ACTIVE NaCl ABSORPTION ACROSS SPLIT LAMELLAE OF POSTERIOR GILLS OF THE CHINESE CRAB Eriocheir sinensis: STIMULATION BY EYESTALK EXTRACT

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Summary

Split lamellae of the posterior gills of freshwater-adapted Chinese crabs (Eriocheir sinensis) were mounted in a modified Ussing-type chamber, and active and electrogenic absorption of Na⁺ and Cl⁻ were measured as positive (I\textsubscript{Na}) or negative (I\textsubscript{Cl}) short-circuit currents. Haemolymph-side addition of eyestalk extract stimulated I\textsubscript{Cl} by increasing both the transcellular Cl⁻ conductance and the electromotive force for Cl⁻ absorption. The effect was dose-dependent. Boiling the eyestalk extract did not change its effectiveness. The stimulating factor passed through dialysis tubing, indicating that it has a molecular mass of less than 2 kDa. RpCAMPs, a blocker of protein kinase A, reduced the stimulated I\textsubscript{Cl}. Eyestalk extract stimulated I\textsubscript{Na} by increasing the transcellular Na⁺ conductance at constant electromotive force. Amiloride-induced current-noise analysis revealed that stimulation of I\textsubscript{Na} was accompanied by an increase in the apparent number of open apical Na⁺ channels at a slightly reduced single-channel current. In addition to the electrophysiological experiments, whole gills were perfused in the presence and in the absence of putative transport stimulators, and the specific activities of the V-ATPase and the Na⁺/K⁺-ATPase were measured. Eyestalk extract, theophylline or dibutyryl-cyclic AMP stimulated the activity of the V-ATPase, whereas the activity of the Na⁺/K⁺-ATPase was unaffected. The simultaneous presence of RpCAMPs prevented the stimulation of V-ATPase by eyestalk extract or theophylline.

Key words: Crustacea, Chinese crab, Eriocheir sinensis, crab, gill, split gill lamella, eyestalk extract, theophylline, cyclic AMP, Ussing-type chamber, short-circuit current, current-noise analysis, V-ATPase, Na⁺/K⁺-ATPase.

Introduction

In fresh water, Chinese crabs (Eriocheir sinensis) maintain an outward-directed osmotic gradient of approximately 600 mosmol kg\(^{-1}\) (DeLeersnyder, 1967). The ensuing salt loss is compensated for by active absorption of NaCl across the posterior gills (for reviews, see Péqueux, 1995; Péqueux et al., 1988). The current model of the mechanisms of NaCl absorption was evaluated using split gill lamellae mounted in modified Ussing chambers (for a review, see Onken and Riestenpatt, 1998). In the presence of external Cl⁻, a negative, Cl⁻-dependent short-circuit current (I\textsubscript{Cl}) was observed. On the basis of the effects of various blockers, this current was judged to reflect active, electrogenic Na⁺-independent Cl⁻ absorption via apical Cl⁻/HCO\(_3\)⁻ antiporters and basolateral Cl⁻ channels driven by an apical V-type H⁺ pump (Onken et al., 1991; Onken and Putzenlechner, 1995). In the absence of external Cl⁻, a positive, Na⁺-dependent short-circuit current (I\textsubscript{Na}) was measured. This current probably reflects active and electrogenic Na⁺ absorption via apical Na⁺ channels and the basolateral Na⁺/K⁺-ATPase (Zeiske et al., 1992).
Moreover, the results of that study indicated that the stimulation of Na\(^+\) absorption resulted from an increase in the number of open apical Na\(^+\) channels. The increase in \(I_{C}\), however, appeared to be dependent upon the activation of apical V-ATPases. More recently, Mo et al. (1998) observed that dopamine increased the cyclic AMP concentration and the activity of the Na\(^+\)/K\(^+\)-ATPase in gill tissue and Na\(^+\) influx across perfused gills. Taken together, these studies suggest that dopamine, which is thought to originate primarily from the pericardial organ (Cooke and Sullivan, 1982), initiates a signal cascade, via cyclic AMP and protein kinase A, resulting in stimulation of the active absorption of Na\(^+\) and Cl\(^-\) across the posterior gills of the Chinese crab.

