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Dopaminergic regulation of ion transport in gills of the euryhaline semiterrestrial crab *Chasmagnathus granulatus*: interaction between D1- and D2-like receptors

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Summary

The effects of dopamine (DA) and dopaminergic agonists and antagonists on ion transport were studied in isolated perfused gills of the crab *Chasmagnathus granulatus*. DA applied under steady state conditions (perfusion with hemolymph-like saline) produced a transient increase of the transepithelial potential difference (V_{te}) from 2.2±0.2 to 4.8±0.3 mV, describing an initial cAMP-dependent stimulating phase followed by an inhibitory phase. Spiperone and domperidone (antagonists of D2-like DA receptors in vertebrates) completely blocked the response to DA, while the D1-like antagonist SCH23390 blocked only the inhibitory phase. Theophylline (phosphodiesterase inhibitor) and okadaic acid (protein phosphatases PP1 and PP2A inhibitor) were also able to block the inhibitory phase, suggesting that it depends on adenylyl cyclase inhibition and on protein phosphatases. When the gills were perfused with hypo-osmotic solution, or with the adenylyl cyclase activator forskolin, V_{te} was increased several-fold. DA applied under these stimulated conditions partially reversed the V_{te} increase by 54% and 25%, respectively. Similarly, the D1-like agonist, fenoldopam, produced a 33% reduction in the stimulated V_{te}. We propose that, in *C. granulatus* gills, DA stimulates adenylyl cyclase and therefore ion transport through D1-like receptors linked to a Gs protein, although they respond to antagonists that interact with D2-like receptors in vertebrates. The inhibitory phase seems to be mediated by D2-like receptors linked to a Gi/o protein, which inhibits adenylyl cyclase, although these receptors can be activated or blocked by agonists or antagonists that interact with D1-like receptors in vertebrates and insects.

Key words: dopamine receptor dopamine agonist, dopamine antagonist, cAMP, Na^+/K^+-ATPase, okadaic acid, transepithelial potential differences, crab, *Chasmagnathus granulatus*.

Introduction

Active ion absorption through the posterior gills of euryhaline crabs is regulated by bioamines and peptidic factors secreted by neuroendocrine organs such as pericardial organs (PO), thoracic ganglion (ThG) and sinus glands (Eckhardt et al., 1995; Kamemoto and Oyama, 1985; Mantel, 1985; Onken et al., 2000; Spanings-Pierrot et al., 2000; among others). In early works, PO extract, dopamine (DA), serotonin, octopamine and hemolymph from seawater acclimated crabs have been injected into whole animals or tested in isolated perfused gills of the blue crab *Callinectes sapidus* and the Chinese crab *Eriocheir sinensis*, resulting in increased Na^+ influx and intracellular cyclic adenosine 3′-5′ monophospate (cAMP) content (Kamemoto, and Oyama, 1985; Lohrmann and Kamemoto, 1987; Sommer and Mantel, 1988; Sommer and Mantel, 1991; Bianchini and Gilles, 1990). The activity of gill Na^+/K^+-ATPase was also tested after injection of hemolymph from 30% seawater crabs (Savage and Robinson, 1983), injection of DA and PO extract (Sommer and Mantel, 1988), injection of DA or dibutyryl-cAMP (Morris and Edwards, 1995) and after incubation of gills with cAMP (Mo et al., 1998). All of these experiments have led to short-term

activation of Na^+/K^+-ATPase, and it was concluded that DA activates Na^+/K^+-ATPase in a cAMP-dependent fashion, with the final result of increasing the rate of active Na^+ uptake across the gills of euryhaline aquatic crabs experiencing hypo-osmotic stress (reviewed by Morris, 2001). Moreover, an increase in the DA content of hemolymph and gills of crabs transferred from saltwater to dilute medium has been shown (Zatta, 1987), suggesting that the DA-cAMP-Na^+/K^+-ATPase mechanism is relevant during *in vivo*, physiological conditions.

In addition, cAMP induces an increase in the short circuit current (I_{sc}) in split gill lamellae of the Chinese crab mounted in an Ussing chamber (Riestenpatt et al., 1994). However, Riestenpatt et al. concluded that the effect was not mediated *via* modulation of Na^+/K^+-ATPase activity, but that it was instead mostly due to an increase in apical conductivity for Na^+ and Cl^-, together with an increase in the electromotive force for Cl^- entry. Although the reasons for the discrepancies regarding the Na^+/K^+-ATPase modulation by cAMP are not clear, this reveals that the regulation of ion transport by DA in crab gills is a complex mechanism that affects several ion-transporting proteins.

Chasmagnathus granulatus (Dana 1851) is a hyper-hypo-regulating crab inhabiting estuarine environments of Brazil, Uruguay and Argentina (Boschi, 1964; Charmantier et al., 2002). It is frequently exposed to dilute seawater as well as to rain pools, and is able to compensate the salt loss by active and electrogenic ion absorption through the posterior gills, with the Na^+/K^+-ATPase being the main driving force (Luquet et al., 2002; Onken et al., 2003). Recent work from our laboratory (Halperin et al., 2004) demonstrated that DA produces a transient increase of the transepithelial potential difference (V_{te}) in isolated perfused posterior gills of this species, with maximal effect dose between 10 and 50 μmol l^{-1}. This response is mediated by the cAMP-protein kinase A (PKA) pathway since it can be blocked by the PKA specific inhibitor KT5720, and is accompanied by a transient increase in Na^+/K^+-ATPase activity. These results and our preliminary data suggest that DA modulates ion transport activity in the gills of *C. granulatus* through a complex mechanism, which involves a stimulatory phase mediated by cAMP-PKA and a rapid deactivation phase that brings both V_{te} and Na^+/K^+-ATPase to resting values. These two regulatory phases could be explained by the interaction of two DA receptors linked to different G proteins, such as the D1 and D2 subtypes, extensively reported to modulate Na^+/K^+-ATPase activity in vertebrate tissues (for reviews, see Missale et al., 1998; Therien and Blostein, 2000) and already pharmacologically detected in crustacean tissues (Kuo et al., 1995; Wilkens et al., 1996; Fingerman, 1997).

