INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa • University of Ottawa

PERMISSION DE REPRODUIRE ET DE DISTRIBUER LA THÈSE

PERMISSION TO REPRODUCE AND DISTRIBUTE THE THESIS

NOM DE L'AUTEUR / NAME OF AUTHOR	MONTPETIT, Colin
ADRESSE POSTALE / MAILING ADDRESS:	189 Deschamps St. Vanier, Ontario K1L 5Z3
GRADE / DEGREE:	**ANNÉE D'OBTENTION / YEAR GRANTED**
Ph.D. (Biology)	2003
TITRE DE LA THÈSE / TITLE OF THESIS:	
Neuronal Control of Catecholamine Release in the Rainbow Trout (*Oncorhynchus mykiss*)	

L'auteur permet, par la présente, la consultation et le prêt de cette thèse en conformité avec les règlements établis par le bibliothécaire en chef de l'Université d'Ottawa. L'auteur autorise aussi l'Université d'Ottawa, ses successeurs et cessionnaires, à reproduire cet exemplaire par photograhie ou photocopie pour fins de prêt ou de vente au prix coûtant aux bibliothèques ou aux chercheurs qui en feront la demande.

Les droits de publication par tout autre moyen et pour vente au public demeureront la propriété de l'auteur de la thèse sous réserve des règlements de l'Université d'Ottawa en matière de publication de thèses.

The author hereby permits the consultation and the lending of this thesis pursuant to the regulations established by the Chief Librarian of the University of Ottawa. The author also authorizes the University of Ottawa, its successors and assignees, to make reproductions of this copy by photographic means or by photocopying and to lend or sell such reproductions at cost to libraries and to scholars requesting them.

The right to publish the thesis by other means and to sell it to the public is reserved to the author, subject to the regulations of the University of Ottawa governing the publication of theses.

N.B. LE MASCULIN COMPREND ÉGALEMENT LE FÉMININ

Dec. 5, 2002
DATE

Colin Montpetit
(AUTEUR) SIGNATURE (AUTHOR)

Université d'Ottawa · University of Ottawa

Université d'Ottawa · University of Ottawa

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

MONTPETIT, Colin J.

AUTEUR DE LA THÈSE - AUTHOR OF THESIS

Ph.D. (Biology)

GRADE - DEGREE

Biology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Neuronal Control of Catecholamine Release in the Rainbow Trout (*Oncorhynchus mykiss*)

Steve Perry

DIRECTEUR DE LA THÈSE - THESIS SUPERVISOR

EXAMINATEURS DE LA THÈSE - THESIS EXAMINERS

J. Fenwick

T. Moon

K. Olson

J.-M. Weber

B. Willmore

J.-M. De Koninck, Ph.D.

LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

SIGNATURE

DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

NEURONAL CONTROL OF CATECHOLAMINE RELEASE

IN THE RAINBOW TROUT

(*Oncorhynchus mykiss*)

By

Colin J. Montpetit

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
Ph.D. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
la Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention du doctorat à

L'Institut de biologie d'Ottawa-Carleton

Doctor of Philosophy (2002), University of Ottawa (Biology)

Title: Neuronal control of catecholamine release in the rainbow trout (*Oncorhynchus mykiss*).

Author: Colin J. Montpetit, B.Sc. (University of Ottawa)

Thesis advisor: Dr. Steve F. Perry, Ph.D. Professor, University of Ottawa.

Research funded in part by:

Ontario Graduate Scholarship – Science and Technology (OGSST)

National Science and Engineering Research Council – Post-Graduate Scholarship (NSERC)

Ontario Graduate Scholarship (OGS)

ABSTRACT

The aim of this thesis was to study aspects of the neuronal control of catecholamine secretion in a teleost, the rainbow trout. The development and validation of a nerve stimulation technique made it possible to determine that a portion of the neuronal control of catecholamine release, which prevailed at low frequency stimulation, could be attributed to vasoactive intestinal polypeptide (VIP) and/or pituitary adenylate cyclase activating polypeptide (PACAP). On the other hand, cholinergic stimulation predominated during higher levels of neuronal activity. Fluorescent histochemical techniques in combination with pharmacological approaches provided direct evidence that VIP and PACAP can elicit the secretion of adrenaline, only, from the chromaffin tissue via specific VIP binding sites that exhibited properties of VPAC receptors.

Using *in situ* perfused posterior cardinal vein preparations, evidence was provided that while the nicotinic receptor appears to be the predominant pathway mediating the effects of acetylcholine on catecholamine secretion, muscarinic receptor stimulation may augment the cellular response to nicotinic receptor activation. Under extreme conditions, muscarinic receptors may directly elicit the secretion of catecholamines.

The impact of extracellular catecholamines on catecholamine secretion from chromaffin cells was also investigated. Results revealed that the mechanisms of adrenergic inhibition of catecholamine secretion in response to cholinergic stimulation include activation of chromaffin cell membrane β_2-receptors and presynaptic α_2-adrenergic receptors. However, catecholamine release in response to VIP appears to be insensitive to the adrenergic negative feedback mechanisms.

Finally, despite the rapid progress in cDNA cloning, molecular information on the receptors mediating the effects of VIP and PACAP in fish is scant. In this thesis, I report preliminary findings of the cloning of the trout PAC_1, $VPAC_1$, and $VPAC_2$ receptors from brain cDNA.

In summary, VIP and PACAP appear to function as neurotransmitters in the neuronal regulation of catecholamine release in this species. The apparent complexity of the mechanisms regulating the secretion of catecholamines from trout chromaffin cells may reflect the precise control required for these hormones to play their role in physiological and biochemical homeostasis in these organisms.

RÉSUMÉ

Le but principal de cette thèse était d'étudier certains aspects du contrôle nerveux de la sécrétion des catécholamines chez un poisson téléostéen, la truite. Le développement d'une manipulation neuronale nous a permis de déterminer qu'une portion du contrôle nerveux de la sécrétion, s'exprimant à de basse fréquence de stimulation neuronale, pouvait être attribué aux neurotransmetteurs tels que le peptide intestinale vasoactif (VIP) et/ou le peptide activant l'adenylate cyclase pituitaire (PACAP). Au contraire, la stimulation cholinergique prédominait lors de stimulation à fréquence élevée. Des techniques histochimiques ont fourni des preuves direct que le VIP et le PACAP sont capable d'induire la sécretion de l'adrénaline par l'intermédiaire des récepteurs spécifiques pour le VIP.

Quoique les récepteurs cholinergiques du type nicotinique paraît être le réseau prédominant effectuant les effets de l'acetylcholine, des résultats en provenance d'une préparation *in situ* de perfusion de la veine cardinale postérieure ont fourni des preuves que la stimulation des récepteurs muscariniques semble augmenter la réponse cellulaire suivant la stimulation des récepteurs nicotiniques. Par contre, sous conditions extrêmes, les récepteurs muscariniques peuvent influencer directement la sécrétion des catécholamines.

L'impact des catécholamines sur la sécrétion des catécholamines fut aussi étudié. Les résultats démontrent que l'inhibition adrénergiques de la sécretion des catécholamines suivant la stimulation cholinergique impliquent l'activation des récepteurs β_2-adrénergiques sur la membrane des cellules chromaffins et les récepteurs

α_2-adrénergiques présynaptiques. Par contre, la sécretion des catécholamines en réponse au VIP paraît insensible à l'inhibition adrénergique.

Finalement, en dépis du progrès rapide du cloning de l'ADN_c, l'information moléculaire sur les récepteurs exprimant les effets du VIP and PACAP chez les poissons demeure minime. Dans cette thèse, je révêle des résultats préliminaires pour le cloning des récepteurs PAC_1, $VPAC_1$, et $VPAC_2$ en provenance de l'ADN_c du cervaux de la truite.

En conclusion, le VIP et le PACAP semble fonctionner comme neurotransmetteurs dans la régulation neuronale de la sécrétion des catécholamines chez la truite. La complexité apparente des méchanismes de régulation de la sécrétion des catécholamines réflête probablement le contrôle précis requis pour que les catécholamines jouent leurs rôles dans l'homéostasie physiologique et biochimique chez cet organisme.

ACKNOWLEDGEMENTS

There are numerous people whom I would like to thank for making my time in the department an enjoyable and rewarding experience. First, I would like to thank my thesis advisor for recognizing my hockey talent and giving me the opportunity to pursue graduate studies in his lab. Certainly, his passion and dedication to his work has enabled me to discover the field of comparative research and the power of science.

I would like to thank Drs Jim Fenwick, James Cheetham, Vance Trudeau, and Ken Marshall for serving on my advisory committee. I wish to thank Dr. Dave Brown and Andrew Ochalsky for their assistance with the microscopy work and generous gift of secondary antibodies for the work presented in chapter 5. I am much indebted to Arash Shasavarani and Mustafa Baya for their guidance and valuable suggestions concerning the molecular cloning aspects of my thesis (chapter 6). Without your help, the project would not have been a valuable learning experience.

Many thanks to past and present labmates for providing a dynamic working environment and a lifetime of memories. To Alex Julio, Marosh Furimsky, John McKendry, Pat Desforges, Stuart Harman, Tina Georgalis, Arash Shasavarani, Mustafa Baya, Brian McNeil, Christian Bordeleau, Caroline Mimeault, Natasha Galant, Mike Lortie, Stephen Dugan, Gavin Harman, thanks for everything.

Finally, this thesis would not have been possible without the personal support and encouragement from my families, the Montpetit's and Lamontagne's. Thank you for giving me the strength and belief to undertake such a challenge.

"SI TU LE VEUX.....

....TU LE PEUX".

(Robert Montpetit)

TABLE OF CONTENTS

ABSTRACT iii

RÉSUMÉ v

ACKNOWLEDGMENTS vii

TABLE OF CONTENTS ix

LIST OF FIGURES xii

LIST OF TABLES xx

LIST OF ABBREVIATIONS xxi

CHAPTER 1. GENERAL INTRODUCTION 1

RATIONALE FOR THE THESIS 2

BACKGROUND KNOWLEDGE:

Humoral catecholamines 4

Mechanisms of catecholamine release 5

Neuronal control of catecholamine release 8

Cholinergic control of catecholamine release 9

Non-cholinergic control (VIP and PACAP) of catecholamine release 11

AIM AND OUTLINE OF THE THESIS 14

CHAPTER 2. Development and validation of an *in situ* saline perfused posterior cardinal vein – nerve stimulating technique 17

INTRODUCTION 18

MATERIALS AND METHODS 20

RESULTS 25

DISCUSSION 37

CHAPTER 3. Cholinergic control of catecholamine secretion from chromaffin cells in the rainbow trout (*Oncorhynchus mykiss*) **40**

INTRODUCTION **41**

MATERIALS AND METHODS **44**

RESULTS **47**

DISCUSSION **56**

CHAPTER 4. VIP and PACAP-mediated control of catecholamine release from the chromaffin tissue in the rainbow trout (*Oncorhynchus mykiss*) **63**

INTRODUCTION **64**

MATERIALS AND METHODS **66**

RESULTS **71**

DISCUSSION **92**

CHAPTER 5. Localisation and characterisation of vasoactive intestinal polypeptide (VIP) receptors in chromaffin cells of the rainbow trout (*Oncorhynchus mykiss*) **99**

INTRODUCTION **100**

MATERIALS AND METHODS **102**

RESULTS **108**

DISCUSSION **120**

CHAPTER 6. Molecular cloning and tissue expression of trout (*Oncorhynchus mykiss*) $VPAC_1$, $VPAC_2$, and PAC_1 receptors **125**

INTRODUCTION **126**

MATERIALS AND METHODS **128**

RESULTS **135**

DISCUSSION **156**

CHAPTER 7. Adrenergic regulation of catecholamine secretion from trout (*Oncorhynchus mykiss)* chromaffin cells **161**

INTRODUCTION **162**

MATERIALS AND METHODS **164**

RESULTS **168**

DISCUSSION **191**

CHAPTER 8. GENERAL DISCUSSION **197**

SUMMARY OF PRINCIPLE FINDINGS **198**

DISCUSSION:

Part I. *In situ* saline perfused posterior cardinal vein preparation **200**

Part II. Neuronal Control of Catecholamine Release:

Cholinergic control-Acetylcholine **200**

Non-cholinergic control- VIP and PACAP **203**

VPAC and PAC_1 receptors **205**

Relative contribution of cholinergic and non-cholinergic neurotransmitters .. **206**

Part III. Adrenaline versus noradrenaline secretion **208**

Part IV. Adrenergic regulation of catecholamine release **210**

Part V. Significance of the neuronal control of catecholamine release **213**

CONCLUSION **214**

REFERENCES **215**

LIST OF FIGURES

CHAPTER 2

Figure 2.1

Voltage (V)-response curve of adrenaline (unfilled circles), and of noradrenaline (filled circles) secretion rates. Each preparation was stimulated for a period of 30 sec at 20 pps, and 1 msec duration..... **page 27**

Figure 2.2

The effects of pre-treatment with saline (N = 6, white bars), or saline containing 100 μM neostigmine (N = 6, black bars) on (A) adrenaline or (B) noradrenaline secretion in response to field stimulation..... **page 29**

Figure 2.3

The effects of pre-perfusion with hexamethonium (10^{-3} M) on (A) adrenaline and (B) noradrenaline secretion rates in response to field stimulation..... **page 31**

Figure 2.4

The effects of pre-perfusion with Na^{+}-free saline on (A) adrenaline and (B) noradrenaline secretion rates in response to field stimulation..... **page 33**

Figure 2.5

The effects of spinal cord removal on (A) adrenaline and (B) noradrenaline secretion rates in response to field stimulation..... **page 35**

CHAPTER 3

Figure 3.1

The effects of cholinergic antagonists on adrenaline (white bars) and noradrenaline (black bars) secretion rates in response to field stimulation ..... **page 50**

Figure 3.2

The effects of cholinergic antagonists on adrenaline (white bars) and noradrenaline (black bars) secretion rates in response to bolus injections of carbachol (10^{-5} mol kg^{-1}) **page 52**

Figure 3.3

The effects of cholinergic agonists on adrenaline (white bars) and noradrenaline (black bars) secretion rates..... **page 54**

CHAPTER 4

Figure 4.1

In situ adrenaline (*black columns*) and noradrenaline (*unfilled columns*) secretion rates in response to bolus injections of A) VIP (10^{-9} mol kg^{-1}; $N = 6$) and B) PACAP-27 (10^{-10} mol kg^{-1}; $N = 6$) ..... **page 72**

Figure 4.2

In situ peak adrenaline (*black columns*) and noradrenaline (*unfilled columns*) secretion rates in response to bolus injections of A) VIP and B) PACAP-27. Values are shown as means ± 1 SEM, *(N = 6-10* different fish for each dose administered). An asterisk denotes a significant difference in peak secretion when compared to the saline injected group ($P<0.05$). *Inserts*, doses of ckVIP (A) and hPACAP-27 (B) required to cause half-maximal release (ED_{50}) were calculated from Hill plots ..... **page 74**

Figure 4.3

The effects of bolus injections of VIP (10^{-9} mol kg^{-1}, *black column, N=6*), and VIP (10^{-9} mol kg^{-1}) in combination with hexamethonium (*Hex*) (10^{-3} M) plus atropine (*Atr*) (10^{-5} M) (*gray column, N = 6*), PACAP 6-27 (10^{-8} M) (*cross hatched column, N = 6*), or VIP6-28 (10^{-6} M) (*hatched column, N = 6*) on *in situ* adrenaline secretion rates..... **page 76**

Figure 4.4

The effects of bolus injections of PACAP-27 (10^{-10} mol kg^{-1}) (*black column, N=6*), and PACAP-27 (10^{-10} mol kg^{-1}) in combination with hexamethonium (*Hex*) plus atropine (*Atr*) (*gray column, N=6*), PACAP6-27 (10^{-8} M) (*crossed hatched column, N=6*), or VIP6-28 (10^{-6} M) (*hatched column, N=6*) on *in situ* adrenaline secretion rates ... **page 78**

Figure 4.5

The effects of bolus injections of carbachol (10^{-5} mol kg^{-1}, *black columns, N=6*), and carbachol (10^{-5} mol kg^{-1}) in combination with VIP 6-28 (10^{-6} M) (*gray columns, N=6*) or hexamethonium (*Hex*) (10^{-3} M) plus atropine (*Atr*) (10^{-5} M) (*unfilled columns, N=6*), on *in situ* (A) noradrenaline and (B) adrenaline secretion rates..... **page 81**

Figure 4.6

Maximal adrenaline (*unfilled bars*) and noradrenaline (*filled bars*) secretion rates in response to an injection of nicotine (10^{-7} mol kg^{-1}) following 20 min pre-perfusion with control saline (*N=6*), Ca^{2+}-free saline (*N=6*) or saline containing thapsigargin.... **page 83**

Figure 4.7

Maximal adrenaline (*unfilled bars*) and noradrenaline (*filled bars*) secretion rates in response to an injection of ckVIP (10^{-9} mol kg^{-1}) following 20 min pre-perfusion with control saline (*N=6*), Ca^{2+}-free saline (*N=6*) or saline containing thapsigargin.... **page 85**

Figure 4.8

Frequency/response curve for adrenaline (*filled circles*) and noradrenaline (*unfilled circles*) secretion rates ..... **page 88**

Figure 4.9

The effects of pre-treatment with saline (*N=6, unfilled columns*), saline containing hexamethonium (10^{-3} M) plus atropine (10^{-5} M) (*N=6, black columns*), or saline containing VIP 6-28 (10^{-6} M) (*N=6, gray columns*) on *in situ* (A) noradrenaline and (B) adrenaline secretion rates in response to low (1 Hz) and high (20 Hz) frequencies of stimulation. ..... **page 90**

CHAPTER 5

Figure 5.1

Identification and localisation of chromaffin cells within the posterior cardinal vein (PCV) tissue of rainbow trout ..... **page 109**

Figure 5.2

Mean tissue concentrations (μg catecholamine g^{-1} tissue) of noradrenaline (open columns) and adrenaline (filled columns) in rainbow trout ..... **page 111**

Figure 5.3

Localisation of VIP binding sites to the chromaffin cell subtypes in the PCV of rainbow trout using triple labelling ..... **page 113**

Figure 5.4

Secretion rates of adrenaline (filled columns) and noradrenaline (unfilled columns) in response to a bolus injection of saline (inset; *N* = *6*) or biotinylated-VIP (10^{-9} mol kg^{-1}; *N* = *6*) using an *in situ* PCV preparation of rainbow trout ..... **page 116**

Figure 5.5

Pharmacological assessment of the VIP binding sites. A-E, labelling of VIP binding sites following treatment with different VPAC and PAC receptor agonists and antagonists. A) Control, labelling of VIP binding sites..... **page 118**

CHAPTER 6

Figure 6.1

PCR amplification of the putative trout VPAC (A) and PAC_1 (B) receptors from brain cDNA..... **page 136**

Figure 6.2

The partial nucleotide and deduced amino acid sequences of the trout (*Oncorhynchus mykiss*) $VPAC_1$ receptor..... **page 138**

Figure 6.3

The partial nucleotide and deduced amino acid sequences of the trout (*Oncorhynchus mykiss*) $VPAC_2$ receptor..... **page 140**

Figure 6.4

The partial nucleotide and deduced amino acid sequences of the trout (*Oncorhynchus mykiss*) PAC_1 receptor..... **page 142**

Figure 6.5

Comparison of partial amino acids sequences of the putative trout $VPAC_1$ (A), $VPAC_2$ (B), and PAC_1 (C) receptors with those of selected vertebrates..... **page 146**

Figure 6.6

Phylogenetic tree of the $VPAC_1$, $VPAC_2$, and PAC_1 receptors using available vertebrate amino acid sequences from GenBank..... **page 149**

Figure 6.7

Tissue distributions of the putative trout $VPAC_1$ and $VPAC_2$ receptors as revealed by RT-PCR ..... **page 152**

Figure 6.8

Tissue distribution of the putative trout PAC_1 receptor as revealed by RT-PCR. **page 154**

CHAPTER 7

Figure 7.1

The effects of elevated concentrations of B) noradrenaline or C) adrenaline on basal catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout..... **page 169**

Figure 7.2

The effects of elevated catecholamine concentrations on carbachol (Cch)-evoked (10^{-6} mol kg^{-1}, final volume 0.3 ml) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout ..... **page 171**

Figure 7.3

The effects of elevated catecholamine concentrations on neuronal (60 V, 20 pps, 0.1 msec, 30 sec duration) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout ..... **page 173**

Figure 7.4

The effects α-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by cholinergic stimulation (carbachol (Cch); 10^{-6} mol kg^{-1}, 0.3 ml)..... **page 177**

Figure 7.5

The effects α-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by electrical neuronal stimulation ..... **page 179**

Figure 7.6

The effects β-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by cholinergic stimulation (carbachol (Cch); 10^{-6} mol kg^{-1}, 0.3 ml)..... **page 181**

Figure 7.7

The effects β-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by electrical neuronal stimulation ..... **page 183**

Figure 7.8

The effects of elevated dibutyril-cAMP concentrations on carbachol (Cch)-evoked (10^{-6} mol kg^{-1}, final volume 0.3 ml) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*)..... **page 185**

Figure 7.9

The effects of elevated dibutyril-cAMP concentrations on VIP-evoked (ckVIP; 10^{-9} mol kg^{-1}, final volume 0.3 ml) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*)..... **page 187**

Figure 7.10

The effects of the β_2-adrenergic receptor agonist salbutamol on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release in response to A) angiotensin II (ANG II; 10^{-9} mol kg^{-1}, 0.3 ml) or B) vasoactive intestinal polypeptide (VIP; 10^{-9} mol kg^{-1}, 0.3 ml)..... **page 189**

LIST OF TABLES

CHAPTER 2

None

CHAPTER 3

Table 3.1

The effects of prior treatment with saline (control), hexamethonium (10^{-3} M), or atropine (10^{-4} M), on total maximal (Max) catecholamine (noradrenaline plus adrenaline) secretion (nmol min^{-1}) elicited by bolus injections of nicotine (10^{-7} mol kg^{-1}) or methacholine (10^{-3} mol kg^{-1})..... **page 49**

CHAPTER 4

None

CHAPTER 5

None

CHAPTER 6

Table 6.1

Forward and reverse primer sequences used in PCR amplification of VPAC and PAC_1 receptor cDNA..... **page 133**

CHAPTER 7

None

LIST OF ABBREVIATIONS

α, Greek letter alpha

Ach, acetylcholine

Ang., angiotensin

ANOVA, Analysis of variance

ATR, atropine

β, Greek letter beta

body wt, body weight

bp, base pair

BSA, bovine serum albumin

cAMP, cyclic adenosine monophosphate

cDNA, complementary DNA

Cy3, cyanine-3

DAPI, 4,6-diamidino-2-phenlidole

DβH, dopamine β-hydroxylase

DHBA, 3,4-dihydroxybenzylamine

DTAF, dichlorotriazinyl aminofluorescein

ED_{50}, half maximal effective dose

EDTA, ethylenediaminetetraacetic acid

FITC, fluorescein isothyocyanate

GSP, gene specific primer

HPLC, high pressure liquid chromatography

Hz, Hertz

mRNA, messenger RNA

N, number of samples

NMDG, N-methyl D-glucamine

PAC_1, PACAP preferring receptor.

PACAP, pituitary adenylate cyclase activating polypeptide

PACAP6-27, PAC_1 receptor antagonist

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PCV, posterior cardinal vein

PNMT, phenylethanolamine-N-methyltransferase

pps, pulses per second

RT-PCR, reverse transcriptase PCR

SEM, standard error of the mean

SNS, sympathetic nervous system

TH, tyrosine hydroxylase

VIP, vasoactive intestinal polypeptide

VIP6-28, VPAC receptor antagonist

$VPAC_1$, VIP and PACAP receptor type 1

$VPAC_2$, VIP and PACAP receptor type 2

V, volts

CHAPTER 1

GENERAL INTRODUCTION

RATIONALE FOR THE THESIS

In fish, as in other vertebrates, the catecholamine hormones (adrenaline and noradrenaline) are released from chromaffin tissue (adrenal homologue; Epple et al., 1995) into the circulation during exposure to a wide range of stressors (Wendelaar Bonga, 1997; Reid et al., 1998). Once in the circulation, these hormones function to reduce the detrimental consequences often associated with stressful situations. Indeed, the primary role of catecholamines is to modulate cardiovascular and respiratory functions (Randall and Perry, 1992), as well as mobilize energy stores to provide for increased energy demands that may accompany stress (Fabbri et al., 1998). The control of catecholamine release from the chromaffin tissue is therefore central to an organism's ability to cope with potentially life-threatening events.

In vertebrates, the release of humoral catecholamine from chromaffin cells is controlled by numerous physiological stimuli including increased neuronal stimulation and humoral signals of circulatory, paracrine or autocrine origin (Ungar and Phillips, 1983; Livett and Marley, 1993). Current evidence suggests that the hypothalamic sympathetic chromaffin cell axis is the predominant pathway involved in the stimulation of humoral catecholamine release. After the discovery of sympathetic preganglionic cholinergic nerve fibres innervating vertebrate chromaffin tissue (Young 1939; Coupland 1963; Nilsson 1976), research into the control of catecholamine secretion has focused extensively on the involvement of cholinergic mechanisms (Ungar and Phillips, 1983; Burgoyne 1991; Edwards and Jones, 1993; Reid et al., 1998).