In addition to the pericardial organ, the eyestalks of Crustacea are known to be neuroendocrine centres. It is well-established that the X-organ–sinus gland complex of the eyestalks produces and liberates peptides that are involved in such diverse functions as the regulation of haemolymph glucose levels (crustacean hyperglycaemic hormone, CHH), moult (moulting-inhibiting hormone, MIH), reproduction (vitellogenesis-inhibiting hormone, VIH) and chromatophore function (red pigment concentrating hormone, RPCH, and pigment-dispersing hormone, PDH; for reviews, see Fingerman, 1987; Keller, 1992). In addition, other substances, such as proctolin (Wood et al., 1996), orcokinin (Bungart et al., 1994), FMRFamid-like peptides (Sithigorngul et al., 1998), enkephalins (Jaros et al., 1985) and biogenic amines (Rodriguez-Sosa et al., 1997; Schmidt, 1997), have been identified in the eyestalks. Biogenic amines and enkephalins were shown to act as neurotransmitters and/or modulators in the eyestalks (Sarojini et al., 1995; Kuo and Yang, 1999), and the same seems to be true of proctolin, orcokinin and FMRFamide-like peptides (Keller, 1992). However, a neurohormonal function cannot be excluded for these substances. Except for enkephalins, the above compounds have been demonstrated to act as cardioactive and/or myotropic neurohormones, although in this case they seem to be liberated from the pericardial organ or the abdominal nerve cord, respectively (Cooke and Sullivan, 1982; Keller, 1992; Saver and Wilken, 1998).

A number of studies with intact animals have clearly indicated that the eyestalks are involved in the control of osmoregulation when Crustacea enter hypo-osmotic media (for reviews, see Mantel and Farmer, 1983; Mantel, 1985; Fingerman, 1987). More recently, extracts of sinus glands have been shown to affect the transport parameters of crab gills. The transbranchial potential difference and the Na\(^+\) influxes across perfused posterior gills of the Chinese crab were increased in a dose-dependent manner by a heat-stable peptide present in a sinus gland extract and with a molecular mass exceeding 5 kDa (Pierrot et al., 1994; Eckhardt et al., 1995). The present study investigated the influence of eyestalk extract on the transport characteristics of split gill lamellae and on the activities of the V-ATPase and the Na\(^+\)/K\(^+\)-ATPase in perfused gills of Chinese crabs.

Some of these data were reported at annual meetings of the Deutsche Zoologische Gesellschaft (Schöbel et al., 1994; Putzenlechner and Graszynski, 1995) and some were included in a recent review (Onken and Riestenpatt, 1998).

**Materials and methods**

### Crabs

Chinese crabs (*Eriocheir sinensis* Milne-Edwards) were obtained from commercial fisherman. The animals were caught in the rivers Eider (Schleswig-Holstein, Germany) or Havel (Brandenburg, Germany). Before use in the laboratory, the animals were kept at 10–14°C for at least 4 weeks in running tap water (containing in mmol l\(^{-1}\): Na\(^+\), 2.0; K\(^+\), 0.08; Ca\(^{2+}\), 3.0; Cl\(^-\), 1.7). Twice a week, the crabs were fed with Ewos fish food (Bertels GmbH, Germany), frozen fish or bovine heart. The crabs were killed rapidly by destroying the ventral ganglia with a large pair of scissors. The carapace was lifted, and the three posterior gills were removed on each side.

### Electrophysiological experiments

For the electrophysiological experiments, single gill lamellae were isolated and split under a microscope (see Schwarz and Graszynski, 1989). Preparations obtained in this fashion consist of a single layer of epithelium and the adherent cuticle (Schwarz, 1990). The split gill lamellae were mounted in a modified Ussing chamber. An epithelial area of 0.01 cm\(^2\) was exposed to the chamber compartments (volume approximately 50 \(\mu\)l) bathing the external and internal sides of the tissue. Continuous perfusion of both chamber compartments with aerated saline was achieved by gravity flow (approximately 2 ml min\(^{-1}\)). All electrophysiological experiments were conducted at room temperature (20°C).

To measure the transepithelial potential difference (\(PD_{ew}\)), calomel electrodes were connected via agar bridges (3% agar in 3 mol l\(^{-1}\) KCl) to both sides of the preparation (distance to the tissue less than 1 mm). The reference electrode was in the internal bath. Silver wires coated with AgCl served as electrodes to apply current for short-circuiting the \(PD_{ew}\) (to measure the short-circuit current, \(I_{sc}\)) from an automatic clamping device (VCC 600, Physiologic Instruments, USA). The transepithelial conductance (\(G_{ew}\)) was calculated from imposed voltage pulses (\(\Delta PD_{ew}\)) and the resulting current deflections (\(\Delta I\)).