In vertebrates, D1-like receptors have been defined as those stimulating adenylyl cyclase upon stimulation with DA, while D2-like DA receptors are those that inhibit adenylyl cyclase (Missale et al., 1998). Recently, three groups of invertebrate DA receptors have been characterized, particularly in insects and in *Caenorhabditis elegans*. Two of these groups respond as D1-like, since they increase intracellular cAMP when stimulated with DA (Mustard et al., 2005). In particular, Trausch et al. have been able to block DA effects in the gills of the Chinese crab with the DA antagonist chlorpromazine (Trausch et al., 1989) and Morris and Edwards inhibited gill Na^+/K^+-ATPase activity in the amphibious crab *Leptograpsus variegatus* by injecting the DA antagonist butaclamol hydrochloride (Morris and Edwards, 1995). More recently, Mo et al. pharmacologically identified D1-like receptors in the gills of the *E. sinensis* (Mo et al., 2002). However, to our knowledge, little is known about the physiological effects mediated by the different subtypes of DA receptors in crab gills. The coupling of these receptors with intracellular signal transduction pathways also remains unexplored.

Our objective was to study the effects of DA on ion absorption (estimated from V_{te}) in isolated gills of *C. granulatus* subjected to different physiological conditions. To achieve a stimulated state of ion transport, gills were perfused with a hypo-osmotic solution, known to produce an increase in the V_{te}, I_{sc} and Na^+/K^+-ATPase activity (Tresguerres et al., 2003), or with solutions containing cAMP agonists, which increase V_{te}, Na^+ influx and Na^+/K^+-ATPase in isolated gills (Halperin et al., 2004). We also investigated the signal transduction pathways involved in the V_{te} response to DA, as well as the effects of agonists and antagonists of D1 and D2 dopamine receptor groups. Our results suggest that DA can either stimulate or inhibit ion uptake across *C. granulatus* gills depending on the gill physiological condition, and that at least part of this modulation takes place through an interaction between D1-like and D2-like receptors, the cAMP-PKA pathway and protein phosphatases.

Materials and methods

Animals

Adult male crabs *Chasmagnathus granulatus* (Dana 1851) in intermolt stage C (Drach and Tchernigovtzeff, 1967) were collected at Punta Rasa, San Clemente del Tuyú, Argentina. Animals were kept in plastic containers with aerated artificial brackish water of 10‰ salinity, fed twice a week with pellets of rabbit food and kept at room temperature of 20±1°C under a 12 h:12 h L:D photoperiod. Crabs of 30–33 mm carapace width were chosen for the study.

Gill perfusion

Crabs were sacrificed by destroying the nervous system with large scissors. After removing the dorsal carapace, gills number 6 (the largest among posterior gills) were gently excised and prepared according to Siebers et al. (Siebers et al., 1985). The afferent and efferent vessels were connected by fine polyethylene tubing (∅=0.4 mm) to a peristaltic pump (afferent) and to a collecting tube (efferent). The tubing was held in position by an acrylic clamp covered with smooth neoprene to minimize gill damage. The preparation was bathed in a beaker containing 25 ml of aerated saline and was perfused at a rate of 0.1 ml min^{-1}. Under these conditions the preparations remained viable for several hours; gills not

Table 1. *Composition of the saline solutions used in the experiments*

Perfusion saline	Salt concentration (mmol l^{-1})						Osmolarity (mOsm l^{-1})
	NaCl	KCl	$MgCl_2$	$CaCl_2$	Hepes	$NaHCO_3$	
20‰	312.00	6.30	4.66	8.35	5.00	7.00	698.60
30‰	468.00	9.46	7.50	12.53	5.00	7.00	1045.00

Salines were adjusted with Tris base to the physiological pH of *Chasmagnathus granulatus*, 7.75 (Luquet and Ansaldo, 1997). Perfusates also included 2 mmol l^{-1} glucose.

showing stable V_{te} after the usual stabilization period were discarded.

Transepithelial potential difference (V_{te})

Ag/AgCl electrodes were connected *via* agar bridges to the external bath and to the collecting tube (internal side). Potential differences (outside–inside) were recorded using a millivoltmeter and a chart recorder.

Effects of dopamine and dopaminergic effectors on V_{te}

Gill perfusions were performed in symmetrical conditions with isosmotic 30‰ saline solution (Table 1) until V_{te} was stabilized (about 30 min). This perfusion protocol led to a low V_{te} value, which was considered as reflecting the 'steady state' conditions. Then, the drugs and drug combinations corresponding to each experiment were applied dissolved in the perfusion saline at the final concentrations detailed below. DMSO (0.05–0.1%) or ethanol (0.6%), which were used as vehicles for some drugs, were previously tested alone and did not affect V_{te}. In some experiments, the perfusion saline was changed to a hypo-osmotic saline (20‰), which resembles the hemolymph composition of crabs acclimated to oligohaline medium (Charmantier et al., 2002; Tresguerres et al., 2003) to reach 'stimulated' conditions before applying the specific treatment.

Chemicals

Dopamine (10 or 50 μmol l^{-1}) was a gift from C. J. Calvete, Fabra, Argentina. Okadaic acid ($C_{44}H_{67}O_{13}Na$, 60 nmol l^{-1}, predissolved in ethanol) was a gift from Alomone Labs, Jerusalem, Israel; NaCl, KCl, $CaCl_2$, $MgCl_2$ and glucose were purchased from Merck (Buenos Aires, Argentina). $NaHCO_3$, and EDTA were from Mallinckrodt (New York, NY, USA). Hepes, forskolin (10 μmol l^{-1}, predissolved in DMSO), SCH23390 (10 μmol l^{-1}, predissolved in distilled water), fenoldopam (15 μmol l^{-1}, predissolved in distilled water), and spiperone (50 μmol l^{-1}, predissolved in DMSO) were obtained from Sigma (St Louis, MO, USA). Domperidone (10 μmol l^{-1}, predissolved in DMSO), ethanol and DMSO were purchased from ICN Biomedical Inc. (Irvine, CA, USA), Sintorgan (Buenos Aires, Argentina), and Carlo Erba (Milan, Italy) respectively. Theophylline (2.5 mmol l^{-1}) and Tris were from Serva (Heidelberg, Germany).

