* **267**, 22740–22746.

NEER, E. J. AND CLAPHAM, D. E. (1988). Roles of G protein subunits in transmembrane signalling. *Nature* **333**, 129–134.

ONKEN, H., GRASZYNSKI, K. AND ZEISKE, W. (1991*a*). Na^+-independent electrogenic Cl^- uptake across the posterior gills of the Chinese crab (*Eriocheir sinensis*): Voltage clamp and microelectrode studies. *J. comp. Physiol.* **161**B, 293–301.

ONKEN, H., RIESTENPATT, S. AND ZEISKE, W. (1991*b*). Na^+ absorption across the gill epithelium of the Chinese crab: Characteristics of the apical Na^+ channel. *Verh. dt. zool. Ges.* **84**, 417–418.

ONKEN, H. AND SIEBERS, D. (1992). Voltage-clamp measurements on single split lamellae of posterior gills of the shore crab *Carcinus maenas*. *Mar. Biol.* **114**, 385–390.

PALMER, L. G. (1988). Regulation of NaCl transport in tight epithelia. In *Advances in Comparative and Environmental Physiology*, vol. 1 (ed. R. Greger), pp. 291–309. Berlin: Springer.

PEQUEUX, A. AND GILLES, R. (1992). Calmodulin as a modulator of NaCl transport in the posterior salt-transporting gills of the chinese crab *Eriocheir sinensis*. *Mar. Biol.* **113**, 65 – 69.

PEQUEUX, A., GILLES, R. AND MARSHALL, W. S. (1988). NaCl transport in gills and related structures. In *Advances in Comparative and Environmental Physiology*, vol. 1 (ed. R. Greger), pp. 1–73. Berlin: Springer.

PUTZENLECHNER, M., ONKEN, H., KLEIN, U. AND GRASZYNSKI, K. (1992). Electrogenic Cl^--uptake across the gill epithelium of *Eriocheir sinensis*: energized by a V-type proton-ATPase? *Verh. dt. zool. Ges.* **85**, 160.

REUSS, L., SEGAL, Y. AND ALTENBERG, G. (1991). Regulation of ion transport across gallbladder epithelium. *A. Rev. Physiol.* **53**, 361–373.

SCHLATTER, E. AND GREGER, R. (1985). Cyclic AMP increases the basolateral Cl^--conductance in the isolated perfused medullary thick ascending limb of Henle's loop of the mouse. *Pflügers Arch.* **405**, 367–376.

SCHWARTZ, G. J. AND AL-AWQATI, Q. (1986). Regulation of transepithelial H^+ transport by exocytosis and endocytosis. *A. Rev. Physiol.* **48**, 153–161.

SCHWARZ, H.-J. (1990). Elektrophysiologische Untersuchungen des transepithelialen Natrium-Transportes isolierter, halbierter Kiemenplättchen posteriorer Kiemen der Wollhandkrabbe *Eriocheir sinensis* und der Winkerkrabbe *Uca tangeri*. PhD thesis, Free University Berlin.

SCHWARZ, H.-J. AND GRASZYNSKI, K. (1989). Ion transport in crab gills: A new method using isolated half platelets of *Eriocheir* gills in an Ussing-type chamber. *Comp. Biochem. Physiol.* **92**A, 601–604.

SKOU, J. C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across the cell membrane. *Physiol. Rev.* **45**, 596–617.

SOMMER, M. J. AND MANTEL, L. H. (1988). Effect of dopamine, cyclic AMP and pericardial organs on sodium uptake and Na/K-ATPase activity in the gills of the green crab, *Carcinus maenas* (L). *J. exp. Zool.* **248**, 272–277.

STETSON, D. L. AND STEINMETZ, P. R. (1983). Role of membrane fusion in CO_2 stimulation of proton secretion by turtle bladder. *Am. J. Physiol.* **245**, C113–C120.

TRAUSCH, G., FORGET, M.-C. AND DEVOS, P. (1989). Bioamines-stimulated phosphorylation and (Na^+,K^+)-ATPase in the gills of the Chinese crab *Eriocheir sinensis*. *Comp. Biochem. Physiol.* **94**B, 487–492.

VAN DRIESSCHE, W. AND ERLIJ, D. (1991). Cyclic AMP increases electrical capacitance of apical membrane of toad urinary bladder. *Archs int. Physiol. Biochim.* **99**, 409–411.

VAN DRIESSCHE, W. AND LINDEMANN, B. (1978). Low-noise amplification of voltage and current fluctuations arising in epithelia. *Rev. scient. Instrum.* **49**, 52–57.

YONATH, J. AND CIVAN, M. M. (1971). Determination of the driving force of the Na^+ pump in toad bladder by means of vasopressin. *J. Membr. Biol.* **5**, 366–385.

ZATTA, P. (1987). Dopamine, noradrenaline and serotonin during hypo-osmotic stress of *Carcinus maenas*. *Mar. Biol.* **96**, 479–481.

ZEISKE, W., ONKEN, H., SCHWARZ, H.-J. AND GRASZYNSKI, K. (1992). Invertebrate epithelial Na^+ channels: amiloride-induced current-noise in crab gill. *Biochim. biophys. Acta* **1105**, 245–252.