For the current-noise analysis experiments, we used a specially constructed low-noise voltage-clamp modified from the original version by Van Driessche and Lindemann (1978). Amlorile-induced fluctuations in \(I_{sc}\) 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 were obtained by adding amlorile (5 \(\mu\)mol l\(^{-1}\)) to the external bathing solution. Blocker noise was analysed using the two-state model, as described previously (Zeiske et al., 1992), and produced the Lorentzian variables (low frequency plateau in the power density spectrum) and \(f_c\) (corner frequency). Preliminary experiments showed that \(f_c\) is linearly related to...
the amiloride concentration under control conditions and after current stimulation with eyestalk extract. Therefore, the Na+ channel open probability ($P_o$) can be expressed as $P_o = I_{Na(Ami)}/I_{Na(Cutl)}$, where $I_{Na(Ami)}$ is the Na$^+$-specific $I_o$ in the presence of 5 mmol l$^{-1}$ amiloride and $I_{Na(Cutl)}$ is that in its absence. For further details and for the calculation of the single-channel current ($i$) and the channel density ($M$), see Zeiske et al. (1992).

The internal saline was a haemolymph-like NaCl saline (with or without eyestalk extract), containing in mmol l$^{-1}$: NaCl, 300; KCl, 6; NaHCO$_3$, 2; Hepes, 5; calcium gluconate, 8; glucose, 2 (pH 7.6, adjusted with Tris). The active absorption of Na$^+$ ($I_{Na}$) and Cl$^-$ ($I_{Cl}$), the corresponding transcellular conductances ($G_{Na}$ and $G_{Cl}$) and the leak conductance ($G_l$) were identified as previously described in detail (Zeiske et al., 1992; Onken, 1996): $I_{Na}$ and $G_{Na}$ were identified as the amiloride-sensitive (100 mmol l$^{-1}$) fractions of the positive $I_o$ and $G_o$, respectively, in the presence of Cl$^-$free Na$^+$-containing saline (chlorides replaced by nitrates) in the external bath. $I_{Cl}$ and $G_{Cl}$ were identified as the Cl$^-$-dependent parts of the negative $I_o$ and $G_o$, respectively, in the presence of external Na$^+$-free Cl$^-$-containing saline (Na$^+$ replaced by choline or K$^+$, respectively). $G_l$ was identified as the remaining conductance in the presence of NaCl-free saline (NaCl replaced with N-methylglucamine nitrate) plus amiloride (100 mmol l$^{-1}$) in the external bath.

**Biochemical experiments**

For the biochemical experiments, pairs of posterior gills were perfused simultaneously using two channels of a peristaltic pump (Miniplus 2, Gilson, USA). Needle tubes connected to the pump were inserted into the afferent vessels of the gills. After rinsing with NaCl saline, the needles were fastened in position with thread, closing the efferent vessels. The efferent vessels were severed close to the threads, allowing complete perfusion of the gills at a rate of 0.3 ml min$^{-1}$. One gill was perfused and bathed with haemolymph-like NaCl saline (see above) containing 4.5 mmol l$^{-1}$ mercaptoethanol. The other gill was perfused and bathed with experimental saline containing the effectors being tested (see Results). After 30–60 min of incubation, both gills were perfused for 10 min with a hypotonic saline (2 mmol l$^{-1}$ Tris, 4.5 mmol l$^{-1}$ mercaptoethanol, pH 7.6). Preliminary experiments ($N=5$) showed that this procedure decreased the cytosolic protein content. The total protein content and the activity of lactate dehydrogenase decreased by 45±6% and 67±10%, respectively (means ± S.E.M.). Total V-ATPase activity was not affected. Because of the reduction in cytosolic protein content, however, the specific activity of V-ATPase increased.

The perfused portions of the gills were homogenized with a Potter S homogenizer (Braun, Germany; 1400 revs min$^{-1}$, 25 strokes) in 2 ml of ice-cold buffer, containing 250 mmol l$^{-1}$ sucrose, 10 mmol l$^{-1}$ Hepes, 5 mmol l$^{-1}$ EDTA, 4.5 mmol l$^{-1}$ mercaptoethanol and 0.05% polyoxyethylene-10-laurylareth. The homogenates were centrifuged for 30 s at approximately 8000 g in a desk centrifuge (Centrifuge 3200, Eppendorf, Germany). The pellets were discarded. The supernatant was used to measure protein content and the activities of the V-ATPase and the Na$^+$/K$^+$-ATPase.