In fish, the neuronal regulation of catecholamine release is controlled by the activity of preganglionic nerves that innervate the chromaffin tissue that line the walls of

the posterior cardinal vein (PCV) (Nilsson et al., 1976; Abrahamsson, 1979; Perry et al., 1991; Abele et al., 1998). The prevailing view is that excitation of these nerves causes the release of acetylcholine (Ach) which then activates the cholinergic receptors of the chromaffin cells to evoke the secretion of catecholamines (Nilsson et al., 1976, Abrahamsson, 1979; Abele et al., 1998). In addition to cholinergic control of catecholamine release, a previous study has shown that in several fish species, a number of polypeptides exist in nerves in the vicinity of chromaffin cells and that they may act as neurotransmitters or neuromodulators (Reid et al., 1995). However, until physiological experiments are performed, their role in the neuronal regulation of chromaffin cell activity in fish will remain unknown. The above background obviously raises an important question as to whether, in addition to Ach, other excitatory substances are released from the preganglionic nerves to trigger the secretion of humoral catecholamines. Therefore, this thesis has examined neuronal aspects of catecholamine release from teleost fish chromaffin cells with emphasis on non-cholinergic pathways.

BACKGROUND KNOWLEDGE

Humoral catecholamines

Historically, adrenaline was discovered as a physiologically active substance from mammalian adrenomedullary extracts (reviewed by Nilsson and Holmgren, 1989). The original name epinephrine (Greek *epi* = on; *nephros* = kidney) has been retained in American terminology, while the Latin name for the adrenal (Latin *ad* = near; *renes* = kidney) forms the basis of the name adrenaline (Nilsson and Holmgren, 1989). The latter terminology (along with noradrenaline) is employed in this thesis.

Humoral catecholamines are synthesized and stored in chromaffin cells from which they can be released into the blood and function as hormones. The biosynthesis of catecholamines in fish, as in other vertebrates, is achieved via a series of enzymatic reactions known as the Blaschko pathway (Nilsson 1983; Randall and Perry, 1992). Unlike in the chromaffin tissue of other vertebrates, the chromaffin cells of fish are not organized into a distinct adrenal gland (reviewed by Reid et al., 1998). Instead, aggregations of chromaffin cells are intimately associated with the vascular system (Nilsson 1983) and also to the head kidney and sympathetic ganglia. In teleosts, catecholamines released into the circulation are secreted from chromaffin cells that are located within the walls of the posterior cardinal vein (PCV) and its branches within the kidney tissue (Nilsson 1976; Mastrolia et al., 1984; Reid et al., 1995; Furimsky et al., 1996). Synaptic overflow of catecholamines from sympathetic nerve endings, which forms a substantial part of noradrenaline release into the circulation of mammals, does not contribute significantly to circulating catecholamines in fish (Perry et al., 1991).

The various tissue responses elicited by catecholamine release are aimed at allowing organisms to cope with environmental and physiological stressors. Fish only release appreciable levels of catecholamines during conditions of acute severe stress (e.g. air exposure, hypoxia and hypercarbia, metabolic acidosis, physical disturbances, anemia, and anesthesia), which generally require the modulation of cardio-respiratory function and the mobilization of energy reserves (Randall and Perry, 1992; Perry and Bernier, 1998; Reid et al., 1998). Indeed, humoral catecholamines contribute to the maintenance or increase in blood oxygen carrying capacity by increasing gill-diffusing capacity, stimulating the release of erythrocytes from the spleen, and by increasing red blood cell hemoglobin-O_2 affinity (Perry and Wood, 1989; Randall and Perry, 1992; Thomas and Perry, 1992, Wendelaar Bonga, 1997; Perry and Bernier, 1998). Additionally, humoral catecholamines can modulate vascular resistance as well as cardiac and venous functions (Capra and Satchell, 1977; Olson et al., 1997; Bernier and Perry, 1999; Bernier et al., 1999). Metabolically, catecholamines are involved in glucose production and lipid mobilization (Fabbri et al., 1998).

Mechanisms of catecholamine release

The magnitude of the elevation in plasma catecholamine levels can vary depending upon their clearance from the circulation, the type and severity of the stress, and the ability of chromaffin cells to secrete a particular catecholamine in response to different hormones and neurotransmitters. Once in the circulation, catecholamines are rapidly cleared via the combination of tissue uptake mechanisms and metabolic degradation predominantly involving the gills, liver, and kidney (Nekvasil and Olson,

1986a, b). Although neuronal stimulation is presumably the predominant mechanism regulating the release of catecholamines, experimental evidence suggests that other factors may play a role (stimulatory or modulatory) in the secretion of catecholamines from chromaffin cells in teleosts (Reid et al., 1998).

Non-endocrine agents that are known to elicit or modulate catecholamine release in fish include hypoxaemia, blood acidosis and potassium. In the Atlantic cod (*Gadus morhua*), perfusion of the PCV with hypoxic blood caused the release of adrenaline (Perry et al., 1991). In rainbow trout, moderate hypoxia enhanced the responsiveness of the chromaffin tissue to cholinergic stimulation (Montpetit and Perry, 1998). Furthermore, exposure of cannulated lamprey (*Petromyzon marinus*) to CO_2 is associated with a significant increase in plasma catecholamines (Dashow and Epple, 1985). However it remains to be determined whether this is due to a direct effect of blood acidosis on the chromaffin tissue. Acidosis, while not influencing basal catecholamine release in trout, modulates the cholinergic control of catecholamine release by enhancing the response of chromaffin cells to nicotinic receptor stimulation (Julio et al., 1998). In spiny dogfish (*Squalus acanthias*), physiological doses of potassium elicit a dose dependent increase in plasma catecholamines (Opdyke et al., 1981; Opdyke et al., 1983a).

Activation of the hypothalamo-pituitary axis and the release of ACTH into the circulation is an integral part of the primary stress response in fish. ACTH, in addition to stimulating the release of corticosteroids from the interrenal cells of the head kidney has also been implicated in the control of catecholamine release in both the rainbow trout (Reid et al., 1996) and the Atlantic hagfish *Myxine glutinosa* (Perry et al., 1993).

Although there is immunohistochemical evidence for the presence of natriuretic peptides in the chromaffin cells of the common carp *Cyprinus carpio* (Kloas et al., 1994), overall results from *in situ* and *in vivo* experiments in rainbow trout and American eels (*Anguilla rostrata*) do not support a role for natriuretic peptides in the control of catecholamine release in teleosts (McKendry et al., 1999). On the other hand, intravenous injection of C-type natriuretic peptide in the spiny dogfish elicits an increase in plasma noradrenaline (McKendry et al., 1999; Montpetit et al., 2001). There is considerable evidence that the regulatory peptides of the renin-angiotensin system, angiotensins, may contribute to the regulation of catecholamine secretion from the chromaffin tissue in fish. Angiotensin II (Ang II) is a potent secretagogue of humoral catecholamine release in rainbow trout and dogfish (Bernier and Perry, 1999; Bernier et al., 1999). In both species, Ang II plays a significant role in the control of catecholamine release during acute hypotension. Serotonin, stored in high concentrations in the vicinity of the chromaffin tissue in the head kidney, has been shown to elicit catecholamine release both *in situ* and *in vivo* in the trout, as well as in the Atlantic hagfish (Fritsche et al., 1993; Bernier et al., 1996). Additionally in Atlantic cod, high concentrations of a particular catecholamine (adrenaline or noradrenaline) flowing past the chromaffin cells appears to auto-inhibit its own release from the chromaffin cells of the PCV through a negative feedback mechanism (Perry et al., 1991). In contrast, in the lamprey (*Petromyzon marinus*) and American eel there is *in vivo* evidence that catecholamines are catecholaminotropic (Dashow and Epple, 1983; Epple and Nibbio, 1985).

Neuronal control of catecholamine release

In the most "primitive" fish, hagfishes and lampreys, the principal catecholamine storing tissue, the chromaffin cells of the systemic heart (Augustinsson et al., 1956; Perry et al., 1993), is not innervated (Green 1902). All other vertebrates, however, appear to possess mechanisms allowing for the neuronal activation of catecholamine secretion. The autonomic nervous systems of teleosts resemble that of higher vertebrates, however, a unique feature of the teleosts include well developed sympathetic chains bearing ganglia that continue forward into the head (Nilsson 1976). Anatomical descriptions of the sympathetic nervous system (SNS) indicate that chromaffin cells that line the wall of the PCV are innervated by preganglionic sympathetic nerve fibres. In the Atlantic cod, there is a discrete innervation of the chromaffin cells by preganglionic nerves passing through the sympathetic chain ganglia at the level of the 3rd spinal autonomic nerve and entering the walls of the PCV (Nilsson 1976). In these fish, *in vivo* bilateral sectioning of the first four spinal nerves reduced catecholamine release in response to air exposure or acute hypoxia (Nilsson et al., 1976; Perry et al., 1991). Furthermore, in the trout and eel, microscopic evidence reveals that all chromaffin cells are in close contact with one or more nerve terminals which ultrastructurally appear to be identical to those found in the adrenomedullary chromaffin cells of higher vertebrates (Mastrolia et al., 1984; Hathaway et al., 1989; Scheuermann 1993).

While the cholinergic neural pathway has been identified as an important route for catecholamine release, there is increasing evidence that a number of non-cholinergic neurotransmitters or neuromodulators also play an important role in the neuronal control of catecholamine release from fish chromaffin cells (Reid et al., 1998). In agreement with

those observations, nerve fibres displaying vasoactive intestinal polypeptide (VIP)-like and pituitary adenylate cyclase activating polypeptide (PACAP)-like immunoreactivity were identified in the vicinity of the chromaffin tissue of Atlantic cod, rainbow trout, European eel and spiny dogfish (Reid et al., 1995). In the axillary bodies of the dogfish, nerve fibres exhibiting substance P-like and galanin-like immunoreactivity were also demonstrated (Reid et al., 1995). Although the significance of these observations remains speculative in fish, there is increasing evidence that VIP, PACAP and tachykinins have the ability to stimulate and/or modulate the secretion of catecholamines under physiological conditions in amphibians and mammals (Wakade et al., 1991; Yamaguchi 1993; Yon et al., 1994; Reid et al., 1998).

Cholinergic control of catecholamine release

Current evidence suggests that the neuronal control of catecholamine release in both teleosts and elasmobranchs is mediated primarily via preganglionic cholinergic nerve fibres that release the neurotransmitter Ach. Ultrastructural histochemistry for acetylcholinesterase activity indicates that the nerve fibres innervating the chromaffin tissue are cholinergic in nature (Nilsson 1976; Mastrolia et al., 1984). Electrical stimulation of the nerve fibres innervating the chromaffin tissue of teleosts (cod and eel) and elasmobranchs (dogfish) elicits the secretion of catecholamines from *in situ* preparations, which can be blocked by prior treatment with the ganglionic blocker, hexamethonium (Nilsson et al., 1976; Abrahamsson 1979; Opdyke et al., 1983b; Perry et al., 1991; Abele et al., 1998). Likewise, exogenous administration of Ach or cholinergic agonists to perfused or perifused preparations of trout, cod, dogfish and eel, causes the

secretion of catecholamines that also is prevented by hexamethonium (Nilsson et al., 1976; Perry et al., 1991; Reid et al., 1995; Al-Kharrat et al., 1997; Gfell et al. 1997). In dogfish, *in vivo* administration of hexamethonium reduces catecholamine release following bouts of intense exercise (Opdyke et al., 1983).

Ach interacts with two distinct cholinergic receptor sub-types, nicotinic and muscarinic receptors. Nicotinic receptors are believed to be the predominant cholinergic receptors involved in the neuronal control of catecholamine release from chromaffin cells (Nilsson et al., 1976; Perry et al., 1991; Reid et al., 1995; Al-Kharrat et al., 1997; Gfell et al., 1997; Julio et al., 1998). Nicotinic receptor stimulation is coupled to the opening of receptor-linked Na^+ channels and the resultant membrane depolarization causes an opening of voltage-dependant Ca^{2+} channels (Brandt et al., 1976; Wada et al., 1985; Liu and Kao, 1990). The subsequent influx of Ca^{2+} is thought to trigger the cascade of events leading to the secretion of catecholamines (Burgoyne et al., 1993).

Experiments designed to investigate the involvement of muscarinic receptors in the release of catecholamines from chromaffin cells of mammals and amphibians have produced mixed results. Indeed, in these organisms, stimulation with muscarinic receptor agonists can enhance nicotinic-evoked release (Role and Perlman, 1983; Accordi 1988; Nassar-Gentina et al., 1997), inhibit nicotinic-evoked release (Swilem and Hawthorn, 1983; Cheek and Burgoyne, 1985; Nassar-Gentina et al., 1991), stimulate catecholamine release (Wakade and Wakade, 1983; Knight and Baker, 1986; Ballesta et al., 1989; Xu et al., 1991; Tobin et al., 1992; Chowdhury et al., 1994) or exert no effect (Liang and Perlman, 1979; Mckay et al., 1991). It would appear as if the presence of muscarinic receptors on chromaffin cells in fish might also exhibit substantial variability. Previous

studies using American eels have provided conflicting evidence. Reid and Perry (1995) were unable to demonstrate any involvement of muscarinic receptors, whereas other studies have documented an inhibitory role of basal secretion (Al-Kharrat et al., 1997), or a positive modulatory role under electrical stimulation of the sympathetic fibres (Abele et al., 1998). Fritsche et al. (1993) demonstrated using an *in situ* saline PCV preparation of rainbow trout, that pre-treatment with hexamethonium prevented the carbachol-induced release of noradrenaline but failed to "completely" block the release of adrenaline. Moreover, Julio et al. (1998) demonstrated that the muscarinic receptor agonist pilocarpine was capable of directly eliciting the release of adrenaline and noradrenaline from *in situ* perfused PCV preparations of trout. Therefore in trout, cholinergic stimulation of catecholamine release may include stimulation of muscarinic in addition to nicotinic receptors.

Non-cholinergic control (VIP and PACAP) of catecholamine release

VIP and PACAP are two closely related neuropeptides that belong to the secretin/ glucagon/ GH-releasing hormone superfamily of peptide hormones (Harmar et al., 1998). VIP was first isolated from porcine intestine as a 28 amino acid polypeptide capable of inducing vasodilation in the canine femoral artery (Said and Mutt, 1970, 1972). PACAP, a peptide first identified from ovine hypothalamus that stimulated adenylyl cyclase in rat anterior pituitary cells occurs in two molecular forms of 27 and 38 amino acids (Miyata et al., 1989; Miyata et al., 1990). Since their discoveries, immunolabeling, *in situ* hybridization, and ligand binding investigations indicate that the neuropeptides are

widely distributed in the central and peripheral nervous systems and in some cases, displaying overlapping distributions (Harmar et al., 1998; Vaudry et al., 2000).

In fish, as in other vertebrates, VIP and PACAP have been implicated as neurotransmitters or neuromodulators in the control of various physiological processes including cardiovascular function, respiration, digestion and glandular secretions (Holmgren, 1985; Dimaline et al., 1987; Aldman and Holmgren, 1992; Holmgren, 1995; Wong et al., 1998; Vaudry et al., 2000; Johnsson et al., 2001). They exert tissue specific effects by interacting with three distinct receptors that belong to a seven transmembrane spanning domain G-protein coupled receptor superfamily that are coupled to adenylyl cyclase and the production of intracellular cAMP (Harmar et al., 1998). Two of these receptors, $VPAC_1$ and $VPAC_2$, are activated with comparable potencies by VIP and PACAP, whereas the third receptor, PAC_1, preferentially binds PACAP (Harmar et al., 1998; Vaudry et al, 2000).

The ability of VIP and PACAP to directly stimulate catecholamine release from chromaffin cells was first demonstrated in rat (Malhotra and Wakade, 1987). Since then, a large number of studies on other mammals and amphibians have reached the same conclusion (Misbahuddin et al., 1988; Yamaguchi 1993; Yon et al., 1994; Tanaka et al., 1996; Lamouche et al., 2001). In mammals, PAC_1 receptors appear to be the predominant receptor mediating the stimulatory effects of VIP and PACAP on catecholamine release (Vaudry et al., 2000). Indeed, while VIP and PACAP elicit the secretion of catecholamines from canine chromaffin cells, only specific PAC_1 receptor antagonists were capable of suppressing the response (Lamouche et al., 2001). Furthermore, PACAP is 1000 times more potent than VIP in eliciting the secretion of adrenaline from bovine

and rat chromaffin cells (Wakade et al., 1991; Watanabe et al., 1992; Isobe et al., 1993; Guo and Wakade, 1994; Watanabe et al., 1995). 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 the PAC_1 receptor mRNA, investigations have also revealed the presence of $VPAC_1$ and $VPAC_2$ receptor mRNA (Usdin et al., 1994).

Research on the relative involvement of cholinergic and non-cholinergic (VIP and PACAP) neurotransmitters in the control of catecholamine secretion from chromaffin cells and the physiological conditions under which they function have been performed on mammals. Guo and Wakade (1994) demonstrated that in the rat adrenal medulla, low frequency nerve stimulation of the splanchnic nerve caused the neural release of VIP from the nerve, which in turn stimulated the secretion of adrenaline from the adrenal medulla. Upon high frequency stimulation, Ach was also released and caused the release of adrenaline and noradrenaline. Although immunolabeling experiments have identified the presence of these neuropeptides in the proximity of chromaffin cells in a number of fish, the physiological significance of these observations remain to be established. The close structural and functional relationship between VIP and PACAP and the coexistence in nerve fibres in the vicinity of chromaffin cells would suggests a role for these neuropeptides in the regulation of catecholamine secretion in fish species.

AIM AND OUTLINE OF THE THESIS

The general aim of this thesis was to investigate the relative contribution of cholinergic and non-cholinergic neurotransmitters in the control of humoral catecholamine secretion in fish, predominantly the rainbow trout. The rainbow trout was chosen because of the extensive background literature existing for this species and its ability to mount a robust adrenergic stress response. Furthermore, the demonstration of sympathetic innervation of the chromaffin tissue and the presence of neuropeptide neurotransmitters within these nerve fibres made this species a suitable candidate to study non-cholinergic neuronal aspects of catecholamine release in fish. All of the components of this thesis are encompassed under the general hypothesis that the neuronal control of catecholamine secretion from piscine chromaffin cells is not confined to nicotinic receptor evoked events.

As such, experimental approaches were designed to answer the following questions:

1) Does cholinergic stimulation of catecholamine release include activation of muscarinic receptors in addition to nicotinic receptors?

2) Do VIP and PACAP elicit the secretion of catecholamines from trout chromaffin cells, and if so, which receptor among PAC_1, $VPAC_1$, and $VPAC_2$ mediate the response?

3) Are acetylcholine (Ach) and VIP/PACAP released from the preganglionic nerves during neuronal stimulation of the chromaffin cells? Can catecholamine secretion be inhibited by receptor antagonists specific for Ach and the neuropeptides under these conditions?

4) And finally, what are the impacts of high levels of catecholamines on the subsequent release of additional catecholamines? Do they affect catecholamine secretion in response to neuronal stimulation?

To evaluate the relative contribution of cholinergic (Ach) and non-cholinergic (VIP and/or PACAP) neurotransmitters, a nerve stimulating technique capable of specifically activating the nerve fibres innervating the chromaffin cells was developed and validated. This technique is described in **CHAPTER 2**.

Although cholinergic stimulation of catecholamine release in fish has been established, a role for muscarinic receptors remains inconclusive. In **CHAPTER 3,** pharmacological experiments were designed to test whether cholinergic stimulation of catecholamine secretion involved muscarinic receptors in addition to nicotinic receptors.

In **CHAPTER 4**, the ability of VIP and PACAP to elicit the secretion of catecholamines from the chromaffin tissue was examined using pharmacological approaches. Furthermore, the relative contributions of Ach and VIP/PACAP during electrical stimulation were investigated.

In **CHAPTER 5**, light microscopy techniques were used to identify the chromaffin cell fraction of the posterior cardinal vein exhibiting binding sites for VIP. Additionally, pharmacological approaches were utilized to assess the ability of these VIP binding sites to mediate catecholamine secretion in response to VIP and PACAP.

In **CHAPTER 6**, molecular cloning of trout $VPAC_1$, $VPAC_2$, and PAC_1 receptor cDNAs is described.

In **CHAPTER 7**, experiments were designed to investigate the effects of catecholamines on catecholamine release (catecholaminotropic effects). The goals were

to assess the impact of extracellular catecholamines on catecholamine secretion from rainbow trout chromaffin cells in response to different stimuli, and to use pharmacological approaches to identify the underlying mechanisms.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF AN *IN SITU* SALINE PERFUSED POSTERIOR CARDINAL VEIN – NERVE STIMULATING TECHNIQUE

(Based in part on Montpetit CJ and Perry SF. 1999. Journal of Experimental Biology 202, 2059-2069)

INTRODUCTION

Numerous approaches have been used to study catecholamine release in fish including *in vivo* assessments of circulating catecholamine levels (e.g.; Perry et al., 1991; Bernier and Perry, 1999a,b), *in vitro* perifusion techniques (e.g.; Gfell et al., 1997; Abele et al., 1998) and *in situ* saline perfused posterior cardinal vein preparations (e.g. Perry et al., 1991; Fritsche et al., 1993; Reid et al., 1995; Al-Kharrat et al., 1997). Advantages of the latter technique are that catecholamine secretion can be studied in the absence of external stimuli without major disturbance to the chromaffin tissue. Indeed, the *in situ* saline perfused posterior cardinal vein preparation has emerged as a well-established tool to dissect the mechanisms regulating catecholamine secretion in piscine chromaffin cells (see review by Reid et al., 1998).

Using pharmacological approaches, the *in situ* perfused PCV preparation has generated important information on the neuronal and hormonal control of catecholamine release in fish, however, very few studies have attempted to study neuronal mechanisms of catecholamine release using direct electrical stimulation of the sympathetic nerve fibers innervating the chromaffin tissue. In the Atlantic cod, there is a discrete innervation of the chromaffin tissue by preganglionic fibres passing through the sympathetic chain ganglia and entering the walls of the posterior cardinal vein (Nilsson 1976). On the left side, the innervation forms a single nerve, which lends itself to experimental manipulation (Nilsson et al., 1976; Nilsson 1984). In the perfused cod posterior cardinal vein a release of catecholamines from the chromaffin tissue could be demonstrated as a response to electrical stimulation of this nerve (Nilsson et al., 1976; Perry et al., 1991). In the spiny dogfish and eels similar results were obtained by stimulating the spinal cord to a

site behind the hind brain (Abrahamsson 1979; Abele et al., 1998). In all these experiments, the demonstration that addition of the ganglionic blocker hexamethonium to the perfusion medium could abolish the release of catecholamines in response to neuronal stimulation confirmed that the innervation of the chromaffin tissue consisted of preganglionic cholinergic nerve fibres of spinal autonomic origin.

Recently, nerve fibres that were immunoreactive to vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) were demonstrated in the vicinity of the chromaffin tissue of different fish (Reid et al., 1995). While studies have focused predominantly on the cholinergic nature of catecholamine secretion from the chromaffin cells, no studies have yet been performed to evaluate the relative contribution of cholinergic (Ach) and non-cholinergic (VIP and/or PACAP) neurotransmitters during increased neuronal stimulation of the chromaffin cells. To conclusively demonstrate a role for Ach and VIP/PACAP stimulation of catecholamine secretion during neuronal excitation of trout chromaffin tissue *in situ*, it was first necessary to develop an *in situ* nerve-stimulating protocol able to specifically elicit catecholamine secretion from the chromaffin tissue in the trout. As such, a field stimulation technique was developed and validated. This chapter presents a general description of this preparation.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout of either sex weighing between 250 and 350g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and held in large fiberglass tanks supplied with dechlorinated city of Ottawa tap water maintained at 15°C. They were allowed to acclimate to the aquaria for at least 3 wk before experimentation. Fish were maintained on a 12L: 12D photoperiod and fed daily to satiation with a commercial fish diet.

In situ saline perfused posterior cardinal vein preparation

Fish were killed by a sharp blow to the head, weighed and placed on ice. A ventral incision was made along the entire length of the animal, and the tissue overlying the heart was removed by blunt dissection to expose the ventricle and the bulbus arteriosis. The posterior cardinal vein and ventricle were catheterized (Clay-Adams PE 160 polyethylene tubing) and served as the inflow and outflow, respectively, of the perfusion fluid. Each preparation was perfused for 20 min with modified aerated Cortland (control) saline (Wolf, 1963; 125 mM NaCl, 2.0 mM KCl, 2.0 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 7.5 mM glucose, 2.0 $CaCl_2$, and 1.25 mM KH_2PO_4, final pH 7.8) to allow catecholamine secretion to stabilize. Perfusion was accomplished by siphon resulting from a positive pressure difference between the surface of the saline and the outflow cannula, which resulted in a flow rate of approximately 1.5 ml min^{-1}.

After the stabilization period, a control pre-stimulation sample was collected in a pre-weighed microcentrifuge tube to assess basal catecholamine secretion rate. After collection of the pre-stimulation sample, the preparations were electrically stimulated in accordance with the treatments described below. All stimulations were performed by administering an electrical current through a pair of wire electrodes connected to a Grass-SD9 stimulator. In total, five post-stimulation samples were collected 1,2,3,4 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.

