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# Localisation of VIP-binding sites exhibiting properties of VPAC receptors in chromaffin cells of rainbow trout (*Oncorhynchus mykiss*)

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**Summary**

**The current model for the neuronal control of catecholamine release from piscine chromaffin cells advocates that the neurotransmitters vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are co-released with acetylcholine from preganglionic fibres upon nerve stimulation. Both VIP and PACAP elicit the secretion of exclusively adrenaline from rainbow trout chromaffin cells, which presumably arises from the activation of VPAC type receptors. Thus, the goals of the present study were (1) to localise VPAC receptors in the chromaffin cell fraction of the posterior cardinal vein (PCV) of trout and (2) to test the hypothesis that the selective secretion of adrenaline elicited by VIP could be explained by the absence of the VPAC receptors from the noradrenaline-containing cells.**

**Fluorescent labelling of chromaffin cells using aldehyde-induced fluorescence of catecholamines and antisera raised against dopamine β-hydroxylase (DβH) revealed a distinct layer of chromaffin cells lining the walls of the PCV. Furthermore, specific VIP-binding sites were demonstrated on chromaffin cells using a biotinylated VIP that was previously established as being bioactive. Although multiple labelling experiments revealed that a number of DβH-positive cells were immunonegative for phenylethanolamine *N*-methyl transferase (PNMT; noradrenaline-containing cells *versus* adrenaline-containing cells, respectively), labelling of VIP-binding sites was similar to that of DβH labelling, suggesting that all chromaffin cells possess VIP-binding sites. Pharmacological assessment of the VIP-binding sites indicated that they exhibited characteristics of VPAC receptors. Specifically, the labelling of VIP-binding sites was prevented after pre-treatment of PCV tissue sections with unlabelled VIP, PACAP or the specific VPAC receptor antagonist VIP 6-28. By contrast, sections pre-treated with the $PAC_1$ receptor blocker PACAP 6-27 displayed normal labelling of VIP-binding sites. Finally, partial cDNA clones for the trout $VPAC_1$ and $VPAC_2$ receptor were obtained and sequenced. Tissue distribution experiments using RT-PCR revealed the presence of $VPAC_1$ receptor mRNA but not that of the $VPAC_2$ receptor in the PCV tissue. The results provide direct evidence that VIP and PACAP can elicit the secretion of adrenaline from the chromaffin tissue *via* specific VIP-binding sites that exhibit properties of VPAC receptors. However, the selective secretion of adrenaline by VIP or PACAP cannot be explained by a lack of VIP-binding sites on the noradrenaline-containing cells.**

Key words: catecholamine, adrenaline, noradrenaline, dopamine β-hydroxylase, phenylethanolamine *N*-methyl transferase, chromaffin cells, VIP, vasoactive intestinal polypeptide, PACAP, pituitary adenylate cyclase-activating polypeptide, fluorescent histochemistry, stress.

## Introduction

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are members of a superfamily of structurally related peptide hormones that include glucagon, glucagon-like peptides, secretin and growth hormone-releasing hormone (Harmar et al., 1998). Moreover, VIP and PACAP exert tissue-specific effects by interacting with three distinct receptors that belong to a seven trans-membrane spanning domain G-protein-coupled receptor superfamily (Harmar et al., 1998). Two of these receptors, $VPAC_1$ and $VPAC_2$, bind VIP and PACAP with equal affinity, whereas a third receptor, $PAC_1$, preferentially binds PACAP. Since their discoveries (Said and Mutt, 1972; Miyata et al., 1989), VIP and PACAP have been implicated as neurotransmitters in various physiological processes, including cardiovascular function, respiration, digestion and glandular secretions in different vertebrate species (reviewed by Nilsson and Holmgren, 1994; Harmar et al., 1998).

Catecholamines that are secreted into the circulation are stored in separate populations of adrenaline- and

noradrenaline-containing chromaffin cells, but, unlike in mammals, the chromaffin cells of fish are not organised into a distinct adrenal gland (reviewed by Reid et al., 1998). In teleost fish, chromaffin cells line the walls of the posterior cardinal vein (PCV). Nerve fibres exhibiting immunoreactivity to VIP and PACAP have been identified in the vicinity of the chromaffin tissue of fish, including cod (*Gadus morhua*), trout (*Oncorhynchus mykiss*), eel (*Anguilla anguilla*) and dogfish (*Squalus acanthias*) (Reid et al., 1995). Because VIP and PACAP can evoke the secretion of adrenaline from *in situ* saline-perfused PCV preparations of trout (Montpetit and Perry, 1999, 2000), it is conceivable that the neuronal control of catecholamine release in fish may include VIP and/or PACAP in addition to acetylcholine. In contrast to mammals, the effects of VIP and PACAP appear to be mediated by receptors exhibiting properties of VPAC receptors in rainbow trout (Montpetit and Perry, 2000). In mammalian and amphibian adrenal chromaffin cells, $PAC_1$ receptors are believed to mediate the VIP/PACAP-elicited secretion of catecholamines. However, there is evidence that VPAC receptors may also participate in this response. Indeed, while the adrenal medulla of mammals exhibits a pronounced expression of $PAC_1$ receptor mRNA, investigations have also revealed the presence of $VPAC_1$ and $VPAC_2$ receptor mRNA (Usdin et al., 1994; Vaudry et al., 2000).

In fish, the secretory profile of catecholamines during cholinergic stimulation of chromaffin tissue features the release of both adrenaline and noradrenaline (Nilsson et al., 1976; Fritsche et al., 1993; Reid and Perry, 1995; Al-Kharrat et al., 1997; Gfell et al., 1997; Montpetit and Perry, 1999). By contrast, however, administration of a range of VIP and PACAP doses selectively causes the release of adrenaline from the chromaffin cells of all vertebrate species studied to date (Guo and Wakade, 1994; Montpetit and Perry, 2000). These findings suggest that the variations in the secretion of adrenaline and noradrenaline in response to acetylcholine and VIP/PACAP may reflect their ability to specifically stimulate adrenaline- *versus* noradrenaline-containing chromaffin cells. Thus, the goal of the present study was to determine whether VIP-binding sites are present on rainbow trout chromaffin cells and if they exhibit a differential spatial localization among different chromaffin cell subtypes. This was achieved using a combination of fluorescent histochemical techniques and pharmacological approaches.

## Materials and methods

### *Experimental animals*

Rainbow trout (*Oncorhynchus mykiss* L.) of both sexes were obtained from Linwood Acres Trout Farm (Campellcroft, ON, Canada). The fish were held in large fibreglass tanks supplied with dechlorinated City of Ottawa tapwater (13°C) and were fed daily to satiation with a commercial fish diet (supplied by fish supplier). Fish were maintained on a 12 h:12 h L:D photoperiod and allowed to acclimate to the aquaria for at least three weeks prior to experimentation.