Statistics

Data were analyzed by repeated-measures analysis of variance (RM-ANOVA), followed by Newman–Keuls multiple comparisons. All data are presented as mean ± standard error of mean (s.e.m.). Differences were considered significant at $P<0.05$.

Results

Effects of dopamine on V_{te}

In gills perfused in steady state conditions (saline 30‰), the addition of 10 μmol l^{-1} DA to the perfusate produced an initial increase of about 120% in V_{te}. This effect was spontaneously reversed and V_{te} returned to control values between 40 and 60 min after the start of the treatment (Fig. 1A,B). Similar effects were seen when gills were perfused with 50 μmol l^{-1} DA (data not shown).

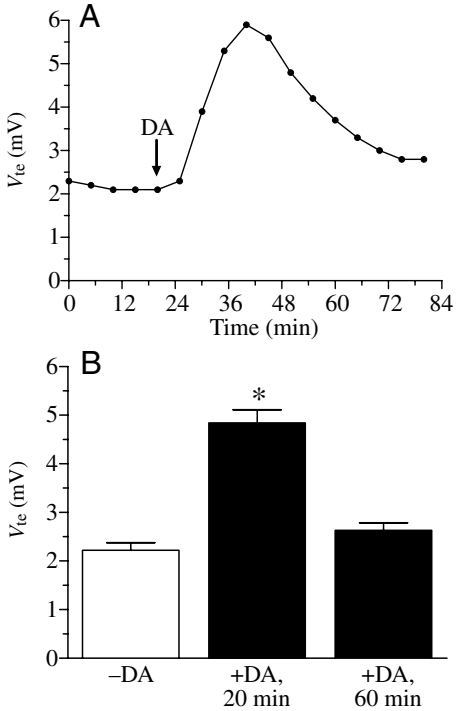

Fig. 1. Effect of the addition of 10 μmol l^{-1} dopamine (DA) to 30‰ saline solution in isolated perfused posterior gills of *Chasmagnathus granulatus*. (A) Time course of a representative experiment. (B) Means plot. Dopamine significantly induces transepithelial potential differences (V_{te}) (*$P<0.01$; $N=5$). After 40–60 min of dopamine application, V_{te} becomes not significantly different from the control value.

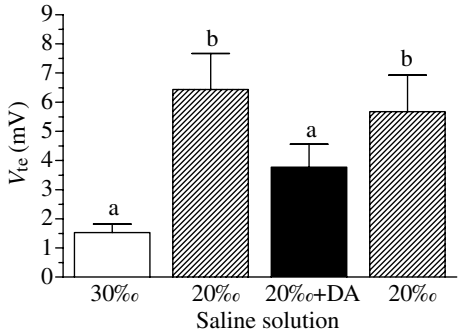

Fig. 2. Transepithelial potential differences (V_{te}) of posterior gills of *Chasmagnathus granulatus*. Gills were initially perfused with 30‰ saline and then perfused with 20‰ saline (hypo-osmotic shock), means were calculated 30 and 90–100 min after the beginning of the experiment. Then, 50 μmol l^{-1} dopamine (DA) was added to the perfusion saline and reversibly reduced the stimulatory effect of hypo-osmotic perfusion. In these cases means were calculated 150–160 and 210–230 min after the beginning of the experiment. Significant differences are noted by different letters (a and b) (P<0.05; N=7).

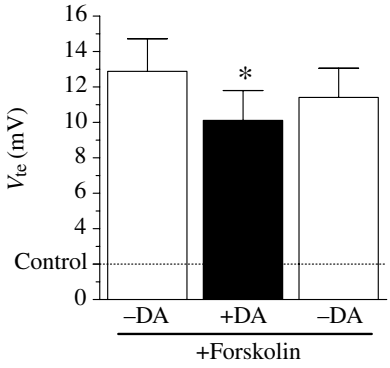

Fig. 3. Effect of DA on posterior gills previously stimulated with the adenylyl cyclase activator forskolin (10 μmol l^{-1}); mean was calculated 40–60 min after the beginning of the experiment. Perfusion with 10 μmol l^{-1} DA did not produce consistent effects; 50 μmol l^{-1} partially reversed the stimulating effect of forskolin on V_{te} (P<0.05; N=7, mean was obtained 80–100 min after the beginning of the experiment). Dotted line represents mean control value obtained after 20 min of perfusion with 30‰ saline. Asterisk, significantly different from –DA values.

When the perfusion saline was changed to hypo-osmotic 20‰ saline, V_{te} was significantly hyper-polarized from 1.5±0.29 mV to 6.4±1.2 mV, bath-positive (stimulated conditions). This effect lasted for as long as the hypo-osmotic saline was applied, in accordance with Tresguerres et al. (Tresguerres et al., 2003). Under these conditions, perfusion with 10 μmol l^{-1} DA produced inconsistent effects on V_{te}. Increasing the DA concentration to 50 μmol l^{-1} caused a reduction of 54% of the stimulation caused by the hypo-osmotic medium. Wash-out with fresh hypo-osmotic saline reversed the effect of DA (Fig. 2).

In a similar experiment, perfusion with the adenylyl cyclase activator forskolin pre-dissolved in DMSO (Laurenza et al., 1989) produced a marked increase of V_{te} from 2.0±0.23 mV to 12.89±1.84 mV. Addition of 50 μmol l^{-1} DA to the perfusion saline reversed the stimulatory effect of forskolin by 25% (Fig. 3). Removing DA produced a recovery of the stimulated V_{te}.