Nerve Stimulation Protocols

Different protocols known to induce neuronally evoked secretion of catecholamines *in situ* in fish (cod, dogfish, and eels) was performed on trout (Nilsson et al., 1976; Abrahamsson 1979; Abele et al., 1998). Because unlike in the cod, it was difficult to trace the neuronal supply to the chromaffin tissue of trout, different approaches were taken. In the first protocol, given that bilateral sectioning of the first four spinal nerves inhibited catecholamine release in response to acute stress in cod *in vivo*, a pair of wire electrodes was placed under the first four spinal nerves near the spine. In a second protocol, similar to that described for eels and dogfish, nerve stimulation of the spinal cord to a site behind the hind brain was performed. In each case, following collection of a pre-stimulation sample, direct electrical stimulation was performed using a range of voltages (1-60V) in combination with a range of frequencies (1-20 pulses per

sec^{-1}). In each case, perfusate catecholamine levels were not significantly elevated over baseline levels. As such, a field stimulating protocol was developed and validated.

Field Stimulation Protocol-Validation

Voltage/response curve: Fish were electrically stimulated using a pair of electrodes sutured to the body wall on either side of the fish in the anterior region of the posterior cardinal vein. After collection of the pre-stimulation sample, each preparation was stimulated only once at 0, 10, 30, 45, 60, or 80 V. Field stimulation was carried out at a frequency of 20 pulses per sec^{-1}, 1 ms in duration for a period of 30s, and collection of post-stimulation samples began immediately at the onset of stimulation. Stimulation at 60 V was determined to be the lowest stimulus amplitude capable of eliciting maximal catecholamine secretion (Fig. 1) and was subsequently utilized for all experiments involving field stimulation.

To assess whether field stimulation causes non-specific depolarization of chromaffin cells via surface current or neuronally mediated catecholamine secretion, the following series of experiments was performed.

Series 1: Effects of neostigmine on electrically stimulated catecholamine secretion. Using the same procedures as described above, the effects of the anticholinesterase agent neostigmine on electrically evoked catecholamine secretion were assessed in preparations perfused with saline or saline containing neostigmine bromide (10^{-4} M).

Series 2: Neuronally evoked catecholamine secretion versus non-specific depolarization. Two sets of experiments were performed in this series using the protocol of series 1. In the first, fish were perfused with saline or saline containing the nicotinic

receptor antagonist hexamethonium (10^{-3} M). In the second, fish were perfused with saline or Na^+-free saline containing 125 mM NMDG (N-methyl, D-glucamine), 2 mM KCl, 2 mM $MgSO_4$, 5 mM $KHCO_3$, 7.5 mM glucose, 2 mM $CaCl_2$, and 1.25 mM KH_2PO_4.

Series 3: Effects of spinal cord removal on electrically stimulated catecholamine secretion. Using the same procedures as in series 1, electrical stimulation was applied to fish in which the anterior third portion of the spinal cord had been removed. For sham preparations, the spinal cord was exposed but not removed.

Analytical Procedures

Perfusate adrenaline and noradrenaline concentrations were determined on alumina-extracted samples (200 µl) using high-pressure liquid chromatography (HPLC) with electrochemical detection. The HPLC consisted of a Varian Star 9012 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, NJ). The extracted samples were passed through an Ultratechsphere ODS-C_{18} 5 µm column (HPLC Technology Ltd., Macclesfield, U.K.) and the separated amines were integrated using the Star Chromatography software program (version 4.0, Varian). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations.

Statistical Analysis

The data are presented as the mean ± 1 standard error of the mean (SEM). Where appropriate, the data were statistically analyzed by a one-way repeated measures ANOVA followed by Dunnett's test for comparison with pre-stimulation values, or a one-way ANOVA followed by Dunnett's test for multiple comparisons; if the normality test failed, an ANOVA on ranks was performed followed by Dunnett's multiple comparison test. In other instances, data were analyzed by Student's t-test or paired t-test, and if the normality test failed, a Mann-Whitney rank sum test was performed. 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

Nerve stimulating protocols

Electrical stimulation of the spinal nerves or nerve cord did not elicit the secretion of either noradrenaline or adrenaline at any level of stimulation. Therefore nerve stimulating protocols known to induce the secretion of catecholamines in cod, eel, or dogfish were unsuccessful in trout. As such, a field stimulation protocol was developed and validated.

Validation of Field Stimulation Protocol

Voltage/response curve. Field stimulation ranging from 0 to 80 V elicited a voltage-dependent secretion of both catecholamines (Fig. 2.1). Significant elevation of the rate of adrenaline and noradrenaline secretion occurred at 45 V and reached a maximum at 60 V.

Series 1: Effects of neostigmine on electrically stimulated catecholamine secretion. Field stimulation caused a significant increase in the rate of adrenaline secretion in both saline-perfused and neostigmine-perfused preparations (Fig. 2.2A). However, in neostigmine-treated preparations, secretion remained elevated at 2 and 3 min post-stimulation; over the 5 min post-stimulation period, total adrenaline secretion was nearly doubled (Fig. 2.2A). Pre-treatment with neostigmine did not effect noradrenaline secretion (Fig. 2.2B).

Series 2: Neuronally evoked catecholamine secretion versus non-specific depolarization. Perfusion with saline containing hexamethonium reduced the rate of adrenaline and noradrenaline secretion in response to field stimulation (Fig. 2.3). Adrenaline secretion, although reduced in the presence of hexamethonium, was

significantly increased from pre-stimulation values (Fig. 2.3A). In contrast, perfusion with Na^+-free saline eliminated both adrenaline and noradrenaline secretion during field stimulation (Fig. 2.4).

Series 3: Effects of spinal cord removal on electrically stimulated catecholamine secretion. Removal of the spinal cord totally prevented the secretion of both adrenaline and noradrenaline (Fig. 2.5) in response to field stimulation.

Figure 2.1 Voltage (V)-response curve of adrenaline (unfilled circles), and of noradrenaline (filled circles) secretion rates. Each preparation was stimulated for a period of 30 sec at 20 pps, and 1 msec duration. Values are shown as means ± 1 SEM, (N = 6 fish for each voltage). An asterisk denotes a significant difference from unstimulated values (0 V). Curves were constructed by fitting the data to a sigmoidal function using an iterative curve-fitting function in a commercial software program (Sigmaplot 4.0)

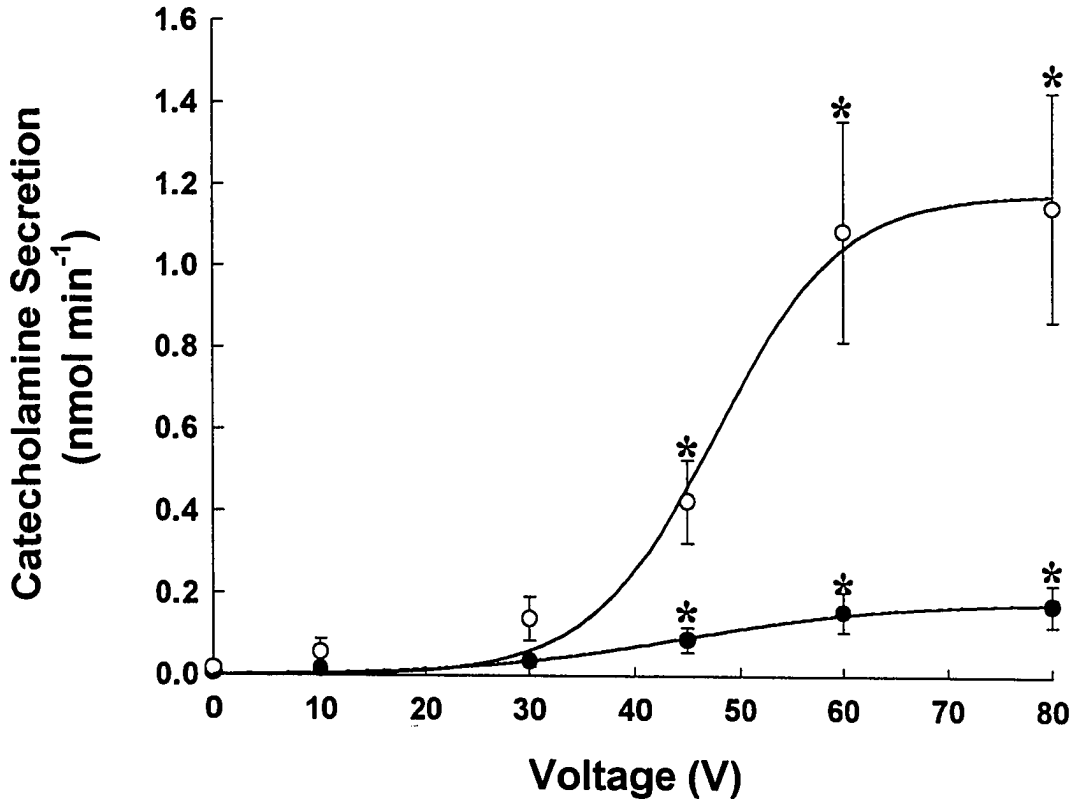

Catecholamine Secretion
(nmol min-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
Voltage (V)

Figure 2.1

Figure 2.2 The effects of pre-treatment with saline (N = 6, white bars), or saline containing 100 μM neostigmine (N = 6, black bars) on (A) adrenaline or (B) noradrenaline secretion in response to field stimulation. Each preparation was stimulated at 60 V at 20 pps, and 1 msec duration, for 30 sec. The inset graphs in each panel illustrate the total quantity of catecholamine secreted over the 5 min collection period. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from the pre-stimulation values (pre); a dagger denotes a significant difference between the control and neostigmine treated group.

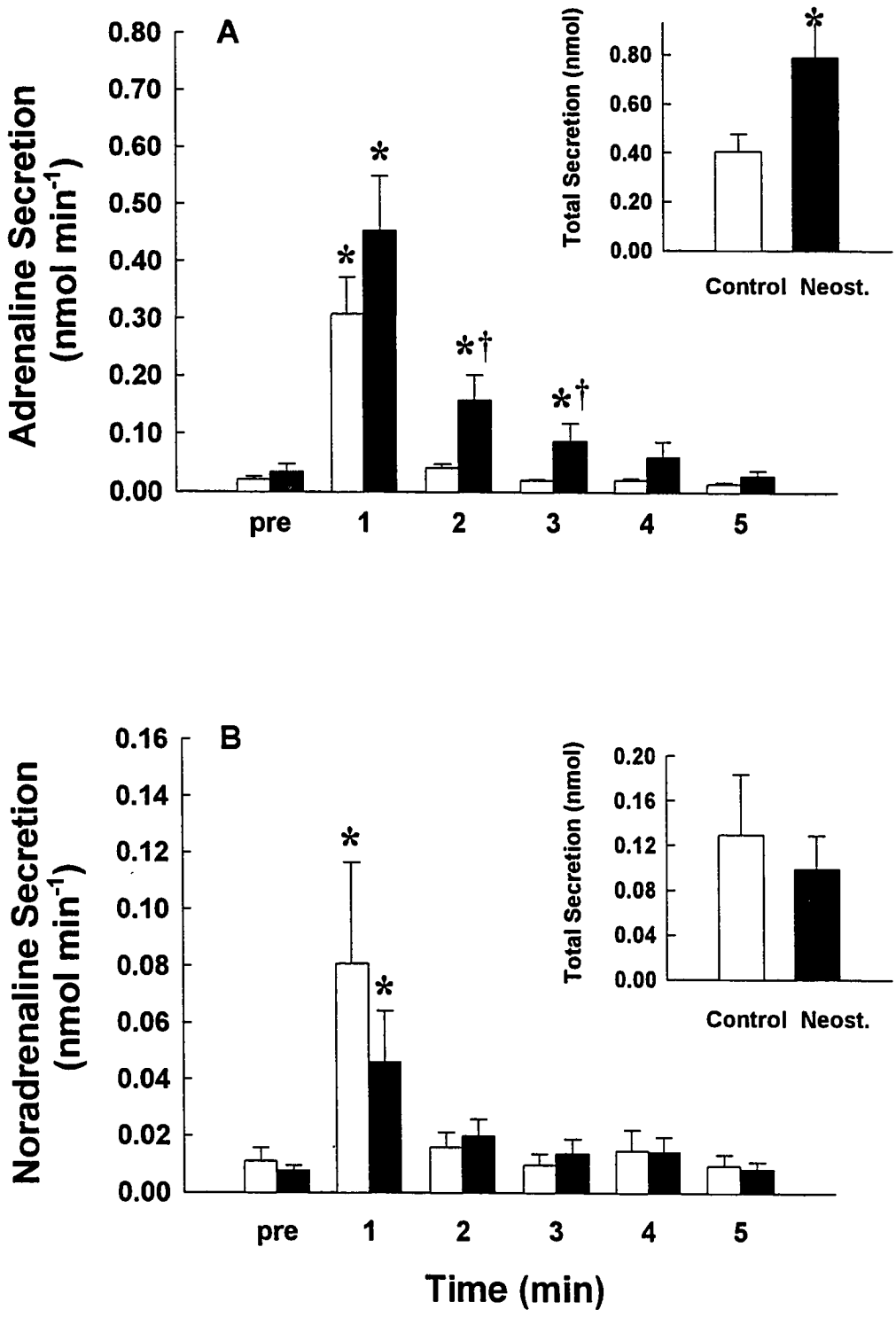

A
Adrenaline Secretion (nmol min-1)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
pre
1
2
3
4
5
Total Secretion (nmol)
0.80
0.60
0.40
0.20
0.00
Control
Neost.
B
Noradrenaline Secretion (nmol min-1)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Total Secretion (nmol)
0.20
0.16
0.12
0.08
0.04
0.00
Time (min)

Figure 2.2

Figure 2.3 The effects of pre-perfusion with hexamethonium (10^{-3} M) on (A) adrenaline and (B) noradrenaline secretion rates in response to field stimulation (N = 8). Control fish (N = 8) were pre-perfused with saline. Each preparation was stimulated at 60 V at 20 pps, and 1 msec duration for 30 sec. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from pre-stimulation values (white bars); a dagger denotes a significant difference between the stimulated values (black bars).

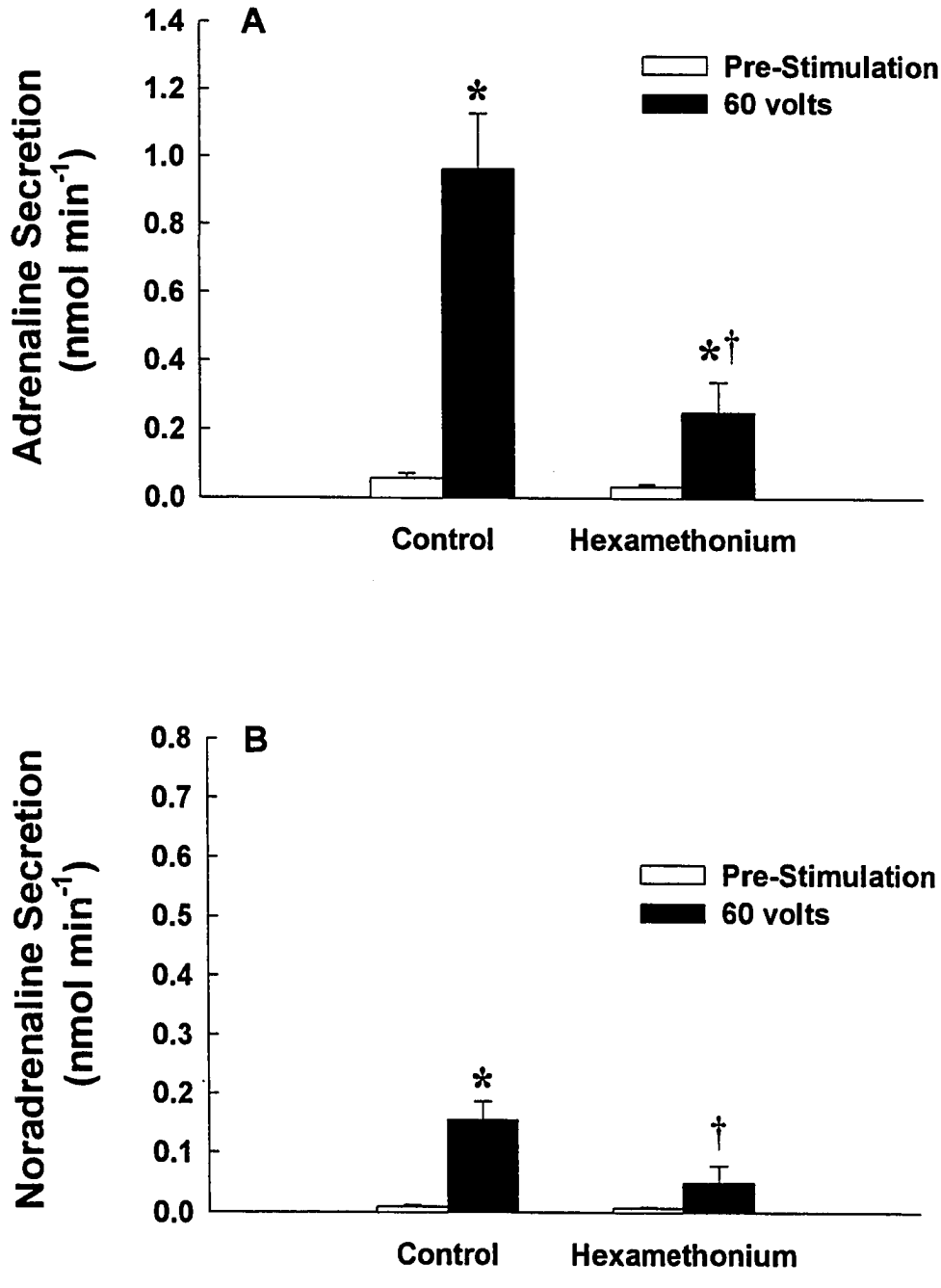

A
Adrenaline Secretion (nmol min-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*†
Pre-Stimulation
60 volts
Control
Hexamethonium
B
Noradrenaline Secretion (nmol min-1)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
†
Pre-Stimulation
60 volts
Control
Hexamethonium

Figure 2.3

Figure 2.4 The effects of pre-perfusion with Na^+-free saline on (A) adrenaline and (B) noradrenaline secretion rates in response to field stimulation (N = 8). Control fish (N = 8) were pre-perfused with saline. Each preparation was stimulated at 60 V at 20 pps, and 1 msec duration for 30 sec. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from pre-stimulation values (white bars); a dagger denotes a significant difference between the stimulated values (black bars).

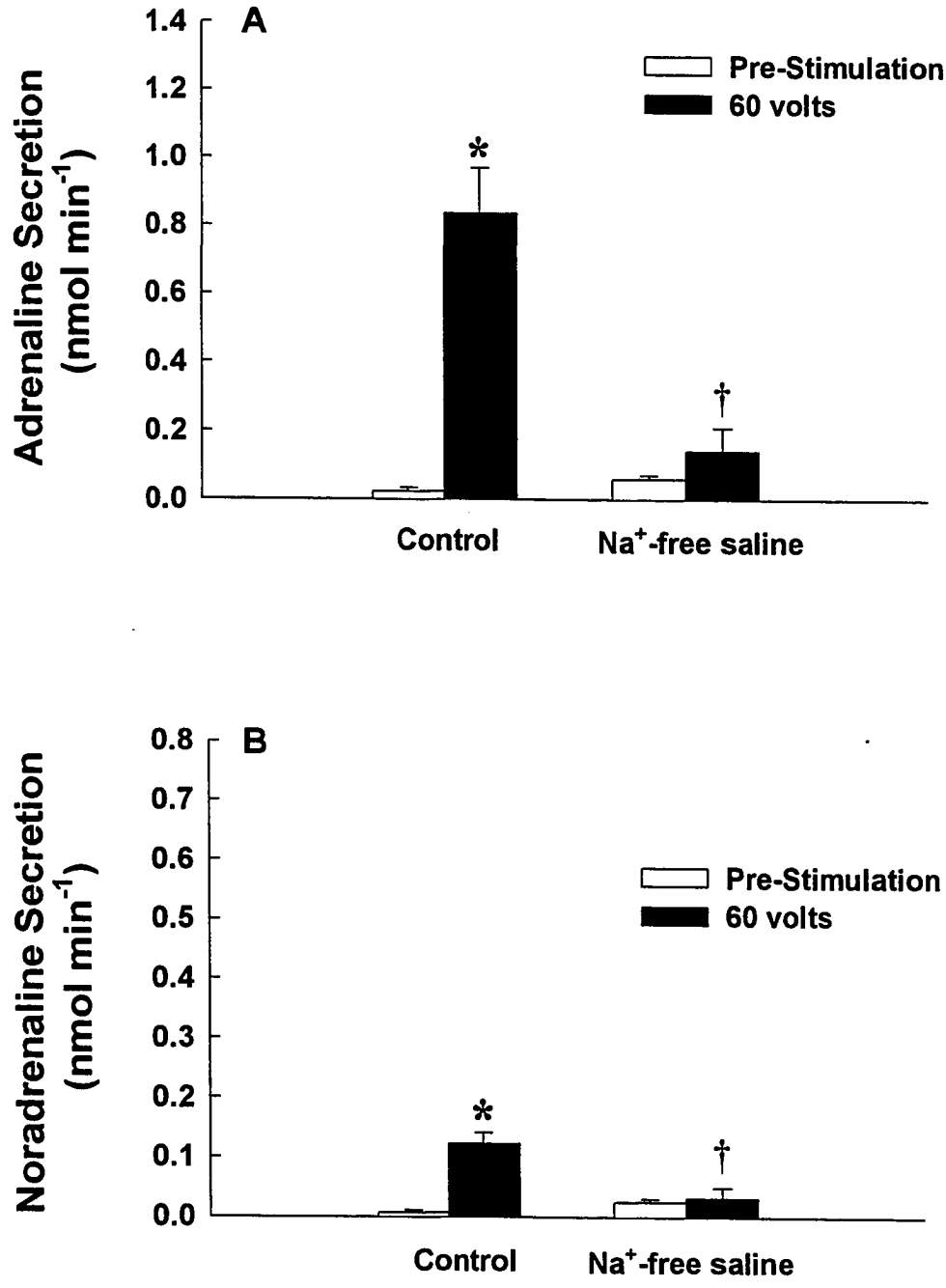

A
Adrenaline Secretion (nmol min-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pre-Stimulation
60 volts
*
†
Control
Na+-free saline
B
Noradrenaline Secretion (nmol min-1)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Pre-Stimulation
60 volts
*
†
Control
Na+-free saline

Figure 2.4

Figure 2.5 The effects of spinal cord removal on (A) adrenaline and (B) noradrenaline secretion rates in response to field stimulation (N = 6). For sham preparations (N = 6), the spinal cord was dissected but not removed. Each preparation was stimulated at 60 V at 20 pps, and 1 msec duration for 30 sec. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from pre-stimulation values (white bars); a dagger denotes a significant difference between the stimulated values (black bars).

