Neurohypophysial hormone receptors and second messengers in trout hepatocytes

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Abstract

Neurohypophysial hormone receptors and second messengers were studied in trout (*Oncorhynchus mykiss*) hepatocytes. Arginine vasotocin (AVT) and isotocin (IT) elicited a concentration-dependent inhibition of cAMP accumulation in the presence of 5×10^{-8} M glucagon (maximal effect for 4.5×10^{-7} M and 1.4×10^{-7} M, half-maximal effect for 2.1×10^{-8} M and 0.7×10^{-8} M, AVT and IT respectively). The effect of glucagon was inhibited up to 90% by AVT and 80% by IT. While AVT inhibited (up to 50%) the basal cAMP production, IT had no such action.

Specific V_1 or V_2 analogues (with reference to vasopressin in mammals) were used for pharmacological characterization of the type of neurohypophysial hormone receptor involved in this inhibition. The V_1 agonist [Phe², Orn⁸]-oxytocin inhibited the glucagon-stimulated cAMP production with a maximal effect for 6×10^{-7} M and a half-maximal effect for 0.9×10^{-8} M concentrations of the analogue. While the V_1 agonist reduced the glucagon-stimulated cAMP level by 70%, it showed only a tendency to reduce the basal level. The V_2 agonist [deamino¹, Val⁴, D-Arg⁸]-vasopressin had no effect either on basal or on glucagon-stimulated cAMP production. The V_1

antagonist [d(CH₂)₅¹, O-Me-Tyr², Arg⁸]-vasopressin totally reversed the 10^{-8} M AVT-induced inhibition of 5×10^{-8} M glucagon-stimulated cAMP production, whereas the V_2 antagonist [d(CH₂)₅¹, D-Ile², Ile⁴, Arg⁸, Ala⁹]-vasopressin had no such effect. In this particular case, maximal and half-maximal effects of the V_1 antagonist were obtained for 2.3×10^{-6} M and 1.2×10^{-6} M respectively.

Changes in intracellular calcium content were measured using the fluorescent probe FURA-2/AM. AVT and IT elicited a concentration-dependent increase in Ca²⁺ accumulation. The comparison of the effect of 10^{-8} M agonists versus AVT showed the following order of potency: AVT = IT > V_1 agonist > V_2 agonist. The V_1 antagonist reversed the AVT-induced Ca²⁺ accumulation whereas the V_2 antagonist had no such effect.

These results are taken as evidence for the presence in trout hepatocytes of neurohypophysial hormone receptors functionally close to the V_{1a} -type linked to cAMP production and Ca²⁺ mobilization.

Journal of Endocrinology (2000) **167**, 137–144

Introduction

In mammals, vasopressin (AVP) possesses a glycogenolytic effect and its mechanism of action has been well defined in hepatocytes. In the rat, labelled AVP binds to specific receptors located on intact cells or isolated membranes and coupled to a G protein (Cantau *et al.* 1980, Fitzgerald *et al.* 1986). This receptor was pharmacologically characterized as a V_{1a} -type (Fahrenholz *et al.* 1984, Sato *et al.* 1992); it was recently cloned and shown to possess seven transmembrane domains (Morel *et al.* 1992, 1993). The V_{1a} receptor was shown to mediate vasopressin-induced glycogenolytic responses by enhancing polyphosphoinositide breakdown and by increasing inositol triphosphate (IP₃) and diacylglycerol (DAG) (Thomas *et al.* 1984, Cerpovicz & Ochs 1992). The IP₃ formed induces an increase in the

concentration of intracellular ionic calcium (Thomas *et al.* 1984) due to the liberation of calcium from intracellular stores (Hajnoczky & Thomas 1997) and sustained by an extracellular calcium influx (Garrison *et al.* 1979, Berven *et al.* 1994, Duszynski *et al.* 1995). DAG activates a protein kinase C (Tang & Houslay 1992). The intervention of protein kinases leads to protein phosphorylation (Morris *et al.* 1994) and to activation of mitogen-activated protein kinase (Romanelli & Van de Werve 1997). Another effect of vasopressin is to inhibit, in a dose-dependent manner, the cAMP accumulation induced by forskolin or glucagon whether cells are depleted in calcium or not (Morgan *et al.* 1983).

In amphibian hepatocytes, arginine vasotocin (AVT) or AVP (the heterologous hormone, generally used) produces a dose-dependent stimulation of glycogenolysis (Janssens

et al. 1983, Ade *et al.* 1995) mediated by cAMP accumulation (Janssens *et al.* 1986) and not by IP₃ production (Kleineke & Janssens 1993). Kleineke and Janssens (1993) showed that AVT induced an increase in intracellular concentration of calcium, directly due to an extracellular calcium influx. They concluded that AVT stimulated cAMP production which not only induced glycogenolysis but also regulated the opening of an ionic channel allowing calcium influx. The stimulatory effect of AVT on cAMP accumulation and the pharmacological study carried out on primary cultures of *Xenopus laevis* hepatocytes led authors to classify this receptor as a V₂-type receptor (Ade *et al.* 1995).