Role of protein phosphatases

Okadaic acid (OA) is a specific, potent and cell permeant inhibitor of PP1 and PP2A protein phosphatases (Cohen et al., 1990). Pre-treatment of perfused gills with 60 nmol l^{-1} OA did not affect the steady state V_{te}. DA applied together with OA caused a significantly more pronounced and prolonged stimulation than DA alone. Furthermore, the presence of OA almost completely blocked the second (inhibitory) phase of the DA effect (Fig. 4).

Effect of theophylline

This experiment was designed to test whether the inhibitory phase of DA can be prevented by maintaining high intracellular concentrations of cAMP. Theophylline has been used as an effective inhibitor of phosphodiesterase in crab gill preparations (Onken et al., 2000; Tresguerres et al., 2003). When this drug was applied to perfused gills of *C. granulatus* it produced a slight increase in $V_{te.}$ Addition of DA in the presence of theophylline produced a further hyperpolarization, which was stable for at least 60 min and remained stable at the same level even when DA was removed. Applying DA after removing theophylline rapidly reduced V_{te} to control values, showing that the DA transduction system was intact (Fig. 5).

Dopamine receptors

To test whether the stimulatory and inhibitory effects of DA were executed by binding of this bioamine to two different receptors, we applied agonists and antagonists of the DA receptor groups 1 and 2 (D1 and D2). The D1 antagonist SCH23390 (Iorio et al., 1983) was applied at a final

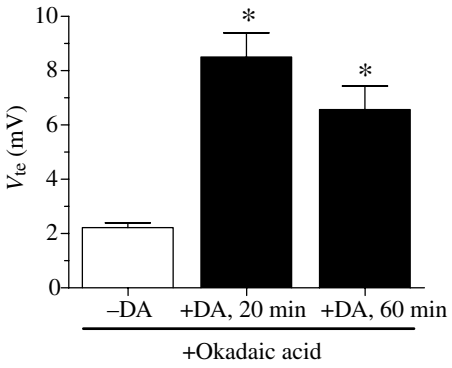

Fig. 4. Effects of 10 μmol l^{-1} dopamine (DA) on transepithelial potential differences (V_{te}) of posterior gills of *Chasmagnathus granulatus,* after pretreatment with the protein phosphatase inhibitor, okadaic acid (60 nmol l^{-1}). V_{te} is strongly and permanently stimulated. (*P<0.001; N=5).

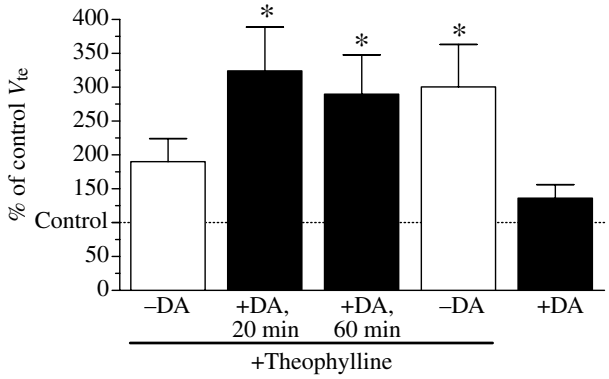

Fig. 5. Effects of 10 μmol l^{-1} dopamine (DA) on transepithelial potential differences (V_{te}), of posterior gills of *Chasmagnathus granulatus* previously perfused with the phosphodiesterase inhibitor theophylline (T), as a percentage of control values (30‰ saline perfusate). Gills were perfused with 30‰ saline solution plus 2.5 mmol l^{-1} T, then 10 μmol l^{-1} DA was added. After stabilization, DA was withdrawn. Finally DA was applied alone (*P<0.05; N=6).

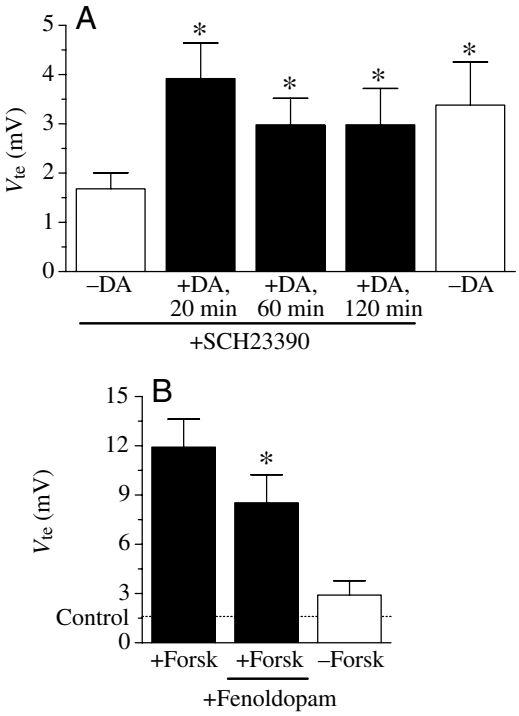

Fig. 6. Effects of D1-like dopamine (DA) receptor agonists and antagonists on transepithelial potential differences (V_{te}) of posterior gills of *Chasmagnathus granulatus*. (A) 10 μmol l^{-1} DA applied after pretreatment with the D1 antagonist, SCH23390 (10 μmol l^{-1}) produced the typical stimulatory phase, increasing V_{te} (*P<0.05; N=5), but not the subsequent inhibitory phase observed in control conditions. (B) Addition of the D1 agonist fenoldopam (15 μmol l^{-1}) to posterior gills previously stimulated with the adenylyl cyclase activator forskolin (Forsk; 10 μmol l^{-1}) produced a partial reversion of the forskolin-induced increase of V_{te} (*P<0.05; N=6; mean was obtained 100–120 min after the beginning of the experiment). Dotted line represents mean control value obtained after 20 min of perfusion with 30‰ saline.

concentration of 10 μmol l^{-1} before and during perfusion with DA, under steady state conditions. The stimulatory phase was not affected while the inhibitory phase was almost totally blocked (Fig. 6A). Addition of 15 μmol l^{-1} of the D1 agonist fenoldopam (Hussain and Lokhandwala, 1997) reversed the stimulating effect of forskolin by 33% (Fig. 6B). The effect of fenoldopam on forskolin-stimulated gills was very similar to the effect previously obtained by perfusing DA in the same conditions (Fig. 3).