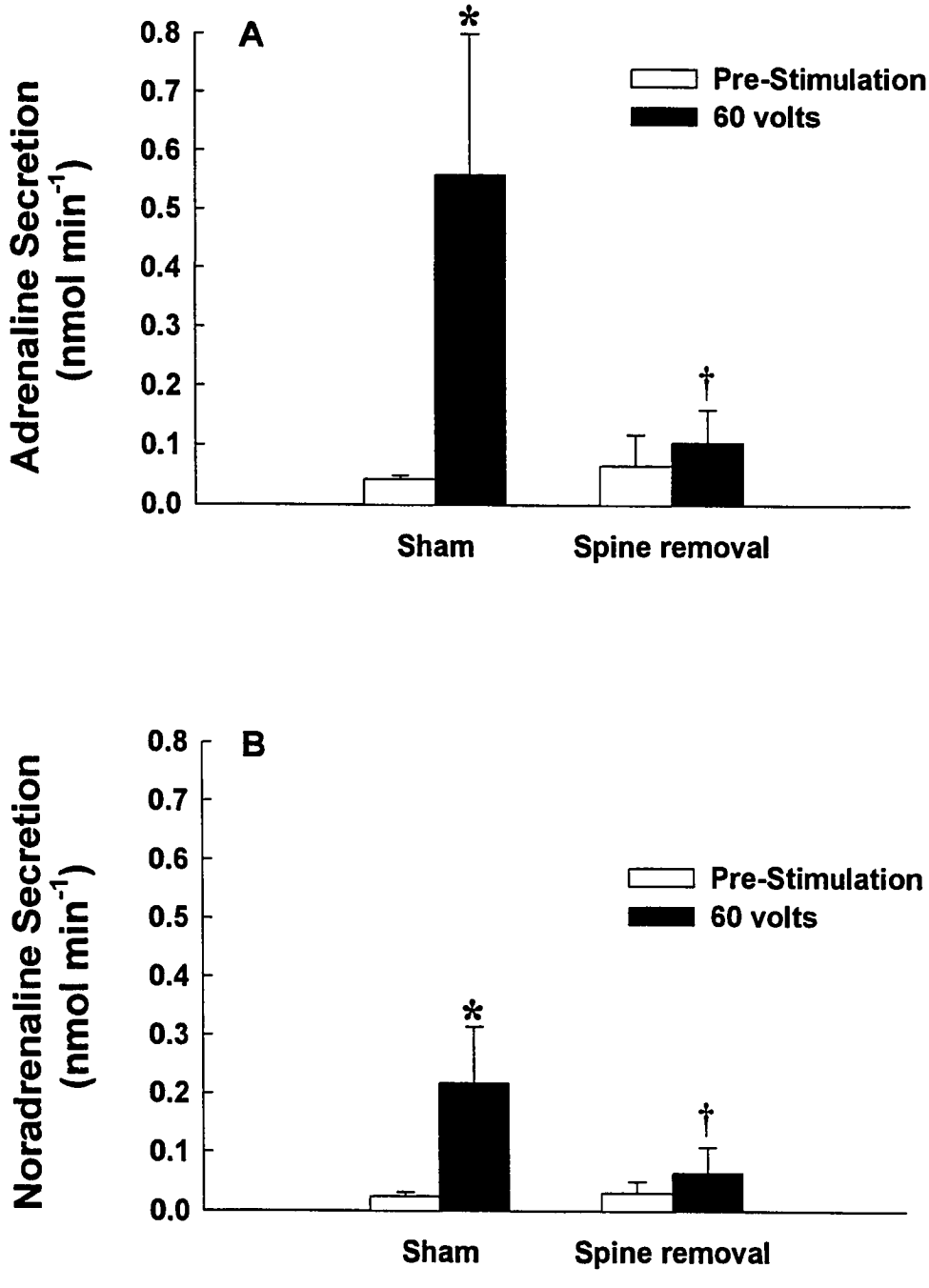

A
Adrenaline Secretion (nmol min-1)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
†
Pre-Stimulation
60 volts
Sham
Spine removal
B
Noradrenaline Secretion (nmol min-1)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
†
Pre-Stimulation
60 volts
Sham
Spine removal

Figure 2.5

DISCUSSION

Validation of the field stimulation technique

In contrast to the situation in the cod (Nilsson 1976), it was technically difficult to exclusively identify and stimulate the fine branch of nerves that supplies the chromaffin tissue in trout. Furthermore, repeated efforts to stimulate the nerves innervating the chromaffin cells of trout by direct application of electrical current to the spinal nerves or spinal cord were unsuccessful. Therefore, procedures known to induce neuronally induced catecholamine secretion in cod (Nilsson et al., 1976), dogfish (Abrahamsson 1979), or eel (Abele et al., 1998) *in situ*, were ineffective in trout. As such a field stimulation protocol was developed and validated.

Field stimulation of *in situ* saline-perfused posterior cardinal vein preparations of trout could induce catecholamine secretion in one of three possible ways. First, conduction of electrical current to the spinal cord could generate action potentials in the preganglionic sympathetic fibers known to innervate the chromaffin cells and ultimately lead to the release of acetylcholine (among other neurotransmitters and neuromodulators). Second, the electrical current could cause direct excitation of the pre-ganglionic nerve terminals without intervention of the spinal cord. Third, catecholamine secretion could be caused by non-specific depolarization of the chromaffin cells in response to the electrical current. In the present study, data obtained using neostigmine, hexamethonium, Na^+-free saline and from fish in which a portion of the spinal cord was removed demonstrated conclusively that catecholamine secretion in response to field stimulation was neuronally induced and dependent upon an intact spinal cord. Furthermore, the contribution of non-specific depolarization of chromaffin cells towards secretion appears to be negligible.

The specificity of the field stimulation technique was confirmed by demonstrating that cholinergic catecholamine secretion was reduced in the presence of the nicotinic receptor antagonist hexamethonium, and that adrenaline secretion was prolonged and more than doubled in the presence of neostigmine over the 5 min period of sample collection. The absence of an effect of neostigmine on noradrenaline secretion during field stimulation was unexpected. However, if the rate of secretion of noradrenaline were already maximal during field stimulation, then any effects of neostigmine on stabilizing acetylcholine levels would go unnoticed. Nevertheless, these results together with the observation that secretion was prevented in fish in which the spinal cord had been removed, indicate that catecholamine secretion elicited by field stimulation is neuronally mediated.

To address the possibility that catecholamine secretion occurred as a result of non-specific depolarization, fish were perfused with Na^+-free saline. It is believed that nicotinic receptor stimulation is coupled to the opening of receptor-linked Na^+ channels and that the resultant membrane depolarization causes an opening of voltage-dependant Ca^{2+} channels (Brandt et al., 1976; Wada et al., 1985; Liu and Kao, 1990). The subsequent influx of Ca^{2+} is thought to trigger the cascade of events leading to the secretion of catecholamines (Burgoyne et al., 1993). The absence of a response during field stimulation in preparations perfused with Na^+-free saline and from which the spinal cord had been removed indicates that the electrical current, in itself, was unable to elicit sufficient chromaffin cell membrane depolarization as to initiate catecholamine secretion.

The results of these validation experiments demonstrate that the field stimulation protocol causes specific neuronally mediated secretion of catecholamines from

chromaffin cells that line the walls of the PCV in trout. As such, this preparation appears to be a valid tool to examine various aspects of neuronal mechanisms regulating the release of catecholamines from the chromaffin cells in trout.

CHAPTER 3

CHOLINERGIC CONTROL OF CATECHOLAMINE SECRETION FROM CHROMAFFIN CELLS IN THE RAINBOW TROUT (*Oncorhynchus mykiss*)

(Based in part on Montpetit CJ and Perry SF. 1999. Journal of Experimental Biology 202, 2059-2069)

INTRODUCTION

In teleosts, the principal source of circulating catecholamines are chromaffin cells located within the walls of the posterior cardinal vein (Nandi, 1961; Gallo et al., 1993). The primary mechanism leading to their secretion is believed to be increased neuronal stimulation by preganglionic nerve fibers (from the sympathetic nervous system) innervating the chromaffin cells (Nilsson, 1983). Upon stimulation, the release of acetylcholine (Ach) stimulates cholinergic receptors ultimately initiating a series of Ca^{2+}-dependent events leading to the secretion of catecholamines (Nilsson et al., 1976; Burgoyne et al., 1993; Fritsche et al., 1993; Furimsky et al., 1996). In teleost fish, it appears that nicotinic receptors are the predominant cholinoceptor present on chromaffin cells (reviewed by Reid et al., 1998).

The nicotinic nature of cholinergic catecholamine secretion has been demonstrated in several teleosts including rainbow trout (*Oncorhynchus mykiss*; Fritsche et al., 1993; Julio et al., 1998), American eel (*Anguilla rostrata*; Reid and Perry, 1995; Al-Kharrat et al., 1997), Atlantic cod (*Gadus morhua*; Nilsson et al., 1976) and carp (*Cyprinus carpio*; Gfell et al., 1997). In the Atlantic cod, electrical stimulation of the sympathetic fibers or application of Ach to the head kidney caused the secretion of catecholamines from *in situ* perfused preparations (Nilsson et al., 1976). Furthermore, bolus injections of the non-selective cholinergic agonist carbachol to *in situ* perfused preparations of American eels caused the secretion of both noradrenaline and adrenaline (Reid and Perry, 1995). In those studies, the secretion of catecholamines in response to either stimulus was prevented by prior treatment with the nicotinic receptor antagonist hexamethonium.

In rainbow trout, nicotinic receptor stimulation is believed to be the primary mediator of cholinergic induced secretion of catecholamines (Reid et al., 1998). Recent evidence, however, suggests that muscarinic cholinergic stimulation may also contribute to catecholamine release. For example, in isolated trout chromaffin cells, hexamethonium reduced the rise in $[Ca^{2+}]_i$ during nicotine administration, but was without effect on $[Ca^{2+}]_i$ in the presence of carbachol (Furimsky et al., 1996). Furthermore, Fritsche et al., (1993) demonstrated using an *in situ* saline perfused posterior cardinal vein preparation of rainbow trout, that pre-treatment with hexamethonium prevented the carbachol-induced release of noradrenaline but failed to "completely" block the release of adrenaline.

Together, the results of these studies suggest a role for muscarinic receptors in the control of catecholamine secretion from trout chromaffin cells. More recently, Julio et al., (1998) demonstrated that the muscarinic receptor agonist pilocarpine was capable of directly eliciting the release of adrenaline and noradrenaline from *in situ* perfused posterior cardinal vein preparations of trout. Selective muscarinic receptor antagonists (e.g. atropine), however, were not used in any of these studies, thus casting doubt on a specific involvement of muscarinic receptor stimulation. Studies designed to evaluate the contribution of the muscarinic receptor in catecholamine secretion in other teleosts (eel and carp) have produced conflicting results (Reid and Perry, 1995; Al-Kharrat et al., 1997; Gfell et al., 1997; Abele et al., 1998). Moreover, while prior experiments have indirectly (Fritsche et al., 1993; Furimsky et al., 1996) or directly (Julio et al., 1998) suggested the presence of muscarinic receptors, no studies have yet been performed to evaluate the relative involvement of muscarinic receptors in catecholamine secretion

during neuronal stimulation of rainbow trout chromaffin cells. Thus, the goal of the present investigation was to determine if neuronal cholinergic stimulation of catecholamine secretion in rainbow trout involves muscarinic receptors.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout weighing between 250 and 350g were obtained and maintained under conditions outlined in Chapter 2.

In Situ Saline Perfused Posterior Cardinal Vein Preparation

The posterior cardinal vein tissue was perfused as outlined in Chapter 2. After the 20-min stabilization period, a control sample was collected in a pre-weighed micro-centrifuge tube to assess basal catecholamine secretion rate. After collection of the pre-sample, whole-body field electrical stimulation (as described in Chapter 2) or a bolus injection of agonist was given to the preparations in accordance with the treatments described below. Agonists were delivered by bolus injection (final volume = 0.3 ml) 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-treatment samples were collected 1,2,3,4 and 5 min after intervention.

Cholinergic evoked catecholamine secretion

The following series of experiments were performed to study the contributions of nicotinic and muscarinic receptor stimulation to catecholamine secretion from the chromaffin tissue.

Series 1: Effects of cholinergic antagonists on electrical and cholinergic agonist-evoked catecholamine secretion. After collection of the pre-sample, bolus injection (0.3 ml) of the non-selective cholinergic agonist carbachol (10^{-5} mol kg^{-1} body wt) or

electrical stimulation was administered to preparations perfused with saline or saline containing the muscarinic receptor antagonist atropine (10^{-4} M), the nicotinic receptor antagonist hexamethonium (10^{-3} M), or both hexamethonium (10^{-3} M) and atropine (10^{-4} M). Furthermore, bolus injection (0.3 ml) of nicotine (10^{-7} mol kg^{-1}), or the muscarinic receptor agonist methacholine (10^{-3} mol kg^{-1}) was administered to preparations perfused with saline, or saline containing hexamethonium (10^{-3} M), or atropine (10^{-4} M), to confirm the specificity of the cholinergic receptor antagonists.

Series 2: Effects of nicotinic and muscarinic receptor agonists on catecholamine secretion. After collection of the pre-sample, preparations were given a bolus injection (0.3 ml) of the nicotinic receptor agonist nicotine (10^{-7} mol kg^{-1} body wt), the muscarinic receptor agonists oxotremorine sesquifumarate (10^{-4} mol kg^{-1} body wt) or methacholine (10^{-3} mol kg^{-1} body wt), or a cocktail of nicotine (10^{-7} mol kg^{-1} body wt) and oxotremorine (10^{-4} mol kg^{-1} body wt) or nicotine and methacholine (10^{-3} mol kg^{1} body wt).

Series 3: Effects of atropine and/or hexamethonium on muscarinic cholinergic stimulation of catecholamine secretion. Bolus injections (0.3 ml) of methacholine (10^{-3} mol kg^{-1} body wt) or oxotremorine (10^{-4} mol kg^{-1} body wt) were administered to preparations perfused with saline, saline containing atropine (10^{-4} M), or saline containing hexamethonium (10^{-3} M).

Analytical Procedures

Determination of perfusate catecholamine levels was performed as outlined in Chapter 2.

Statistical Analysis

The data are presented as the mean ± 1 standard error of the mean (SEM). Where appropriate, the data were statistically analyzed by a one-way repeated measures ANOVA followed by Dunnett's test for comparison with pre-stimulation values, or a one-way ANOVA followed by Dunnett's test for multiple comparisons; if the normality test failed, an ANOVA on ranks was performed followed by Dunnett's multiple comparison test. In other instances, data were analyzed by Student's t-test or paired t-test, and if the normality test failed, a Mann-Whitney rank sum test was performed. 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

Series 1: Effects of cholinergic antagonists on electrical and cholinergic agonist-evoked catecholamine secretion. A set of experiments was performed to confirm the specificity of the cholinergic receptor antagonists, hexamethonium and atropine; the results are presented in Table 3.1. Nicotine evoked catecholamine secretion was abolished by pre-treatment with hexamethonium and unaffected by pre-treatment with atropine. Conversely, methacholine (cholinergic receptor agonist) evoked catecholamine secretion was unchanged in the presence of hexamethonium but was blocked by atropine. These results indicate that at the concentrations employed in the present study, hexamethonium (10^{-3} M) and atropine (10^{-4} M) were acting as specific nicotinic and muscarinic receptor antagonists, respectively.

Both adrenaline and noradrenaline secretion rates were increased over baseline levels in response to field stimulation or bolus injections of carbachol (Fig 3.1A and 3.2A). In fish given carbachol, secretion of both catecholamines remained above resting levels for the entirety of the experiment (Fig. 3.2A). Prior treatment with atropine significantly reduced adrenaline secretion in response to both stimuli (Fig. 3.1B and 3.2B). Pre-treatment with hexamethonium significantly lowered adrenaline secretion in response to field stimulation (Fig 3.1C). However, hexamethonium entirely prevented adrenaline secretion in fish given carbachol (Fig. 3.2C). Similar results were obtained in the presence of both antagonists (Fig 3.1D and 3.2D), i.e. adrenaline secretion was markedly reduced during electrical stimulation (Fig. 3.1D) and abolished during application of carbachol (Fig. 3.2D). Prior treatment with any of the antagonists was without effect on noradrenaline secretion during electrical stimulation (Fig. 3.1B,C, D).

On the other hand, atropine lowered noradrenaline secretion in response to bolus injections of carbachol, while hexamethonium totally prevented its release (Fig.3.2B,C, and D).

Series 2: Effects of nicotinic and muscarinic receptor agonists on catecholamine secretion. Figure 3.3 illustrates the effects of bolus injections of nicotine, oxotremorine (muscarinic agonist), and a cocktail of both agonists on catecholamine secretion in the perfused posterior cardinal vein preparation. Nicotine or oxotremorine administration predominantly affected adrenaline secretion. Noradrenaline secretion was only slightly affected, reaching about 0.1 nmol min^{-1} in response to nicotine or oxotremorine. When administered together, nicotine and oxotremorine appeared to have a simple additive effect on adrenaline secretion. The effect on noradrenaline secretion on the other hand was greater than additive and was more than tripled in the presence of both agonists. Application of another muscarinic agonist, methacholine (10^{-3} mol kg^{-1}), had similar effects on adrenaline secretion (see Table 3.1). Nicotine-evoked adrenaline and noradrenaline secretion occurred transiently. Oxotremorine administration caused a sustained secretion of both adrenaline and noradrenaline, albeit at a lower level.

Series 3: Effects of atropine and hexamethonium on methacholine- and oxotremorine-evoked catecholamine secretion. Prior treatment with atropine or hexamethonium had no effect on maximal secretion rates in response to bolus injection of oxotremorine (data not shown). On the other hand, atropine totally prevented methacholine-evoked secretion (Table 3.1).

Table 3.1 The effects of prior treatment with saline (control), hexamethonium (10^{-3} M), or atropine (10^{-4} M), on total maximal (Max) catecholamine (noradrenaline plus adrenaline) secretion (nmol min^{-1}) elicited by bolus injections of nicotine (10^{-7} mol kg^{-1}) or methacholine (10^{-3} mol kg^{-1}). Pre-injection (Pre) values are included; * denotes a significant difference between the pre-injection value and the maximal response. N = 6 different preparations for each combination of agonist/saline and antagonist.

Agonist	Control		Hexamethonium (10^{-3} M)		Atropine (10^{-4} M)	
	Pre	Max	Pre	Max	Pre	Max
Nicotine (10^{-7} mol kg^{-1})	0.05 ± 0.01	0.86 ± 0.21*	0.03 ± 0.01	0.03 ± 0.01	0.08 ±0.02	1.06 ± 0.33 *
Methacholine (10^{-3} mol kg^{-1})	0.04 ± 0.01	0.14 ± 0.04*	0.04 ± 0.004	0.09 ± 0.01*	0.05 ± 0.01	0.06 ± 0.01

Note: Values are shown as means ±1 SEM.

Figure 3.1 The effects of cholinergic antagonists on adrenaline (white bars) and noradrenaline (black bars) secretion rates in response to field stimulation. Each preparation was pre-treated with (A) saline (Control; N = 6), or saline containing (B) atropine (10^{-5} M; N = 6), (C) hexamethonium (10^{-3} M; N = 6), or (D) atropine (10^{-5} M) plus hexamethonium (10^{-3} M) (N=6) in the solution. Electrical stimulation was carried out at 60 V at 20 pps, and 1msec duration for 30 sec. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from pre-stimulation (pre) values; a dagger denotes a significant difference from the control values (A).

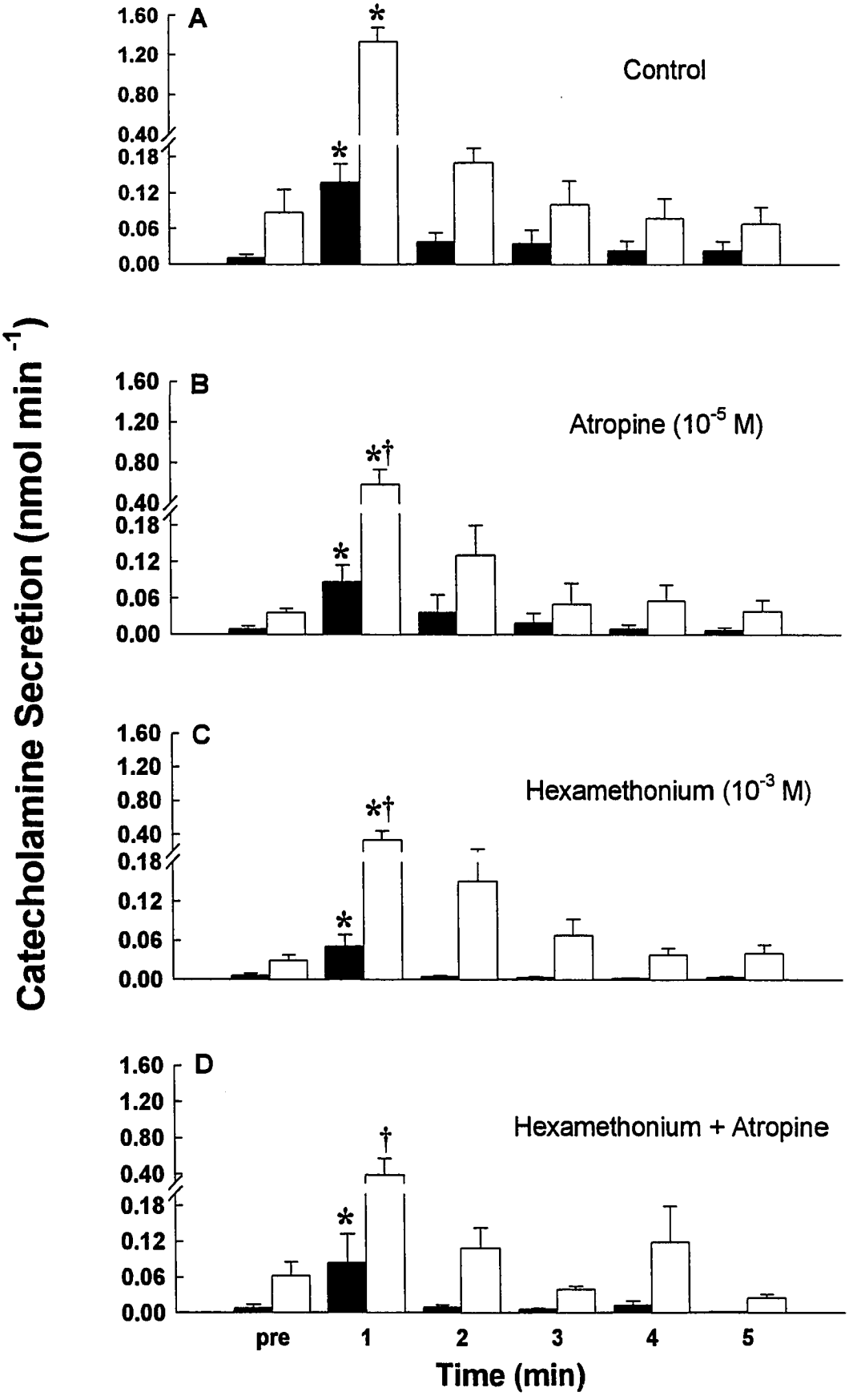

A
Control
B
Atropine (10^{-5} M)
C
Hexamethonium (10^{-3} M)
D
Hexamethonium + Atropine
1.60
1.20
0.80
0.40
0.18
0.12
0.06
0.00
*
*†
†
pre
1
2
3
4
5
Time (min)
Catecholamine Secretion (nmol min^{-1})

Figure 3.1

Figure 3.2. The effects of cholinergic antagonists on adrenaline (white bars) and noradrenaline (black bars) secretion rates in response to bolus injections of carbachol (10^{-5} mol kg^{-1}). Each preparation was pre-treated with (A) saline (control; N = 6), or saline containing (B) atropine (10^{-4} M; N = 6), (C) hexamethonium (10^{-3} M; N = 6), or (D) atropine (10^{-5} M) plus hexamethonium (10^{-3} M) (N = 6) in the solution. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from the pre-stimulation (pre) value; a dagger denotes a significant difference with the control values (A).

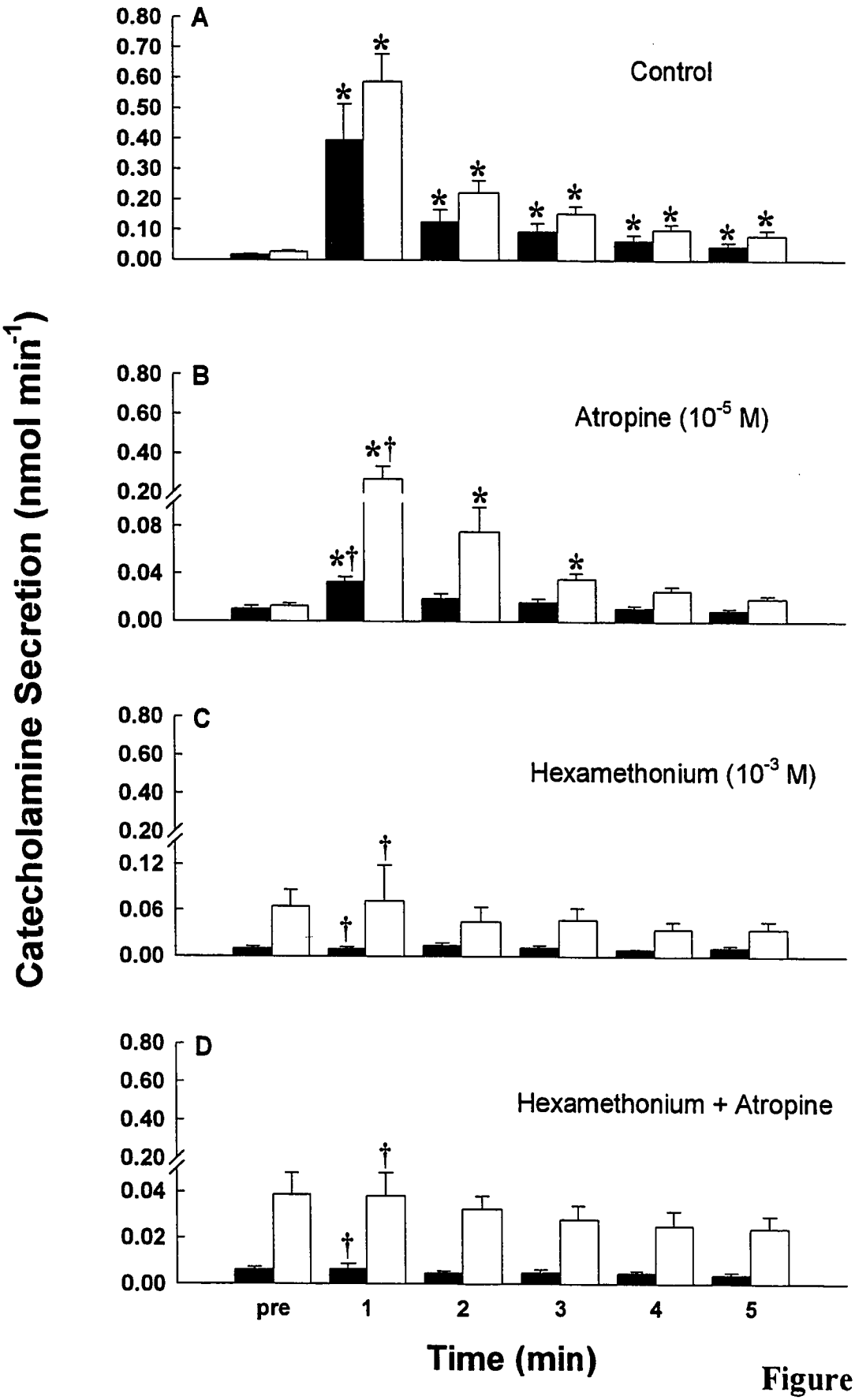

A
Control
B
Atropine (10^{-5} M)
C
Hexamethonium (10^{-3} M)
D
Hexamethonium + Atropine
Catecholamine Secretion (nmol min^{-1})
Time (min)
pre
1
2
3
4
5

Figure 3.2

Figure 3.3 The effects of cholinergic agonists on adrenaline (white bars) and noradrenaline (black bars) secretion rates. Each preparation was given a bolus injection of (A) nicotine (10^{-7} mol kg^{-1}; N = 6), (B) oxotremorine (10^{-4} mol kg^{-1}; N = 6), or (C) a cocktail of nicotine (10^{-7} mol kg^{-1}) and oxotremorine (10^{-4} mol kg^{-1}) (N = 6). Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from the pre-stimulation (pre) value.