In contrast to other vertebrates, little is known in fish concerning the mechanism of action of neurohypophysial peptides on hepatocytes (Lahlou *et al.* 1988, Moon & Mommsen 1990). Moon and Mommsen (1990) showed that neurohypophysial peptides activated the glycogenolysis in eel and to a lesser extent in trout liver. In the same study, AVT induced in eel a dose-dependent stimulation of cAMP concentration. However, the concentrations of AVT used in their study were relatively high (10⁻⁷ to 10⁻⁵ M). In contrast, Lahlou *et al.* (1988) showed in the trout that neurohypophysial peptides at concentrations of 5 × 10⁻⁷ to 5 × 10⁻⁶ M (concentrations also relatively high) inhibited the cAMP accumulation stimulated by 4 × 10⁻⁸ M glucagon (Lahlou *et al.* 1988).

Taken together, these data led us to investigate further the mechanism of action of neurohypophysial peptides in trout hepatocytes. The aim of the present study was (1) to analyse the nature of second messengers (cAMP and Ca²⁺) involved in the transduction of the neurohypophysial peptide effect, and (2) to characterize pharmacologically the type(s) of peptide receptor(s) present in trout hepatocytes by using selected specific V₁ or V₂ analogues. Results are considered with regard to the phylogenetic evolution of neurohypophysial peptide receptors in the vertebrate series.

Materials and Methods

Animals

Rainbow trout, *Oncorhynchus mykiss* (average weight 220 g), were purchased from a fish farm and maintained in the laboratory at 12–15 °C, under constant photoperiod (12 h light per day).

Chemicals

Pure arginine vasotocin (AVT), isotocin (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 and colleagues (Manning &

Sawyer 1984, Manning *et al.* 1993): [Phe², Orn⁸]-oxytocin (V_{1a} agonist), [deamino¹, Val⁴, D-Arg⁸]-vasopressin (V₂ agonist), [d(CH₂)₅¹, O-Me-Tyr², Arg⁸]-vasopressin (V_{1a} antagonist), [d(CH₂)₅¹, D-Ile², Ile⁴, Arg⁸, Ala⁹]-vasopressin (V₂ antagonist). HANK's minimum essential medium (HMEM), bovine serum albumin fraction V (BSA) and collagenase H were obtained from Boehringer Mannheim (Illkirch, France) and heparin LEO (5000 UI/ml) from LEO Laboratory (St-Quentin-les-Yvelines, France). Glucagon was supplied by NOVO Nordisk Pharmaceutique S.A. (Boulogne, France). Theophylline (cAMP phosphodiesterase inhibitor) was purchased from Sigma-Aldrich Chimie (L'Isle d'Abeau Chesnes, France). FURA-2/AM and pluronic acid were obtained from Molecular Probes (Interchim, France). The [³H]cAMP radioimmunoassay kit was obtained from Amersham (Les Ulis, France).

Preparation of hepatocytes

The basic technique has been described previously (Porthé-Nibelle & Lahlou 1981). Briefly, the trout liver was perfused via the portal vein for 5 min with a perfusion buffer (in mM: NaCl 66, KCl 66, glucose 5.5, Hepes 10, pH 7.4) and then for 1 h with collagenase (0.8 mg/ml perfusion buffer). Hepatocytes were then collected after 2 passages through a 3-layer gauze, rinsed twice with perfusion buffer and re-suspended in HMEM (300 mosmol/l; pH 7.4). Under these conditions, the intracellular ion concentrations in the isolated cells are not in steady state, although they are viable at room temperature. Consequently, the cells were then preincubated for 40 min in HMEM prior to use. This treatment restores and stabilizes the cellular electrolyte concentrations for several hours (Lahlou *et al.* 1988). Cell viability was checked using 0.04% Trypan blue and preparations containing more than 15% stained cells were discarded.