Protein content was measured according to the method of Bradford (1976), modified according to Sedmark and Grossberg (1977), with bovine serum albumin as the standard.

ATPase activities were measured, according to Onishi et al. (1976), by determining the amount of inorganic phosphate liberated into the homogenate. SDS activation and measurement of Na$^+$/K$^+$-ATPase activity were conducted according to Kosiol et al. (1988). In each assay, 10–20 μg of protein was used, and the Na$^+$/K$^+$-ATPase activity was calculated as the difference in the amount of inorganic phosphate liberated in the presence and the absence of 5 mmol l$^{-1}$ ouabain.

For the measurements of V-ATPase activity, 50–100 μg of protein was preincubated in a mixture of 50 mmol l$^{-1}$ Mops-Tris, 1 mmol l$^{-1}$ sodium orthovanadate (to block P-type ATPases), 1 mmol l$^{-1}$ sodium azide (to block F$_1$F$_0$-ATPase), 5 mmol l$^{-1}$ MgCl$_2$ and 0.01% polyoxyethylene-10-laurylareth (pH 8.0) at 30°C for 10 min. The reaction was initiated by adding 3 mmol l$^{-1}$ Tris-ATP. After 15 min, ATPase activity was measured as liberated phosphate (see above). V-ATPase activity was defined as the difference between the activities obtained in the presence and in the absence of 1 mmol l$^{-1}$ bafilomycin A$_1$ (Bowman et al., 1988).

**Eyestalk extract**

Eyestalks were cut off directly after Chinese crabs adapted to fresh water had been killed. If the eyestalks were not used immediately, they were stored at ~28°C. For the preparation of eyestalk extract, 10 eyestalks were thoroughly ground in a mortar containing quartz sand and 4–10 ml of haemolymph-like NaCl saline. After centrifugation (Centrifuge 3200, Eppendorf, Germany) for 2 min at approximately 8000 g, the pellet was discarded. The volume of the supernatant was increased to 50 ml with NaCl saline, resulting in a solution containing extract from two eyestalks per 10 ml of saline. Measurement of pH and osmolality showed no detectable differences from pure NaCl saline. In preliminary experiments, the effects of Ca$^{2+}$ and Mg$^{2+}$ on $I_{Cl}$ were tested. Mg$^{2+}$ had no effect. An increase in [Ca$^{2+}$] from 8 to 12 mmol l$^{-1}$ resulted in a small and slow increase in $I_{Cl}$. Increasing [Ca$^{2+}$] to higher values decreased $I_{Cl}$.

**Chemical reagents**

All reagents were of analytical grade. All substances not mentioned below were purchased from Merck. Dibutyryl-cyclic AMP, choline chloride, Tris-ATP, 2-mercaptoethanol, sodium orthovanadate and EDTA were obtained from Sigma. Hepes and theophylline were purchased from Serva. Calcium gluconate, sodium nitrate and potassium nitrate were purchased from Fluka. The R$_p$ isomer of cyclic adenosine-3’,5’-phosphorothioate (R$_p$CMP) was obtained from Biomol. Amiloride was a gift from Merck, Sharp and Dohme.
Bafilomycin was obtained from Dr K. Altendorf (University of Osnabrück, Germany) and was predissolved in dimethylsulphoxide (DMSO) to give a stock solution of 0.1 mol l\(^{-1}\). At the final concentration (0.001 %), DMSO alone had no effect on ATPase activity. The concentration of bafilomycin \(A_1\) was determined by spectrophotometry according to Bowman et al. (1988).

**Statistical analyses**

All results are given as means ± standard error of the mean (S.E.M.). At \(N\geq3\), differences between groups were tested using the paired (electrophysiological tests on the same preparations) or unpaired (biochemical measurements on gill pairs) Student’s \(t\)-test. Statistical significance was assumed for \(P<0.05\).