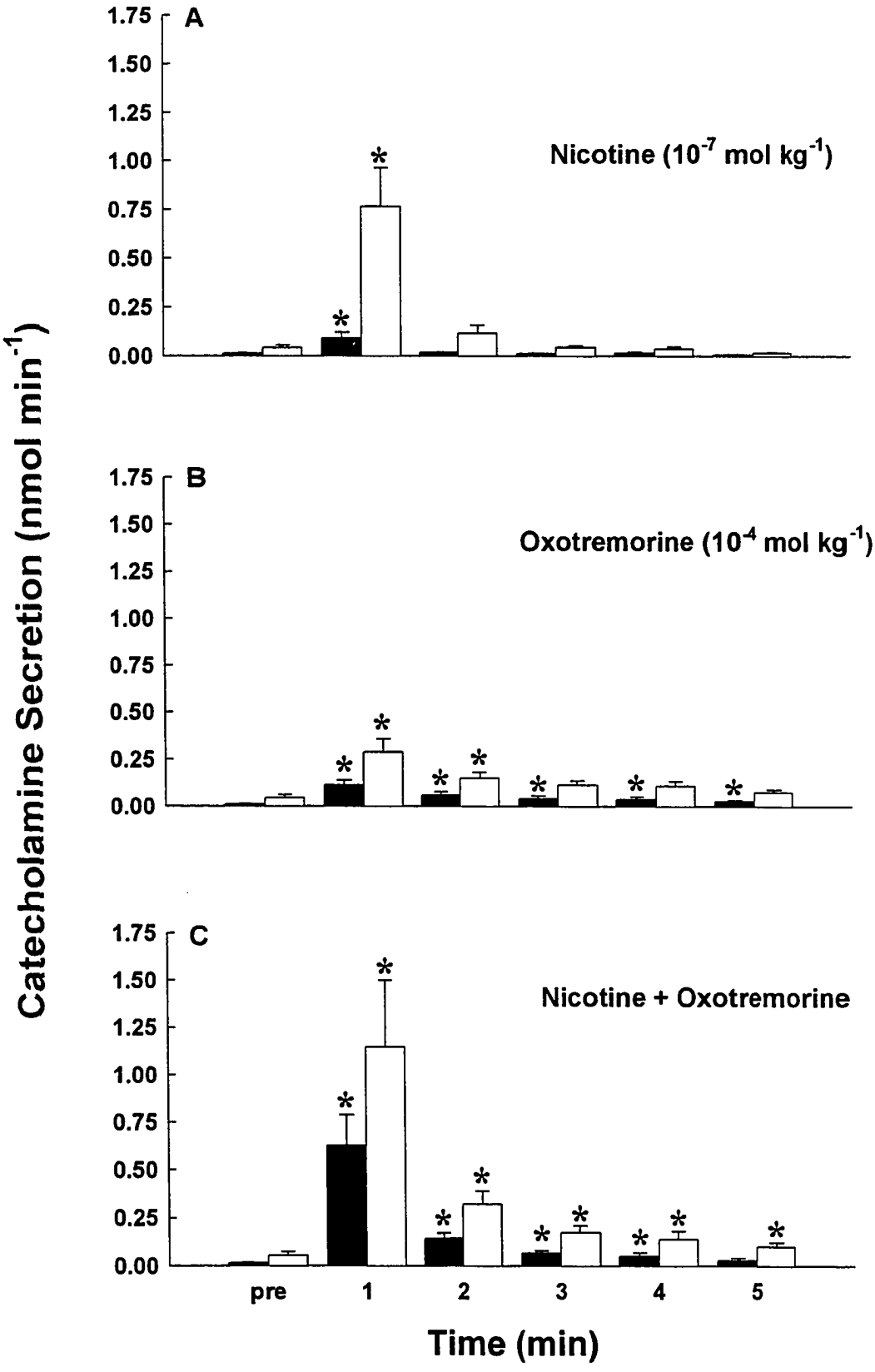

A
Nicotine (10^{-7} mol kg^{-1})
B
Oxotremorine (10^{-4} mol kg^{-1})
C
Nicotine + Oxotremorine
Catecholamine Secretion (nmol min^{-1})
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
pre
1
2
3
4
5
Time (min)

Figure 3.3

DISCUSSION

During periods of acute severe stress, teleost fish release noradrenaline and adrenaline into the circulation (reviewed by Randall and Perry, 1992; Thomas and Perry, 1992; Wendelaar Bonga, 1997; Reid et al., 1998). The subsequent elevation of plasma catecholamine levels is believed to initiate a series of physiological adjustments aimed at enhancing blood O_2 transport and gill O_2 diffusing capacity (reviewed by Randall and Perry, 1992; Epple, 1993). In teleosts, the secretion of catecholamines from chromaffin cells is under the control of numerous physiological stimuli. These include stimulation by pre-ganglionic sympathetic nerve fibers (Nilsson et al., 1976; Abele et al., 1998), localized changes in blood chemistry (Opdyke et al., 1983; Perry et al., 1991), activation of the renin-angiotensin system (Opdyke et al., 1981; Bernier and Perry, 1997), serotonin (Fritsche et al., 1993; Reid et al., 1996), and adrenocorticotropic hormone (Reid et al., 1996; Bernier and Perry, 1996). Of these, increased neuronal stimulation of the chromaffin cells by preganglionic cholinergic fibers is thought to be the predominant mechanism promoting catecholamine secretion (reviewed by Epple et al., 1995; Reid et al., 1998).

Based on the results of the present investigation, it appears that cholinergic control of catecholamine secretion from trout chromaffin cells is not confined to nicotinic receptor evoked events. Indeed, this is the first study to conclusively demonstrate a role for muscarinic receptors in catecholamine secretion from rainbow trout chromaffin cells. An involvement of muscarinic receptors in the catecholamine secretion process is not without precedent. In the common carp (*Cyprinus carpio*), the muscarinic receptor agonists, muscarine and pilocarpine, elicited catecholamine secretion in perifused head

kidney preparations (Gfell et al., 1997). Previous studies using American eels have provided conflicting evidence. Reid and Perry (1995) were unable to demonstrate any involvement of muscarinic receptors whereas other studies have documented an inhibitory role of basal secretion (Al-Kharrat et al., 1997), or a positive modulatory role under electrical stimulation of the sympathetic fibers (Abele et al., 1998). Moreover, there are numerous reports that demonstrate a role of muscarinic receptors in catecholamine secretion in non-piscine vertebrates although the results are highly variable. Indeed, these investigations have demonstrated that muscarinic receptor stimulation can enhance nicotinic evoked release (Role and Perlman, 1983; Accordi 1988; Nassar-Gentina et al., 1997), inhibit nicotinic evoked release (Swilem and Hawthorn, 1983; Cheek and Burgoyne, 1985; Nassar-Gentina et al., 1991), stimulate secretion in the absence of nicotinic agonists (Wakade and Wakade, 1983; Knight and Baker, 1986; Ballesta et al., 1989; Xu et al., 1991; Tobin et al., 1992; Chowdhury et al., 1994), or exert no effects on catecholamine secretion (Liang and Perlman, 1979; Mckay et al., 1991). The results of the present investigation suggest that in rainbow trout, muscarinic receptor stimulation enhances nicotine-induced secretion, and that under intense stimulation may directly elicit catecholamine secretion. This was shown by the fact that catecholamine secretion elicited by either field stimulation or carbachol was inhibited by atropine but was not further decreased when added with hexamethonium. The direct effect of muscarinic receptor stimulation on catecholamine secretion was confirmed using the specific muscarinic agonists, oxotremorine and methacholine.

This is the first study (in rainbow trout) to use a muscarinic receptor antagonist (atropine), and the muscarinic receptor agonists methacholine and oxotremorine to dissect

the cholinergic control of catecholamine secretion. Methacholine- and oxotremorine-induced catecholamine secretion, and their inhibition by atropine have been described for avian (Knight and Baker, 1986) and mammalian (Ballesta et al., 1989; Xu et al., 1991; Yamagami et al., 1991; Nassar-Gentina et al., 1997) chromaffin cells. In this study, atropine inhibited the methacholine-evoked secretion but did not reduce catecholamine secretion during oxotremorine administration. The inability of atropine to block oxotremorine-evoked secretion is not understood. However, resistance to muscarinic antagonists has been demonstrated in other systems (Robitaille et al., 1997). The possibility that oxotremorine-evoked secretion was mediated by nicotinic receptor activation can be discounted because the response was insensitive to hexamethonium. Regardless, taken together, the fact that methacholine and oxotremorine elicited secretion and that atropine reduced secretion in response to field stimulation, bolus injections of carbachol and methacholine, clearly indicates the presence of muscarinic receptors on rainbow trout chromaffin cells with a role in the control of catecholamine secretion.

Modulation of presynaptic Ach release via muscarinic receptor stimulation is an alternative explanation for the observations. Muscarinic cholinergic modulation of presynaptic activity has been demonstrated in the central nervous system of non-piscine vertebrates (Raiteri et al., 1990). It is unlikely, however, that the muscarinic effect on catecholamine secretion observed in the present study was mediated through modulation of presynaptic Ach release from nerve fibers innervating the chromaffin cells. First, presynaptic muscarinic receptors generally regulate Ach release through inhibitory actions (Raiteri et al., 1990). Second, the nicotinic receptor antagonist hexamethonium did not affect the oxotremorine-evoked secretion of catecholamines. If, indeed,

stimulation of pre-synaptic muscarinic receptors was causing Ach release, then catecholamine secretion should have been reduced by hexamethonium. Third, Furimsky et al., (1996) demonstrated that when hexamethonium was added to isolated trout chromaffin cells after stimulation with carbachol, there was no significant decrease in $[Ca^{2+}]_i$ as was observed after nicotine administration thus implicating the presence of hexamethonium-insensitive muscarinic receptors.

The stimulation of muscarinic receptors is known to activate phospholipase C to produce IP_3, which subsequently causes the release of Ca^{2+} from intracellular stores within chromaffin cells (Eberhard and Holtz, 1987; O'Sullivan and Burgoyne, 1989; Abad et al., 1992). In some species, the rise in $[Ca^{2+}]_i$ is not sufficient to trigger catecholamine secretion (Cheek and Burgoyne, 1985; Sorimachi et al., 1992). However, in other species, muscarinic receptor stimulation can cause sufficient release of Ca^{2+} from intracellular stores to elicit the secretion of catecholamines (Burgoyne et al., 1993).

In accordance with other studies, high doses of muscarinic agonists were required to elicit catecholamine secretion. In rainbow trout, muscarinic receptor stimulation during field stimulation does not appear to be able to induce secretion in the absence of nicotinic receptor activation. However, under more intense conditions of stimulation, muscarinic receptor stimulation may have direct positive effects on secretion. Using the field stimulation technique, the muscarinic cholinergic evoked increase in $[Ca^{2+}]_i$ presumably was not sufficient to trigger secretion but probably enhanced post-nicotinic receptor Ca^{2+}-dependent events thereby increasing the nicotinic-induced secretion of catecholamines. However, under more intense muscarinic receptor stimulation, these $[Ca^{2+}]_i$ levels may reach threshold and trigger the secretion process. Furthermore,

muscarinic cholinergic stimulation of catecholamine release may help to sustain secretion in the event of nicotinic receptor desensitization (Boksa and Livett, 1984). In this study, oxotremorine, methacholine, and carbachol administration caused a sustained and prolonged secretion of both catecholamines. This response might be useful during prolonged stressful situations when the nicotinic secretory response may be desensitized (Boksa and Livett, 1984).

It is possible that catecholamine secretion also is controlled by non-cholinergic neurotransmitters. Reid et al., (1995) identified a number of neurotransmitters or neuropeptides including vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) in nerve fibers in the vicinity of chromaffin cells in teleost fish. Their role in the secretion process in fish has yet to be investigated, although both are known to have stimulatory effects on catecholamine secretion in non-piscine vertebrates (Watanabe et al., 1995; Yamaguchi, 1993). Similar studies are required to determine their relative contribution to catecholamine secretion in teleost fish. A line of evidence for this occurrence is that in response to field stimulation, pretreatment with hexamethonium plus atropine failed to completely inhibit the secretion of both adrenaline and noradrenaline.

Conclusion

Using a "new" field stimulating technique, this is the first study to directly investigate the possible involvement of muscarinic receptor stimulation of catecholamine secretion from the chromaffin tissue in the rainbow trout. The results of the study support the presence of muscarinic receptors with a functional role in the cholinergic control of

catecholamine secretion. More specifically, muscarinic receptor stimulation enhances the nicotinic evoked secretion of catecholamines, and that under intense stimulation may directly elicit secretion.

CHAPTER 4

VIP AND PACAP-MEDIATED CONTROL OF CATECHOLAMINE RELEASE FROM THE CHROMAFFIN TISSUE IN THE RAINBOW TROUT (*Oncorhynchus mykiss*)

(Based in part on Montpetit CJ and Perry SF. 2000.
Journal of Endocrinology 166, 705-714)

INTRODUCTION

In teleost fish, the secretion of catecholamines from chromaffin tissue, located within the walls of the posterior cardinal vein (Nandi, 1961; Gallo et al., 1993), is under the control of numerous physiological stimuli including signals of humoral, neuronal, and autocrine/paracrine origin (Reid et al., 1998). Although several factors are known to initiate catecholamine secretion, increased neuronal activity of preganglionic sympathetic nerve fibers is thought to be the predominant mechanism for catecholamine secretion during stress (Epple et al., 1995; Reid et al., 1998).

The current model for the neuronal control of catecholamine secretion in fish advocates that a number of non-cholinergic neurotransmitters and/or neuromodulators are co-released with acetylcholine during neuronal activity (Reid and Perry, 1995a; Abele et al., 1998; Montpetit and Perry, 1999). However, this model has not been tested and thus the role of non-cholinergic neurotransmitters in controlling catecholamine secretion in fish is unknown. Reid et al. (1995), using immunohistochemistry, identified a number of neurotransmitters or neuropeptides, including VIP (vasoactive intestinal polypeptide) and PACAP (pituitary adenylate cyclase activating polypeptide) in nerve fibers innervating the chromaffin tissue of Atlantic cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*), American eel (*Anguilla anguilla*), and dogfish (*Squalus acanthias*). While VIP and PACAP mediation of catecholamine secretion have been demonstrated in non-piscine chromaffin cells (Misbuhuddin et al., 1988; Yamaguchi, 1993; Guo and Wakade, 1994; Yon et al., 1994; Watanabe et al., 1995; Tanaka et al., 1996), their involvement in fish is unresolved. Thus, the goal of the first component of the study was to determine whether

the neuropeptides VIP and PACAP are able to directly stimulate the secretion of catecholamines from the chromaffin tissue in rainbow trout.

A recent study (Montpetit and Perry, 1999; Chapter 3) demonstrated that blockade of chromaffin cell cholinergic (nicotinic and muscarinic) receptors failed to completely inhibit the neuronally induced secretion of adrenaline and noradrenaline in rainbow trout. Consequently, the participation of unspecified non-cholinergic neurotransmitters was suggested. Thus, a second goal of the present investigation was to assess the relative contribution of cholinergic and non-cholinergic mechanisms in the control of catecholamine secretion from trout chromaffin cells during neuronal stimulation and to identify the contributing non-cholinergic neurotransmitters. This was accomplished by comparing catecholamine secretion in the presence or absence of cholinergic or VIP/PACAP receptor antagonists during neuronal stimulation of chromaffin tissue *in situ*.

MATERIAL AND METHODS

Experimental Animals

Rainbow trout weighing between 250 and 350g were maintained under conditions outlined in Chapter 2.

In situ saline-perfused posterior cardinal vein preparation

The posterior cardinal vein tissue was perfused as outlined in Chapter 2. After the 20-min stabilization period, a control pre-stimulation sample was collected in a pre-weighed microcentrifuge tube to assess basal catecholamine secretion rate. After collection of the pre-stimulation sample, a bolus injection of agonist or whole field body electrical stimulation (as described in Chapter 2) was given to preparations in accordance with the treatments described below. Agonists were delivered by a bolus injection 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-stimulation samples were collected at 1, 2, 3, 4, and 5 min after intervention.

VIP- and PACAP- induced catecholamine secretion

Series 1: VIP and PACAP evoked catecholamine secretion. To characterize the effects of exogenous ckVIP (chicken VIP, Peninsula Laboratories Inc., San Carlos, CA, USA) and hPACAP-27 (PACAP-27-NH_2 human, ovine, rat, Peninsula Laboratories Inc.) on catecholamine secretion, preparations were given a single bolus injection (final volume, 0.3 ml) of VIP or PACAP-27, at doses ranging from 10^{-13} to $5X10^{-8}$ mol kg^{-1} body mass (N = 6-10 for each dose). In these experiments, only adrenaline secretion was significantly elevated over baseline levels and therefore only dose response curves for ckVIP- and hPACAP-27-induced adrenaline secretion were constructed. Generally, peak

secretion rates were obtained in the second or third minute after agonist administration. Therefore, dose response curves for ckVIP or hPACAP-27-elicited adrenaline secretion were constructed that expressed peak catecholamine secretion rates as a function of dose. The doses of ckVIP and hPACAP-27 required to cause half-maximal release (ED_{50}) were calculated from Hill plots of log (% of maximal secretion / 100-% of maximal secretion) versus log dose. Only those values falling between 5% and 95% were used.

Series 2: effects of VIP 6-28, PACAP 6-27, and cholinergic antagonists on VIP- and PACAP-27-evoked catecholamine secretion. Experiments were performed to evaluate the effects of ckVIP and hPACAP-27 on catecholamine secretion in the presence or absence of VIP (VIP 6-28, Peninsula Laboratories), PACAP (PACAP 6-27, Peninsula Laboratories Inc.), and cholinergic receptor (hexamethonium plus atropine (Sigma, Oaksville, Ontario, Canada)) antagonists. VIP 6-28 and PACAP 6-27 were chosen on the basis of their potent antagonism of VPAC (VIP and PACAP receptors) receptors (Bodanszky et al., 1973; Fishbein et al., 1994) and PAC_1 (PACAP receptors) (Robberecht et al., 1991; Gaspo et al., 1997), respectively. In a first experimental series, different preparations were given a bolus injection (0.6 ml) of ckVIP (10^{-10} mol kg^{-1}) *(N=6)*, a cocktail (0.6 ml) containing ckVIP (10^{-10} mol kg^{-1}; 0.3 ml) plus VIP 6-28 (10^{-6} M; 0.3ml) *(N=6)*, a cocktail (0.6 ml) containing ckVIP (10^{-10} mol kg^{-1}; 0.3 ml) and a mixture of hexamethonium (10^{-3} M) plus atropine (10^{-5} M) (0.3 ml) *(N=6)* or a cocktail (0.6 ml) containing ckVIP (10^{-10} mol kg^{-1}; 0.3 ml) plus PACAP 6-27 (10^{-8} M; 0.3 ml) *(N=6)*. Similarly, in a second experimental series, preparations were given a bolus injection (0.6 ml) containing hPACAP-27 (10^{-10} mol kg^{-1}) or hPACAP-27 (10^{-10} mol kg^{-1}; 0.3 ml) in combination with the antagonists described above (0.3 ml). In a third set of

experiments, bolus injection (0.6 ml) of the VIP or PACAP-27 receptor antagonists, VIP 6-28 (10^{-6} M) and PACAP 6-27 (10^{-8} M), respectively, were administered to preparations to confirm that they were not acting as peptidergic agonists. Each preparation received a single injection.

Series 3: effects of cholinergic receptor antagonists, and of VIP 6-28 on carbachol elicited catecholamine secretion. This series of experiments was performed to confirm the specificity of the various drugs. Preparations were given a bolus injection (0.3 ml) of either 10^{-5} mol kg^{-1}carbachol (non-specific cholinergic receptor agonist; Sigma) or carbachol in combination with a cocktail (0.3 ml) of hexamethonium (nicotinic receptor antagonist; 10^{-3} M) plus atropine (muscarinic receptor antagonist; 10^{-5} M) (0.3 ml). Additionally, preparations were given a bolus injection of carbachol in combination with VIP 6-28 (10^{-6} M).

Series 4: effects of Ca^{2+}-free saline and internal Ca^{2+} stores depletion on nicotine and VIP elicited secretion of catecholamines. Owing to the requirement of Ca^{2+} in secretory processes, experiments were performed to ascertain the sources of Ca^{2+} that are recruited following cholinergic (nicotinic) and VIP stimulation of catecholamine secretion. A single bolus injection (0.3 ml) of either 10^{-7} mol kg^{-1} nicotine (nicotinic cholinergic receptor agonist; Sigma) or 10^{-9} mol kg^{-1} ckVIP was administered to preparations prior perfused with control saline, Ca^{2+}-free saline, or saline containing thapsigargin (10^{-6} M; internal Ca^{2+} store releaser).

Series 5: Contribution of cholinergic versus non-cholinergic neurotransmitters on catecholamine secretion. To compare catecholamine secretion in the presence or absence of cholinergic and non-cholinergic receptor antagonists during low and high levels of neuronal activity, it was first necessary to establish frequency response curves. Each preparation was given a single electrical stimulation (see above), at frequencies ranging from 0 to 20 Hz.

This series of experiments was performed to determine the relative contribution of cholinergic and non-cholinergic neurotransmitters on catecholamine secretion at different levels of neuronal activity. To accomplish this, each preparation was electrically stimulated (see above) only once at a low (1 Hz) or high (20 Hz) frequency of stimulation, in the presence or absence of cholinergic and VIP receptor antagonists. Preparations were perfused with control saline for 15-20 min, followed by a switch to saline containing VIP 6-28 (10^{-6} M), prior to electrical stimulation. Given the observation that PACAP 6-27 was without effect on either VIP- or PACAP-elicited adrenaline secretion, only the effects of the VIP receptor antagonist, VIP 6-28, on catecholamine secretion were evaluated.

Analytical Procedures

Determination of perfusate catecholamine levels was performed as outlined in Chapter 2.

Statistical analysis

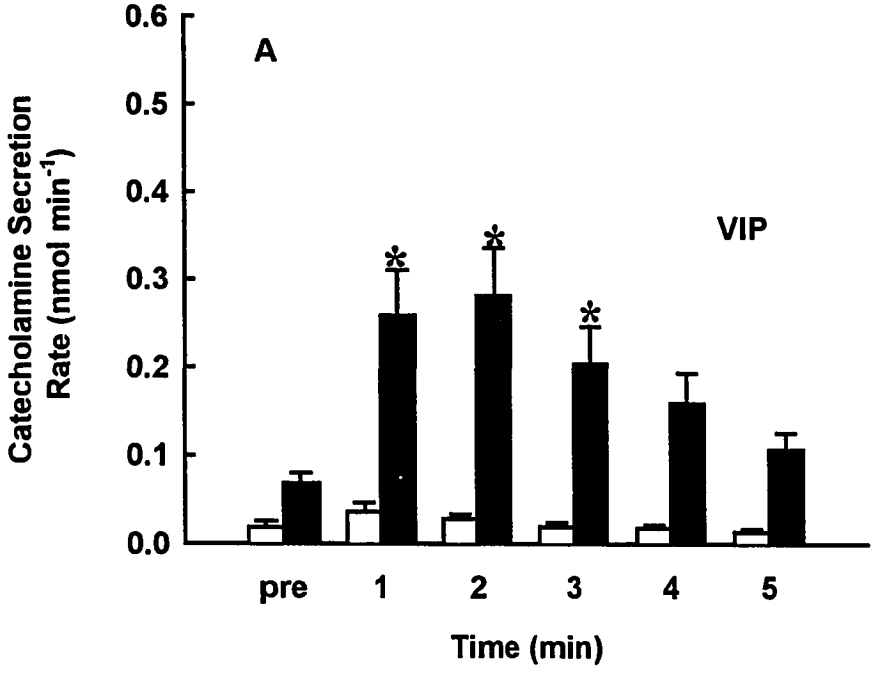
The data are presented as means ± 1 standard error of the mean (SEM). Where appropriate, the data were analyzed statistically using one-way repeated measures analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison to pre-stimulation values. In other instances, a one-way ANOVA followed by Dunnett's test for multiple comparisons was performed; if the normality test failed, an ANOVA on ranks was performed followed by Dunnett's test for multiple comparisons. The fiducial limits of significance were set at 5%. Significance of ED_{50} values was determined by calculating the 95% confidence intervals of the Hill plot regressions (SigmaPlot 4.0). All statistical tests were performed using a commercial statistical software package (SigmaStat, Jandel Scientific, version 2.03).