### *Fluorescent histochemistry*

#### *Localization of chromaffin cells and VIP-binding sites within the PCV tissue*

Posterior cardinal veins, in the anterior region of the head kidney, were dissected and collected in 0.1 mol $l^{-1}$ phosphate-buffered saline (PBS; adjusted to pH 7.2, containing 0.5 mol $l^{-1}$ NaCl). The catecholamine-containing cell fraction of the PCV was identified by detection of catecholamines using aldehyde-induced green fluorescence (Furness et al., 1977; Lacoste et al., 2001). Each PCV was cut into 5-mm pieces and incubated for 24 h at 4°C in a solution of 4% paraformaldehyde and 0.55% glutaraldehyde (prepared in PBS). Tissues were washed with PBS and cryoprotected by immersion in a series of PBS solutions containing 5%, 10%, 15% and 20% sucrose (w/v) for 1 h each. Tissues were embedded in Cryomatrix (OCT-compound; Shandon, Pittsburgh, PA, USA) and quick-frozen in liquid $N_2$. Cross-sections (10 μm) were prepared using a cryostat, cooled to –17°C, and thaw-mounted on poly-L-lysine-coated slides. Following rehydration in PBS (3×5 min), stained cells were visualized in the PCV sections using fluorescence microscopy (see below). Other sections were kept in the dark at –20°C until needed for immunostaining of enzymes known to be involved in the biosynthesis of catecholamines.

Characterisation of chromaffin cells was confirmed by immunolabelling of dopamine β-hydroxylase (DβH) using the methods of Bernier and Perry (1997). Following rehydration in PBS, sections were incubated with 5% bovine serum albumin (BSA; ICN Biomedical, Aurora, OH, USA) in PBS for 1 h. Sections were then incubated for 12 h with mouse monoclonal anti-DβH (dilution 1:500; Chemicon, Temecula, CA, USA) and further incubated for 2 h with the Cy3-conjugated donkey anti-mouse secondary antibody (dilution 1:400; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Sections were then examined after a final rinse in PBS (3×5 min).

To determine whether VIP-binding sites were differentially distributed amongst the different chromaffin cell subtypes, sections were triple labelled for DβH (an enzyme found in all chromaffin cells), phenylethanolamine *N*-methyltransferase (PNMT; an enzyme found only in adrenaline-containing cells) and VIP-binding sites, according to the protocols described below. Following rehydration in PBS (3×5 min), sections were reduced in $NaBH_4$ (3×5 min), to decrease autofluorescence, and immunolabelled for DβH as described above. Sections were then incubated in rabbit polyclonal anti-bovine PNMT (dilution 1:100; ProtosTech International, New York, NY, USA) and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit secondary antibody (dilution 1:200; Jackson Immunoresearch Laboratories) using the same protocol as for DβH immunolabelling.

To visualise VIP-binding sites within the PCV, tissue sections (previously immunostained for DβH and PNMT)

were incubated with a solution of biotinylated hVIP ($2.58\times10^{-6}$ mol $l^{-1}$ biotinyl-VIP human, porcine, rat; Peninsula Laboratories Inc., San Carlos, CA, USA) for 2 h and further incubated with Cascade blue-conjugated streptavidin (dilution 1:500; Jackson Immunoresearch Laboratories) for an additional 2 h. Stained cells were then visualised in the PCV tissues using fluorescence microscopy.

*Receptor pharmacology*

These experiments were performed to evaluate the pharmacological properties of the VIP-binding sites. Sections were reduced in $NaBH_4$ (3×5 min). VIP-binding sites (assumed to be $PAC_1$ or VPAC receptors) were then saturated by incubating sections with solutions containing excess VIP receptor agonist ckVIP ($10^{-5}$ mol $l^{-1}$ chicken VIP; Peninsula Laboratories Inc.), VIP receptor antagonist hVIP 6-28 ($10^{-5}$ mol $l^{-1}$ human VIP 6-28; Peninsula Laboratories Inc.), PACAP receptor agonist hPACAP-27 ($10^{-5}$ mol $l^{-1}$ human PACAP-27; Peninsula Laboratories Inc.) or the PACAP receptor antagonist hPACAP 6-27 ($10^{-5}$ mol $l^{-1}$ human PACAP 6-27; Peninsula Laboratories Inc.) for 2 h each prior to incubation with biotinylated VIP (described above). PACAP 6-27 and VIP 6-28 were chosen on the basis of their potent antagonism of $PAC_1$ (Robberecht et al., 1991; Gaspo et al., 1997) and VPAC receptors (Bodanszky et al., 1973; Fishbein et al., 1994), respectively. Excess amounts were determined from previously established dose–response curves for VIP- and PACAP-elicited secretion of catecholamines (Montpetit and Perry, 2000). To visualise binding of biotinylated VIP, sections were then incubated with DTAF (dichlorotriazinyl aminofluorescein)-conjugated streptavidin (dilution 1:500; Jackson Immunoresearch Laboratories) as described earlier. In control experiments, tissue sections were incubated for 2 h with PBS prior to labelling of VIP-binding sites. Experiments were performed on consecutive sections (eight per slide) from the PCV of six different fish.

*Control and specificity experiments*

For the aldehyde-induced fluorescence of catecholamines, sections reduced in $NaBH_4$ (3×5 min) served as a methodological control (Corrodi et al., 1964). Essentially, $NaBH_4$ reduces the aldehyde reaction with the monoamine and eliminates the fluorescence. Heart tissue (lacking chromaffin cells) processed for the aldehyde-induced green fluorescence of catecholamines served as a negative control. Specificity of primary antibodies was demonstrated previously (Reid et al., 1995; Lacoste et al., 2001) and that of secondary antibodies was confirmed by omission of the primary antibody. To distinguish specific from non-specific binding of biotinyl-hVIP, VIP receptors were saturated with an excess of unlabelled ckVIP ($10^{-5}$ mol $l^{-1}$) for 2 h prior to the addition of biotinyl-hVIP. Background fluorescence was determined by replacing the primary antibodies and fluorescent probes with PBS.

*Fluorescence microscopy*

All incubations were performed in a moist sealed chamber at room temperature (approximately 20°C), consistently using 50-μl aliquots of the various solutions. Sections were mounted using 30 μl of Vectashield mounting medium with or without the nucleic acid dye DAPI (4,6-diamidino-2-phenlidole; Vector Laboratories, Inc., Burlingame, CA, USA). To preserve the slides, cover slips were sealed with nail polish and stored at –20°C. The PBS, $NaBH_4$ and BSA solutions were freshly prepared before use.

Tissue sections were visualised with a Zeiss Axiophot photomicroscope with appropriate filters using X10 Ph1, X16 Ph2 or X40 Ph2 Plan Neofluar objectives. Photographs were taken using Kodak MAX iso400-color 35-mm film. The negatives were developed by a local processing centre and the images were processed using a negative scanner (SprintScan 35, Polaroid) and Paint Shop Pro software (version 5.01; JASC Software Inc., Minneapolis, MN, USA).

*Determination of catecholamine stores*

Measurements were performed to confirm the presence of stored catecholamines in the PCV tissue. The PCV of different fish ($N$=6) was separated from the surrounding kidney tissue and both the vein and the kidney were divided into thirds (anterior, middle and posterior for each). The various tissues were placed into pre-weighed microcentrifuge tubes (1.5 ml), frozen in liquid $N_2$ and stored at –80°C until subsequent determination of catecholamine levels. The tissues were then thawed and washed with 1 ml of Cortland saline (Wolf, 1963; 125 mmol $l^{-1}$ NaCl, 2.0 mmol $l^{-1}$ KCl, 2.0 mmol $l^{-1}$ $MgSO_4$, 5.0 mmol $l^{-1}$ $NaHCO_3$, 7.5 mmol $l^{-1}$ glucose, 2.0 mmol $l^{-1}$ $CaCl_2$ and 1.25 mmol $l^{-1}$ $KH_2PO_4$; final pH, 7.8). After removing the saline by aspiration, the tubes were reweighed to determine tissue masses. Aliquots (1 ml) of 4% perchloric acid containing 2 mg $ml^{-1}$ EDTA and 0.5 mg $ml^{-1}$ sodium bisulphite were added to each tube (Nilsson, 1989), and samples were homogenised with a micro-ultrasonic cell disrupter. The supernatant was diluted 100-fold with a 4% perchloric acid solution containing EDTA and sodium bisulphite (as above) and analysed for catecholamine levels (see below).