Incubation procedure and cAMP determination

The basic technique has been described previously by Lahlou *et al.* (1988). Routinely, the typical incubation medium contained 10 mM theophylline and 5‰ (w/v) BSA in HMEM, pH 7.4. The final volume was 200 µl containing 100 µl equilibrated cell preparation (on average: 3.5 × 10⁶ cells or 0.6 mg protein/100 µl cell preparation) in HMEM and 20 µl hormone(s). Because inhibitions are often larger or better revealed when the target system is stimulated by another hormone (Guibbolini & Lahlou 1987, Lahlou *et al.* 1988), 5 × 10⁻⁸ M glucagon were added to the incubation medium. When hormones and analogues were considered together in the assay, they were added simultaneously in the incubation medium. The reaction was initiated by addition of the cells which were left to incubate for 10 min

at 20 °C under gentle shaking, and was terminated by 3 min heating in boiling water. Samples taken in duplicate were then centrifuged at 1600 g for 10 min at 4 °C, and the supernatants were collected for cAMP measurement. The levels of cAMP were determined in each sample using a [³H]cAMP radioimmunoassay kit as specified by the supplier. Protein quantities were determined in each sample by using the method of Bradford (1976).

Results were expressed as pmoles cAMP/mg protein per 10 min or as a ratio (test/basal, i.e. in the presence/absence of hormone). Because of variations between individual cell preparations, cAMP content was also expressed as a percentage of maximum (the 100% value was that obtained with 5×10^{-8} M glucagon alone).

Results were expressed as means $\pm$ S.E., for *n* independent liver preparations (with each test carried out in duplicate or in triplicate). Statistical significance was determined using the Student's *t*-test applied to comparison between unpaired values, and comparison of means using ANOVA single factor-factorial test (Statview software, Brain Power Inc., Calabasas, CA, USA).

Calcium measurement

All subsequent operations were carried out at 20 °C in the dark and under slow shaking. Cell suspensions (2×10^7 cells in 2 ml HMEM) were incubated for 1 h loading in the presence of FURA-2/AM (5 μ M) and pluronic acid (0.05%). After this incubation period, cells were rinsed twice with HMEM (200 g, 20 s), re-suspended in 2 ml HMEM and left to stabilize for 45 min. Changes in intracellular concentration of calcium ($[Ca^{2+}]_i$) were measured in 1.5 ml hepatocyte suspension in the presence or absence of hormone(s) (15 μ l added), using a Perkin-Elmer spectrofluorimeter. The fluorescence emission of FURA 2, induced by excitation at two wavelengths (340 and 380 nm), was recorded at 510 nm. The fluorescence ratio (F340/F380) was used as an indicator of the variations of $[Ca^{2+}]_i$. In these experiments, increasing hormone concentrations were successively added to the cell suspension. Therefore, the fluorescence ratios obtained represent cumulative responses. For each hormone concentration, the experiment was followed for 2.5 min (5 cycles of 30 s) at room temperature. Maximal effect was obtained between 2 and 2.5 min. Results were expressed as test vs basal ratio, means $\pm$ S.E. (*n* independent preparations). Statistical significance of peptide effects on fluorescence ratios in cell preparations was assessed by using repeated measures ANOVA (SYSTAT 5.2.1 software, Systat Inc., NY, USA). This model was carried out since each cell preparation was submitted to increasing concentrations of AVT, IT or analogues. In addition, for AVT and IT analysis, since the sphericity assumption was not checked, *P* values were corrected using the Greenhouse–Geisser adjustment.

Figure 1 Effect of AVT (■) and IT (●) concentrations on basal cAMP content in trout hepatocytes. Results are means $\pm$ S.E. (*n*=3 for AVT and *n*=3 for IT) and are expressed as test vs basal cAMP ratio. Statistical significance was determined using ANOVA. For AVT, mean values were significantly different from basal for concentrations $\geq 5 \times 10^{-7}$ M ($P<0.05$). IT values were not significantly different from basal. Error bars are not indicated when smaller than the symbol.

Results

cAMP measurements

Effects of hormones The basal cAMP content in hepatocytes was 2.9 ± 0.6 pmoles cAMP/mg protein per 10 min (*n*=14). The effects of AVT and IT in relation to their concentration are illustrated in Fig. 1. In this case, the level of cAMP, as expressed by the test vs basal ratio, was decreased by AVT in a dose-dependent manner. Maximal (D_{max}) and half-maximal (IC_{50}) inhibitions were obtained for $3.0 \pm 1.4 \times 10^{-6}$ M and $4.5 \pm 2.3 \times 10^{-7}$ M concentrations of the peptide (*n*=3). While AVT inhibited (up to 50%) basal cAMP production, IT had no significant effect.