RESULTS

Series 1: VIP and PACAP induced catecholamine secretion and dose response curves. Figure 4.1 illustrates the temporal effects of bolus injections of ckVIP or hPACAP-27 on catecholamine secretion. Significant increases in adrenaline secretion were observed in response to exogenous ckVIP (Fig. 4.1A) and hPACAP-27 (Fig. 4.1B) within 1 min of injection. The increases in adrenaline secretion caused by ckVIP and hPACAP-27 were dose dependent (Fig. 4.2). ED_{50} values obtained from the dose response curves revealed that exogenous ckVIP and hPACAP-27 stimulated adrenaline secretion with similar potency. The ED_{50} values for adrenaline secretion were $1.91X10^{-11}$ and $1.02X10^{-11}$ mol kg^{-1} for ckVIP and hPACAP-27, respectively. Noradrenaline secretion was unaffected by either neuropeptide at all of the doses tested.

Series 2: effects of VIP 6-28, PACAP 6-27, and cholinergic receptor antagonists on VIP- and PACAP-27- evoked adrenaline secretion. The addition of VIP 6-28 (VPAC receptor antagonist) caused significant reductions in adrenaline secretion rates in response to ckVIP (Fig. 4.3) or hPACAP-27 (Fig. 4.4), however PACAP 6-27 (PAC_1 receptor antagonist) was without effect. Blockade of the cholinergic receptor (using hexamethonium plus atropine) did not affect the ckVIP- or hPACAP-27 -evoked secretion of adrenaline. VIP 6-28 or PACAP 6-27, alone, did not affect adrenaline secretion (Figures 4.3 and 4.4).

Figure 4.1 *In situ* adrenaline (*black columns*) and noradrenaline (*unfilled columns*) secretion rates in response to bolus injections of A) VIP (10^{-9} mol kg^{-1}; $N = 6$) and B) PACAP-27 (10^{-10} mol kg^{-1}; $N = 6$). Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from the pre-stimulation (*Pre*) value ($P<0.05$).


A
VIP
Catecholamine Secretion
Rate (nmol min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pre
1
2
3
4
5
Time (min)

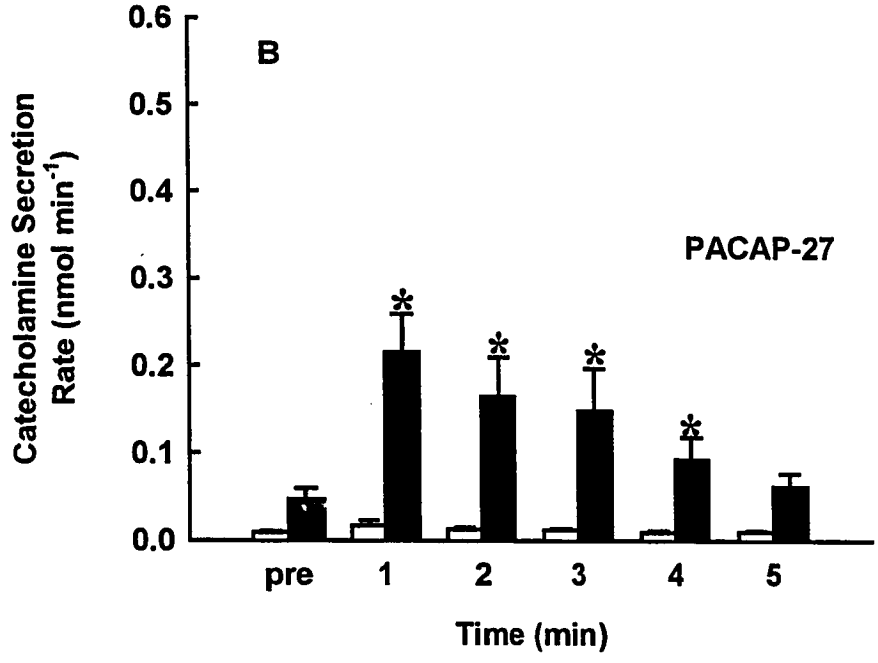

B
PACAP-27
Catecholamine Secretion
Rate (nmol min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pre
1
2
3
4
5
Time (min)

Figure 4.1

Figure 4.2 *In situ* peak adrenaline (*black columns*) and noradrenaline (*unfilled columns*) secretion rates in response to bolus injections of A) VIP and B) PACAP-27. Values are shown as means ± 1 SEM, *(N = 6-10* different fish for each dose administered). An asterisk denotes a significant difference in peak secretion when compared to the saline injected group ($P<0.05$). *Inserts*, doses of ckVIP (A) and hPACAP-27 (B) required to cause half-maximal release (ED_{50}) were calculated from Hill plots that expressed (log [% of maximal secretion / 100- % of maximal secretion] versus log dose). The regression equations and ED_{50}'s are: for VIP, $y=0.298x + 3.11$, $r=0.98$, $ED_{50}= 1.90*10^{-11}$ mol kg^{-1}; for PACAP, $y= 0.5666x + 6.23$, $r=0.99$, $ED_{50}=1.03*10^{-11}$ mol kg^{-1}.

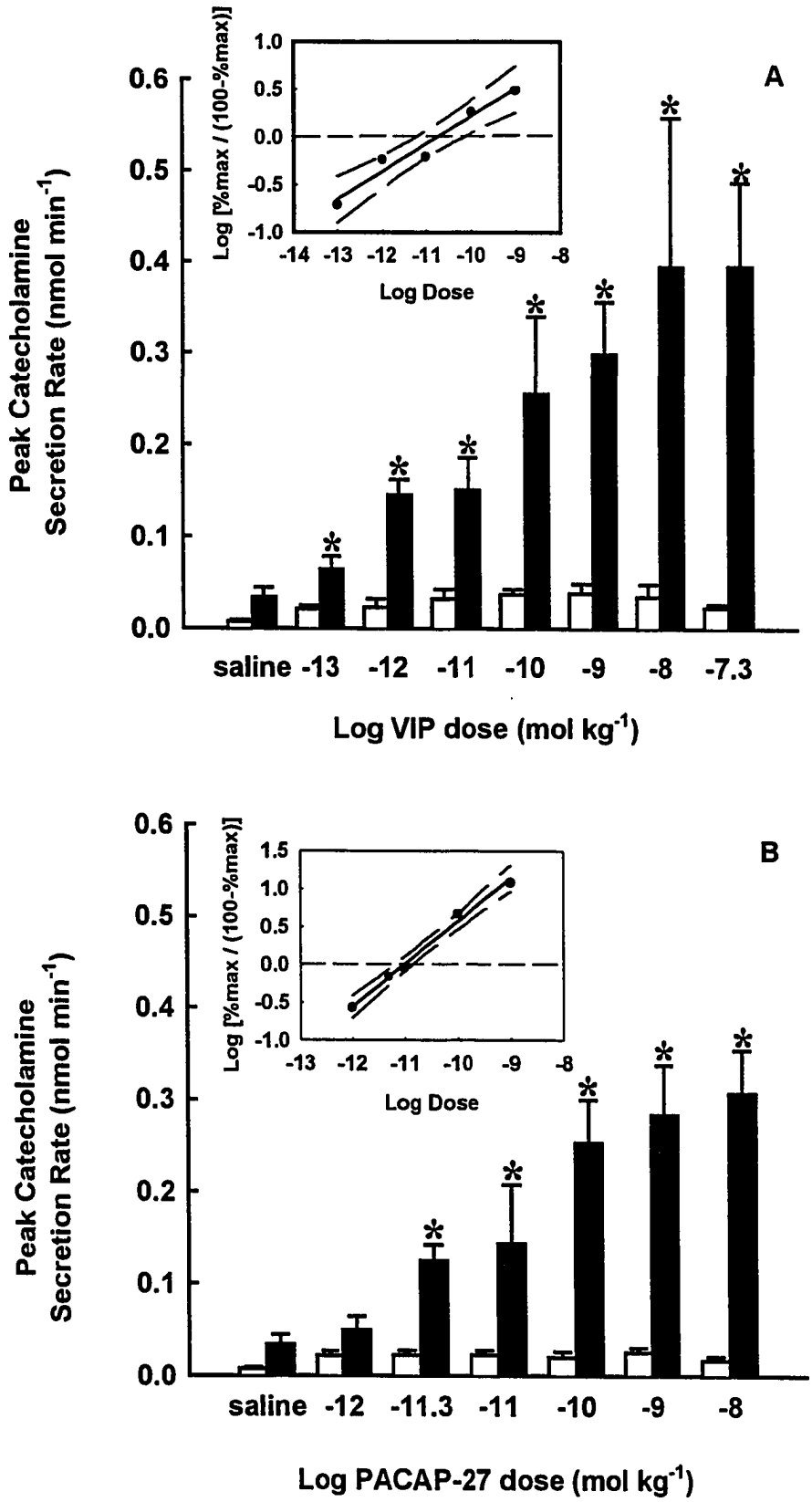

A
Peak Catecholamine Secretion Rate (nmol min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Log [%max / (100-%max)]
1.0
0.5
0.0
-0.5
-1.0
-14 -13 -12 -11 -10 -9 -8
Log Dose
saline -13 -12 -11 -10 -9 -8 -7.3
Log VIP dose (mol kg-1)
B
Peak Catecholamine Secretion Rate (nmol min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Log [%max / (100-%max)]
1.5
1.0
0.5
0.0
-0.5
-1.0
-13 -12 -11 -10 -9 -8
Log Dose
saline -12 -11.3 -11 -10 -9 -8
Log PACAP-27 dose (mol kg-1)

Figure 4.2

Figure 4.3 The effects of bolus injections of VIP (10^{-9} mol kg^{-1}, *black column, N=6*), and VIP (10^{-9} mol kg^{-1}) in combination with hexamethonium (*Hex*) (10^{-3} M) plus atropine (*Atr*) (10^{-5} M) (*gray column, N = 6*), PACAP 6-27 (10^{-8} M) (*cross hatched column, N = 6*), or VIP6-28 (10^{-6} M) (*hatched column, N = 6*) on *in situ* adrenaline secretion rates. In addition, the effects of bolus injections of VIP 6-28 (10^{-6} M) (*unfilled column, N = 6*) alone was evaluated. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference with pre-stimulation (*Pre*) value ($P<0.05$).

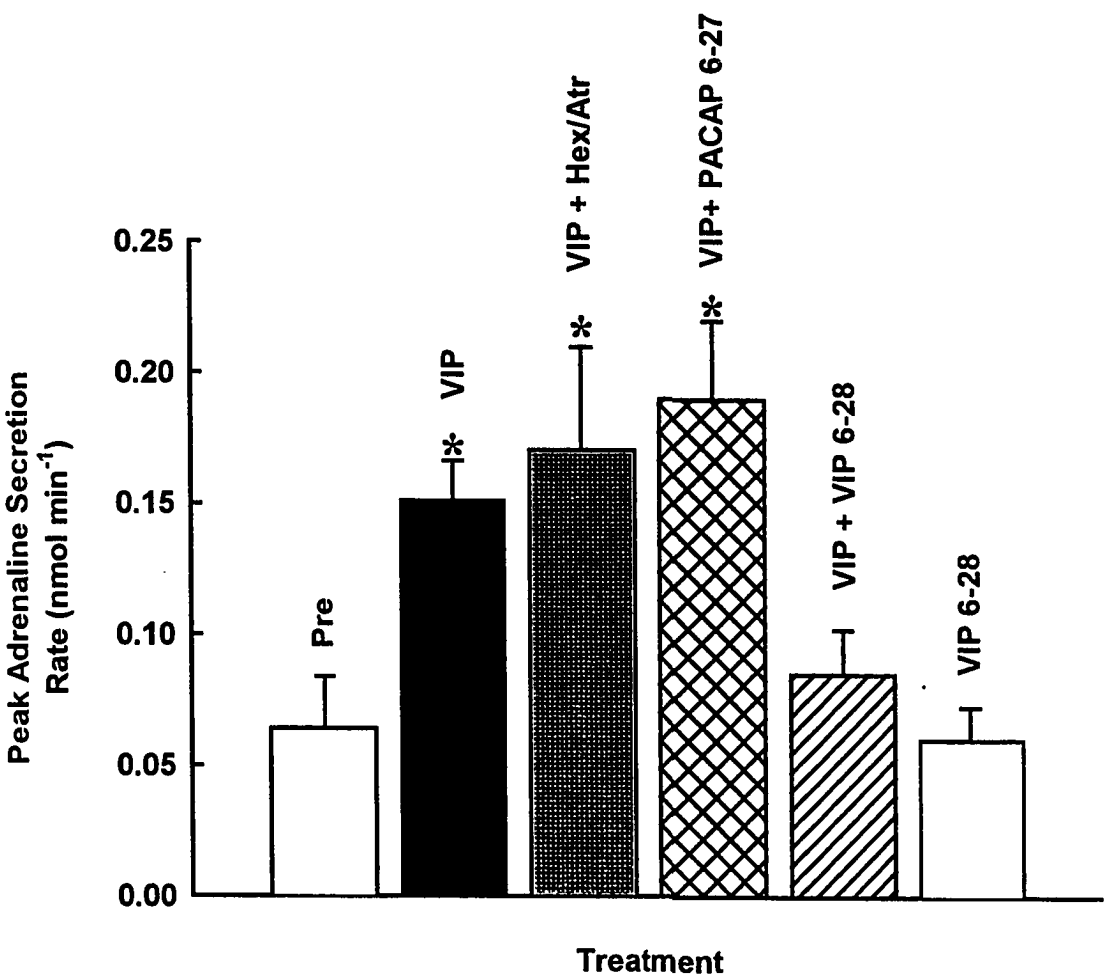

Peak Adrenaline Secretion Rate (nmol min-1)
0.25
0.20
0.15
0.10
0.05
0.00
Pre
VIP
*
VIP + Hex/Atr
*
VIP+ PACAP 6-27
*
VIP + VIP 6-28
VIP 6-28
Treatment

Figure 4.3

Figure 4.4 The effects of bolus injections of PACAP-27 (10^{-10} mol kg^{-1}) (*black column, N=6*), and PACAP-27 (10^{-10} mol kg^{-1}) in combination with hexamethonium (*Hex*) plus atropine (*Atr*) (*gray column, N=6*), PACAP6-27 (10^{-8} M) (*crossed hatched column, N=6*), or VIP6-28 (10^{-6} M) (*hatched column, N=6*) on *in situ* adrenaline secretion rates. In addition, the effects of bolus injections of PACAP6-27 (10^{-8} M) (*unfilled column, N=6*) alone was evaluated. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference with pre-stimulation (*Pre*) values ($P<0.05$).

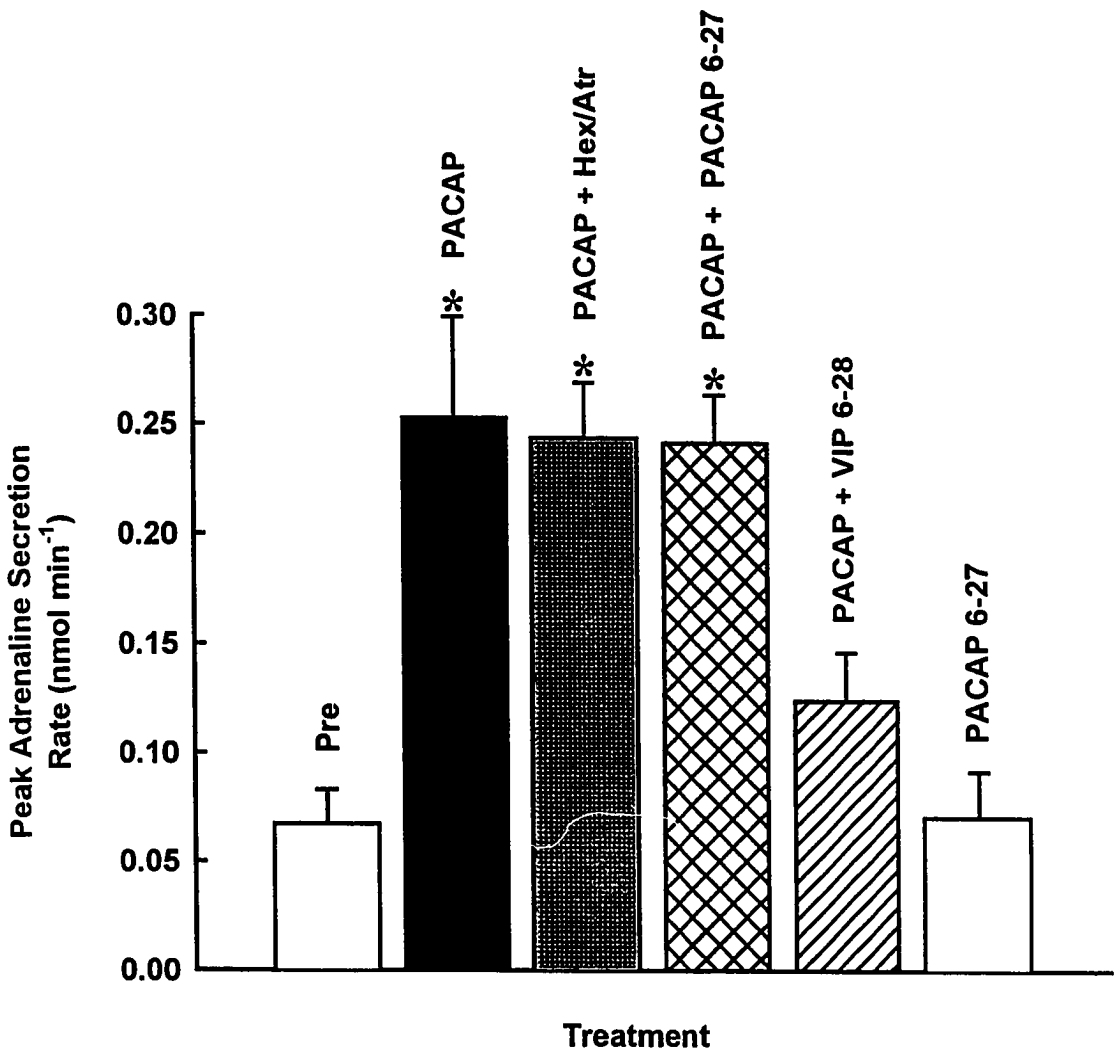

Peak Adrenaline Secretion Rate (nmol min-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Pre
PACAP
PACAP + Hex/Atr
PACAP + PACAP 6-27
PACAP + VIP 6-28
PACAP 6-27
*
*
*
Treatment

Figure 4.4

Series 3: effects of cholinergic receptor antagonists and of VIP 6-28 on carbachol-elicited catecholamine secretion. Carbachol-evoked adrenaline and noradrenaline secretion in the presence of a cocktail of hexamethonium and atropine was markedly diminished (Fig. 4.5). Because PACAP 6-27 had no effect on ckVIP- and hPACAP-27-elicited release, only the effect of VIP 6-28 on carbachol-evoked release was evaluated; VIP 6-28 did not alter the cholinergic secretion of either catecholamine (Fig. 4.5).

Series 4: effects of Ca^{2+}-free saline and internal Ca^{2+} stores depletion on nicotine and VIP elicited secretion of catecholamines. Nicotine-evoked adrenaline and noradrenaline was prevented with the removal of Ca^{2+} from the perfusion media (Fig. 4.6). Prior treatment with thapsigargin, did not alter catecholamine secretion in response to nicotinic receptor stimulation (Fig. 4.6). On the other hand, while pre treatment with Ca^{2+} free saline did not alter adrenaline secretion in response to ckVIP, depletion of internal Ca^{2+} stores prevented it (Fig. 4.7).

Figure 4.5 The effects of bolus injections of carbachol (10^{-5} mol kg^{-1}, *black columns, N=6*), and carbachol (10^{-5} mol kg^{-1}) in combination with VIP 6-28 (10^{-6} M) (*gray columns, N=6*) or hexamethonium (*Hex*) (10^{-3} M) plus atropine (*Atr*) (10^{-5} M) (*unfilled columns, N=6*), on *in situ* (A) noradrenaline and (B) adrenaline secretion rates. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference from pre-stimulation (*Pre*) values ($P<0.05$).

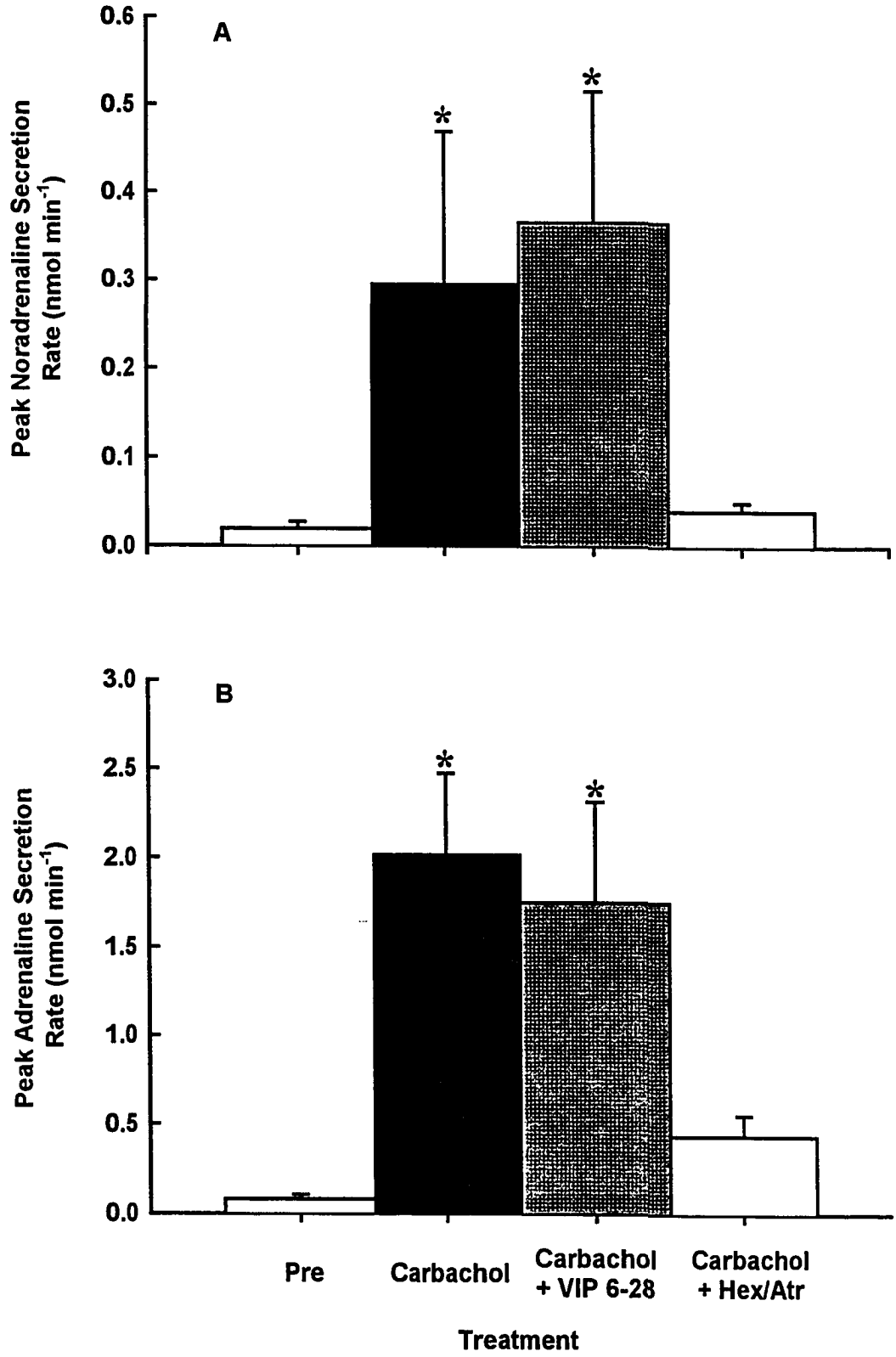

A
Peak Noradrenaline Secretion Rate (nmol min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
B
Peak Adrenaline Secretion Rate (nmol min-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Pre
Carbachol
Carbachol + VIP 6-28
Carbachol + Hex/Atr
Treatment

Figure 4.5

Figure 4.6 Maximal adrenaline (*unfilled bars*) and noradrenaline (*filled bars*) secretion rates in response to an injection of nicotine (10^{-7} mol kg^{-1}) following 20 min pre-perfusion with control saline (*N=6*), Ca^{2+}-free saline (*N=6*) or saline containing thapsigargin (10^{-6} M; *N=6*). The pre-value (*Pre*) represents catecholamine secretion rates prior to the injection of nicotine. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference with pre-stimulation (*Pre*) value ($P<0.05$).

