Neurohypophysial hormone regulation of Cl^- secretion: physiological evidence for V1-type receptors in sea bass gill respiratory cells in culture

M E Guibbolini¹ and M Avella

Laboratoire de Physiologie Cellulaire et Moléculaire, UMR CNRS 6548, Université de Nice–Sophia Antipolis, Faculté des Sciences, Parc Valrose, 06108 Nice Cedex 2, France

¹Laboratoire de Physiologie, Réponse des Organismes aux Stress Environnementaux, UMR INRA 1112, Université de Nice–Sophia Antipolis, Faculté des Sciences, Parc Valrose, 06108 Nice Cedex 2, France

(Requests for offprints should be addressed to M E Guibbolini; Email: marielle.guibbolini@unice.fr)

Abstract

Neurohypophysial hormone receptors were studied in primary cultures of sea bass (*Dicentrarchus labrax*) gill respiratory-like cells grown on permeable supports. This preparation was previously shown to provide a functional model for investigating the hormonal regulation of Cl^- secretion.

Under control conditions, the cultured monolayered epithelium had a short-circuit current (I_{SC}) of $3.5 \pm 1.1 \mu\text{A} \times \text{cm}^{-2}$. This current had previously been identified as an active Cl^- secretion. The addition of increasing concentrations of the fish neurohypophysial hormones, arginine vasotocin (AVT) or isotocin (IT), elicited a concentration-dependent stimulation of the I_{SC} . Maximal increases of $60.9 \pm 12.1\%$ and $117.7 \pm 28.0\%$ above the basal I_{SC} value were obtained for 10^{-7} M AVT and IT respectively. Half-maximal effects were obtained for 3.1×10^{-9} M AVT and for 1.4×10^{-9} M IT. Mucosal application of 1.0 mM diphenylamine-2-carboxylic acid (a specific blocker of Cl^- channels) after serosal addition of 5×10^{-8} M AVT or IT inhibited not only the basal but also the stimulated current, revealing a correlation with a hormone-dependent Cl^- transport.

Specific V1 or V2 receptor analogues of vasopressin (mammalian hormone) were used to characterize the type of neurohypophysial hormone receptors pharmaco-

logically. While the V1 agonist [Phe²,Orn⁸]-oxytocin stimulated the basal Cl^- secretion with a similar profile to that of AVT or IT, the V2 agonist [Deamino¹,Val⁴,D-Arg⁸]-vasopressin had no effect. The V1 antagonist [d(CH₂)₅¹,O-Me-Tyr²,Arg⁸]-vasopressin used at a concentration of 5×10^{-7} M totally reversed the 10^{-8} M AVT-stimulated Cl^- secretion, whereas the V2 antagonist [d(CH₂)₅¹,D-Ile²,Ile⁴,Arg⁸,Ala⁹]-vasopressin used at the same concentration had no significant effect. In contrast, similar experiments carried out in the presence of 10^{-8} M IT showed that both antagonists significantly reduced the IT-stimulated Cl^- secretion, with the efficiency of the V1 receptor antagonist being significantly greater than that of the V2.

This study provides evidence for neurohypophysial hormone control of Cl^- secretion in fish cultured gill respiratory cells. It suggests that on a physiological basis the hormonal effect is shared by the two peptides present in fish neurohypophysis (AVT and IT), acting by means of two distinct, although pharmacologically similar, V1-type receptors (according to the mammalian classification). These specific receptors are expected to play an important role in controlling ion homeostasis in seawater fish.

Journal of Endocrinology (2003) **176**, 111–119

Introduction

In teleost fishes, faced with a wide range of environmental ion concentrations, osmoregulation depends mainly on the transport activity of the gills. Direct exploration of ion transport pathways through the branchial epithelium has long been impeded by the anatomical complexity of this tissue. Primary gill cell cultures designed from a freshwater (FW) fish, the rainbow trout (*Oncorhynchus mykiss*) and a seawater (SW) fish, the European sea bass (*Dicentrarchus*

labrax) by Pärt *et al.* (1993) and Avella *et al.* (1994) respectively have provided a new approach.

Primary cultures of sea bass gill cells exhibit the morphological (Avella *et al.* 1994) and functional (Avella & Ehrenfeld 1997) characteristics of a Cl^- secretory epithelium. Gill cells grown on permeable supports form a confluent, highly polarized (serosal positive) tight epithelium composed of a single cell type exhibiting the morphological characteristics of gill respiratory cells which have therefore been termed 'respiratory-like cells' (Avella

et al. 1994, Avella & Ehrenfeld 1997). The first exhaustive electrophysiological study of this epithelium demonstrated a short-circuit current (I_{SC}) that correlated with an active Cl^- secretion as measured by ^{36}Cl fluxes (Avella & Ehrenfeld 1997). Pharmacological evidence led us to establish a new model defining separately basolateral and apical components of this transepithelial Cl^- secretion (Avella & Ehrenfeld 1997). Recent research carried out on this model provided strong evidence for a hormonal control of the Cl^- secretion (Avella *et al.* 1999).

In mammals, vasopressin, although displaying a wide variety of physiological properties, has been shown to play a major role in water and ion transport. Three typical vasopressin (AVP) receptors have been characterized by their tissue localization, signal transduction pathways, genetic structure, and functional and pharmacological parameters. In short, activation of V1a receptors increases inositol 1,4,5 triphosphate (IP_3) and Ca^{2+} mobilization, that of V1b increases Ca^{2+} mobilization (and cAMP in the presence of the corticotrophin-releasing factor) and that of V2 leads to cAMP synthesis.