In situ *saline-perfused PCV preparation*

The bioactivity of the biotinylated VIP probe was tested using an *in situ* saline-perfused PCV previously described by Montpetit and Perry (2000). Briefly, the PCV and the ventricle of the heart were catheterised (Clay-Adams PE 160 polyethylene tubing) to serve as the inflow and outflow of the perfusion fluid, respectively. Each preparation was perfused in the anterograde direction for 20 min with modified aerated Cortland saline (Wolf, 1963) to allow stabilisation of catecholamine secretion.

After the stabilisation period, a control (pre-treatment) sample was collected in a pre-weighed microcentrifuge tube to assess basal catecholamine secretion rate. After collection of the pre-sample, a single bolus injection of biotinylated hVIP ($2.58\times10^{-6}$ mol $l^{-1}$; final volume 0.3 ml) was delivered through a three-way valve connected to the inflow catheter. A period

of 1 min was allowed for the drug to be delivered to the chromaffin tissue before post-samples were collected in pre-weighed tubes. In total, five post-samples were collected 1 min, 2 min, 3 min, 4 min and 5 min after intervention. All samples were frozen in liquid $N_2$ after collection and stored at –80°C until determination of catecholamine levels. Perfusate samples were reweighed before catecholamine analysis to permit an estimation of perfusion flow rates and thus allow the calculation of catecholamine secretion rates.

*Determination of perfusate and tissue catecholamine levels*

Perfusate and tissue adrenaline and noradrenaline levels were determined on alumina-extracted samples (200 µl) using high-pressure liquid chromatography (HPLC) with electrochemical detection according to Montpetit and Perry (2000). The HPLC consisted of a Varian Star 9012 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, NJ, USA). The extracted samples were passed through an Ultratechsphere ODS-$C_{18}$ 5-µm column (HPLC Technology Ltd, Macclesfield, UK) using Mobile Phase for catecholamines (Chromosystems GmBh, München, Germany), and the separated amines were integrated using the Star Chromatography software program (version 4.0; Varian Chromatography Systems, Walnut Creek, CA, USA). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide as an internal standard in all assays.

*Cloning of $VPAC_1$ and $VPAC_2$ receptor partial cDNAs*

Partial cDNA clones corresponding to the third and seventh transmembrane domains (TMDs) of the putative trout $VPAC_1$ and $VPAC_2$ were obtained based on PCR amplification using degenerate primers (Chow, 1997). Briefly, degenerate primers were designed according to conserved regions within the third and seventh TMD of the VPAC ($VPAC_1$ and $VPAC_2$) receptors of various vertebrates. Approximately 5 µg of total RNA from trout brain was reverse-transcribed into cDNA using oligo-(deoxythymidine)$_{12–18}$ primer and SuperScript II reverse transcriptase (Gibco-BRL Life Technologies, Burlington, Ontario, Canada) in a 20-µl mixture. PCR amplification of first strand cDNA (1 µl) was performed in 50-µl reaction mixtures containing 0.2 µmol $l^{-1}$ of each primer (forward and reverse), 2 mmol $l^{-1}$ $MgCl_2$, 0.2 mmol $l^{-1}$ of each dNTP and 2.5 units of Taq DNA polymerase (Gibco-BRL Life Technologies) in 10× PCR buffer (supplied with the enzyme). Following a denaturation step at 94°C for 5 min, the reaction was subjected to 34 cycles of 94°C for 40 s, 60.5°C for 40 s and 72°C for 40 s, with a final extension for 10 min at 72°C in a thermal cycler (Eppendorf Mastercycler).

For the first round of amplification, 1 µl of brain cDNA was used as template with the following primer pairs: VPAC F2 (FOR) 5′-TTCTGGCTKCTRGTGGAAGG-3′ and VPAC R7 (REV) 5′-ACCACAAADCCCTGRAADGABCC-3′. PCR yielded single products of 490 bp. Validity of the PCR products was assessed on the basis of a semi-nested PCR amplification of the products obtained in the initial PCRs. For the second round, 1 µl of the first PCR reaction was used as template with the following primer pairs: VPAC F3 (FOR) 5′-GAGMGGAARTAYTTCTGGKGGTACAT-3′ and the VPAC R7 (REV) primer (see above). Amplification profiles were performed as described above. Each reaction was submitted to electrophoresis on a 1.25% agarose gel stained with ethidium bromide. Gels were visualised under ultraviolet light (BioRad Chemi Doc attached to a camera) and digital images processed using commercial software (Quantity One software, version 4.1.1; BioRad).

PCR products of interest were agarose gel purified (GenElute Gel purification kit; Sigma, St Louis, MO, USA) and cloned into pCR2.1 vector (TOPO TA Cloning kit; Invitrogen, Burlington, Ontario, Canada). Plasmids from selected positive clones were purified using GeneElute Plasmid purification kit (Sigma) or Promega Wizard kit (Fisher Scientific Ltd, Nepean, Ontario, Canada) and the DNA was sequenced on both strands using Li-Cor 4200L DNA sequencer technology (Canadian Molecular Research Services, CMRSinc; Ottawa, Ontario, Canada). Of the different positive clones that were analysed, two groups could be identified after sequencing and determination of the highest nucleotide sequence identity with other known $VPAC_1$ and $VPAC_2$, respectively. As such, they were termed trout $VPAC_1$ and trout $VPAC_2$ receptor cDNA clones, respectively.

*Sequence analysis*

GenBank searches were performed with the standard BLAST algorithms at the National Center for Biotechnology Information (NCBI) using the default settings (Altschul et al., 1997). Sequence alignments were carried out on derived protein sequences using the default settings in CLUSTAL W with DNAMAN software (Lynnon Biosoft, Quebec, Canada). The same software was used for sequence editing and translation. All sequences used for BLAST searches and alignments were edited to include only the area of the trout $VPAC_1$ and $VPAC_2$ receptor sequence, which was amplified in between the primer sites used to obtain the clones.

*Tissue distribution of VPAC1 and VPAC2 receptors by RT-PCR*

Total RNA was isolated from various trout tissues (brain, PCV, kidney, gill, spleen, heart, liver, intestine and blood) and reverse-transcribed as described above. 1 µl of first strand product was then used as template for PCR. PCR was performed using sequence-specific primers. The reaction conditions consisted of 5 min of denaturation at 94°C, followed by 40 s at 94°C, 40 s at 62°C and 40 s extension at 72ºC for 34 cycles, and a 10 min extension at 72°C on the final cycle. Furthermore, β-actin control RT-PCR was performed to verify the quality and integrity of first strand cDNAs produced from the various tissues. The reaction conditions were similar except for 40 s annealing at 55°C. Minus template and single primer control PCRs were also performed.

### *Statistical analysis*

The data are presented as means ± 1 S.E.M. Where appropriate, the data were statistically analysed by a two-way repeated-measures analysis of variance (ANOVA) followed by Dunnett's test for comparison with pre-stimulation values. The fiducial limits of significance were set at 5%. All statistical tests were performed using a commercial statistical software package (SigmaStat version 2.03).