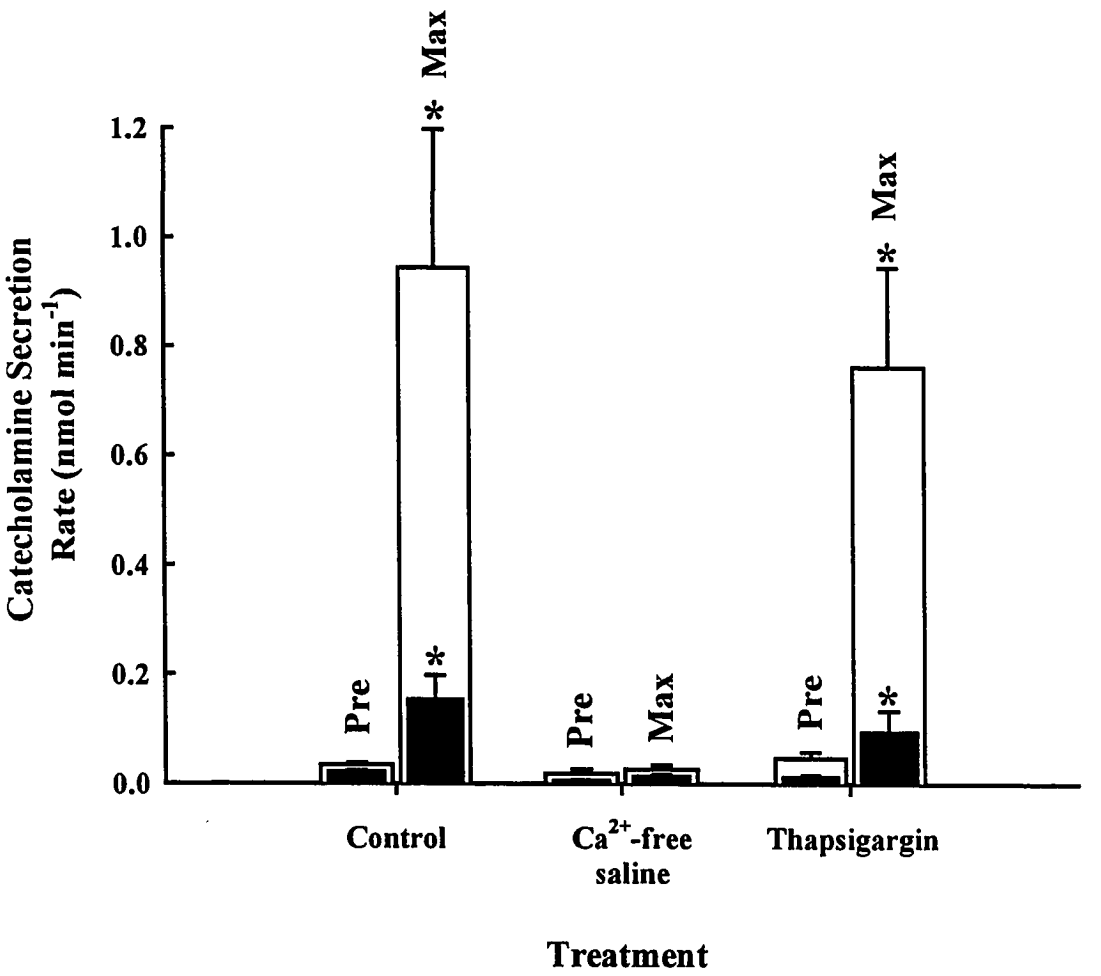

Catecholamine Secretion Rate (nmol min-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Max
*
Pre
*
Pre
Max
Pre
Max
*
Control
Ca2+-free saline
Thapsigargin
Treatment

Figure 4.6

Figure 4.7 Maximal adrenaline (*unfilled bars*) and noradrenaline (*filled bars*) secretion rates in response to an injection of ckVIP (10^{-9} mol kg^{-1}) following 20 min pre-perfusion with control saline (*N=6*), Ca^{2+}-free saline (*N=6*) or saline containing thapsigargin (10^{-6} M; *N=6*). The pre-value (*Pre*) represents catecholamine secretion rates prior to the injection of nicotine. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference with pre-stimulation (*Pre*) value ($P<0.05$).

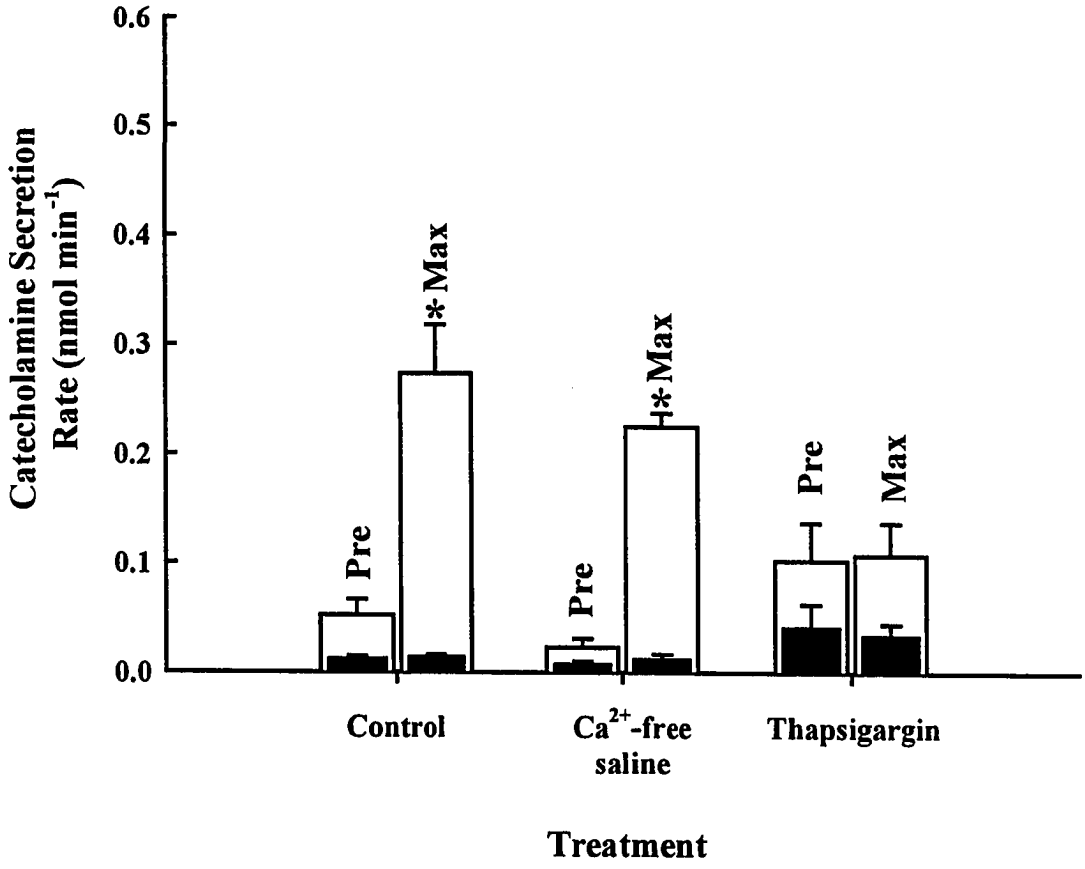

Catecholamine Secretion Rate (nmol min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
* Max
* Max
Pre
Pre
Pre
Max
Control
Ca2+-free saline
Thapsigargin
Treatment

Figure 4.7

Series 5: Contribution of cholinergic versus non-cholinergic neurotransmitters on catecholamine secretion. Catecholamine secretion during field stimulation, *in situ*, was frequency dependent (Fig. 4.8). Significant elevation of the rate of adrenaline and noradrenaline secretion occurred at 1 Hz and reached a maximum at 10 Hz. Given that 1 Hz elicited significant increases in catecholamine secretion and that 20 Hz induced maximal secretion, these frequencies were chosen for subsequent experiments to evaluate the relative contribution of non-cholinergic neurotransmitters during high and low levels of neuronal activity.

To evaluate the involvement of cholinergic and non-cholinergic neurotransmitters at different levels of neuronal activity, preparations were stimulated at low (1 Hz) or high (20 Hz) frequencies in the presence or absence of cholinergic receptor blockade (using hexamethonium plus atropine) or VPAC receptor blockade (using VIP 6-28) (Fig. 4.9). Given the inability of PACAP 6-27 to reduce the VIP- and PACAP-elicited adrenaline secretion, only VIP 6-28 was used in this series of experiments. During stimulation carried out at 1 Hz, noradrenaline secretion was significantly reduced in preparations perfused with saline containing hexamethonium plus atropine. In preparations perfused with saline containing VIP 6-28, both the rate of noradrenaline and adrenaline secretion was significantly reduced. At 20 Hz, the rate noradrenaline and adrenaline secretion were significantly reduced in preparations perfused with saline containing hexamethonium plus atropine. In preparations perfused with saline containing VIP 6-28, secretion of both catecholamines was unaffected.

Figure 4.8 Frequency/response curve for adrenaline (*filled circles*) and noradrenaline (*unfilled circles*) secretion rates. Each preparation was stimulated at different frequencies (*Hz, Hertz*) of stimulation for a period of 30 s at 60V and for 1msec. Values are shown as means ± 1 SEM ($N=6$ fish for each frequency). An asterisk denotes a significant difference with non-stimulated values (0V) ($P<0.05$).

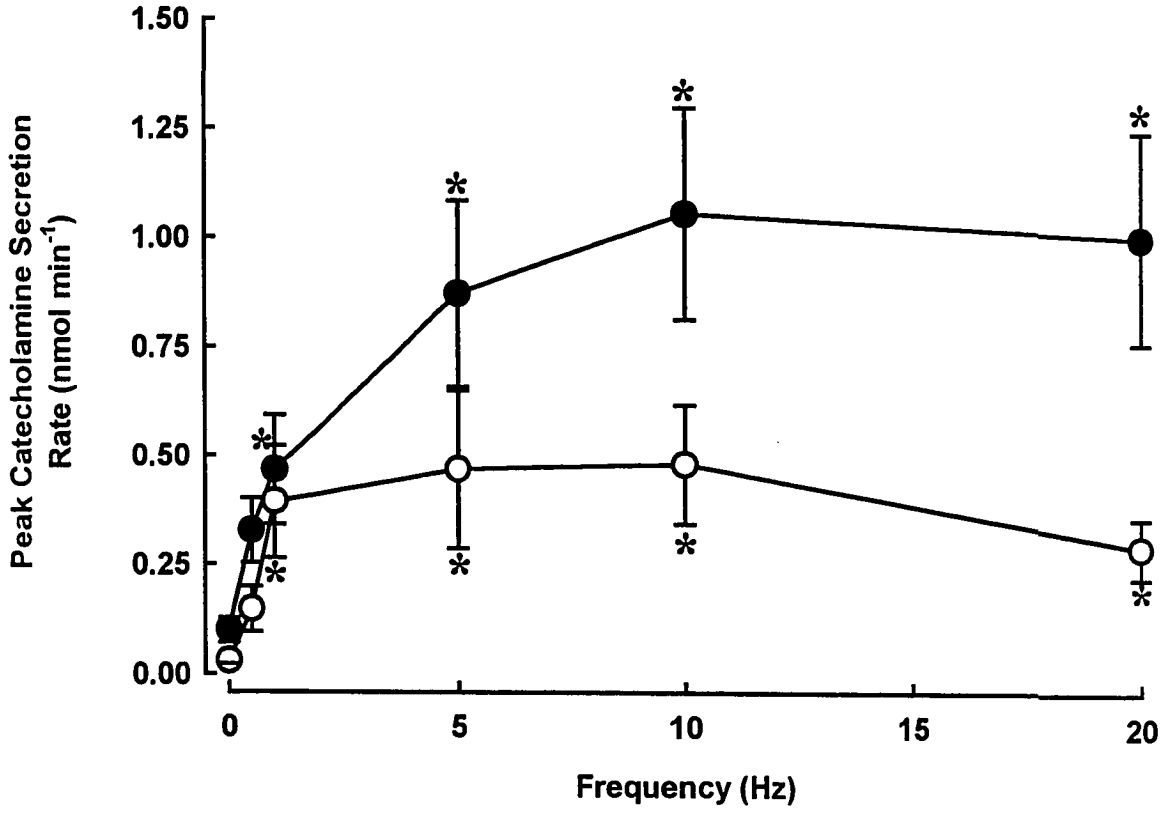

Peak Catecholamine Secretion Rate (nmol min-1)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
Frequency (Hz)

Figure 4.8

Figure 4.9 The effects of pre-treatment with saline (*N=6, unfilled columns*), saline containing hexamethonium (10^{-3} M) plus atropine (10^{-5} M) (*N=6, black columns*), or saline containing VIP 6-28 (10^{-6} M) (*N=6, gray columns*) on *in situ* (A) noradrenaline and (B) adrenaline secretion rates in response to low (1 Hz) and high (20 Hz) frequencies of stimulation. Values are shown as means ± 1 SEM. An asterisk denotes a significant difference with the pre-stimulation (*Pre*) values ($P<0.05$); a cross denotes a significant difference with the control saline treated group ($P<0.05$).

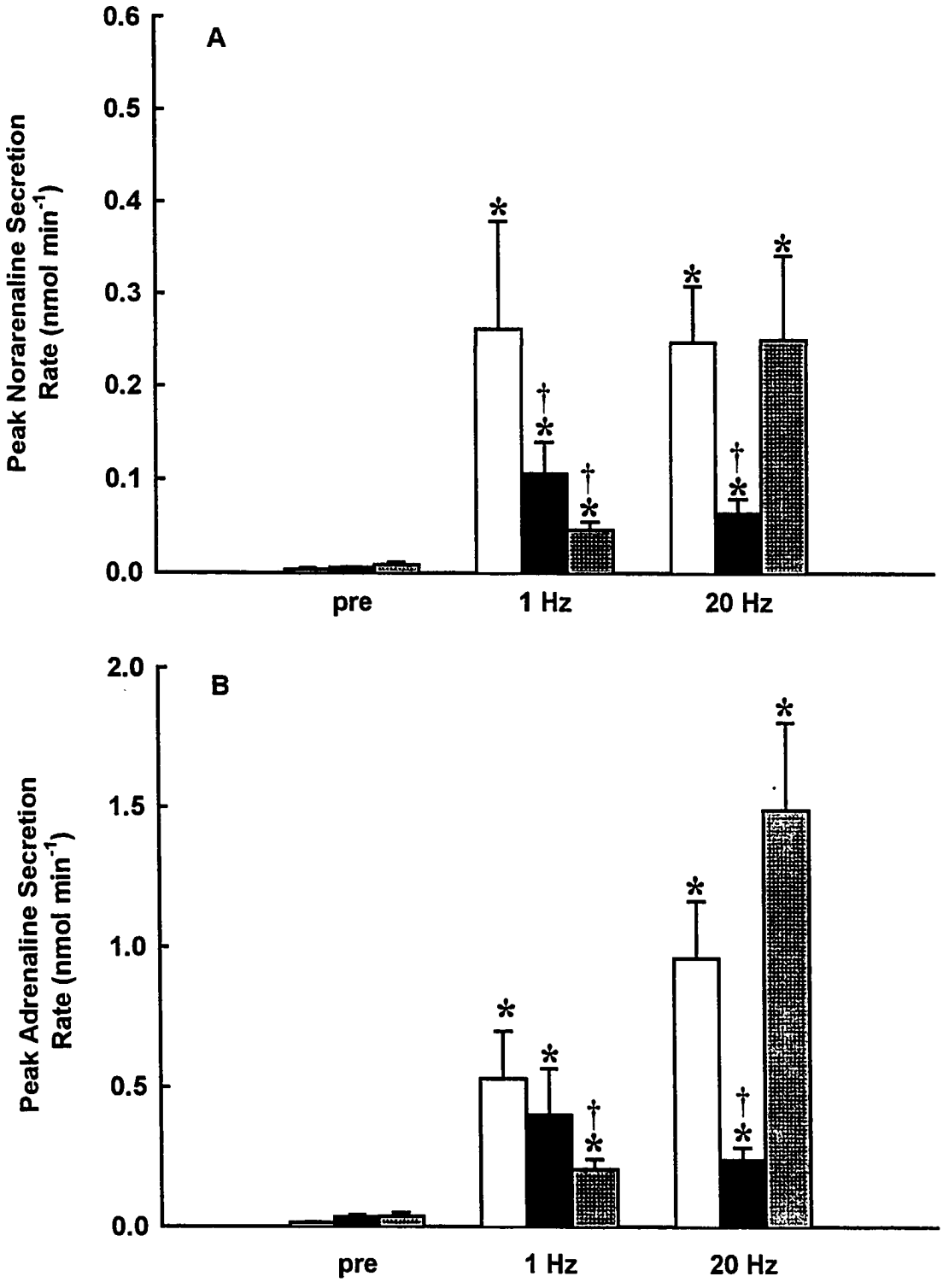

A
Peak Norarenaline Secretion Rate (nmol min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pre
1 Hz
20 Hz
B
Peak Adrenaline Secretion Rate (nmol min-1)
2.0
1.5
1.0
0.5
0.0
pre
1 Hz
20 Hz

Figure 4.9

DISCUSSION

In fish, the neuropeptides VIP and PACAP are found throughout the central and peripheral nervous systems. VIP and PACAP have been implicated as neurotransmitters or neuromodulators in the control of various physiological processes including cardiovascular function, respiration, digestion and glandular secretions (Holmgren, 1985; Dimaline et al., 1987; Aldman and Holmgren, 1992; Holmgren, 1995; Wong et al., 1998). On the basis of the results of the present investigation, a role for VIP and/or PACAP can be extended to the regulation of catecholamine secretion from chromaffin cells in the rainbow trout.

Direct effects of exogenous VIP and PACAP on *in situ* catecholamine secretion.

The ability of the neuropeptides VIP and PACAP to directly stimulate the secretion of adrenaline from piscine chromaffin cells is reported for the first time. In agreement with prior studies using non-piscine vertebrates (Misbahuddin et al., 1988; Wakade et al., 1991; Isobe et al., 1993; Guo and Wakade, 1994; Yon et al., 1994; Watanabe et al., 1995), bolus injections of VIP or PACAP caused a dose-dependent release of adrenaline from the chromaffin tissue associated with the posterior cardinal vein. The ED_{50} values for ckVIP and hPACAP-27 showed that both neuropeptides were able to stimulate adrenaline secretion with a similar degree of potency. The results also indicated that the increase in adrenaline secretion in response to VIP or PACAP was significantly reduced by the VPAC receptor antagonist, VIP 6-28, but unaffected by the PAC_1 receptor antagonist, PACAP 6-27, or the cholinergic receptor antagonists, hexamethonium and atropine. Thus, the results of this study suggest that the neuronal

control of trout chromaffin cell activity may include both cholinergic and peptidergic components (see below).

In agreement with prior studies conducted in several teleosts including rainbow trout, American eel, Atlantic cod, and the common carp (Nilsson et al., 1976; Fritsche et al., 1993; Reid and Perry, 1995b; Al-Kharrat et al., 1997; Gfell et al., 1997; Montpetit and Perry, 1999), the carbachol-evoked elevation of adrenaline and noradrenaline secretion was significantly reduced in the presence of hexamethonium (nicotinic receptor antagonist) and atropine (muscarinic receptor antagonists). Because cholinoceptor blockade did not affect ckVIP- or hPACAP-27-evoked adrenaline secretion, it was clear that the effects of the neuropeptides on trout chromaffin cells occur independently of cholinergic stimulation. Moreover, the observation that the VPAC receptor antagonist, VIP 6-28, significantly attenuated the ckVIP- and hPACAP-27-elicited secretion of adrenaline indicates that these neuropeptides specifically stimulate VPAC receptors. In accordance with prior studies, the secretory profile during cholinergic stimulation includes the release of both adrenaline and noradrenaline (Fritsche et al., 1993; Montpetit and Perry, 1999). VIP and PACAP, however preferentially stimulated the secretion of adrenaline (see also Guo and Wakade, 1994). These findings imply that the variations in the secretion of adrenaline and noradrenaline in response to Ach and VIP/PACAP may not reflect their potency but their ability to specifically stimulate adrenaline- versus noradrenaline-containing chromaffin cells.

In the present study, PACAP-27 and VIP were equipotent in stimulating adrenaline secretion. This is in marked contrast to the situation in non-piscine chromaffin cells where PACAP is 1000 times more potent than VIP (Wakade et al., 1991; Watanabe

et al., 1992; Isobe et al., 1993; Guo and Wakade, 1994; Watanabe et al., 1995). It has been previously demonstrated in fish and higher vertebrates, that two different types of receptors may mediate the biological effects of VIP and PACAP. PAC_1 receptors have a much greater affinity for PACAP (1000 times) than VIP, whereas the VPAC receptors bind VIP and PACAP with similar affinity (Chow, 1997; Wei et al., 1998; Wong et al., 1998). In mammals, the adrenal medulla exhibits a pronounced expression of the PAC_1 receptor, while the VPAC receptor is undetectable (Watanabe et al., 1992; Spengler et al., 1993), or expressed at much lower levels. Moreover, pharmacological approaches have revealed that the VIP and PACAP mediated effects on catecholamine release from adrenomedullary chromaffin cells occurs through activation of PAC_1 receptors. Yet, given the fact that both VIP and PACAP elicited the secretion of adrenaline with equipotency, and that the VPAC receptor antagonist (VIP6-28) reduced secretion in response to both neuropeptides, it appears that in rainbow trout, the VIP and PACAP elicited secretion of adrenaline may be mediated by VPAC type receptors. Although Reid et al. (1995) identified VIP- and PACAP-immunoreactive like substances in nerve fibers in the vicinity of piscine chromaffin tissue, it is unclear as to which one or both of these neuropeptides play a role in catecholamine secretion. However, given the inability of the specific PAC_1 receptors antagonist (PACAP 6-27) to reduce adrenaline secretion in response to VIP and PACAP, it is possible that VIP, rather than PACAP, is responsible for the non-cholinergic component of the neuronal regulation of adrenaline secretion from trout chromaffin cells.

The precise mechanisms underlying the initiation of catecholamine secretion by VIP/PACAP from trout chromaffin cells are not known. However, owing to the specific

requirement of Ca^{2+} in the secretory process (Burgoyne et al., 1993; Furimsky et al., 1996), pathways for increasing intracellular Ca^{2+} are likely responsible. VIP and PACAP receptors belong to a seven transmembrane spanning domain G-protein coupled receptor superfamily and are coupled to adenylyl cyclase and phospholipase C (Ishihara et al., 1992; Lutz et al., 1993; Spengler et al., 1993; Wong et al., 1998). Indeed, in non-piscine chromaffin cells, VIP or PACAP receptor stimulation is associated with an elevation of cAMP, inositol 1,4,5-trisphosphate and diacylglycerol, which are believed to promote the entry of extracellular Ca^{2+} as well as stimulating the release of Ca^{2+} from intracellular stores (Malhotra et al., 1989; Isobe et al., 1993; Przywara et al., 1996; Tanaka et al., 1996). The ability of VIP to elicit the secretion of adrenaline in fish perfused with Ca^{2+}-free saline, together with the inability of the chromaffin cells to secrete adrenaline following depletion of internal calcium stores is consistent with the recruitment of Ca^{2+} from intracellular stores.

The use of heterologous peptides in this study may explain some of the inter-species differences in the degree of potency of VIP and PACAP in stimulating adrenaline secretion from chromaffin cells. This is unlikely, however, because the amino acid sequence of different vertebrate VIP's has been strongly conserved and most substitutions are conservative suggesting that the evolutionary pressure to conserve the VIP molecule has been strong. Indeed, in trout, the VIP amino acid sequence differs from the cod, dogfish, porcine and chicken VIP's at the 3, 6, 4, and 1 positions, respectively (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 been analyzed, PACAP's throughout other vertebrates (rat, sheep, mouse, human, amphibian, chicken,

and teleost fish) also share a high degree of sequence similarity (Wong et al., 1998). Additionally, PACAP shares 66-68% sequence similarity with VIP. In mammalian and fish species, these structural differences appear to have little effect on their biological functions. Indeed, porcine, cod, and dogfish VIP were equipotent in stimulating amylase secretion and displacement of radioligand from guinea pig pancreatic acini (Dimaline et al., 1987). Moreover, porcine VIP was able to inhibit cholecystokinin-induced gall bladder contraction in trout (Aldman and Holmgren, 1992) and to relax smooth muscle in the rectum and swim bladder in the cod (Lundin and Holmgren, 1984). Similarly, different forms of PACAP (frog, zebrafish, and ovine) were able to stimulate growth hormone secretion with more or less similar potencies from goldfish pituitary cells (Wong et al., 1998).

Relative contribution of cholinergic and non-cholinergic neurotransmitters in the neuronal control of catecholamine secretion.

The differential participation of cholinergic and peptidergic neurotransmitters in the neural control of catecholamine secretion in response to different levels of preganglionic nerve activity has been reported for mammals (Malhotra and Wakade, 1987; Wakade et al., 1991; Guo and Wakade, 1994). In agreement with those investigations, the present study demonstrated that cholinergic stimulation predominated during high frequencies of electrical stimulation while the non-cholinergic component prevailed at low frequencies of stimulation.