In teleost fish, the neurohypophysis produces arginine vasotocin (AVT) and isotocin (IT), peptides that differ from those of mammals. AVT is considered to be the ancestral peptide in the vertebrate neurohypophysial family and IT occurs in teleost fish only. The physiological functions controlled by these hormones remain uncertain, although AVT in particular is believed to play a key role in salt and water regulation (Maetz & Lahlou 1974, Takei 1993). From *in vivo* studies on fish, a number of physiological functions, such as vasopressor and diuretic effects, and hormonal control of water and ion exchanges across the gills, have been ascribed to these peptides (Babiker & Rankin 1972, Henderson & Wales 1974, Maetz & Lahlou 1974, Henderson *et al.* 1986). However, most of these observations were obscured by concurrent haemodynamic effects of these hormones. *In vitro* studies showed that labelled AVT binds to specific receptors on eel gill cells (Guibbolini *et al.* 1988). In rainbow trout gill plasma membranes, the AVT or IT binding was followed by a strong inhibition of the adenylate cyclase activity (in the absence of Ca^{2+}) mediated by a Gi protein sensitive to pertussis toxin and guanine nucleotides (Guibbolini & Lahlou 1987, 1992). The use of specific neurohypophysial analogues indicated that the effects of AVT and IT were mediated by a new type of receptor, functionally similar to the mammalian neurohypophysial V1a type (Guibbolini & Lahlou 1990). Although the latter results favoured a direct effect of these peptides on gill cells, they could not define the exact target cells (gill chloride, respiratory, pillar or mucous cells), the function of these peptides, or the different types of neurohypophysial peptide receptors present in the branchial epithelium.

The present work represents a detailed study on primary cultures of sea bass gill cells which shows that (1) the respiratory-like cells are effective targets for the neuro-

hypophysial peptides and (2) both AVT and IT stimulate Cl^- secretion, and (3) suggests that this response is mediated by two different types of specific V1-like receptors. This investigation proposes that neurohypophysial hormones participate directly in the control of ion transport across fish gills.

Materials and Methods

Fish

European sea bass (*D. labrax*) of 35 g average weight were obtained from a local sea farm (Société 3A, Antibes, France). They were kept at ambient temperature (14–18 °C) and natural lighting in a semi-open circuit (water completely renewed every 6 h) in 1 m³ tanks containing Mediterranean SW. Fish were fed daily with Aqualim pellets (Biomar, Nersac, France) at 2.5% of their body weight.

Primary cell culture

Cell culture protocol Before culture preparation, fish were kept for 2 h in a 10 litre tank of aerated SW containing antibiotics and fungicides: Furaladone 0.02% (Sigma, St Louis, MO, USA) and Temerol 0.02% (Francodex, Carros, France). They were killed by a blow to the head and decerebrated. They were then decapitated and the gills excised. The cartilage of the gill arches was removed and the filaments remaining were dipped into a 'washing medium' (see below). They were washed under gentle automatic shaking (5 × 10 min, 100 agitations/min). Single cell suspensions were prepared by a technique modified from that of Pärt *et al.* (1993), as described by Avella & Ehrenfeld (1997), which consists of successive steps of trypsinization, filtration, centrifugation and resuspension in the 'culture medium' (see below). Finally, cells were seeded in Costar-Transwell 0.45 µm pore inserts (25 mm diameter, 4.8 cm² surface; Corning Costar Corporation, Cambridge, MA, USA) in six-well Costar plates at a high density (2 × 10⁶ cells/cm²) and maintained in a low-temperature incubator (Jouan, St Nazaire, France) at 18 °C, in humidified air (i.e. atmospheric pCO₂). After 24 h, cells reached confluency. The medium was changed every second day and the cells were used in the present experiments from days 6 to 9.

Solutions The solutions and culture conditions used have been described by Avella *et al.* (1994). In brief, the 'washing medium' consisted of Leibovitz L15 (ICN Pharmaceuticals, Orsay, France) supplemented with NaCl (20 mM), fungizone (0.1 µg/ml), penicillin (200 IU/ml), streptomycin (200 µg/ml) and gentamycin (400 µg/ml). All antibiotics were supplied by Sigma. The 'culture medium' consisted of Leibovitz L15 supplemented with

10% sea bass serum (see Avella & Ehrenfeld 1997 for preparation), NaCl (20 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml) and gentamycin (200 µg/ml). The final pH of both washing and culture media was adjusted to 7.8.

Electrical measurements The inserts containing the gill cell monolayer were placed in a modified Ussing chamber (Avella & Ehrenfeld 1997) in order to measure the transepithelial electrical potential (V_t), I_{SC} and resistance (R_t) of gill cells in culture. The volumes of the apical and basolateral bathing solutions were 2 ml and 4 ml respectively. The spontaneous V_t was measured with Agar-KCl salt bridges and was clamped at 0 V, through platinum electrodes, using an automatic voltage clamp (Model DVC-1000; World Precision Instruments, Sarasota, FL, USA). The sign of the transepithelial potential difference was determined by referring the basal side to the apical side (the latter taken as reference). The I_{SC} was continuously recorded on a chart paper recorder (SEFRAM, Metrix Electronics Ltd, Basingstoke, UK).