**Results**

**Electrophysiological measurements**

In nine split lamella preparations, \(I_{Cl}\) and \(G_{Cl}\) (for definitions see Materials and methods) were measured before and after internal application of eyestalk extract (see Fig. 1). \(I_{Cl}\) was significantly increased by eyestalk extract from \(-58±17\,\mu A\,cm\(^{-2}\) to \(-184±34\,\mu A\,cm\(^{-2}\) (\(P<0.05\)). Simultaneously, \(G_{Cl}\) was significantly increased from \(2.48±0.26\,mS\,cm\(^{-2}\) to \(3.43±0.36\,mS\,cm\(^{-2}\) (\(P<0.05\)). The electromotive force for active \(Cl^-\) absorption (\(E_{Cl} = I_{Cl}/G_{Cl}\)) was increased by eyestalk extract from \(-25±7\,mV\) to \(-52±7\,mV\) (\(P<0.05\)). The effects of eyestalk extract on active \(Cl^-\) absorption were reversible (see Fig. 1).

In three preparations, \(I_{Cl}\) was measured as a function of the concentration of eyestalk extract in the internal saline. The original eyestalk extracts (two eyestalks per 10 ml of NaCl saline) were diluted with NaCl saline to obtain different concentrations of eyestalks. In all three experiments, a stepwise increase in eyestalk content resulted in a stepwise, but saturating, increase in \(I_{Cl}\). Half-maximal \(I_{Cl}\) stimulation was achieved at \(0.43±0.06\) eyestalks per 10 ml of saline.

In a further series of experiments (\(N=5\)), the effect of eyestalk extract on \(I_{Cl}\) was observed before and after boiling the eyestalk extract twice for 10 min. After boiling, the eyestalk extract was centrifuged (to eliminate precipitated protein) and the original volume was restored with distilled water. Under control conditions, \(I_{Cl}\) was \(-85±13\,\mu A\,cm\(^{-2}\). Untreated eyestalk extract significantly increased \(I_{Cl}\) to \(-221±51\,\mu A\,cm\(^{-2}\) (\(P<0.05\)). After rinsing with NaCl saline, the \(I_{Cl}\) values returned to control levels. Subsequent addition of boiled eyestalk extract resulted in a similar stimulation of \(I_{Cl}\) to \(-221±19\,\mu A\,cm\(^{-2}\) (\(P<0.05\)).

To estimate roughly the molecular mass of the stimulating factor in the eyestalk extract, a concentrated and boiled extract (14 eyestalks in 7 ml of NaCl saline) was dialysed for 3 days at 4 °C in 50 ml of NaCl saline. According to the producer of the dialysis tubing (Sigma), it is ‘useful for separating compounds with a molecular weight of \(\leq1200\) from compounds with a molecular weight of \(>2000\)’. In three experiments, the bathing solution stimulated \(I_{Cl}\) from \(-93±18\,\mu A\,cm\(^{-2}\) to \(-429±95\,\mu A\,cm\(^{-2}\), indicating that the factor had passed through the dialysis tubing.

In three preparations, the effects of the \(R_p\) diastereomer of cyclic adenosine-3’,5’-phosphorothioate (R\(_p\)cAMPS), an inhibitor of protein kinase A (Rothermel et al., 1984), on the stimulation of \(I_{Cl}\) induced by eyestalk extract were tested. The mean control \(I_{Cl}\) of \(-30±23\,\mu A\,cm\(^{-2}\) was increased by eyestalk extract to \(-109±22\,\mu A\,cm\(^{-2}\). Addition of 45 \(\mu\)mol l\(^{-1}\) R\(_p\)cAMPS resulted in a decrease in \(I_{Cl}\) to \(-61±9\,\mu A\,cm\(^{-2}\). Similar R\(_p\)cAMPS-induced depressions of \(I_{Cl}\) were observed after stimulation of \(I_{Cl}\) with theophylline (5 mmol l\(^{-1}\); \(N=1\)) or dibutyryl-cyclic AMP (60 \(\mu\)mol l\(^{-1}\); \(N=5\)).

In six split lamella preparations, \(I_{Na}\) and \(G_{Na}\) were measured before and after internal application of eyestalk extract (see Fig. 2). \(I_{Na}\) was significantly increased by eyestalk extract from \(170±43\,\mu A\,cm\(^{-2}\) to \(288±39\,\mu A\,cm\(^{-2}\) (\(P<0.05\)). Simultaneously, \(G_{Na}\) was significantly increased from \(2.32±0.32\,mS\,cm\(^{-2}\) to \(3.92±0.35\,mS\,cm\(^{-2}\) (\(P<0.05\)). The electromotive force for active \(Na^+\) absorption (\(E_{Na} = I_{Na}/G_{Na}\)) was unaffected by eyestalk extract (71±7 mV before and 72±6 mV after addition of eyestalk extract). The effects of eyestalk extract on active and electronegative \(Na^+\) absorption were reversible (see Fig. 2). Like \(I_{Cl}\) (see above), \(I_{Na}\) could also be stimulated with boiled eyestalk extract (\(N=2\)) or by using the bathing medium of the dialysis tubing as internal perfusion medium (\(N=3\)).