The effects of AVT and IT were studied in the presence of 5×10^{-8} M glucagon and the results are shown in Fig. 2. We have previously shown that this concentration of glucagon was sufficient to yield an accurately measurable increase in cAMP (Lahlou *et al.* 1988). In the present experiments, 5×10^{-8} M glucagon stimulated cAMP accumulation by a factor of 3.9 ± 0.7 (*n*=14) for an average absolute cAMP level of 11.3 ± 3.2 pmoles cAMP/mg protein per 10 min (*n*=14). In these conditions, both peptides elicited a concentration-dependent inhibition of cAMP accumulation. For AVT, the maximum effect was obtained with $4.5 \pm 2.1 \times 10^{-7}$ M and the half-maximum effect was obtained with $2.1 \pm 3.4 \times 10^{-8}$ M (*n*=7); for IT, D_{max} was $1.4 \pm 1.0 \times 10^{-7}$ M and IC_{50} was $0.7 \pm 0.35 \times 10^{-8}$ M (*n*=3). Stimulation by glucagon was inhibited up to 90% by AVT and 80% by IT.

Effects of synthetic analogues Specific V_1 and V_2 analogues were used to relate the AVT effect to the

Figure 2 Effect of (A) AVT and (B) IT concentrations on 5×10^{-8} M glucagon-stimulated cAMP accumulation in trout hepatocytes. Results are means $\pm$ S.E. ($n=7$ for AVT and $n=3$ for IT) and are expressed as percentages of maximal effect. The maximal effect (taken as 100%) was that obtained with 5×10^{-8} M glucagon alone. Statistical significance was determined using ANOVA. For AVT, mean values were significantly different from glucagon alone between 10^{-8} and 5×10^{-6} M ($P<0.01$). For IT, mean values were significantly different from glucagon alone for 10^{-8} M and 3×10^{-8} M ($P<0.05$) and between 10^{-7} and 3×10^{-6} M ($P<0.01$). Error bars are not indicated when smaller than the symbol.

presence of receptors functionally close to V_1 and/or V_2 type(s).

Agonists The effects of the V_1 and V_2 agonists on basal (Table 1) and on 5×10^{-8} M glucagon-stimulated cAMP production (Fig. 3) revealed that the V_1 agonist inhibited the glucagon-stimulated activity with maximal and half-maximal inhibitions obtained for $6.0 \pm 2.7 \times 10^{-7}$ M and $0.9 \pm 0.4 \times 10^{-8}$ M concentrations of the analogue ($n=6$). While the V_1 agonist reduced the glucagon-stimulated level of cAMP by 70%, it only showed a slight, not significant tendency to reduce the basal level. The V_2

Table 1 Effect of the V_1 agonist ([Phe², Orn⁸]-oxytocin) and the V_2 agonist ([deamino¹, Val⁴, D-Arg⁸]-vasopressin) concentrations on basal cAMP content in trout hepatocytes. Results (means $\pm$ S.E., $n=4$ for the V_1 agonist and $n=3$ for the V_2 agonist) are expressed as percentage of basal cAMP content

	V_1 agonist (%)	V_2 agonist (%)
Basal Agonist	100	100
10^{-9} M	94 ± 7	90 ± 21
10^{-8} M	78 ± 14	95 ± 11
10^{-7} M	78 ± 13	83 ± 15
10^{-6} M	75 ± 14	99 ± 32

Values were not statistically significantly different from basal (ANOVA).

agonist produced no effect in any of the experimental conditions.

Antagonists The concentration of 10^{-8} M AVT was chosen in this set of experiments because it corresponds to the IC_{50} of this hormone challenging the effect of 5×10^{-8} M glucagon (Fig. 2A). The V_1 antagonist was able to reverse totally the inhibition caused by 10^{-8} M AVT on the 5×10^{-8} M glucagon-stimulated production of cAMP (Fig. 4A). Maximal and half-maximal effects of the V_1 antagonist were obtained with $2.3 \pm 0.8 \times 10^{-6}$ M and $1.2 \pm 0.6 \times 10^{-6}$ M, respectively ($n=6$). In contrast, the V_2 antagonist (Fig. 4B) did not produce this effect, and even showed a slight tendency to decrease further the glucagon-stimulated/AVT-inhibited cAMP accumulation. This tendency was noted for 10^{-8} M V_2 analogue only and was not reproduced at higher concentrations.

Ca^{2+} measurements

The results we obtained with cAMP led us to undertake experiments on Ca^{2+} , the other main second messenger. Data concerning the effect of the neurohypophysial peptides on Ca^{2+} mobilization are illustrated in Fig. 5. AVT and IT (in the range of 10^{-10} to 10^{-6} M) elicited an increase in intracellular Ca^{2+} content in hepatocytes. The analysis of the responses of these two peptides showed that AVT and IT exerted a similar dose-dependent effect on Ca^{2+} increase.

The use of the same neurohypophysial peptide analogues as in the cAMP measurements gave results summarized in Table 2. For the same concentration as AVT (10^{-8} M), the V_1 agonist also increased the intracellular Ca^{2+} ratio significantly from the basal level. The V_2 agonist had a less pronounced effect than the V_1 agonist. The V_1 antagonist reversed the AVT effect on Ca^{2+} accumulation whereas the V_2 antagonist had no such effect.