The cell culture medium (antibiotics and serum free) was used on both sides of the monolayer to avoid possible intracellular ion and volume changes due to different experimental and culture media. In order to prevent electrical artifacts in the chambers, media were not withdrawn between the addition of agents and increasing concentrations of each agent were successively added to the appropriate side. Blockers or hormone antagonists were applied in the presence of the initial hormone. Hormones and pharmacological substances were prepared in the cell culture medium described above. Membranes were given vehicle alone (cell culture medium) before starting experiments.

Hormones and drugs Diphenylamine-2-carboxylic acid (DPC) was purchased from Sigma. Pure AVT, IT and vasopressin analogues were obtained from Bachem (Torrance, CA, USA). The following analogues were selected for their high pharmacological specificity in mammals, as established by Manning & Sawyer (1984) and Manning *et al.* (1993): [Phe², Orn⁸]-oxytocin (V1a agonist), [Deamino¹, Val⁴, D-Arg⁸]-vasopressin (V2 agonist), [d(CH₂)₅¹, O-Me-Tyr², Arg⁸]-vasopressin (V1a antagonist) and [d(CH₂)₅¹, D-Ile², Ile⁴, Arg⁸, Ala⁹]-vasopressin (V2 antagonist).

Statistics Experimental data correspond to I_{SC} values taken at stabilization (steady state). For the sake of uniformity, results are presented in the Figures as I_{SC} ratios (test/basal, i.e. after compared with before the addition of the agent). Data variability is expressed as the S.E. of the mean. The *n* values given in the Figure legends and Table 1 represent the number of filters used from at least three independent fish cell cultures. Considering the relatively high variability observed in I_{SC} control levels, the effects

of hormones, analogues and blocker were analysed by Student's paired *t*-test to compare individual data differences (test minus basal). Statistical comparisons between V1 and V2 antagonist effects were determined with experimental data treated as ratios (hormone/hormone+V1 antagonist versus hormone/hormone+V2 antagonist) using Student's unpaired *t*-test.

Results

In control conditions, the I_{SC} of the cultured monolayered epithelium decreased slightly in the first 5–10 min and then remained stable for hours ($I_{SC}=3.5 \pm 1.1 \mu A \times cm^{-2}$, 49 filters from 13 independent fish). The V_t after the stabilization period was 12.8 ± 1.7 mV (49 filters, serosal side positive) and the R_t was $7925 \pm 878 \Omega/cm^2$ (49 filters). The stability of the cell spontaneous electrophysiological parameters facilitated the assessment of the neurohypophysial peptide effects and receptors involved in Cl^- secretion.

Effects of AVT and IT on Cl^- secretion

Figure 1 illustrates I_{SC} changes as a function of time in the presence of 5×10^{-9} M AVT (Fig. 1A) or IT (Fig. 1B). The increase in the I_{SC} was quasi-immediate after the addition of the peptides. The maximum was reached 10 min after the addition of AVT and 12 min after that of IT. In the presence of AVT or IT, these effects remained stable for at least 16 and 40 min respectively. The stabilization of I_{SC} obtained in the presence of the peptides permitted us to carry out the experiments with blocker and antagonists. In the following experiments, the stimulated I_{SC} values were recorded 15 min after the addition of the peptide when the effect had stabilized at its maximum.

Dose-response curves of AVT (Fig. 2A) and IT (Fig. 2B) on basal I_{SC} were established over a wide range of concentrations, from 10^{-10} to 5×10^{-8} M. We did not use peptide concentrations higher than 5×10^{-8} M, considered less physiological (see Discussion). All ratios (test/basal) were above 1, indicating a stimulation of the I_{SC} . Application of increasing concentrations of hormones elicited dose-response curves marked by a significant I_{SC} increase for concentrations as low as 5×10^{-9} M for AVT and 5×10^{-10} M for IT. The current increased to a maximum of $60.9 \pm 12.1\%$ and $117.7 \pm 28.0\%$ above the basal level for 10^{-7} M AVT ($n=6$) and 10^{-7} M IT ($n=5$) respectively. Half-maximal I_{SC} stimulation was observed at AVT and IT concentrations of 3.1×10^{-9} M and 1.4×10^{-9} M respectively.

Apical application of 1.0 mM DPC (a specific blocker of Cl^- channels) on 5×10^{-8} M AVT- or IT-treated cells totally inhibited the peptide-stimulated I_{SC} and reduced the basal I_{SC} (control) by approximately 75% (Fig. 3).

Figure 1 Stimulatory effect of neurohypophysial peptides on I_{sc} as a function of time in sea bass gill cells in primary cultures. (A) AVT or (B) IT at a final concentration of 5×10^{-9} M were added to the serosal side of the preparation. I_{sc} is expressed in $\mu A \times cm^{-2}$ as a function of time (min). A representative experiment (selected from a set of six for AVT and five for IT) is given for both peptides.