In a further set of eight experiments, eyestalk extract was used to stimulate \(I_{Na}\). Before and after addition of eyestalk extract, the single-channel current (\(i\)) and the number of open channels (\(M\)) were determined using amiloride-induced current-noise analysis (see Zeiske et al., 1992; Riestenpatt et
Biochemical experiments

In the first series of experiments, 15 pairs of gills were studied. The control gills were bathed and perfused with NaCl saline for 60 min. The experimental gills were bathed and perfused for 60 min with NaCl saline containing eyestalk extract (two eyestalks per 10 ml of NaCl saline; N=5), theophylline (2.5 mmol l\(^{-1}\); N=6) or dibutyryl-cyclic AMP (500 \(\mu\)mol l\(^{-1}\); N=4). After homogenization and centrifugation (see Materials and methods), the protein content and the activities of the V-type ATPase and the Na\(^+/K\(^+\) -ATPase were measured. The results are summarized in Table 2. Neither the protein content nor the activity of the Na\(^+/K\(^+\) -ATPase was significantly influenced by eyestalk extract, theophylline or dibutyryl-cyclic AMP. However, the activity of the V-ATPase was significantly stimulated in all three cases (\(P<0.05\), unpaired Student’s t-tests). In a further series of experiments, the perfusion time was reduced to 30 min, and similar stimulations of V-ATPase activity were observed (\(P<0.05\); Table 3).

To study the effects of R\(\rho\)cAMPS on the stimulation of V-ATPase activity, a preincubation period was recommended by Rothermel et al. (1984). We chose a 30 min preincubation in the presence or absence of 12 \(\mu\)mol l\(^{-1}\) R\(\rho\)cAMPS on ice, followed by a 30 min incubation with eyestalk extract or 400

![Graph showing time-course of the short-circuit current (I\(_{sc}\)) with Cl\(^-\)-free NaNO\(_3\) saline as the external bathing medium (internal NaCl saline). The vertical current deflections are due to voltage pulses of 10 mV. The length of these deflections is directly proportional to the transepithelial conductance (G\(_{te}\)). Before and after internal (i) addition of eyestalk extract (two eyestalks per 10 ml of NaCl saline), 100 \(\mu\)mol l\(^{-1}\) amiloride was added to the external (o) medium to measure the amiloride-sensitive part of the short-circuit current (I\(_{Na}\)) and the transepithelial Na\(^+\) conductance (G\(_{Na}\)).

Fig. 2. Time-course of the short-circuit current (I\(_{sc}\)) with Cl\(^-\)-free NaNO\(_3\) saline as the external bathing medium (internal NaCl saline). The vertical current deflections are due to voltage pulses of 10 mV. The length of these deflections is directly proportional to the transepithelial conductance (G\(_{te}\)). Before and after internal (i) addition of eyestalk extract (two eyestalks per 10 ml of NaCl saline), 100 \(\mu\)mol l\(^{-1}\) amiloride was added to the external (o) medium to measure the amiloride-sensitive part of the short-circuit current (I\(_{Na}\)) and the transepithelial Na\(^+\) conductance (G\(_{Na}\)).
theophylline and in the presence or absence of 6 μmol l⁻¹ RₚcAMPS at room temperature (20 °C). The results are summarized in Table 4 and show that the treatment with RₚcAMPS prevented the stimulation of the V-ATPase activity by eyestalk extract or theophylline. In preliminary experiments, we had determined that preincubation on ice reduced the stimulation of V-ATPase activity by eyestalk extract or theophylline. However, there was still significant stimulation (P<0.05, unpaired Student’s t-tests). Moreover, we confirmed that RₚcAMPS has no effect on unstimulated ATPase activity when present during the incubation period and/or during the preincubation period (eight pairs of gills). Furthermore, no inhibitory effect of RₚcAMPS (50 μmol l⁻¹, N=6; 100 μmol l⁻¹, N=3) was observed when it was added to a homogenate of gills in which V-ATPase activity had been stimulated with theophylline.