## Results

### *Catecholamine fluorescence histochemistry*

The chromaffin cell-containing fraction of the PCV was identified using the aldehyde-induced green fluorescence that is characteristic of catecholamines (Fig. 1A–C). The pattern of labelling revealed a distinct layer of catecholamine-containing cells lining the wall of the PCV in the immediate vicinity of the lumen, with many fewer chromaffin cells lying away from this grouping. In other sections, PCV tissue was double labelled using the aldehyde-induced fluorescence of catecholamines and immunostaining for DβH (Fig. 1D,E). Co-localisation of catecholamine-containing and DβH-positive cells confirmed that there was a high density of chromaffin cells adjacent to the lumen of the PCV. The presence of chromaffin cells was also verified by measuring tissue catecholamine content in the PCV and surrounding kidney tissue (Fig. 2). While low concentrations of catecholamines were detected in the kidney tissue, high levels were measured in the PCV, with the highest concentration being located in the anterior region. Adrenaline was the predominate catecholamine stored in the PCV.

Multi-labelling experiments revealed that only a small number of DβH-positive cells were immunonegative for PNMT (Fig. 3). This finding indicates that, in the chromaffin tissue of trout, there are relatively few numbers of cells containing only noradrenaline. Labelling with biotinylated

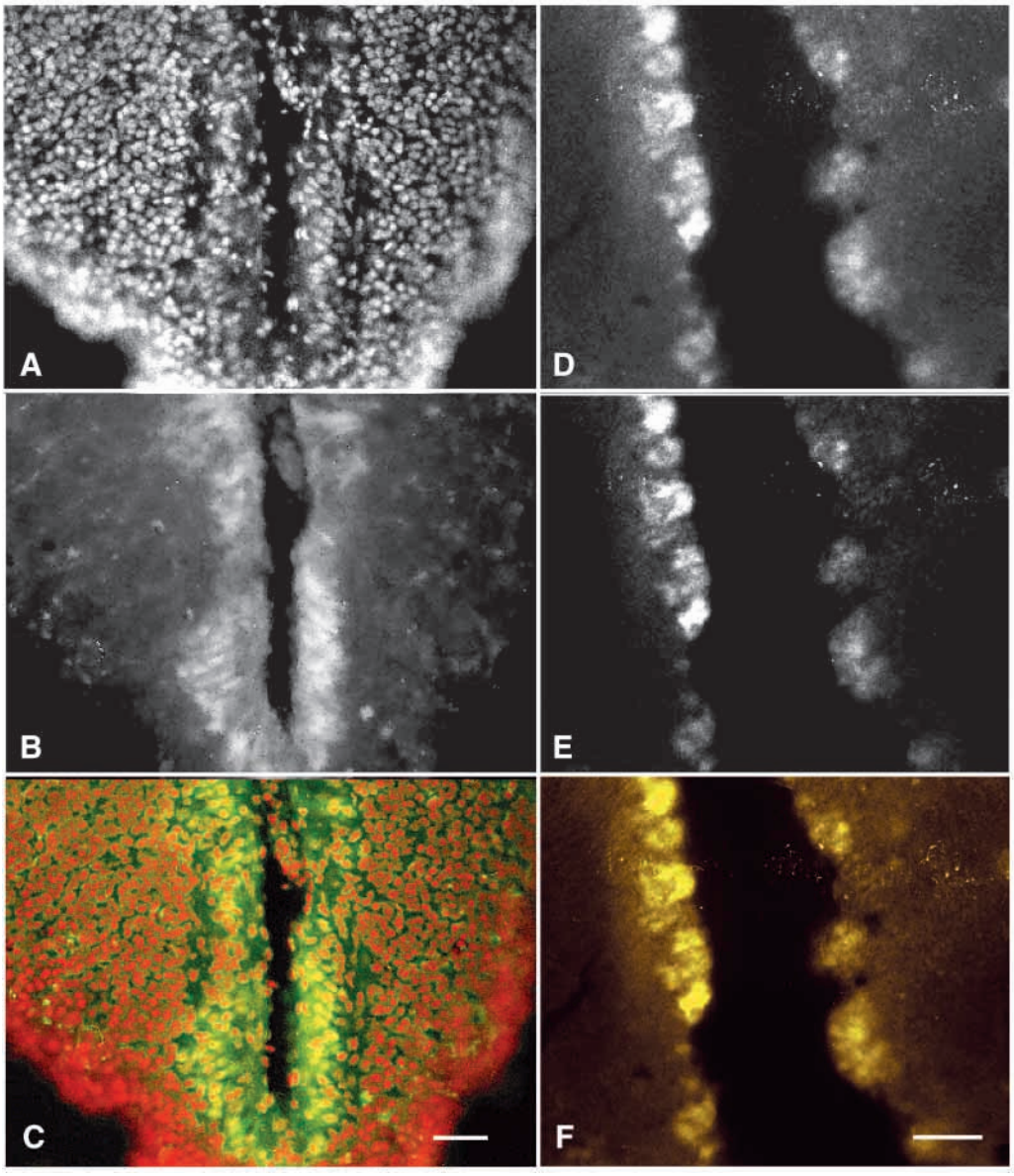


Fig. 1. Identification and localisation of chromaffin cells within the posterior cardinal vein (PCV) tissue of rainbow trout based on the double labelling of (A) nucleic acids and (B) catecholamines or the double labelling of (D) dopamine β-hydroxylase (DβH) and (E) catecholamines. Panel C is an overlay image of the sections shown in A and B (nucleic acids and catecholamines appear in red and green/yellow, respectively). Panel F is an overlay image of the sections shown in D and E (DβH and catecholamines appear in red and green, respectively; yellow indicates co-localisation of catecholamines and DβH). Note that the labelling patterns for DβH and catecholamines are identical. Scale bars D–F, 100 μm; scale bars A–C, 50 μm.

VIP revealed that all DβH-positive (chromaffin) cells appeared to possess VIP-binding sites. Moreover, the pattern of labelling of VIP demonstrated that VIP-binding sites were present on both PNMT-positive (adrenaline-containing) and PNMT-negative (noradrenaline-containing) chromaffin cells.

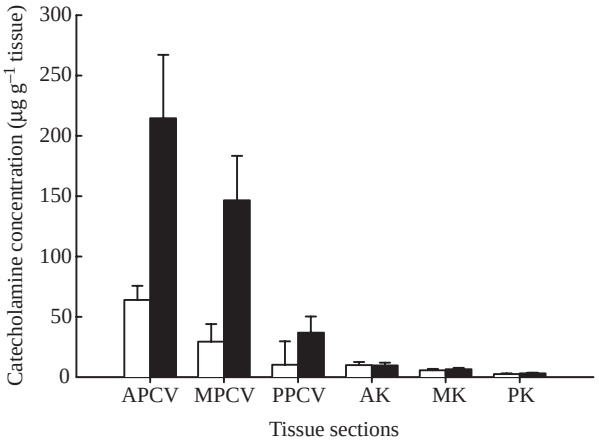


Fig. 2. Mean tissue concentrations (μg catecholamine $g^{-1}$ tissue) ± 1 S.E.M. of noradrenaline (open columns) and adrenaline (filled columns) in rainbow trout (*N*=6). Abbreviations: APCV, anterior posterior cardinal vein; MPCV, middle posterior cardinal vein; PPCV, posterior posterior cardinal vein; AK, anterior kidney; MK, middle kidney; PK, posterior kidney.

Control tissue sections exhibited dull non-specific autofluorescence in comparison with the bright specific fluorescence illustrated in Figs 1, 3. Excess unlabelled ckVIP eliminated the specific fluorescence obtained using the streptavidin–biotinylated VIP probe complex on chromaffin cells (see below). Incubation of sections without primary antisera did not produce any specific labelling.