Effects of neurohypophysial analogues on Cl^{-} secretion

Effects of agonists The effects of synthetic vasopressin analogues were next considered to characterize pharmacologically the receptors involved in the gill response to the fish peptides. The V1 agonist (Fig. 4A), used over a wide range of concentrations (10^{-10} to 10^{-7} M), increased the basal I_{sc} in a dose-dependent manner, yielding an effect similar to those obtained with AVT and IT. In these conditions, maximal ($25.6 \pm 6.5\%$ above basal, $n=4$) and half-maximal stimulatory effects were obtained at V1 agonist concentrations of 10^{-7} M and approximately 5×10^{-9} M respectively (Table 1). While the V1 agonist stimulated basal Cl^{-} secretion, the V2 agonist,

Figure 2 Effects of AVT and IT concentrations on basal I_{sc} in sea bass gill cells in primary cultures. Increasing concentrations (10^{-10} to 5×10^{-8} M) of (A) AVT or (B) IT were successively applied to the serosal side. Results are means $\pm$ S.E. ($n=6$ for AVT and $n=5$ for IT) and are expressed as test vs basal I_{sc} ratios. Statistical significance was determined using Student's paired *t*-test (one tail). Comparisons were made by taking into account the experimental I_{sc} values ($\mu A \times cm^{-2}$). Results obtained in the presence of peptides were statistically different from the basal values, with $P < 0.05$ for AVT concentrations $\geq 5 \times 10^{-9}$ M and IT concentrations $\geq 5 \times 10^{-10}$ M. Error bars are not indicated when smaller than the symbol.

used within the same range of concentrations, produced no significant effect (Fig. 4B and Table 1).

Effects of antagonists Specific V1 and V2 receptor antagonists were used to relate the effects of AVT and IT to the presence of receptors functionally close to V1 and/or V2 type(s).

The effects of the antagonists were considered on 10^{-8} M AVT- (Fig. 5) or IT- (Fig. 6) treated cells. The V1 or V2 antagonists used at 5×10^{-7} M were added to the serosal side of the preparation after the effects of AVT or IT had stabilized at their maximum.

In the presence of AVT, the V1 antagonist was able to reverse totally the stimulation induced by AVT (Fig. 5A). The V2 antagonist, used in the same conditions did not

Figure 3 Effects of DPC on AVT- and IT-stimulated I_{sc} in sea bass gill cells in primary cultures. AVT or IT at a concentration of 5×10^{-8} M was added to the serosal side of the cells grown on permeable filters. In each stimulatory condition (i.e. after AVT or IT addition), DPC (1.0 mM) was added to the mucosal side after the current had reached a stabilized maximal response. Results are means $\pm$ S.E. and are expressed as test vs basal I_{sc} ratios. The number of experiments in the absence (open and hatched bars) or the presence (solid bars) of DPC were six for control, nine for AVT and five for IT. Statistical significances and comparisons were determined using the experimental I_{sc} values ($\mu A \times cm^{-2}$) with Student's paired *t*-test (one tail). Results obtained in the presence of AVT and IT were statistically different from the basal I_{sc} values ($P < 0.05$). Results obtained with DPC were significantly different from both basal and stimulated conditions ($P < 0.0001$).

produce any significant effect on the peptide-stimulated Cl^{-} secretion (Fig. 5B).

A similar study was carried out in the presence of 10^{-8} M IT (Fig. 6). Under these conditions, although the V1 antagonist used at a concentration of 5×10^{-7} M significantly reduced the IT-stimulated I_{sc} by $65.36 \pm 3.4\%$, the V2 antagonist used at the same concentration was able to reduce it by $38.7 \pm 5.2\%$ only. Moreover, these antagonistic effects were significantly different from each other ($P < 0.01$).

Discussion

Analysis of the electrophysiological parameters found in the present work reveals that the I_{sc} of the cell monolayer was lower than that observed in previous studies (Avella & Ehrenfeld 1997, Avella *et al.* 1999). The tissue resistance found here, a little higher than the one described previously, is characteristic of a very tight epithelium. The slight variations observed between the studies were probably due to different fish populations, and periods of the year for culture and fish serum preparations, the latter being essential for cell differentiation as shown by Avella & Ehrenfeld (1997).

AVT and IT

Since the present work is concerned with physiological experiments, it is relevant to compare the peptide con-

Figure 4 Stimulatory effects of V1 and V2 agonists on I_{sc} in sea bass gill cells in primary cultures. Increasing concentrations (10^{-10} to 10^{-7} M) of the (A) V1 or (B) V2 agonist were added successively (arrowheads) to the serosal side. I_{sc} is expressed in $\mu A \times cm^{-2}$ as a function of time (min). Results presented are from a single representative experiment selected from a set of four for the V1 agonist and three for the V2 agonist.

centrations used in the present study with the circulating AVT and IT levels and with previous results obtained in gills. The concentrations used in our work are within the range of the circulating peptide levels reported in fish (Henderson *et al.* 1986, Perrott *et al.* 1991, Warne *et al.* 1994, Pierson *et al.* 1995). As for the gills of SW-adapted rainbow trout, Guibbolini & Lahlou (1987) have shown that AVT and IT produced a maximal inhibition of membrane adenylate cyclase activity at 10^{-11} to 10^{-10} M. Sainsbury & Balment (1991) confirmed, on the same material, a similar maximal inhibitory effect of AVT at 10^{-10} to 10^{-9} M on cellular cAMP content. The present results obtained with sea bass gill cells showed a significant stimulation of I_{sc} starting at 5×10^{-9} M of AVT and 5×10^{-10} M of IT, which compare approximately with the doses producing a maximal inhibitory effect in rainbow trout gill preparations. Our study also shows that half-maximal I_{sc} stimulation was observed with AVT and IT concentrations of 3.1×10^{-9} M and