Finally, two D2 receptor antagonists, spiperone (50 μmol l^{-1}) and domperidone (10 μmol l^{-1}) (Kuo et al., 1995; Rodriguez et al., 2002), added before and during DA perfusion, completely blocked the DA effects. No DA-dependent stimulation was seen after applying either of these antagonists. As a positive control, at the end of the experiments the drugs were washed out with fresh saline solution; then if the gills had been previously treated with domperidone, DA added alone produced the stimulatory effect described in previous experiments. On the other hand, the effect of spiperone was irreversible, since no further effect of DA could be observed after washing out the antagonist (Fig. 7A,B; refer to Fig. 1).

Discussion

Dopaminergic regulation of gill ion transport has been studied in many euryhaline crab species with different respiratory habits. The general trend, with the exception of the fully terrestrial species, is that DA stimulates Na^+/K^+-ATPase and other ion-transporting proteins through an increase in the intracellular cAMP (Morris, 2001).

A recent paper of our laboratory (Halperin et al., 2004) shows that in *C. granulatus*, DA produces a transient increase in the transepithelial potential difference (V_{te}) and in the rate of ^{22}Na uptake in isolated gills of this species perfused with a 30‰ saline solution. Furthermore, our results suggest that the effects are mediated by a cAMP-PKA-dependent stimulation of Na^+/K^+-ATPase. We have also established that perfusion of *C. granulatus* gills with hypo-osmotic saline solution (20‰) strongly stimulates V_{te} and short-circuit current, at least in part through a cAMP-dependent mechanism that involves increased Na^+/K^+-ATPase activity (Tresguerres et al., 2003).

Since the response to both DA and hypo-osmotic perfusion seems to affect the same cellular components and to be mediated by similar cell signaling events, we studied the interaction between both stimuli. For this purpose we have defined 'steady state conditions' when the gills are perfused with 30‰ saline solution, which is isosmotic to the hemolymph of estuarine and seawater acclimated crabs, and 'stimulated conditions' when the gills are perfused with a hypo-osmotic saline (20‰), similar to the hemolymph of crabs acclimated to oligohaline medium. The steady state conditions are characterized by a low rate of adenylyl cyclase activity, since the addition of the phosphodiesterase inhibitors IBMX

and theophylline produces much lower stimulation than perfusion with hypo-osmotic solution or with the adenylyl cyclase activator forskolin (Tresguerres et al., 2003; Halperin et al., 2004; present study).

DA applied to posterior gills of *C. granulatus* under steady state conditions causes a transient stimulation of V_{te}, as previously reported (Halperin et al., 2004). In contrast, upon stimulation by either hypo-osmotic perfusion or by the addition of forskolin, DA reduces V_{te}. This suggests that the inhibitory phase of DA effect occurs through an active component, which antagonizes the effects of other cAMP-mediated stimuli, and not merely through receptor saturation or deactivation. The blockade of this phase by the phosphodiesterase inhibitor theophylline (see Fig. 5) suggests that DA acts upstream of PKA, likely by inhibiting adenylyl cyclase. However, direct measurements of cAMP levels are needed to support this idea.

The role of protein phosphatases (PPs) in modulation of Na^+/K^+-ATPase has not yet been reported in crustacean gills. In contrast, there are many reports for other ion transporting epithelia like renal tubules of mammals. In these cases the PPs are known to dephosphorylate the Na^+/K^+-ATPase, regulating pump activity (Ewart and Klip, 1995). Regulation of these enzymes by DA is also known to exist in mammalian kidneys e.g. through the regulatory protein DARP-32 (Bertorello and Katz, 1995; Therien and Blostein, 2000). Our results support the involvement of PPs in the regulation of Na^+/K^+-ATPase by DA in *C. granulatus* gills, since their inhibition with okadaic acid blocks the inhibitory phase of the DA effect. Furthermore, since okadaic acid also reinforces the stimulating effect of DA, we can speculate that the PPs are already active under steady state conditions. In order to confirm this model, further experiments testing a possible direct relationship between DA on PPs activity are necessary.

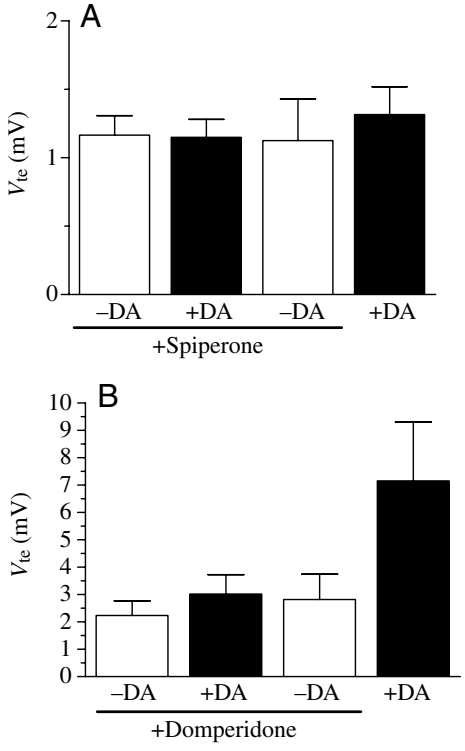

Fig. 7. Effects of D2-like dopamine (DA) antagonists, spiperone (A) (10 μmol l^{-1}; N=6) and domperidone (B) (50 μmol l^{-1}; N=6) on transepithelial potential differences (V_{te}) of posterior gills of *Chasmagnathus granulatus*. Mean control values (antagonist alone), were obtained 40–50 min after the beginning of the experiment. Both antagonists completely blocked the DA (10 μmol l^{-1}) stimulating effect; means were calculated 60–80 min after the beginning of the experiment. Only when domperidone was used was the blockade reversible.