### Discussion

**Eyestalk extract and electrogenic NaCl absorption across split gill lamellae of Chinese crabs**

In the present study, active and electrogenic absorption of Na⁺ and Cl⁻ (Iₓ and Iₐ), the respective transcellular conductances (Gₓ and Gₐ) and the most likely paracellular leak conductance (Gù) across split lamellae of the posterior gills of Chinese crabs adapted to fresh water were measured, as in a number of previous studies (Onken et al., 1991; Zeiske et al., 1992; Riestenpatt et al., 1994; Onken and Putzenlechner, 1995; Onken, 1996). Addition of eyestalk extract to the haemolymph-side medium markedly stimulated Iₓ and Iₐ (see Figs 1, 2). The magnitudes, time courses and reversibility of the current stimulations induced by eyestalk extract were very similar to those observed after internal application of membrane-permeant dibutyryl-cyclic AMP or theophylline (Riestenpatt et al., 1994), a reagent known to increase intracellular cyclic AMP levels by blocking its degradation by the phosphodiesterase (Johnson and Nielsen, 1978). The circuit analyses of the changes in Iₓ and Iₐ induced by eyestalk extract, theophylline and dibutyryl-cyclic AMP followed the same pattern: the stimulation of Iₓ was due to increases in Gₓ and Eₓ, whereas the stimulation of Iₐ was based on an increase in Gₐ at constant Eₐ (see Results and Riestenpatt et al., 1994). The leak conductance was never affected.

As in other tight epithelia, the apical fractional resistance of Chinese crab split gill lamellae was high (Onken et al., 1991, 1995). Consequently, Gₓ and Gₐ mainly reflect the respective apical conductances, and marked changes in these parameters, as observed in the presence of eyestalk extract (see Results), dibutyryl-cyclic AMP or theophylline (Riestenpatt et al., 1994), suggest modulations of the electrogenic transporters in the apical membrane. Thus, according to the transport model (see Introduction), it is likely that Na⁺ channels and the V-ATPases are affected. Since the electromotive force of an electrogenic transport can be related to the respective ATPase that supplies the driving force (see Macknight et al., 1980), the above assumption is also consistent with the finding that the stimulators of the currents increased Eₓ, but did not affect Eₐ (see Results and Riestenpatt et al., 1994).

**Eyestalk extract and amiloride-induced current-noise**

For a more detailed investigation of the changes in apical Na⁺ conductance induced by eyestalk extract, amiloride-
induced current-noise analysis was used. The background and applicability of this technique in Chinese crab split gill lamellae have previously been addressed in detail (Zeiske et al., 1992; Riestenpatt et al., 1994) and will not be discussed here. With theophylline as the stimulator of \( I_{\text{Na}} \), Riestenpatt et al. (1994) observed large increases in the number of open apical Na\(^+\) channels, whereas the single-channel current was not significantly affected. Nevertheless, in five out of seven experiments, the single-channel current was reduced by between 10 and 50\% (see Table 1 in Riestenpatt et al., 1994).

The results obtained in the present study are very similar (see Table 4). Simultaneously with the stimulation of \( I_{\text{Na}} \) by eyestalk extract (mean 2.4-fold), a large increase in the number of open channels (mean 2.9-fold) and a slight reduction in the single-channel current (mean approximately 20\%) were observed. The increase in the apparent number of open apical Na\(^+\) channels may due to opening of pre-existing channels (Li et al., 1982), to the insertion of channel-containing vesicles into the apical membrane (Van Driessche and Erlij, 1991) or to a large increase in the open probability of a constant population of spontaneously opening and closing channels (Eaton and Marunaka, 1990). The reduction in single-channel current may be due to depolarization of the transapical voltage at increased \( I_{\text{Na}} \) and \( G_{\text{Na}} \). Nevertheless, one must also consider that the ‘new’ channels may have a lower single-channel conductance than the channels that were active before stimulation. Riestenpatt et al. (1994) observed that the affinity of the apical channel population for Na\(^+\) and amiloride was changed after stimulation of \( I_{\text{Na}} \) with theophylline, indicating that the channels recruited/modulated by increasing cellular cyclic AMP concentration differ from those that were active before \( I_{\text{Na}} \) stimulation. The decrease in \( f_c \), observed after stimulation of \( I_{\text{Na}} \) with eyestalk extract (see Table 1) or theophylline, may reflect a decreased blocking rate by amiloride at an increased affinity for the competing Na\(^+\) (Riestenpatt et al., 1994).