Table 1 Effects of increasing concentrations of V1 and V2 agonists on I_{SC} in sea bass gill cells in primary culture. Values are means ± S.E.M. (n=4 for V1 and n=3 for V2 agonists)

V1 agonist				V2 agonist			
	I _{SC} ratio (test/basal)	I _{SC} (μA × cm ⁻²)	I _{SC} difference (V1 agonist–basal) (μA × cm ⁻²)		I _{SC} ratio (test/basal)	I _{SC} (μA × cm ⁻²)	I _{SC} difference (V2 agonist–basal) (μA × cm ⁻²)
Basal I _{SC}	(a)	1.000	2.03 ± 0.43		1.000	2.37 ± 0.33	
Agonist							
5 × 10 ⁻¹⁰ M	(b)	1.108 ± 0.091	2.14 ± 0.38	0.11 ± 0.06 (b–a)	1.023 ± 0.013	2.44 ± 0.37	0.06 ± 0.04 (b–a)
1 × 10 ⁻⁹ M	(c)	1.117 ± 0.092	2.15 ± 0.38	0.12 ± 0.06 (c–a)	1.032 ± 0.018	2.46 ± 0.38	0.09 ± 0.06 (c–a)
5 × 10 ⁻⁹ M	(d)	1.142 ± 0.094	2.20 ± 0.40	0.17 ± 0.07 (d–a)*	1.073 ± 0.041	2.57 ± 0.46	0.20 ± 0.13 (d–a)
1 × 10 ⁻⁸ M	(e)	1.166 ± 0.091	2.26 ± 0.42	0.23 ± 0.08 (e–a)*	1.101 ± 0.047	2.64 ± 0.86	0.27 ± 0.15 (e–a)
5 × 10 ⁻⁸ M	(f)	1.234 ± 0.072	2.43 ± 0.48	0.40 ± 0.08 (f–a)*	1.139 ± 0.057	2.74 ± 0.52	0.37 ± 0.19 (f–a)
1 × 10 ⁻⁷ M	(g)	1.256 ± 0.065	2.48 ± 0.49	0.45 ± 0.08 (g–a)*	1.131 ± 0.060	2.72 ± 0.53	0.35 ± 0.20 (g–a)

The increasing concentrations of the agonists were successively applied to the serosal side.
I_{SC} difference: comparisons between experimental data were made by Student's paired *t*-test (means of individual data differences).
*Data were significantly different from those of the basal period with *P*<0.05 (one tail).

1.4 × 10⁻⁹ M respectively. In this respect, the AVT and IT EC₅₀ values reported here are consistent with the dissociation constants (*K_d* in nM: 3.2 in FW and 1.1 in SW) found previously for AVT in eel isolated gill cells (Guibbolini *et al.* 1988).

The present results from sea bass show that AVT and IT, within a concentration range of 10⁻¹⁰ to 5 × 10⁻⁸ M, are able to produce a large increase in I_{SC}. DPC (a specific Cl⁻ channel blocker) used at 1.0 mM abolished the peptide-stimulated I_{SC} totally and also reduced the basal I_{SC} consistently (by approximately 75%). These latter results are in accordance with those already published (approximately 70%; Avella & Ehrenfeld 1997) using the same concentration of DPC. In this previous study, a dose–response curve for DPC was established in unstimulated conditions and a total inhibition of I_{SC} was found for DPC concentrations higher than the one used here, confirming the correlation between the current measured and the Cl⁻ secretion. In the present study, we did not use such concentrations which were considered to be too high.

The present work shows that both AVT and IT induce a dose-dependent stimulation of Cl⁻ secretion through cultured gill respiratory cells from a marine fish. These results are in agreement with a previous observation in which a single dose of AVT (5 × 10⁻⁸ M) stimulated Cl⁻ secretion through this epithelium (Avella *et al.* 1999). These hormone effects are comparable with those observed in two kidney cell lines (A6 and mIMCD-K2), in which the neurohypophysial peptides (AVT and AVP) were reported to elicit an apical Cl⁻ permeability (Verrey 1994, Kizer *et al.* 1995).

In the present study, the physiological significance of AVT- and IT-stimulated Cl⁻ secretion is consistent with the necessity for fish living in SW to excrete ions through the gills.

Pharmacological characterization of receptors

The use of vasopressin analogues for pharmacological characterization of neurohypophysial peptide receptors in mammals was popularized by Manning & Sawyer (1984) who designed a large number of these compounds. Several investigators have utilized this approach in amphibians (Larcher *et al.* 1992, Butlen *et al.* 1993) and fishes (Pang & Furspan 1984, Guibbolini & Lahlou 1990, Uchiyama & Murakami 1994, Pierson *et al.* 1996, Guibbolini *et al.* 2000). The present study is the first attempt to characterize pharmacologically the neurohypophysial peptide receptors in the gill respiratory cells of a marine fish.