To confirm that biotinylated VIP was indeed binding to VIP receptors, its bioactivity was tested using an *in situ* saline-perfused PCV preparation. Bolus injection of biotinylated VIP caused a significant increase in the rate of secretion of adrenaline; noradrenaline secretion was unaffected (Fig. 4). Administration of saline did not cause the release of either catecholamine.

### *Pharmacological assessment of VIP-binding sites*

To assess the chromaffin cell VIP-binding sites pharmacologically, sections of the PCV were treated with non-conjugated VIP and PACAP receptor agonists/antagonists prior to incubation with biotinylated VIP for visualisation of binding sites (Fig. 5). While prior treatment with the VIP

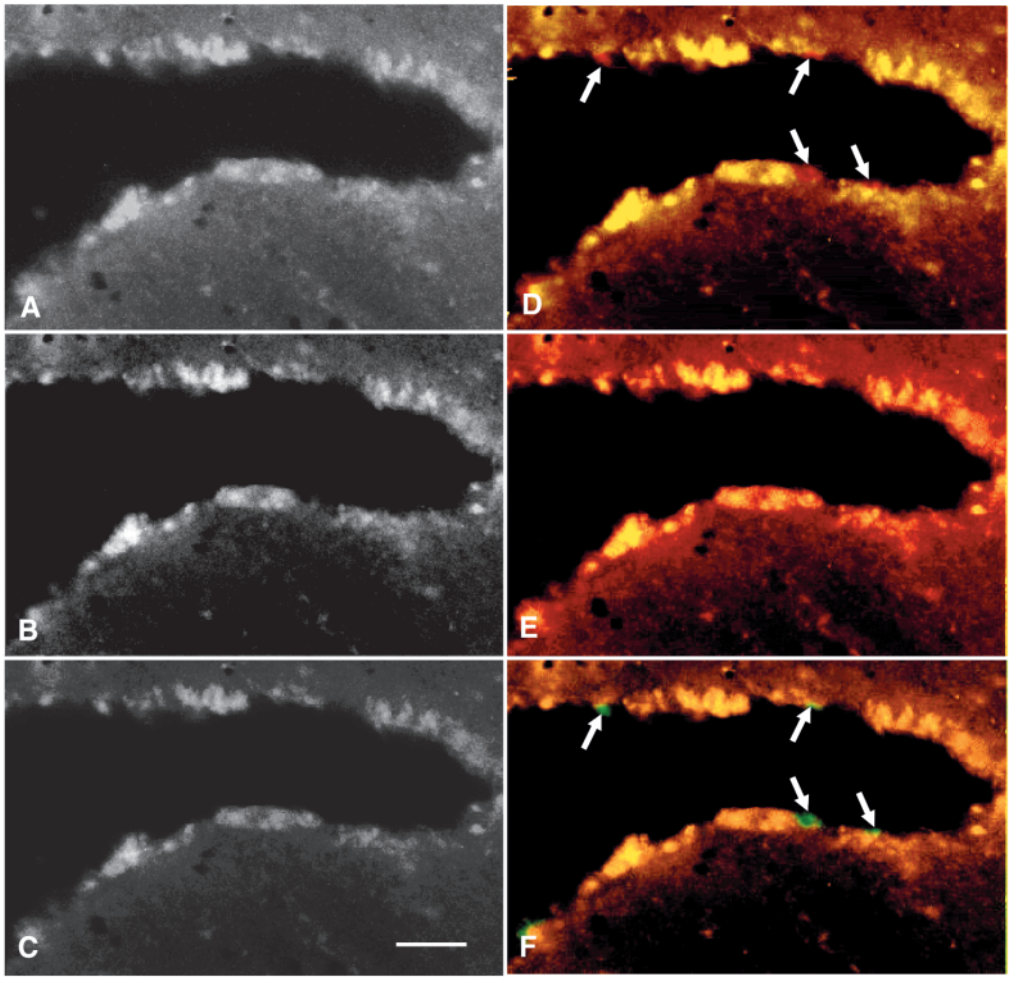


Fig. 3. Localisation of vasoactive intestinal polypeptide (VIP)-binding sites to the chromaffin cell subtypes in the posterior cardinal vein of rainbow trout using triple labelling. (A) Dopamine β-hydroxylase (DβH)-immunoreactive cells (mouse anti-DβH). (B) Phenylethanolamine *N*-methyl transferase (PNMT)-immunoreactive cells (rabbit anti-bovine PNMT). (C) Labelling of VIP-binding sites. (D) Overlay image of the sections shown in A and B (DβH- and PNMT-positive cells); the PNMT-negative cells are indicated by arrows. (E) Overlay image of the sections shown in A and C (DβH and VIP labelling). (F) Overlay image of the sections shown in B and C (PNMT and VIP labelling); the PNMT-negative cells are indicated by arrows. Yellow/orange colour indicates co-localisation. Note that only a few of the DβH-labelled cells were negative for PNMT, thus indicating only a small number of noradrenaline-containing cells. Moreover, the pattern of VIP binding was identical to that of DβH, indicating that all chromaffin cells possess VIP-binding sites. Scale bars A–F, 150 μm.

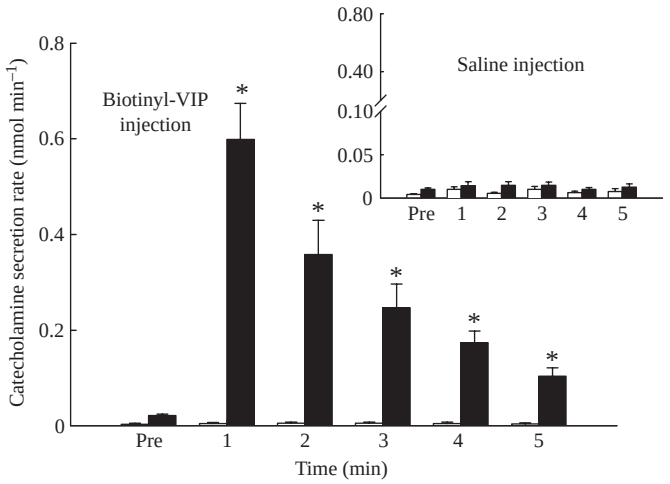


Fig. 4. Secretion rates of adrenaline (filled columns) and noradrenaline (open columns) in response to a bolus injection of saline (inset; $N$=6) or biotinylated vasoactive intestinal polypeptide (VIP) ($10^{-9}$ mol kg$^{-1}$; $N$=6) using an *in situ* posterior cardinal vein preparation of rainbow trout. Values are shown as means ± 1 S.E.M. Asterisks denote a significant difference from the pre-stimulation (Pre) value ($P$<0.05).

receptor agonist ckVIP, the VIP receptor antagonist VIP 6-28 and the PACAP receptor agonist hPACAP-27 eliminated the fluorescence obtained with the DTAF-conjugated streptavidin–biotinylated VIP complex, treatment with the PACAP receptor antagonist PACAP 6-27 had no effect on the binding of biotinylated VIP.