Discussion

The present investigation shows, first, that fish neurohypophysial hormones elicit a dose-dependent inhibition of

Figure 3 Effect of (A) the V₁ agonist ([Phe², Orn⁸]-oxytocin) and (B) the V₂ agonist ([deamino¹, Val⁴, D-Arg⁸]-vasopressin) concentrations on 5 × 10⁻⁸ M glucagon-stimulated cAMP accumulation in trout hepatocytes. Results are means ± S.E. (n=6 for the V₁ agonist and n=7 for the V₂ agonist) and are expressed as percentages of maximal effect. The maximal effect (taken as 100%) was that obtained with 5 × 10⁻⁸ M glucagon alone. Statistical significance was determined using ANOVA. For the V₁ agonist, mean values were significantly different from glucagon alone between 3 × 10⁻⁸ and 3 × 10⁻⁶ M (P<0.05). The V₂ agonist values were not statistically significantly different from glucagon alone. Error bars are not indicated when smaller than the symbol.

cAMP accumulation in trout hepatocytes which is strikingly revealed in the presence of 5 × 10⁻⁸ M glucagon. The stimulation by glucagon was decreased up to 90% by AVT and 80% by IT, for an AVT or IT concentration of 4.5 ± 2.1 × 10⁻⁷ M (n=7) and 1.4 ± 1.0 × 10⁻⁷ M (n=3), respectively. In rat liver, 10⁻⁸ M vasopressin used in similar conditions was only able to reduce by 16% the cAMP accumulation induced by 10⁻⁸ M glucagon (Morgan *et al.* 1983). In rat brain, vasopressin was found partially to prevent the cAMP accumulation induced by noradrenaline, dopamine and forskolin (50%, 45% and 40% by 3 × 10⁻⁷, 5.5 × 10⁻⁷ and 3 × 10⁻⁷ M AVP respectively,

Figure 4 Effect of (A) the V₁ antagonist ([d(CH₂)₅¹, O-Me-Tyr², Arg⁸]-vasopressin) and (B) the V₂ antagonist ([d(CH₂)₅¹, D-Ile², Ile⁴, Arg⁸, Ala⁹]-vasopressin) concentrations on trout hepatic cellular cAMP production stimulated by 5 × 10⁻⁸ M glucagon and inhibited by 10⁻⁸ M AVT. Results are means ± S.E. (n=6 for the V₁ antagonist and n=5 for the V₂ antagonist) and are expressed as percentages of maximal effect. The maximal effect (taken as 100%) was that obtained with 5 × 10⁻⁸ M glucagon alone. ANOVA was used to determine the statistical significance of the effects of the antagonists on the inhibitory action of AVT on glucagon-stimulated cAMP production (P<0.05 for 3 × 10⁻⁶ M V₁ antagonist and 10⁻⁸ M V₂ antagonist). Student's *t*-test applied to comparison of unpaired values was used to compare the AVT effect on glucagon-stimulated cAMP production with that of glucagon alone (P<0.001 and P<0.005 for curves A and B respectively). Error bars are not indicated when smaller than the symbol.

Newman 1985). In our study, not only did the neurohypophysial peptides strongly inhibit the stimulatory effect, but the basal activity (Fig. 1) was also reduced by AVT (50% inhibition), a fact only observed in a few cases: 15–25% inhibition in human platelets (Vanderwel *et al.* 1983), 40% in trout gill plasma membranes (Guibbolini & Lahlou 1987). Secondly, in our model, not only did AVT and IT decrease cAMP production but they also behaved

Figure 5 Effect of (A) AVT and (B) IT concentrations on intracellular Ca²⁺ content in trout hepatocytes. Results are means ± S.E. (*n* = 6 for AVT and *n* = 6 for IT) and are expressed as test vs basal ratio. The dose effect is statistically significant at 0.01 for AVT and at 0.05 for IT (repeated measures ANOVA, Greenhouse–Geisser *P* value of 0.011 for AVT and 0.027 for IT).

like Ca²⁺-mobilizing hormones, as do homologous peptides in rat hepatocytes (Thomas *et al.* 1984, Hajnoczky & Thomas 1997). To our knowledge, the present study is the first showing an effect of IT on second messenger production in fish hepatocytes.

In the present study, neurohypophysial hormones inhibited glucagon-stimulated cAMP accumulation with IC₅₀ values of around 10⁻⁸ M. With regard to circulating hormone levels, those of neurohypophysial peptides have been estimated only recently in fish (Perrott *et al.* 1991, Balment *et al.* 1993, Warne *et al.* 1994, Pierson *et al.* 1995a,b). Our present observations are consistent with our own estimates since, in plasma of trout adapted to various degrees of salinity, AVT and IT measured by ELISA were in the range of 8–26 nM and 3–5.5 nM respectively

Table 2 Effect of the V₁ and V₂ vasopressin receptor agonists and antagonists on calcium content in trout hepatocytes. Results (means ± S.E. of *n* experiments) are expressed as test vs basal ratio.