Agonists

In our experiments, while the V1a agonist [Phe²,Orn⁸]-oxytocin stimulated Cl⁻ secretion over the same range of concentrations as did AVT or IT, the V2 agonist [Deamino¹,Val⁴,D-Arg⁸]-vasopressin, a potent antidiuretic, produced no effect. This agrees with the findings obtained in rainbow trout gill adenylate cyclase by Guibbolini & Lahlou (1990). In the present study, the rank order of potency (affinity and efficacy) was as follows: IT>AVT>V1a agonist>V2 agonist. Taken together, these results suggest the presence of V1-type receptors in sea bass gill respiratory-like cells. Previous studies on gill plasma membranes from SW-adapted rainbow trout had revealed a different sequence: AVT>IT>V1a agonist>V2 agonist (Guibbolini & Lahlou 1987, 1990). Thus, comparison between sea bass and rainbow trout shows differences in the potency order of AVT and IT. This contrast may result from the difference in fish species, and from the presence of hormone receptors differentially sensitive to AVT and IT and/or differently distributed between chloride and respiratory cells. This latter point may be explained by

Figure 5 Effect of V1 or V2 antagonist on 10^{-8} M AVT-stimulated I_{sc} in sea bass gill cells in primary cultures. Results are means $\pm$ S.E. ($n=7$ and $n=6$ respectively for V1 and V2 antagonists) and are expressed as test vs basal I_{sc} ratio. The (A) V1 or (B) V2 antagonist was added at a concentration of 5×10^{-7} M to the serosal side, after AVT-stimulated I_{sc} had reached a stabilized maximal level. Statistical significance was determined using Student's paired *t*-test (one tail). Comparisons were made by taking into account the experimental I_{sc} values ($\mu A \times cm^{-2}$). Data obtained in the presence of AVT alone were significantly different from basal values ($P<0.01$). Results obtained in the presence of V1 antagonist added to AVT-treated cells were significantly different from AVT alone ($P<0.01$) and not significantly different from basal. Results obtained in the presence of V2 antagonist added to AVT-treated cells were not significantly different from AVT alone but significantly different from basal ($P<0.01$). Statistical significance between antagonist effects was determined using Student's unpaired *t*-test (one tail) and revealed that the effect of AVT+V1 antagonist was significantly different from that of AVT+V2 antagonist ($P<0.05$).

variations between the two fish gill preparations: the preparation of trout gill plasma membrane was obtained from both populations of chloride and respiratory cells while the culture of sea bass gill respiratory cells excludes the population of chloride cells. Moreover, work carried out in eel isolated gill cells showed that increasing external salinity produces parallel changes between chloride cell number and size and AVT-binding capacity (Guibbolini *et al.* 1988).

Figure 6 Effect of V1 or V2 antagonist on 10^{-8} M IT-stimulated I_{sc} in sea bass gill cells in primary cultures. Results are means $\pm$ S.E. ($n=5$ and $n=7$ respectively for V1 and V2 antagonists) and are expressed as test vs basal I_{sc} ratio. The (A) V1 or (B) V2 antagonist was added at a concentration of 5×10^{-7} M to the serosal side, after IT-stimulated I_{sc} had reached a stabilized maximal level. Statistical significance was determined using Student's paired *t*-test (one tail). Comparisons were made by taking into account the experimental I_{sc} values ($\mu A \times cm^{-2}$). Data obtained in the presence of IT alone were significantly different from basal values ($P<0.01$). Results obtained in the presence of V1 or V2 antagonist added to IT-treated cells were significantly different from IT alone ($P<0.01$) and from the basal ($P<0.01$). Statistical significance between antagonist effects was determined using Student's unpaired *t*-test (one tail) and revealed that the effect of IT+V1 antagonist was significantly different from that of IT+V2 antagonist ($P<0.01$).

In sea bass, our present results suggest that the V1-type receptors located in gill respiratory cells are predominant over V2 receptors and are more sensitive to IT than to AVT. More generally, the difference in the relative potential effects between the two peptides in various fish (rainbow trout, eel and sea bass) preparations as described above might indicate that, in SW conditions, the V1-type receptors are predominantly more sensitive to AVT in gill chloride cells and to IT in gill respiratory cells. However, this hypothesis cannot rule out the possibility that the two types of receptor can be present in both cell types and that they may display different pharmacological characteristics

in various species or biological preparations. Further experiments using a preparation of homogeneous chloride cells are required to confirm the present hypothesis.