### *Cloning and sequence analysis of the trout VPAC$_1$ and VPAC$_2$ receptor partial clones*

Although an apparently single 490-bp cDNA fragment was amplified following PCR with the degenerate primers designed against the VPAC receptors, two different clones of similar sizes were ultimately isolated. Sequence analyses of the DNA inserts revealed that the partial sequences corresponded to VPAC$_1$ andVPAC$_2$ receptors. As such, the partial cDNA clones were subsequently termed trout VPAC$_1$ and trout VPAC$_2$, respectively. A BLASTn search of the GenBank database using the partial sequences of the trout clones produced matches corresponding to receptors belonging to the VIP/PACAP/glucagon/secretin/parathyroid/calcitonin receptor family. For the trout VPAC$_1$ partial cDNA clone, the best match was to the goldfish VPAC$_1$ receptor (E value, $10^{-29}$). At the protein level (Fig. 6), the trout sequence displayed highest amino acid identity (88%) with the goldfish VPAC$_1$ receptor. For the trout VPAC$_2$ receptor partial cDNA clone, the best match (BLASTn search) was to the rat/mouse/human VPAC$_2$ receptor (E values, $10^{-4}$). Each of the VPAC$_2$ receptor matches showed approximately 60% identity with the trout VPAC$_2$ sequence.

Sequence alignments of the trout sequences indicated that they are homologous to their vertebrate counterparts. Visual inspection of the deduced amino sequences of the trout partial

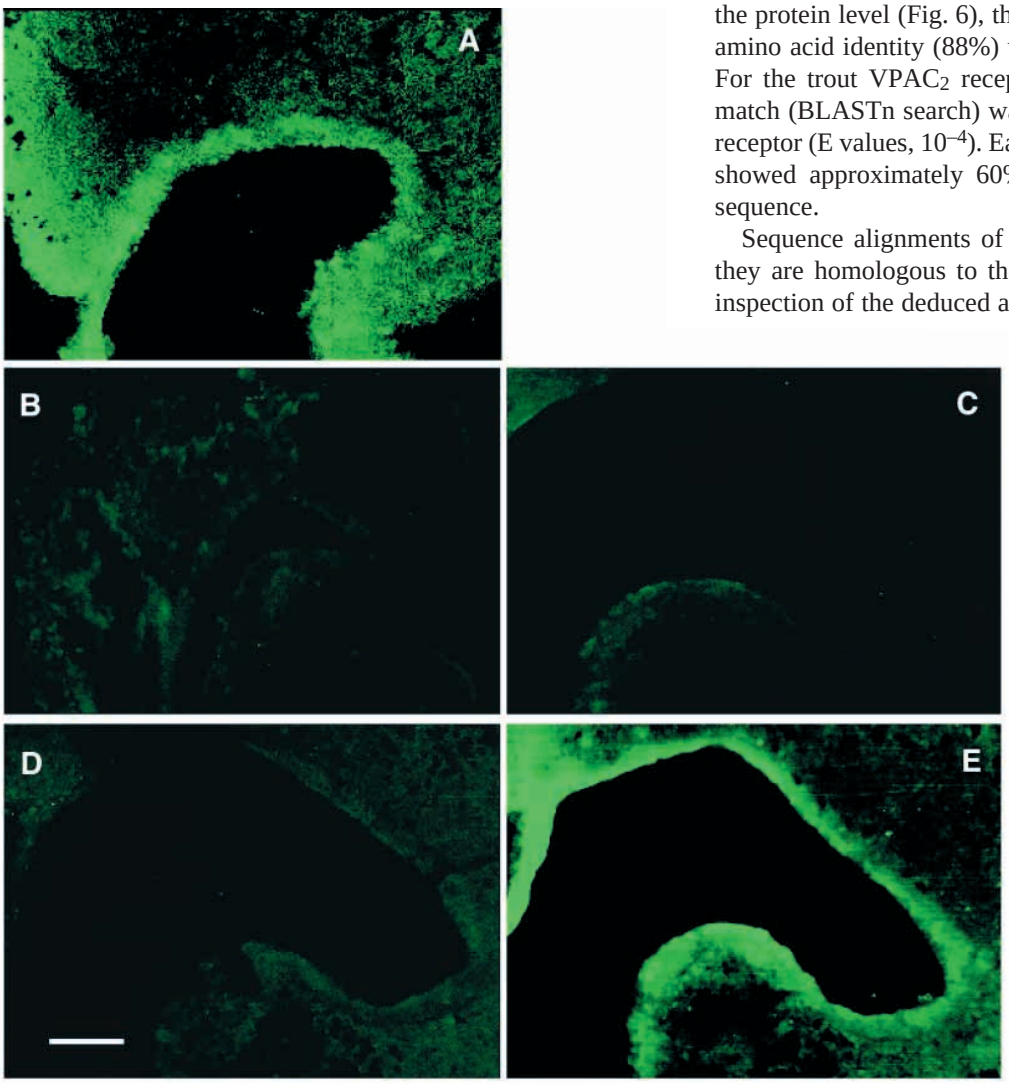


Fig. 5. Pharmacological assessment of the vasoactive intestinal polypeptide (VIP)-binding sites. Labelling of VIP-binding sites following treatment with different VPAC and PAC receptor agonists and antagonists. (A) Control, labelling of VIP-binding sites. (B) Labelling of VIP-binding sites following treatment with ckVIP. (C) Labelling of VIP-binding sites following treatment with the VIP receptor antagonist VIP 6-28. (D) Labelling of VIP-binding sites following treatment with the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor agonist PACAP-27. (E) Labelling of VIP-binding sites following treatment with the PAC$_1$ receptor antagonist PACAP 6-27. Scale bars A–E, 150 μm.

clones (Fig. 6) revealed motifs that are common to $VPAC_1$ and $VPAC_2$. A signature motif for the $VPAC_1$ receptor was identified at a unique cysteine residue within TMD 5, just preceding the 'IIRIL' motif. A consensus phosphorylation sequence for protein kinase C is also located in the 2nd intracellular loop between TMDs 3 and 4 of $VPAC_1$ receptors (Chow 1997; Chow et al., 1997). The conserved 'SE-R/K' motif is also found in several other members in the same receptor family, including the secretin, GHRH (growth hormone releasing hormone), glucagon and PTH (parathyroid hormone) receptors. Another motif, the 'PDI I/V' found only in $VPAC_1$, $VPAC_2$ and $PAC_1$ receptors between the putative TMD 5 and TMD 6 (Chow et al., 1997), was also present in the trout sequences. The 'RLA K/R' motif immediately in front of TMD 6 also matches the consensus found in other members of G-protein-coupled receptors.