	Intracellular Ca ²⁺ ratio (test/basal)
Basal	1
AVT (10 ⁻⁸ M)	1.187 ± 0.041 (<i>n</i> = 6)**
V ₁ agonist (10 ⁻⁸ M)	1.133 ± 0.027 (<i>n</i> = 4)*
V ₂ agonist (10 ⁻⁸ M)	1.070 ± 0.010 (<i>n</i> = 3)
AVT (10 ⁻⁸ M) + V ₁ antagonist (10 ⁻⁶ M)	1.036 ± 0.023 (<i>n</i> = 3)#
AVT (10 ⁻⁸ M) + V ₂ antagonist (10 ⁻⁶ M)	1.118 ± 0.010 (<i>n</i> = 3)

P* < 0.05, *P* < 0.01 compared with basal; #*P* < 0.05 compared with AVT (repeated measures ANOVA).

(Pierson *et al.* 1995a,b). Furthermore, neurohypophysial hormone IC₅₀ values in the present study are in agreement with *K_d* values found in binding experiments in rat intact hepatocytes for AVP (15 nM, Cantau *et al.* 1980) and in eel isolated gill cells for AVT (3.2 to 1 nM in fresh- and sea-water-adapted fish respectively, Guibbolini *et al.* 1988).

The use of vasopressin analogues for pharmacological characterization of neurohypophysial peptide receptors may be extended with some caution to non-mammalian vertebrates. Several investigators used 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). The present study is the first attempt to characterize the neurohypophysial hormone receptor in fish hepatocytes by means of artificial analogues. The V₁ agonist presented a slight tendency to act in a similar manner to AVT on basal cAMP levels, for concentrations within the range of those of AVT and IT. When cAMP accumulation was stimulated by glucagon, the D_{max} for AVT, IT and V₁ agonist decreased to a similar lower value of 1–6 × 10⁻⁷ M and the IC₅₀ for AVT, IT and V₁ agonist followed a similar pattern. When the V₁ antagonist was added, it totally suppressed the AVT-induced inhibition of the glucagon-stimulated activity. In contrast, the V₂ analogues did not show any effect. The slight further decrease only observed in the presence of 10⁻⁸ M V₂ antagonist has already been noticed in mammals with the V₂ antagonist behaving like a V₁ agonist (Ellis *et al.* 1994). With regard to Ca²⁺ accumulation, neurohypophysial peptide agonists at 10⁻⁸ M revealed the following order of potency: AVT = IT > V₁ agonist > V₂ agonist. Concerning the antagonists, while the V₁ antagonist reversed significantly the AVT effect, the V₂ antagonist had no such effect.

Our results taken together (cAMP decrease, Ca²⁺ increase, pharmacological receptor characterization) are in favour of the presence of V_{1a}-type receptors in trout hepatocytes. Some authors have proposed that V₂-type receptors coupled with adenylate cyclase stimulation may

also exist in fish, namely in eel liver (Moon & Mommsen 1990) and in trout kidney (Perrott *et al.* 1993), although the concentrations of hormone used in their experiments were high ($D_{\max}$ between 10^{-6} – 10^{-5} M). Our previous work on peripheral vasotocin receptors in lower vertebrates emphasized the 'V₁' feature of these receptors in such distinct organs as trout pituitary and gill (Guibbolini & Lahlou 1987, Guibbolini *et al.* 1988, Lahlou *et al.* 1988, Guibbolini & Lahlou 1990, 1992, Pierson *et al.* 1996). Recently, in the teleost fish *Catostomus commersoni*, the molecular structure of the vasotocin receptor was established by Mahlmann *et al.* (1994) and that of the isotocin receptor by Hausmann *et al.* (1995). Both AVT and IT receptors transcripts have been found in several bony fish organs (gills, liver, pituitary, lateral line). The AVT receptor presented a sequence homology with mammalian neurohypophysial hormone receptors, particularly with the rat vasopressin V_{1a} receptor (60.9% identity), and to a lesser extent with the human oxytocin receptor (48%) and rat and human vasopressin V₂ receptors (about 40%). The isotocin receptor displays the greatest similarity to the mammalian oxytocin receptor. It can be noted that the sequence homology between receptors may also explain the slight V₁ agonistic effect we observed with the V₂ antagonist on trout hepatocytes. Mahlmann's study (Mahlmann *et al.* 1994) also showed that (1) *Xenopus* oocytes injected with the cloned AVT receptor-encoding cRNA respond to AVT by the induction of membrane chloride currents, (2) the AVT-induced responses are blocked, in particular, by the V₁ specific antagonist, [d(CH₂)₅¹, O-Me-Tyr², Arg⁸]-vasopressin (the same as used in our study), and (3) the transduction of the hormonal message occurs via the phospholipase C and inositol phosphate/calcium pathway, usual characteristics of the V₁ receptor family.