**Eyestalk extract and ATPase activities**

To study the involvement of the basolateral Na\(^+\)/K\(^+\)-ATPase and the apical V-ATPase in the stimulating effect of eyestalk extract, theophylline and dibutyryl-cyclic AMP in greater detail, the specific activities of the two ATPases were studied in homogenates of gills that had been perfused in the presence or in the absence of transport stimulators. As suggested by the electrophysiological data (see above), perfusion of the gills with transport stimulators resulted in marked increases in the activity of the V-ATPase, whereas the activity of the Na\(^+\)/K\(^+\)-ATPase was not affected (see Table 2, 3). It has been shown that apical V-ATPases can be modulated in various ways (Merzendorfer et al., 1997). For the apical V-ATPase of the insect Malpighian tubule, which drives fluid secretion across this epithelium, a cyclic-AMP- and cyclic-GMP-dependent stimulation has been demonstrated (O’Donnell et al., 1996). With respect to the basolateral Na\(^+\)/K\(^+\)-ATPase, our findings seem to contradict the results of Mo et al. (1998). These authors observed that dopamine and dibutyryl-cyclic AMP stimulated Na\(^+\) influx across perfused posterior gills of Chinese crabs. Since perfusion with dopamine also generated increases in intracellular cyclic AMP concentration and Na\(^+\)/K\(^+\)-ATPase activity, it seemed that a cyclic-AMP-dependent activation of the Na\(^+\)/K\(^+\)-ATPase made at least some contribution to the stimulation of active Na\(^+\) absorption. However, cyclic AMP had both inhibitory and stimulatory effects on the Na\(^+\)/K\(^+\)-ATPase of other epithelial tissues. More recently, Cheng et al. (1999) showed that cellular [Ca\(^{2+}\)] modulates the influence of protein kinase A on the Na\(^+\)/K\(^+\)-ATPase. Thus, it may well be that dopamine stimulates active NaCl absorption across Chinese crab gills by activating the basolateral Na\(^+\)/K\(^+\)-ATPase through an increase in cellular cyclic AMP concentration and in the concentration of another cellular factor (e.g. Ca\(^{2+}\)). However, with eyestalk extract and with an augmentation of cellular cyclic AMP level only, we obviously observed a different mode of stimulation.

**The signal transduction pathway activated by eyestalk extract**

The many similarities between the effects of eyestalk extract, theophylline and dibutyryl-cyclic AMP (see Results and Riestenpatt et al., 1994) suggest that the influence of the stimulating factor in the eyestalk extract is mediated by an increase in cellular cyclic AMP concentration. Using Rp-cAMPS, this has been verified for the stimulation of \( I_{\text{Cl}} \) and V-ATPase activity. This inhibitor of protein kinase A (Rothermel et al., 1984) inhibited the stimulated \( I_{\text{Cl}} \) across split
gill lamellae and prevented the stimulation of V-ATPase activity in perfused gills (see Table 4).

The stimulating factor in the eyestalk extract

The stimulating effect of eyestalk extract on $I_{Cl}$ clearly became saturated with increasing concentration, indicating that the extract contains a factor that specifically influences the transport characteristics of the tissue. During the preparation of eyestalk extract, care was taken that neither the pH nor the osmolality of the saline was changed. In preliminary experiments, it was verified that changes in the concentrations of $Ca^{2+}$ or $Mg^{2+}$ could be excluded as stimulators of active NaCl absorption (see Materials and methods). Boiled eyestalk extract still increased $I_{Cl}$ and $I_{Na}$, indicating that the stimulatory factor is not heat-sensitive. After dialysing the eyestalk extract, the stimulatory factor appeared in the bathing medium of the dialysis tubing. According to the specification of the tubing, the stimulatory factor should therefore have a molecular mass of less than 2 kDa. This finding suggests that peptides of the CHH/MH/VH-family with over 70 amino acid residues (see Keller, 1992) are unlikely to be responsible for the transport-stimulating effects of the eyestalk extract. On the basis of the results of the present study, further assumptions about the identity of the transport-stimulating factor in the eyestalks seem hardly useful. Although most of these substances are thought to act only as neurotransmitters/neuromodulators in the eyestalks, all identified smaller peptides (see Introduction) and a number of biogenic amines are possible candidates for the active component of the extract. An as yet undetected hormonal compound may even be responsible for the observed effects. Consequently, identification of the factor in the eyestalks that stimulates electrogenic NaCl absorption across split gill lamellae of Chinese crab gills by increasing the apical Na$^+$ conductance and by activating the apical V-ATPase activity must await future studies.

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References