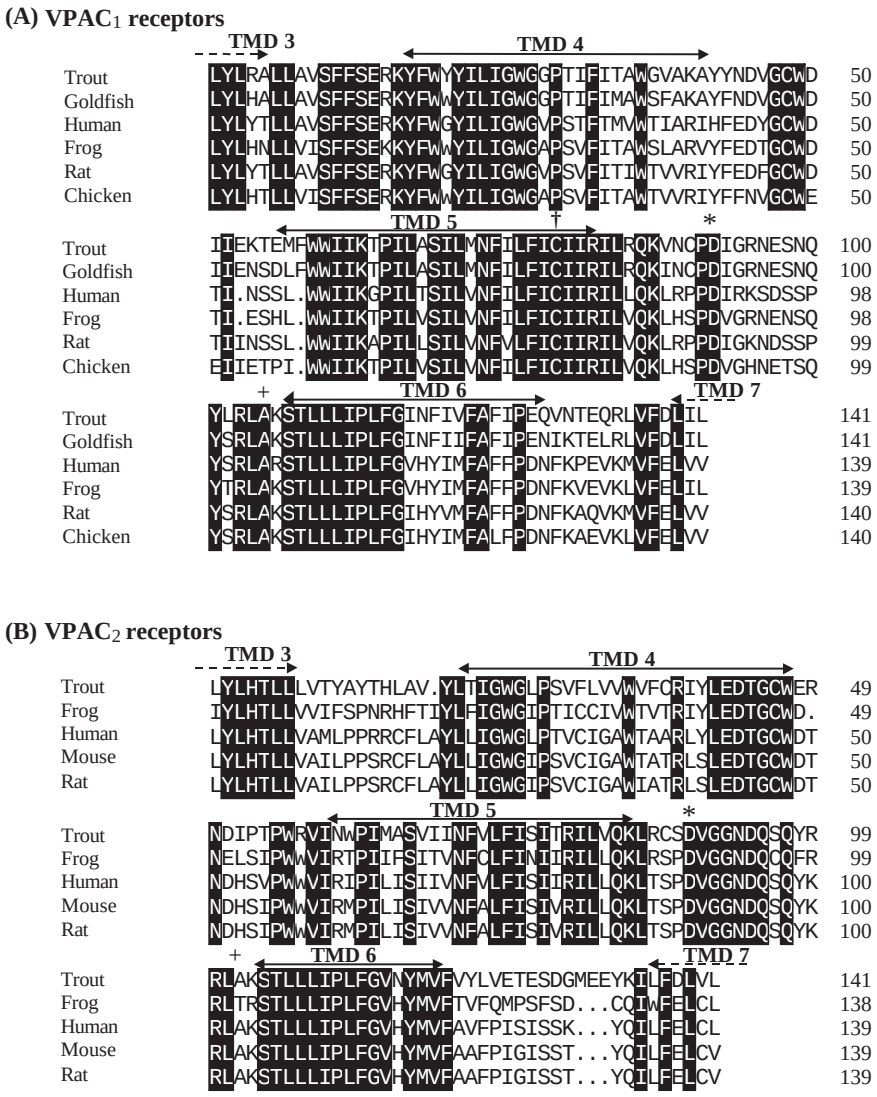


Fig. 6. Comparison of deduced partial amino acid sequences of the putative trout $VPAC_1$ (A) and $VPAC_2$ (B) receptors with those of selected vertebrates. Conserved amino acids (100% identity) among taxa are highlighted. Sequences were aligned using CLUSTAL W and the alignment was prepared from DNAMAN. The putative trans-membrane domains (TMDs) are denoted by horizonatal arrows and were deduced by visual inspection of published reports and by hydrophobocity analysis. The $G_{s\alpha}$-protein coupling motif (basic-L/A L/A/V/S-basic) is shown by a plus. The RLA K/R motif is common to members of the G-protein-coupled receptor family. The PD I/V motif common to vasoactive intestinal polypeptide (VIP)-binding receptors is shown by an asterisk. Finally, the conserved cysteine preceding the IIRIL motif, common only to $VPAC_1$ receptors, is shown by a dagger. Sequences were edited to include only the region spanning the primer sites used to obtain the trout partial cDNA clones.

*Tissue distribution of $VPAC_1$ and $VPAC_2$ receptors by RT-PCR*

Sequence-specific primers were designed for each receptor. Trout $VPAC_1$ receptor mRNA was found in brain, liver and intestine. Additionally, while less-prominent products were observed in the PCV, kidney, gill, spleen and heart, no product was observed in blood (Fig. 7). For trout $VPAC_2$ receptor mRNA, products were observed in the brain, spleen, heart, liver and intestine. No products were evident in the PCV, kidney, gill or blood (Fig. 7).

## Discussion

In a previous study, we demonstrated that VIP and PACAP were able to directly elicit the secretion of adrenaline from trout chromaffin cells (Montpetit and Perry, 2000). The results of that study suggested that VIP- or PACAP-evoked catecholamine secretion was being mediated by VPAC-type receptors. The present study, using fluorescent histochemical techniques, extends these findings by showing that VIP receptors are specifically localised to chromaffin cells concentrated around the lumen of the posterior cardinal vein (PCV). The presence of VIP- and PACAP-binding sites in defined pathways in the central and peripheral nervous systems has been previously demonstrated in higher vertebrates (Vertongen et al., 1997; Becker et al., 2000; Vaudry et al., 2000). In the present study, the identification of specific VIP-binding sites on chromaffin cells is reported for the first time.

In teleost fish, catecholamines that enter the circulation are secreted from chromaffin cells that are located within the walls of the PCV in the region of the head kidney (Reid et al., 1998). The distribution of chromaffin cells in a discrete layer within the walls of the PCV supports the results of several previous histological

studies (Reid et al., 1995; Furimsky et al., 1996; Bernier and Perry, 1997). The pattern of labelling suggests that the majority of chromaffin cells form aggregates adjacent to the lumen of the PCV, although individual cells can also be observed away from these groupings.

In vertebrates, a variety of studies have demonstrated that adrenaline and noradrenaline are stored, at least in part, in separate populations of chromaffin cells. The existence of separate noradrenaline- and adrenaline-containing cells has been previously demonstrated in teleost fish (Mastrolia et al., 1984; Gallo et al., 1993; Kloas et al., 1994; Reid et al., 1995; Abelli et al., 1996). In the present study, the observation that some of the chromaffin cells were immunopositive for DβH but not for PNMT confirmed the presence of a sub-population of chromaffin cells containing only noradrenaline. However, the vast majority of the chromaffin cells in the PCV were PNMT-immunoreactive. Because PNMT catalyses the methylation of noradrenaline to adrenaline, its presence is indicative of adrenaline-containing chromaffin cells. This finding is consistent with previous results demonstrating that adrenaline is the more abundant catecholamine in trout chromaffin tissue (Reid et al., 1995).

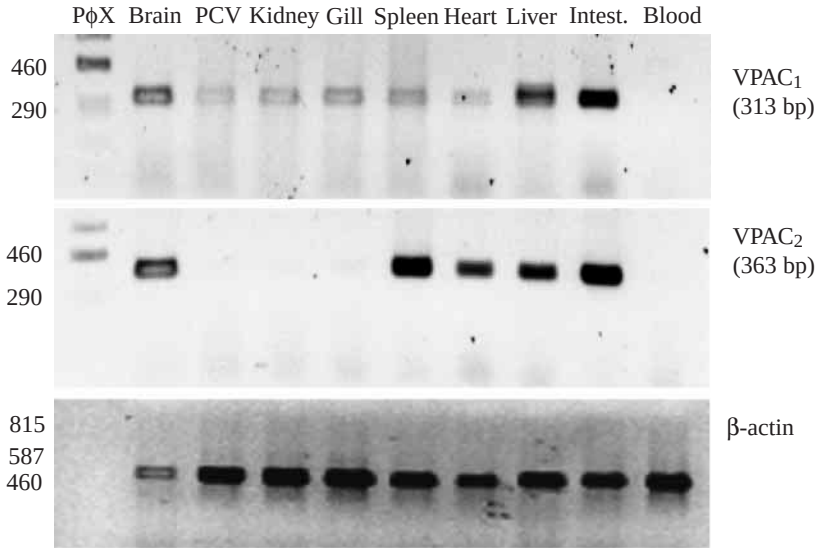


Fig. 7. Tissue distributions of the putative trout $VPAC_1$ and $VPAC_2$ receptors as revealed by RT-PCR. PCR was performed using the sequence-specific primers deduced from the partial cDNA clones for each receptor. Using the sequence-specific primers for trout $VPAC_1$ receptors, an expected 313-bp product was observed in all tissues with the exception of blood. Prominent products were observed in the liver and intestine. Using sequence-specific primers for trout $VPAC_2$ receptors, an expected 363-bp product was observed in brain, spleen, heart, liver and intestine. No products were observed in posterior cardinal vein (PCV), kidney, gill and blood. As an internal control, RT-PCR for trout β-actin was performed. In all tissues, PCR products specific for trout β-actin were consistently observed. PϕX, DNA molecular size ladder.