In summary, trout hepatocytes display a neurohypophysial hormone receptor which is functionally close to the V_{1a} sub-family of vasopressin receptors, as results from pharmacological and second messenger production studies support. Interestingly, the cellular signalling machinery of these cells responds equally to the two peptides, arginine vasotocin and isotocin, present in the teleostean pituitary. The present results sustain the observation that, unlike the mammalian hormones vasopressin and oxytocin, the teleost fish peptides vasotocin and isotocin have not been shown up to now to act on separate target organs and their physiological effects are usually qualitatively similar, although displaying different hormonal potencies. Functional and structural binding studies are awaited to clarify this discrepancy, so as to determine the presence of one or more neurohypophysial peptide receptors within the same target organ.

Acknowledgements

The authors are indebted to Dr B Fossat and Dr J Porthé-Nibelle for helpful advice on hepatocyte iso-

lation and to Dr S Dallot for statistical expertise of data analysis.

References

- Ade T, Segner H & Hanke W 1995 Hormonal response of primary hepatocytes of the clawed toad, *Xenopus laevis*. *Experimental and Clinical Endocrinology and Diabetes* **103** 21–27.
- Balment RJ, Warne JM, Tierney M & Hazon N 1993 Arginine vasotocin and fish osmoregulation. *Fish Physiology and Biochemistry* **11** 189–194.
- Berven LA, Hughes BP & Barritt GJ 1994 A slowly ADP-ribosylated pertussis-toxin-sensitive GTP-binding regulatory protein is required for vasopressin-stimulated Ca²⁺ inflow in hepatocytes. *Biochemical Journal* **299** 399–407.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Butlen D, Ammar A & Roseau S 1993 Pharmacological characterization of vasotocin receptors in the frog nephron. In *Vasopressin*, pp 574. Eds P Gross, D Richter & GL Robertsons. Paris: John Libbey Eurotext.
- Cantau B, Keppens S, De Wulf H & Jard S 1980 [³H]Vasopressin binding to isolated rat hepatocytes and liver membranes: regulation by GTP and relation to glycogen phosphorylase activation. *Journal of Receptor Research* **1** 137–168.
- Cerpovicz PF & Ochs RS 1992 Effects of EGF on the mass of inositol 1,4,5-trisphosphate and SN(1, 2)-diacylglycerol in freshly isolated rat hepatocytes: comparison with vasopressin. *Biochemical and Biophysical Research Communications* **187** 1055–1062.
- Duszynski J, Elensky M, Cheung JY, Tillotson DL & LaNoue KF 1995 Hormone-regulated Ca²⁺ channel in rat hepatocytes revealed by whole cell patch clamp. *Cell Calcium* **18** 19–29.
- Ellis MJ, Mulligan RS, Evans MJ & Donald RA 1994 The effects of corticotrophin-releasing hormone, arginine vasopressin and their antagonists on ACTH release from perfused horse anterior pituitary cells. *Journal of Endocrinology* **143** 85–93.
- Fahrenholz F, Boer R, Crause P, Fritzsch G & Grzonka Z 1984 Interactions of vasopressin agonists and antagonists with membrane receptors. *European Journal of Pharmacology* **100** 47–58.
- Fitzgerald TJ, Uhing RJ & Exton JH 1986 Solubilization of the vasopressin receptor from rat liver plasma membranes. *The Journal of Biological Chemistry* **261** 16871–16877.
- Garrison JC, Borland K, Florio VA & Twible DA 1979 The role of calcium ion as a mediator of the effects of angiotensin II, catecholamines and vasopressin on the phosphorylation and activity of enzymes in isolated hepatocytes. *The Journal of Biological Chemistry* **254** 7147–7156.
- 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 neurohypophysial 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, 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.
- Hajnoczky G & Thomas AP 1997 Minimal requirements for calcium oscillations driven by the IP₃ receptor. *EMBO Journal* **16** 3533–3543.
- 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.