There are many reports on dopaminergic regulation of Na^+/K^+-ATPase activity mediated by protein kinases in different vertebrate tissues. Phosphorylation of Na^+/K^+-ATPase on residues specific for PKA or protein kinase C (PKC) stimulates or inhibits the enzyme's activity in a tissue specific fashion (Therien and Blostein, 2000). Although no study has proven direct phosphorylation of crab gill Na^+/K^+-ATPase, two reports give indirect support to this possibility. Trausch et al. have reported that cAMP, DA and serotonin induce the phosphorylation of proteins collected in the cell fraction expected to contain most of the Na^+/K^+-ATPase activity (Trausch et al., 1989). In the same study, the induced phosphorylation was correlated to increased Na^+/K^+-ATPase activity in db-cAMP perfused gills of *E. sinensis*. In addition, Towle et al. identified one putative site for PKA and several putative sites for PKC phosphorylation in the sequence of *Callinectes sapidus* Na^+/K^+-ATPase α-subunit (Towle et al., 2001). These phosphorylation sites are probably involved in modulating the enzyme activity and/or its targeting to the cell membrane.

Using several of the D1 and D2 effectors most commonly used in vertebrate studies, we have been able to mimic or block both the stimulating and the inhibiting effects of DA, thus supporting the existence of different DA receptor subtypes in the gills of *C. granulatus.* However, the effects of these drugs are clearly different from the pattern described in the literature (Bertorello and Katz, 1995; Missale et al., 1998; Therien and Blostein, 2000; Asghar et al., 2001; Mustard et al., 2005). In the gills of *C. granulatus*, domperidone and spiperone, which are known D2 antagonists in vertebrates, seem to function as D1-like antagonists, since they produce the same effect as the one produced by the PKA inhibitor KT5720, a total blockade of the DA-induced stimulation of V_{te}. Accordingly, Trausch et al. have inhibited the DA induced, PKA-dependent phosphorylation of proteins by adding chlorpromazine another vertebrate D2 receptor antagonist (Trausch et al., 1989). However, these authors have not discussed their results in terms of receptor subtypes. It must be taken into account that in the few DA receptors characterized in invertebrates, particularly in insects, spiperone is effective to inhibit the D1-like response at μmolar concentrations, as in the results of the present paper, whereas the effectiveness of this drug has been tested in only one D2-like invertebrate DA receptor, with weaker effects (Mustard et al., 2005). On the other hand, domperidone is also able to block the D1-like response in *C.*

Table 2. *Effect of different dopamine effectors on vertebrate and invertebrate receptors*

	Antagonists		Agonists	
Receptor type	SCH23390	Spiperone	Dopamine	Fenoldopam
Mammalian D1-like[1]	++++	+/+/–	+/–/+	+++
Mammalian D2-like[1]	+/–	++++/+++	++	++/+
Invertebrate D1-like				
AmDOP1[2]	+	+	+	Nm
CeDOP1[2]	Agonist	Nm	+	Nm
DmDOP1[2]	+	+	+	Nm
AmDOP2[2]	+/–	+	+	Nm
DAMB[2]	+	+/–	+	Nm
CgDR1[3]	–	+	+	Nm
Invertebrate D2-like				
CeDOP2[2]	+	+/–	+/–	Nm
DmDR2[2]	Nm	–	+	Nm
CgDR2[3]	+	Nm	+/–	+

Symbols for quali-quantitative comparison have been assigned from data reviewed by [1](Missale et al., 1998) and [2](Mustard et al., 2005); maximum effectiveness (++++), no effect (–), Nm=not measured.

AmDOP1 and AmDOP2, D1-like receptors of *Apis melifica*; DmDOP1 and DAMB, *Drosophila melanogaster*; CeDOP1, D1-like receptors of *Coenorhabditis elegans*; CeDOP2 and DmDR2, D2-like receptors of *D. melanogaster* and *C. elegans*, respectively. [3]CgDR1 and CgDR2, *Chasmagnathus granulatus* D1-like and D2-like receptors from this paper.

granulatus but is not effective on the insect D1-like receptor AmDop2 (Mustard et al., 2005).

SCH23390, a D1-like antagonist both in vertebrates and insects (Missale et al., 1998; Mustard et al., 2005) blocks most of the inhibitory (D2-like) phase of the DA effect on steady state gills of *C. granulatus*, in the same way as the inhibitors of phosphodiesterase and PPs, theophylline and OA. In the same experiments, SCH23390 does not affect the stimulatory (D1-like) phase. These results are partially coincident with the results obtained for *C. elegans*, in which SCH23390 acts as an antagonist of the D2-like receptor CeDOP2 and as agonist of the D1-like CeDOP1 (for a review, see Mustard et al., 2005). The D1 agonist fenoldopam mimics the inhibitory phase of DA effect, reducing V_{te} on gills of *C. granulatus* previously stimulated with forskolin, in contrast to the report (Hussain et al., 1997) in which bromocriptine (D2 agonist) partially reverts the effect of forskolin on the Na^+/K^+-ATPase of rat renal proximal tubules. D1-like receptors have already been identified in the gills of *E. sinensis* by studying the binding of the D1 antagonist SCH23390 (Mo et al., 2002). However, our results suggest that in crabs SCH23390 could be a D2-like and not a D1-like antagonist. Table 2 summarizes the effects of several DA effectors on vertebrates and invertebrates. Comparative pharmacology of DA receptors is difficult to interpret since the response of the few characterized invertebrate (insect) receptors matches with that in vertebrates only for some effectors like the D1 antagonist SCH23390. By contrast spiperone, which is an effective D2 antagonist in vertebrates, antagonizes D1-like receptors more efficiently than D2-like receptors in invertebrates (Missale et al., 1998; Mustard et al., 2005). As far as we can conclude from our results, the response of *C. granulatus* DA receptors is different from that in vertebrates for these two antagonists and also for domperidone and fenoldopam. Compared to other invertebrates, *C. granulatus* D1-like receptors respond to spiperone in the same way as in insects, while D2-like receptors respond to SCH23390 in a similar fashion as in *C. elegans*.