Antagonists and fish neurohypophyseal peptide receptors

The effects of antagonists on AVT- and IT-induced stimulation of Cl^- secretion reveal the presence of V1-type receptors, but with pharmacological differences depending on the peptide used. Indeed, while the V1a antagonist $[\text{d}(\text{CH}_2)_5^1, \text{O-Me-Tyr}^2, \text{Arg}^8]$ -vasopressin was able to suppress the AVT-stimulated Cl^- secretion totally, it reduced that of IT by 65% only. Moreover, while the V2 antagonist $[\text{d}(\text{CH}_2)_5^1, \text{D-Ile}^2, \text{Ile}^4, \text{Arg}^8, \text{Ala}^9]$ -vasopressin produced no effect on AVT-stimulated Cl^- secretion, it reduced by 39% that of IT. Considering the magnitude of the respective actions of the antagonists on the AVT- or IT-induced effect, the present data are in line with the results obtained previously on rainbow trout gill plasma membranes (Guibbolini & Lahlou 1990). Indeed, in rainbow trout, while the V1a antagonist totally suppressed the effects of AVT and IT on the adenylate cyclase activity, the V2 antagonist showed no effect on either peptide (Guibbolini & Lahlou 1990). Nevertheless, in contrast to the work performed on the heterogeneous population of rainbow trout gill cells, the present study carried out on the homogeneous population of sea bass gill cells in culture reveals some differences of interest. The results suggest the presence of two specific and pharmacologically similar V1-type receptors (according to the mammalian classification) – AVT and IT receptors – and permit us to localize them in a specific cell type, the respiratory-like cells. The presence of two types of neurohypophyseal hormone receptors has already been described in fish gills (Mahlmann *et al.* 1994, Hausmann *et al.* 1995). Indeed, the molecular structure of the vasotocin receptor in the teleost fish *Catostomus commersoni* gills was established by Mahlmann *et al.* (1994) and that of the IT receptor by Hausmann *et al.* (1995). In their studies, the AVT receptor presented a sequence homology with mammalian neurohypophyseal hormone V1-type receptors and the IT receptor displayed the greatest similarity to the mammalian oxytocin receptors. The studies of Mahlmann *et al.* (1994) and Hausmann *et al.* (1995), using the same V1 antagonist as in our work, are also in line with our physiological results. These authors showed that (1) *xenopus* oocytes injected with the cloned AVT or IT receptor-encoding cRNA respond to AVT or IT by an induction of membrane chloride currents and (2) the AVT-induced responses are blocked particularly by the V1 specific antagonist, $[\text{d}(\text{CH}_2)_5^1, \text{O-Me-Tyr}^2, \text{Arg}^8]$ -vasopressin.

The present investigation demonstrates clearly that primary cultures of sea bass gill cells are effective targets for the fish neurohypophyseal peptides, AVT and IT, and that both stimulate branchial Cl^- secretion. This study provides pharmacological evidence and suggests, in a SW fish,

that the physiological effects of AVT and IT in the gills are mediated by two distinct, although pharmacologically similar, V1-type receptors, both located in gill respiratory-like cells. However, we cannot rule out the possibility of the presence of V1 receptor also located in chloride cells.

Previous observations have described complex interactions between cellular signalling pathways involved in the effects of the neurohypophyseal hormone in gill respiratory cells. Indeed, complex features between second messengers were found in sea bass with AVT used together with db-cAMP on I_{SC} (Avella *et al.* 1999) and in rainbow trout where AVT inhibited cAMP and stimulated IP3 production depending on the concentration used (Guibbolini & Pärt 1997, Guibbolini *et al.* 1997). Further investigation is required to analyse the neurohypophyseal hormonal signalling pathways in gill cells.

This work, together with earlier results involving other hormones or fish species (Guibbolini & Pärt 1997, Guibbolini *et al.* 1997, Avella *et al.* 1999), emphasizes the participation of gill respiratory cells as a site for the regulation of ion transport, enlarging the earlier views attributing this transport chiefly to gill chloride cells at the organismic scale.

Acknowledgements

The authors are greatly indebted to Professor B Lahlou (Université de Nice–Sophia Antipolis, France) for invaluable advice and extensive comments throughout this work. They also express their gratitude to Dr P Poujeol (Laboratoire de Physiologie Cellulaire et Moléculaire, Université de Nice–Sophia Antipolis) for his scientific comments. They are also grateful to Dr B Maetz for her critical comments on this manuscript.

References

- Avella M & Ehrenfeld J 1997 Fish gill respiratory cells in culture: a new model for Cl^- -secreting epithelia. *Journal of Membrane Biology* **156** 87–97.
- Avella M, Berhaut J & Payan P 1994 Primary culture of gill epithelial cells from the sea bass *Dicentrarchus labrax*. *In Vitro Cellular and Developmental Biology* **30A** 41–49.
- Avella M, Pärt P & Ehrenfeld J 1999 Regulation of Cl^- secretion in seawater fish (*Dicentrarchus labrax*) gill respiratory cells in primary culture. *Journal of Physiology* **516** 353–363.
- Babiker MM & Rankin JC 1972 Effects of neurohypophyseal hormones on renal function in the freshwater- and seawater-adapted eel (*Anguilla anguilla* L.). *Journal of Endocrinology* **57** xi–xii.
- Butlen D, Ammar A & Roseau S 1993 Pharmacological characterization of vasotocin receptors in the frog nephron. In *Vasopressin*, p 574. Eds P Gross, D Richter & GL Robertsons. Paris and London: John Libbey Eurotext.
- Guibbolini ME & Lahlou B 1987 Neurohypophyseal peptide inhibition of adenylate cyclase activity in fish gills. The effect of environmental salinity. *FEBS Letters* **220** 98–102.
- Guibbolini ME & Lahlou B 1990 Evidence for presence of a new type of neurohypophyseal hormone receptor in fish gill epithelium. *American Journal of Physiology* **258** R3–R9.