- Janssens PA, Caine AG & Dixon JE 1983 Hormonal control of glycogenolysis and the mechanism of action of adrenaline in amphibian liver *in vitro*. *General and Comparative Endocrinology* **49** 477–484.
- Janssens PA, Kleineke J & Caine AG 1986 Calcium-independent stimulation of glycogenolysis by arginine vasotocin and catecholamines in liver of the axolotl (*Ambystoma mexicanum*) *in vitro*. *Journal of Endocrinology* **109** 75–84.
- Kleineke JW & Janssens PA 1993 Hormone-induced rise in cytosolic Ca^{2+} in axolotl hepatocytes: extracellular origin and control by cAMP. *American Journal of Physiology* **265** C1281–C1288.
- Lahlou B, Fossat B, Porthé-Nibelle J, Bianchini L & Guibbolini M 1988 Inhibitory effect of neurohypophyseal peptides on cyclic AMP accumulation by isolated hepatocytes of the trout. *Journal of Endocrinology* **119** 439–445.
- 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.
- 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*. *Annals of the New York Academy of Sciences*, vol 689, pp 219–232. Eds WG North, AM Moses & L Shares. New York: The New York Academy of Sciences.
- Moon TW & Mommsen TP 1990 Vasoactive peptides and phenylephrine actions in isolated teleost hepatocytes. *American Journal of Physiology* **259** E644–E649.
- Morel A, O'Carroll A-M, Brownstein MJ & Lolait SJ 1992 Molecular cloning and expression of a rat V_{1a} arginine vasopressin receptor. *Nature* **356** 523–526.
- Morel A, Lolait SJ & Brownstein MJ 1993 The cloning and structure of the rat V_{1a} AVP receptor. In *Vasopressin*, pp 59–68. Eds P Gross, D Richter & GL Robertsons. Paris: John Libbey Eurotext.
- Morgan NG, Shipp CC & Exton JH 1983 Studies on the mechanism of inhibition of hepatic cAMP accumulation by vasopressin. *Federation of European Biochemical Societies* **163** 277–281.
- Morris NJ, Bushfield M, Lavan BE & Houslay MD 1994 Multi-site phosphorylation of the inhibitory guanine nucleotide regulatory protein Gi-2 occurs in intact rat hepatocytes. *Biochemical Journal* **301** 693–702.
- Newman ME 1985 Vasopressin inhibits cyclic AMP accumulation and adenylate cyclase activity in cerebral preparations. *Federation of European Biochemical Societies* **181** 203–206.
- Pang PKT & Fursan P 1984 The effect of arginine vasotocin on hemodynamics and urine production in the perfused trout trunk. *Federation Proceedings* **43** 720.
- Perrott MN, Carrick S & Balment RJ 1991 Pituitary and plasma arginine vasotocin levels in teleost fish. *General and Comparative Endocrinology* **83** 68–74.
- Perrott MN, Sainsbury RJ & Balment RJ 1993 Peptide hormone-stimulated second messenger production in the teleostean nephron. *General and Comparative Endocrinology* **89** 387–395.
- Pierson PM, Guibbolini ME & Lahlou B 1995a Enzyme linked immunosorbent assay for the neurohypophyseal hormones arginine vasotocin and isotocin. *Journal of Immunoassay* **16** 55–79.
- Pierson PM, Guibbolini ME, Mayer-Gostan N & Lahlou B 1995b 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 hormone-induced ACTH release in trout pituitary. *Journal of Endocrinology* **149** 109–115.
- Porthé-Nibelle J & Lahlou B 1981 Mechanisms of glucocorticoid uptake by isolated hepatocytes of the trout. *Comparative Biochemistry and Physiology B* **69** 425–433.
- Romanelli A & Van de Werve G 1997 Activation of mitogen-activated protein kinase in freshly isolated rat hepatocytes by both a calcium- and a protein kinase C-dependent pathway. *Metabolism* **46** 548–555.
- Sato C, Liu JH, Uchihara M, Izumi N, Yauchi T, Sakaj Y, Asahina Y, Fukuma T, Takano T & Marumo F 1992 V₁-receptor mediated GSH efflux by vasopressin from rat hepatocytes. *Life Sciences* **51** 507–512.
- Tang EKY & Houslay MD 1992 Glucagon, vasopressin and angiotensin all elicit a rapid, transient increase in hepatocyte protein kinase C activity. *Biochemical Journal* **283** 341–346.
- Thomas AP, Alexander J & Williamson JR 1984 Relationship between inositol polyphosphate production and the increase of cytosolic free Ca^{2+} induced by vasopressin in isolated hepatocytes. *The Journal of Biological Chemistry* **259** 5574–5584.
- 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.
- Vanderwel M, Lum DS & Haslam RJ 1983 Vasopressin inhibits the adenylate cyclase activity of human platelet particulate fraction through V₁ receptors. *Federation of European Biochemical Societies* **164** 340–344.
- 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 9 March 2000

Revised manuscript received 15 May 2000

Accepted 5 June 2000