We propose that, in *C. granulatus* gills, DA stimulates adenylyl cyclase and therefore ion transport through D1-like receptors linked to activation of adenylyl cyclase. D2-like receptors appear to inhibit ion transport activity, estimated as V_{te}, through reduction of cAMP levels.

The fact that V_{te} is first increased and then decreased suggests that in the gills of *C. granulatus*, D1-like receptors have higher affinity than D2-like receptors, or the latter are activated by elevated cAMP levels. The first hypothesis offers an explanation for previous results (Halperin et al., 2004) in which DA concentrations higher than 50 μmol l^{-1} produce less pronounced and less sustained activation of V_{te} in isolated perfused gills of this species. Perfusion with a high concentration of DA can rapidly activate D2-like receptors, antagonizing the response to the more sensitive D1-like receptors. This could also explain our results, in which the most stimulating DA concentration reported by Halperin et al. (10 μmol l^{-1}) (Halperin et al., 2004) is not enough to produce a consistent inhibitory effect on hypo-osmotically or forskolin stimulated gills, while 50 μmol l^{-1} DA effectively reduces the hypo-osmotic- or forskolin-induced V_{te}. However, cAMP-mediated phosphorylation-deactivation of D1-like receptors cannot be ruled out from these hypotheses.

As for the role of DA in osmoregulation, it is clear that this neurohormone modulates the gill V_{te} in different ways, depending on the level of adenylyl cyclase activity. When this enzyme works at the steady state level, DA stimulates cAMP production, causing a transient increase in V_{te}, Na^+/K^+-ATPase activity and sodium transport. On the other hand, when gill

adenylyl cyclase has already been activated by either DA, hypo-osmotic medium or any other stimulus involving cAMP transduction, the effect of DA is to reduce its activity level. Further work is required to characterize the molecular nature of dopamine receptors in crustacean gills and their role in osmoregulation.

List of abbreviations

cAMP	cyclic adenosine 3′-5′ monophospate
D1 and D2	DA receptor groups 1 and 2
DA	dopamine
I_{sc}	short circuit current
OA	okadaic acid
PKA	protein kinase A
PKC	protein kinase C
PO	pericardial organs
PPs	protein phosphatases
s.e.m.	standard error of mean
ThG	thoracic ganglion
V_{te}	transepithelial potential difference

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References

Asghar, M., Hussain, T. and Lokhandwala, M. F. (2001). Activation of dopamine D1-like receptor causes phosphorylation of α_1-subunit of Na^+, K^+-ATPase in rat renal proximal tubules. *Eur. J. Pharmacol.* **411**, 61-66.

Bertorello, A. M. and Katz, A. I. (1995). Regulation of Na^+-K^+ pump activity: pathways between receptors and effectors. *News Physiol. Sci.* **10**, 253-260.

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.

Boschi, E. E. (1964). Los crustáceos decápodos brachyura del litoral bonaerense. *Bol. Inst. Biol. Mar.* **164**, 1-34.

Charmantier, G., Gimenez, L., Charmantier-Daures, M. and Anger, K. (2002). Ontogeny of osmoregulation, physiological plasticity and larval export strategy in the grapsid crab *Chasmagnathus granulata* (Crustacea, Decapoda). *Mar. Ecol. Prog. Ser.* **229**, 185-194.

Cohen, P., Holmes, C. F. B. and Tsukitani, Y. (1990). Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* **15**, 98-102.

Drach, P. and Tchernigovtzeff, C. (1967). Sur la méthode de détermination des stades d'intermue et son application générale aux crustacés. *Vie Milieu* **18**, 597-607.

Eckhardt, E., Pierrot, C., Thuet, P., Van Herp, F., Charmantier-Daures, M., Trilles, J. P. and Charmantier, G. (1995). Stimulation of osmoregulating processes in the perfused gill of the crab *Pachygrapsus marmoratus* (Crustacea, Decapoda) by a sinus gland peptide. *Gen. Comp. Endocrinol.* **99**, 169-177.

Ewart, H. S. and Klip, A. (1995). Hormonal regulation of the Na^+-K^+-ATPase: mechanisms underlying rapid and sustained changes in pump activity. *Am. J. Physiol. Cell Physiol.* **269**, 295-311.

Fingerman, M. (1997). Crustacean endocrinology: a retrospective, prospective, and introspective analysis. *Physiol. Zool.* **70**, 257-269.

Halperin, J., Genovese, G., Tresguerres, M. and Luquet, C. M. (2004). Modulation of ion uptake across posterior gills of the crab *Chasmagnathus granulatus* by dopamine and cAMP. *Comp. Biochem. Physiol.* **139A**, 103-109.

Hussain, T. and Lokhandwala, M. F. (1997). Renal dopamine DA1 receptor coupling with G(S) and G(q/11) proteins in spontaneously hypertensive rats. *Am. J. Physiol. Renal Physiol.* **272**, 339-346.

Hussain, T., Abdul-Wahab, R. and Lokhandwala, M. F. (1997). Bromocriptine stimulates Na^+, K^+-ATPase in renal proximal tubules via the cAMP pathway. *Eur. J. Pharmacol.* **321**, 259-263.

Iorio, L. C., Barnett, A., Leitz, F. H., Houser, V. P. and Korduba, C. A. (1983). SCH 23390, a potential benzazepine antipsychotic with unique interactions on dopaminergic systems. *J. Pharmacol. Exp. Ther.* **226**, 462-468.