The concentration of catecholamines within the chromaffin tissue and the extent of their release differ greatly within and between fish species (Reid et al., 1998). Yet, the fact that different secretagogues can preferentially induce the secretion of a particular catecholamine implies an ability to specifically stimulate subpopulations of chromaffin cells. For example, administration of cholinergic agonists to *in situ* preparations causes the release of adrenaline and noradrenaline (Nilsson et al., 1976; Opdyke et al., 1983; Al-Kharrat et al., 1997; Gfell et al., 1997; Montpetit and Perry, 1999; Montpetit et al., 2001). By contrast, while the administration of angiotensin II elicits predominantly the secretion of adrenaline in fish (Bernier and Perry, 1997; Bernier et al., 1999), injection of natriuretic peptide in dogfish (*Squalus acanthias*) causes the release of noradrenaline only (McKendry et al., 1999; Montpetit et al., 2001). However, because the results of the present study demonstrated the presence of VIP receptors on both types of chromaffin cells, the selectivity of VIP and PACAP for adrenaline secretion cannot be explained by differential receptor abundance on the chromaffin cell subtypes. Nor can the greater numbers of PNMT-positive chromaffin cells explain the selective secretion of adrenaline. Indeed, although the PNMT-positive cells are referred to as 'adrenaline cells' (Reid et al., 1995, 1998), they also contain and secrete noradrenaline, albeit at lower levels.

VIP and PACAP exert tissue-specific effects by interacting with three distinct receptors (Harmar et al., 1998). Two of these receptors, $VPAC_1$ and $VPAC_2$, bind VIP and PACAP with equal affinity, whereas a third receptor, $PAC_1$, preferentially binds PACAP. To characterise the receptors that mediate the stimulatory effects of VIP and PACAP on catecholamine release from trout chromaffin cells, several pharmacological approaches were used in a previous study (Montpetit and Perry, 2000). Exogenous administration of a range of doses of VIP and PACAP to *in situ* perfused PCV preparations caused the release of adrenaline in a dose-dependent manner. Evaluation of the effective dose eliciting 50% of the maximal secretion ($ED_{50}$) revealed that VIP and PACAP elicited release of adrenaline with comparable potencies. Furthermore, neuronal stimulation (low frequency) or adrenaline secretion in response to VIP or PACAP was inhibited in the presence of the VPAC receptor antagonist VIP 6-28 (Montpetit and Perry, 2000) while being unaffected by the $PAC_1$ receptor antagonist PACAP 6-27. The results of the present study, employing histochemistry in conjunction with prior application of receptor agonists and antagonists, support the findings of the previous investigation. Indeed, labelling of VIP-binding sites was prevented in tissues pre-treated with unlabelled VIP, VPAC receptor antagonist and PACAP but not with $PAC_1$ receptor antagonist. Together, these results argue for a role of VPAC receptors regulating chromaffin cell activity in the rainbow trout.

In the present study, different forms of VIP were used to

visualise and characterise receptors. The primary sequences of VIPs share considerable sequence similarity among vertebrates, with only minor amino acid changes having been reported. Indeed, the VIP amino acid sequence of fish (dogfish, trout and cod), amphibians, reptiles and birds differs from the mammalian VIP at only 4–5 positions (Nilsson, 1975; Dimaline et al., 1987; Thwaites et al., 1989; Holmgren and Jensen, 1994; Wang and Conlon, 1995). While the trout PACAP amino acid sequence has not yet been ascertained, PACAPs among other vertebrates (rat, sheep, mouse, human, amphibian, chicken and teleost fish) also share a high degree of sequence similarity (Wong et al., 1998). Results indicate that species variations of VIP and PACAP involve conservative substitutions that do not affect the biological activities of the peptides (Lundin and Holmgren, 1984; Dimaline et al., 1987; Aldman and Holmgren, 1992; Chow, 1997; Wong et al., 1998; Alexandre et al., 1999; Montpetit and Perry, 2000; Hoo et al., 2001).

Whether or not trout VPAC receptors respond typically to VIP, PACAP and receptor antagonists is not known. Recently, goldfish $VPAC_1$ and $PAC_1$ receptors were cloned and functionally characterised (Chow, 1997; Chow et al., 1997; Wong et al., 1998). Using heterologous VIPs and PACAPs, the VPAC and $PAC_1$ receptors responded in an expected manner based on the pharmacological characteristics of these receptors (Chow, 1997; Wong et al., 1998). In the present study, three partial clones exhibiting sequence properties of the $VPAC_1$, $VPAC_2$ and $PAC_1$ receptors (data shown for VPAC receptor clones only) were obtained from trout brain. It would appear then that, as in higher vertebrates, trout possess all three receptors.

Tissue distribution experiments using RT-PCR demonstrated the presence of $VPAC_1$ receptors, but not $VPAC_2$ receptors, in the PCV tissue. These preliminary results suggest that, in trout, the chromaffin cell VPAC receptors mediating adrenaline secretion are of the $VPAC_1$ subtype. However, the results of the RT-PCR experiments cannot identify the source of cells expressing the $VPAC_1$ mRNA. Unlike in mammals, fish chromaffin cells are not organised into a distinct gland. Indeed, the heterogeneous populations of cells within the PCV have made it difficult to isolate pure populations of chromaffin cells from the tissue. Therefore, detection of these receptors specifically on chromaffin cells could not be performed.

The inability of prior studies to clone the $VPAC_2$ receptor in lower vertebrates has led to the suggestion of the existence of a unique VPAC receptor in these species (Vaudry et al., 2000). However, recently, a $VPAC_2$ receptor was cloned and functionally characterised in the frog (*Rana tigrina rugulosa*; Hoo et al., 2001). By isolating partial clones for the trout $VPAC_1$, $VPAC_2$ and $PAC_1$ receptors, we have provided direction for future investigations into the VIP and PACAP system and the neuronal control of catecholamine release in fish. Although it seems likely that the partial cDNA clones reported in this paper also correspond to specific $VPAC_1$, $VPAC_2$ and $PAC_1$ receptor types, definite identification of these receptors must await the isolation of complete gene sequences and functional characterisation of the encoded proteins.

The results of this and an earlier study (Montpetit and Perry, 2000) reveal that both VIP and PACAP can potentially contribute to catecholamine secretion from trout chromaffin cells. However, whether or not these neuropeptides *actually* contribute significantly to the afferent limb of the adrenergic stress response *in vivo* has yet to be determined. Indeed, the release of these neuropeptides during sympathetic activation of the chromaffin cell in response to acute stressors such as hypoxia, air exposure or handling remains to be confirmed. However, we have recently shown that cholinergic and VPAC receptor antagonists can inhibit catecholamine secretion during electrically evoked neuronal stimulation of the chromaffin cells in trout, thus implicating the release of acetylcholine, VIP and/or PACAP under these conditions (Montpetit and Perry, 2000). Further research should now address the relative contribution of VIP and PACAP *in vivo* to establish the functional significance of these neuropeptides (and their receptors) in the control of catecholamine secretion in fish.

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