- Guibbolini ME & Lahlou B 1992 Gi protein mediates adenylate cyclase inhibition by neurohypophyseal hormones in fish gill. *Peptides* **13** 865–871.
- Guibbolini ME & Pärt P 1997 Hormonal control of cAMP production in cultured epithelial cells of trout gill. In *Proceedings from the Annual Congress of the Society for Experimental Biology. Cells in Culture*, Canterbury, UK, 7–A1·33. Eds Society of Experimental Biology. York: The Trouser Press.
- Guibbolini ME, Henderson IW, Mosley W & Lahlou B 1988 Arginine vasotocin binding to isolated branchial cells of the eel: effect of salinity. *Journal of Molecular Endocrinology* **1** 125–130.
- Guibbolini ME, Pärt P, Rankin JC, Pierson PM & Lahlou B 1997 Neurohypophyseal hormone receptors in fish tissues. In *Proceedings from the XXXIII International Congress of Physiological Sciences*, St Petersburg, Russia, P005·11. Eds International Union of Physiological Sciences.
- Guibbolini ME, Pierson PM & Lahlou B 2000 Neurohypophyseal hormone receptors and second messengers in trout hepatocytes. *Journal of Endocrinology* **167** 137–144.
- Hausmann H, Meyerhof W, Zwiers H, Lederis K & Richter D 1995 Teleost isotocin receptor: structure, functional expression, mRNA distribution and phylogeny. *FEBS Letters* **370** 227–230.
- Henderson IW & Wales NAM 1974 Renal diuresis and antidiuresis after injections of arginine vasotocin in the freshwater eel (*Anguilla anguilla* L.). *Journal of Endocrinology* **61** 487–500.
- Henderson IW, Hazon N & Hughes K 1986 Hormones, ionic regulation and kidney function in fishes. In *Physiological Adaptations of Marine Animals*, pp 245–265. Ed. MS Laveracks. London: Society for Experimental Biology.
- Kizer NL, Vondorpe D, Lewis B, Bunting B, Russell J & Stanton BA 1995 Vasopressin and cAMP stimulate electrogenic chloride secretion in an IMCD cell line. *American Journal of Physiology* **268** F854–F861.
- Larcher A, Delarue C, Homo-Delarche F, Kikuyama S, Kupryszewski G & Vaudry H 1992 Pharmacological characterization of vasotocin stimulation of phosphoinositide turnover in frog adrenal gland. *Endocrinology* **130** 475–483.
- Maetz J & Lahlou B 1974 Actions of neurohypophyseal hormones in fishes. In *Handbook of Physiology – Endocrinology*, vol 4, part 1, pp 521–544. Eds RO Greep & EB Astwood. Baltimore: American Physiological Society.
- Mahlmann S, Meyerhof W, Hausmann H, Heierhorst J, Schönrock C, Zwiers H, Lederis K & Richter D 1994 Structure, function, and phylogeny of [Arg⁸]vasotocin receptors from teleost fish and toad. *PNAS* **91** 1342–1345.
- Manning M & Sawyer WH 1984 Design and uses of selective agonistic and antagonistic analogs of the neuropeptides oxytocin and vasopressin. *Trends in Neurosciences* **7** 6–9.
- Manning M, Stoev S, Chan WY & Sawyer WH 1993 Receptor-specific antagonists of vasopressin and oxytocin. A current perspective. In *The Neurohypophysis: A Window on Brain Function*, vol 689, pp 219–232. Eds WG North, AM Moses & L Shares. New York: The New York Academy of Sciences.
- Pang PKT & Fursan P 1984 The effect of arginine vasotocin on hemodynamics and urine production in the perfused trout trunk. *Federation Proceedings* **43** 720.
- Pärt P, Norrgren L, Bergström E & Sjöberg P 1993 Primary cultures of epithelial cells from rainbow trout gills. *Journal of Experimental Biology* **175** 219–232.
- Perrott MN, Carrick S & Balment RJ 1991 Pituitary and plasma arginine vasotocin levels in teleost fish. *General and Comparative Endocrinology* **83** 68–74.
- Pierson PM, Guibbolini ME, Mayer-Gostan N & Lahlou B 1995 ELISA measurements of vasotocin and isotocin in plasma and pituitary of the rainbow trout: effect of salinity. *Peptides* **16** 859–865.
- Pierson PM, Guibbolini ME & Lahlou B 1996 A V1-type receptor for mediating the neurohypophyseal-induced ACTH release in trout pituitary. *Journal of Endocrinology* **149** 109–115.
- Sainsbury RJ & Balment RJ 1991 Arginine vasotocin (AVT) modulation of cyclic AMP production in isolated renal tubules and dispersed gill cells of rainbow trout. *Journal of Endocrinology* **131** (Suppl) 123.
- Takei Y 1993 Role of peptide hormones in fish osmoregulation. In *Fish Ecophysiology*, chapter 5, pp 136–160. Eds JC Rankin & FB Jensens. London, UK: Chapman and Hall.
- Uchiyama M & Murakami T 1994 Effects of AVT and vascular antagonists on kidney function and smooth muscle contraction in the river lamprey, *Lampetra japonica*. *Comparative Biochemistry and Physiology* **107A** 493–499.
- Verrey F 1994 Antidiuretic hormone action in A6 cells: effect on apical Cl and Na conductances with aldosterone for NaCl reabsorption. *Journal of Membrane Biology* **138** 65–76.
- Warne JM, Hazon N, Rankin JC & Balment RJ 1994 A radioimmunoassay for the determination of arginine vasotocin (AVT): plasma and pituitary concentrations in fresh- and seawater fish. *General and Comparative Endocrinology* **96** 438–444.

Received in final form 1 October 2002

Accepted 1 October 2002