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.

Kuo, C. M., Hsu, C. R. and Lin, C. Y. (1995). Hyperglycaemic effects of DA in tiger shrimp, *Penaeus monodon*. *Aquaculture* **135**, 161-172.

Laurenza, A., Sutkowski, E. M. and Seamon, K. B. (1989). Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol. Sci.* **10**, 442-447.

Lohrmann, D. M. and Kamemoto, F. I. (1987). The effect of dibutyryl cAMP on sodium uptake by isolated perfused gills of *Callinictes sapidus*. *Gen. Comp. Endocrinol.* **65**, 300-305.

Luquet, C. M. and Ansaldo, M. (1997). Acid-base balance and ionic regulation during emersion in the estuarine intertidal crab *Chasmagnathus granulata* Dana (Decapoda Grapsidae). *Comp. Biochem. Physiol.* **117A**, 407-410.

Luquet, C. M., Postel, U., Halperin, J., Urcola, M. R., Marques, R. and Siebers, D. (2002). Transepithelial potential differences and Na^+ flux in isolated perfused gills of the crab *Chasmagnathus granulatus* (Grapsidae) acclimated to hyper- and hypo-salinity. *J. Exp. Biol.* **205**, 71-77.

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

Missale, C., Nash, S. R., Robinson, S. W., Jaber, J. and Caron, M, G. (1998). Dopamine receptors: from structure to function. *Physiol. Rev.* **78**, 189-225.

Mo, J. L., Devos, P. and Trausch, G. (1998). Dopamine as a modulator of ionic transport and Na^+/K^+-ATPase activity in the gills of the Chinese crab *Eriocheir sinensis*. *J. Crust. Biol.* **18**, 442-448.

Mo, J. L., Devos, P. and Trausch, G. (2002). Dopamine D1 receptors in the gills of Chinese crab *Eriocheir sinensis*. *Comp. Biochem. Physiol.* **131C**, 433-438.

Morris, S. (2001). Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. *J. Exp. Biol.* **204**, 979-989.

Morris, S. and Edwards, T. (1995). Control of osmoregulation via regulation of Na^+/K^+-ATPase activity in the amphibious purple shore crab *Leptograpsus variegatus*. *Comp. Biochem. Physiol.* **112C**, 129-136.

Mustard, J. A., Beggs, K. T. and Mercer, A. R. (2005). Molecular biology of the invertebrate dopamine receptors. *Arch. Insect Biochem. Physiol.* **59**, 103-117.

Onken, H., Schobel, A., Kraft, J. and Putzenlechner, M. (2000). Active NaCl absorption across split lamellae of posterior gills of the Chinese crab *Eriocheir sinensis*: stimulation by eyestalk extract. *J. Exp. Biol.* **203**, 1373-1381.

Onken, H., Tresguerres, M. and Luquet, C. M. (2003). Active NaCl absorption across posterior gills of hyperosmoregulating *Chasmagnathus granulatus*. *J. Exp. Biol.* **206**, 1017-1023.

Riestenpatt, S., Zeiske, W. and Onken, H. (1994). Cyclic AMP stimulation of electrogenic uptake of Na^+ and Cl^- across the gill epithelium of the Chinese crab *Eriocheir sinensis*. *J. Exp. Biol.* **188**, 159-174.

Rodríguez, E. M., Medesani, D. A., López Greco, L. S. and Fingerman, M. (2002). Effects of some steroids and other compounds on ovarian growth of the red swamp crayfish, *Procambarus clarkii*, during early vitellogenesis. *J. Exp. Zool.* **292**, 82-87.

Savage, J. P. and Robinson, G. D. (1983). Inducement of increased gill Na^+/K^+-ATPase activity by a hemolymph factor in hyperosmoregulating *Callinectes sapidus*. *Comp. Biochem. Physiol.* **75A**, 65-69.

Siebers, D., Winkler, A., Lucu, C., Thedens, G. and Weichart, D. (1985). Na-K-ATPase generates an active transport potential in the gills of the hyperregulating shore crab *Carcinus maenas*. *Mar. Biol.* **87**, 185-192.

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 gills of the green crab *Carcinus maenas* (L). *J. Exp. Zool.* **248**, 272-277.

Sommer, M. J. and Mantel, L. H. (1991). Effects of dopamine and acclimation to reduced salinity on the concentration of cyclic AMP in the

gills of the green crab, *Carcinus maenas* (L). *Gen. Comp. Endocrinol.* **82**, 364-368.

Spanings-Pierrot, C., Van Herp, F., Gompel, M., Skaret, G. and Charmantier, G. (2000). Involvement of the crustacean hyperglycemic hormone in the control of gill ion transport in the crab *Pachygrapsus marmoratus*. *Gen. Comp. Endocrinol.* **119**, 340-350.

Therien, A. G. and Blostein, R. (2000). Mechanisms of sodium pump regulation. *Am. J. Physiol. Cell Physiol.* **279**, 541-566.

Towle, D. W., Paulsen, R. S., Weihrauch, D., Kordylewski, M., Salvador, C., Lignot, J. H. and Spanings-Pierrot, C. (2001). Na^++K^+-ATPase in gills of the blue crab *Callinectes sapidus*: cDNA sequencing and salinity-related expression of α-subunit mRNA and protein. *J. Exp. Biol.* **204**, 4005-4012.

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.* **94B**, 487-492.

Tresguerres, M., Onken, H., Pérez, A. F. and Luquet, C. M. (2003). Electrophysiology of posterior, Na-Cl absorbing gills *Chasmagnathus granulatus*: rapid responses to osmotic variations. *J. Exp. Biol.* **206**, 619-626.

Wilkens, J. L., Kuramoto, T. and McMahon, B. R. (1996). The effects of six pericardial hormones and hypoxia on the semi-isolated heart and sternal arterial valve of the lobster *Homarus americanus*. *Comp. Biochem. Physiol.* **114C**, 57-65.

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