In addition to assessing the relative contribution of cholinergic and non-cholinergic neurotransmitters in the neural control of catecholamine secretion from trout

chromaffin cells, this study has identified a non-cholinergic component. The observed reduction in catecholamine secretion during low frequency stimulation in preparations previously treated with the VIP receptor antagonist, VIP 6-28, strongly suggests the participation of the neuropeptides, VIP and/or PACAP. The reduction in noradrenaline secretion was, however, unexpected given the predominant effect of VIP and PACAP on adrenaline secretion. It is clear that cholinergic stimulation, although less prevalent, participates at low frequencies of stimulation with respect to noradrenaline secretion. Based on these results, direct VIP receptor stimulation may interact with cholinergic pathways to regulate noradrenaline secretion from trout chromaffin cells. The precise mechanisms of this interaction remain unknown. To conclusively determine the relative involvement of VIP and PACAP, future studies need to identify which peptides are secreted from the preganglionic nerve terminals during electrical stimulation of these nerves.

Overall, the results of the present investigation demonstrate that the neuronal control of catecholamine secretion in teleost fish may not be confined to cholinergic-evoked events. Indeed, the results suggest that VIP and/or PACAP may act as neurotransmitters and be directly involved in the neuronal stimulation of adrenaline secretion from piscine chromaffin cells, especially at low levels of neuronal activity. Furthermore, the fact that the VIP receptor antagonist (VIP 6-28) greatly reduced adrenaline secretion in response to both VIP and PACAP, together with the observation that both neuropeptides stimulated the secretion of adrenaline with similar potencies, suggest that the VIP- and PACAP-evoked secretion was mediated by VPAC type receptors. This is in marked contrast to the situation in non-piscine chromaffin cells in

which PACAP rather than VIP is believed to be more important in mediating the neuronal non-cholinergic component to adrenaline secretion.

CHAPTER 5

LOCALISATION AND CHARACTERISATION OF VASOACTIVE INTESTINAL POLYPEPTIDE (VIP) RECEPTORS IN CHROMAFFIN CELLS OF THE RAINBOW TROUT (*Oncorhynchus mykiss*)

(Based in part on Montpetit CJ and Perry SF. 2002. Journal of Experimental Biology, Submitted)

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). 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 (reviewed by Nilsson and Holmgren, 1994; Harmar et al., 1998). Recently, nerve fibres exhibiting immunoreactivity to VIP and PACAP were identified in the vicinity of the chromaffin tissue of fish including *Gadus morhua, Oncorhynchus mykiss, Anguilla anguilla,* and *Squalus acanthias* (Reid et al., 1995).

Catecholamines that are secreted into the circulation are stored in separate populations of adrenaline- and noradrenaline-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). 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 (Ach). In contrast to mammals, the effects of VIP and PACAP appear to be mediated by VPAC type receptors in rainbow trout (Montpetit and Perry, 2000). In mammalian and amphibian adrenal chromaffin cells, PAC_1 receptors are thought to mediate the stimulatory effects of VIP and PACAP on catecholamine secretion (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). In 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 were present on rainbow trout chromaffin cells and if they exhibited 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 weighing between 250 and 350g were obtained and maintained under conditions outlined in Chapter 2.

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 M phosphate-buffered saline (PBS; adjusted to pH 7.2). To identify the catecholamine containing cell fraction of the PCV, the aldehyde-induced green fluorescence of catecholamines method (Furness et al., 1977; Lacoste et al., 2001) was used. 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 (wt/vol) for 1 h each. Tissues were embedded in Cryomatrix (OCT-compound, Shandon, Pittsburgh, PA) 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 X 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 et al. (1997). Following

rehydration in PBS, sections were incubated with 5% bovine serum albumin (BSA; ICN Biomedical, Aurora, OH) in PBS for 1 h. Sections were then incubated for 12 h with anti-dopamine β-hydroxylase (DβH; mouse monoclonal anti-DβH; dilution 1:500; Chemicon), and further incubated for 2 h with the Cy3-conjugated donkey anti-mouse secondary antibody (dilution 1:400; Jackson Immunoresearch Laboratories, West Grove, PA). Sections were then examined after a final rinse in PBS (3 X 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 X 5 min), sections were reduced in $NaBH_4$ (3 X 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) and fluorescein isothyocyanate (FITC)-conjugated donkey anti-rabbit secondary antibody (dilution 1:200; Jackson Immunoresearch Laboratories, West Grove, PA) using the same protocol as for DβH immunolabelling.

To visualise VIP binding sites within the PCV, tissue sections (previously immunostained for DßH and PNMT) were incubated with a solution of biotinylated hVIP (2.58×10^{-6} M biotinyl-VIP human, porcine, rat; Peninsula Laboratories Inc.) for 2 h and further incubated with Cascade blue-conjugated streptavidin (dilution 1:500; Jackson Immunoresearch Laboratories, West Grove, PA) 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 X 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} M chicken VIP; Peninsula Laboratories Inc.), VIP receptor antagonist hVIP-6-28 (10^{-5} M humanVIP6-28 Peninsula Laboratories Inc.), PACAP receptor agonist hPACAP-27 (10^{-5} M human PACAP-27; Peninsula Laboratories Inc.) or the PACAP receptor antagonist hPACAP6-27 (10^{-5} M human PACAP6-27; Peninsula Laboratories Inc.) for 2 h each prior to incubation with biotynilated-VIP probe (described above). To visualise binding of the probe, sections were then incubated with DTAF (dichlorotriazinyl aminofluorescein)-conjugated streptavidin (dilution 1:500; Jackson Immunoresearch Laboratories, West Grove, PA) as described earlier. In control experiments, tissue sections were incubated for 2 h with PBS prior to labelling of VIP binding sites.

Control and specificity experiments

For the aldehyde induced fluorescence of catecholamines, sections reduced in $NaBH_4$ (3 X 5min) served as a methodological control. 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) but was confirmed in the present study 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} M chicken VIP; Peninsula Laboratories Inc.) 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 (~20° C), consistently using 50-µl aliquots of the various solutions. Sections were mounted using 30µl of Vectashield mounting medium with or without the nucleic acid dye DAPI (4,6-diamidino-2-phenlidole; Vector Laboratories, Inc., Burmingham, CA). To preserve the slides, coverslips 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 35mm 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.).

Determination of catecholamine stores

Measurements were performed to confirm the presence of stored catecholamines in the PCV tissue. The PCV of different fish 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 micro-centrifuge 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). After removing the saline by aspiration, the tubes were reweighed to determine tissue weights. 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 posterior cardinal vein preparation**

The bioactivity of the biotinylated-VIP probe was tested using an *in situ* saline perfused PCV previously described by Montpetit and Perry (2000). The posterior cardinal vein tissue was perfused as outlined in Chapter 2. After the 20-min stabilisation period, a control (pre-treatment) sample was collected in pre-weighed micro-centrifuge tube to assess basal catecholamine secretion rate. After collection of the pre-sample, a single bolus injection of biotinylated-hVIP (2.58 X 10^{-6} M; 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, 2, 3, 4 and 5 min after intervention.

Determination of perfusate and tissue catecholamine levels

Perfusate and tissue adrenaline and noradrenaline levels were determined by HPLC as outlined in Chapter 2.

Statistical analysis

The data are presented as the mean ± 1 standard error of the mean (SEM). Where appropriate, the data were statistically analysed by a two-way repeated measures 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. 5.1A-C). This was achieved by comparing the fluorescent labelling of nucleic acids with that of catecholamines. 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. Most of these chromaffin cells formed aggregates in this area, with fewer chromaffin cells lying outside of these clusters. In other sections, PCV tissue was double labelled using the aldehyde-induced fluorescence of catecholamines and immunostaining for DβH (Fig. 5.1D-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. 5.2). 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.

Multiple labelling experiments revealed that only a small number of DβH - positive cells were immunonegative for PNMT (Fig. 5.3). This finding indicates that in the chromaffin tissue of trout, there are relatively few numbers of cells containing only noradrenaline. Labelling with the biotinylated-VIP probe revealed that all DβH-positive (chromaffin) cells appeared to possess VIP binding sites. The pattern of labelling of VIP,

Figure 5.1. 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) catecholamines and E) DβH. Panel **C** is an overlay image of the sections shown in A & B (nucleic acids and catecholamines appear in red and green/yellow, respectively). Panel F is an overlay image of the sections shown in D & E (catecholamines and DβH appear in green and red, respectively; yellow indicates co-localisation of catecholamines and DβH). Note that the labelling patterns for catecholamines and DβH are identical. *Bars* D-F, 100 μm; *Bars* A-C, 50 μm.

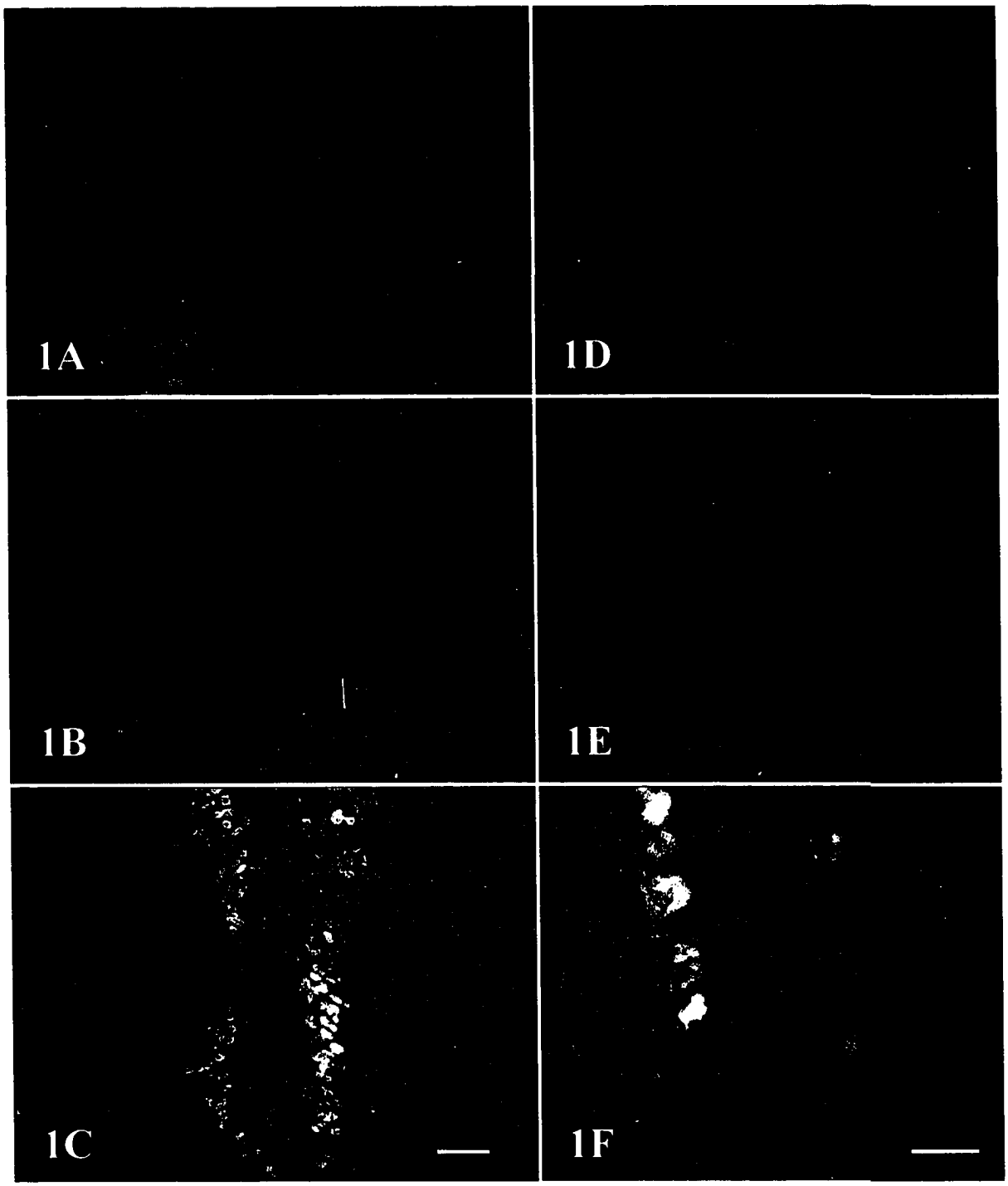


Figure 5.1

Figure 5.2. Mean tissue concentrations (μg catecholamine g^{-1} tissue) of noradrenaline (open columns) and adrenaline (filled columns) in rainbow trout (N = 6). Values are shown as means ± 1 SEM. Abbreviations: APCV-anterior posterior cardinal vein, MPCV-middle posterior cardinal vein, and PPCV-posterior posterior cardinal vein; AK-anterior kidney, MK-middle kidney, and PK-posterior kidney.

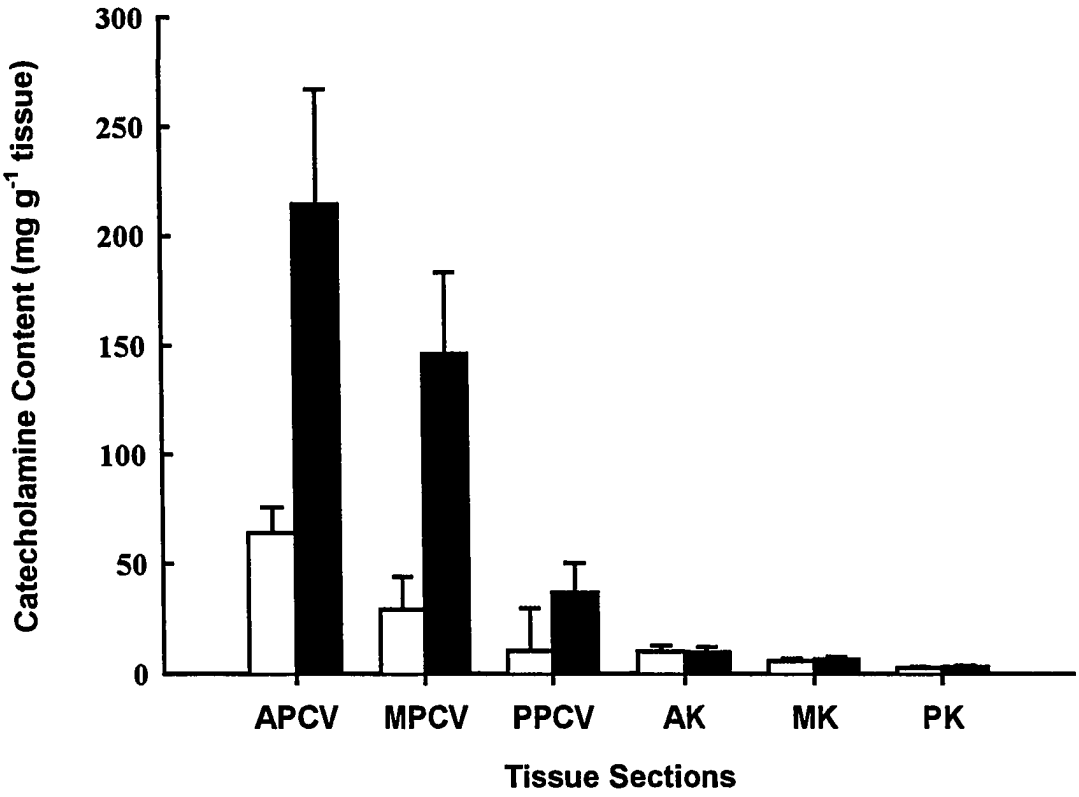

300
250
200
150
100
50
0
Catecholamine Content (mg g-1 tissue)
APCV
MPCV
PPCV
AK
MK
PK
Tissue Sections

Figure 5.2

Figure 5.3. Localisation of VIP binding sites to the chromaffin cell subtypes in the PCV of rainbow trout using triple labelling.

A) DβH-immunoreactive cells (mouse anti- DβH). **B)** PNMT-immunoreactive cells (rabbit anti-bovine PNMT). **C)** Labelling of VIP-binding sites.

D) Overlay image of the sections shown in A & B (DβH and PNMT positive cells); the PNMT-negative cells are indicated by arrows. **E)** Overlay image of the sections shown in A & C (DβH and VIP labelling). **F)** Overlay image of the sections shown in B & C (PNMT and VIP labelling); the PNMT-negative cells are indicated by arrows. Yellow/bright 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. *Bars* A-F, 150μm.

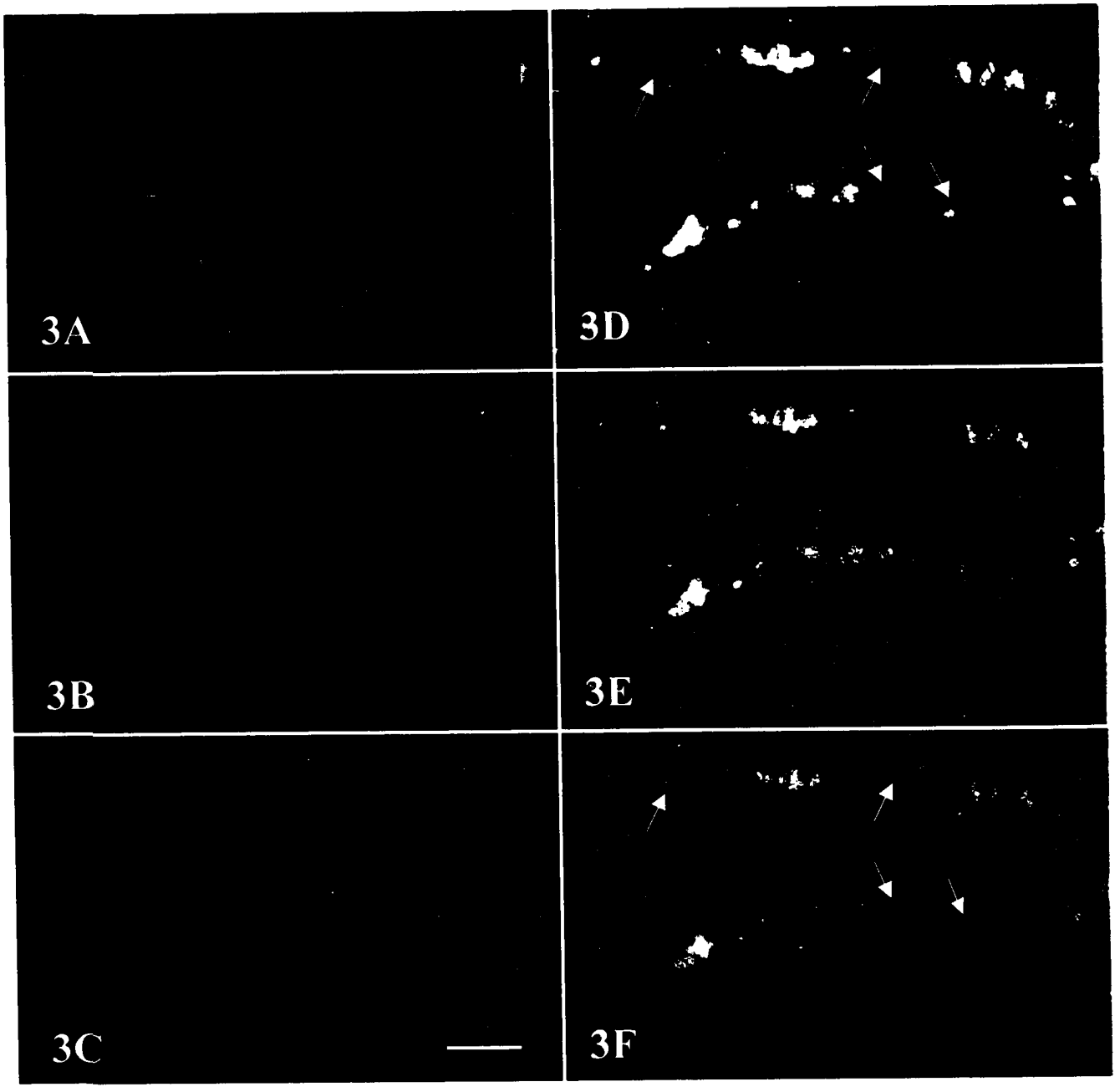


Figure 5.3

however, demonstrated that VIP binding sites were present on both PNMT-positive (adrenaline-containing) and negative (noradrenaline-containing) chromaffin cells.

Control tissue sections exhibited dull non-specific autofluorescence in comparison to the bright specific fluorescence illustrated in Figs. 5.1 and 5.3. Excess unlabeled 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 the biotinylated VIP probe 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. 5.4). Administration of saline did not cause the release of either catecholamine.

Pharmacological assessment of VIP binding sites

To pharmacologically assess the chromaffin cell VIP-binding sites, sections of the PCV were treated with non-conjugated VPAC and PAC_1 receptor agonists/antagonists prior to incubation with biotinylated-VIP for visualisation of binding sites (Fig. 5.5). While prior treatment with the VIP receptor agonist, ckVIP, the VIP receptor antagonist VIP6-28, 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, PACAP6-27, was without effect on the binding of the biotinylated-VIP probe.

Figure 5.4. Secretion rates of adrenaline (filled columns) and noradrenaline (unfilled columns) in response to a bolus injection of saline (inset; $N = 6$) or biotinylated-VIP (10^{-9} mol kg^{-1}; $N = 6$) using an *in situ* PCV preparation of rainbow trout. Values are shown as means ± S.E.M. An asterisk denotes a significant difference from the pre-stimulation (Pre) value ($P<0.05$).

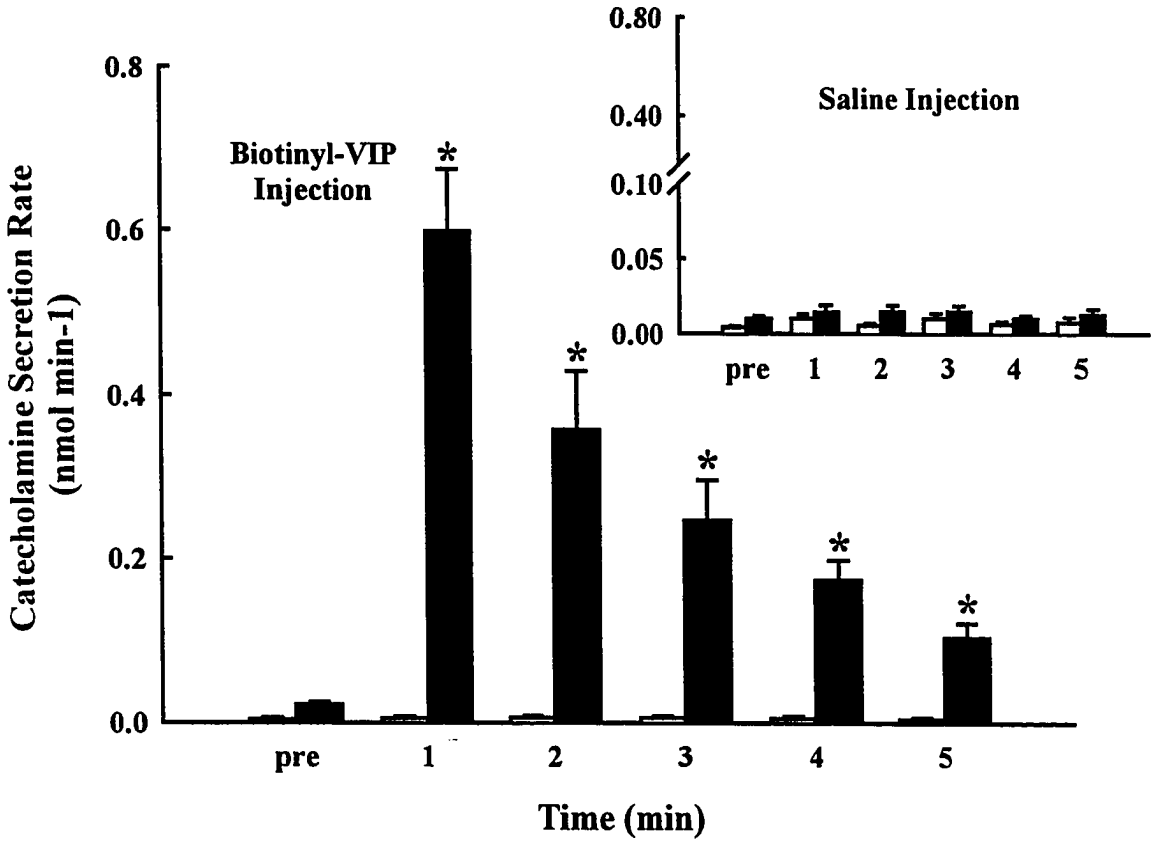

Catecholamine Secretion Rate
(nmol min-1)
0.8
0.6
0.4
0.2
0.0
Biotinyl-VIP
Injection
*
*
*
*
*
pre
1
2
3
4
5
Time (min)
0.80
0.40
0.10
0.05
0.00
Saline Injection
pre
1
2
3
4
5

Figure 5.4

Figure 5.5. Pharmacological assessment of the VIP binding sites. A-E, 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 VIP receptor antagonist, VIP6-28. **D)** Labelling of VIP binding sites following treatment with PACAP-27. **E)** Labelling of VIP binding sites following treatment with PAC_1 receptor antagonist, PACAP6-27. *Bars* A-E, 150 μm.

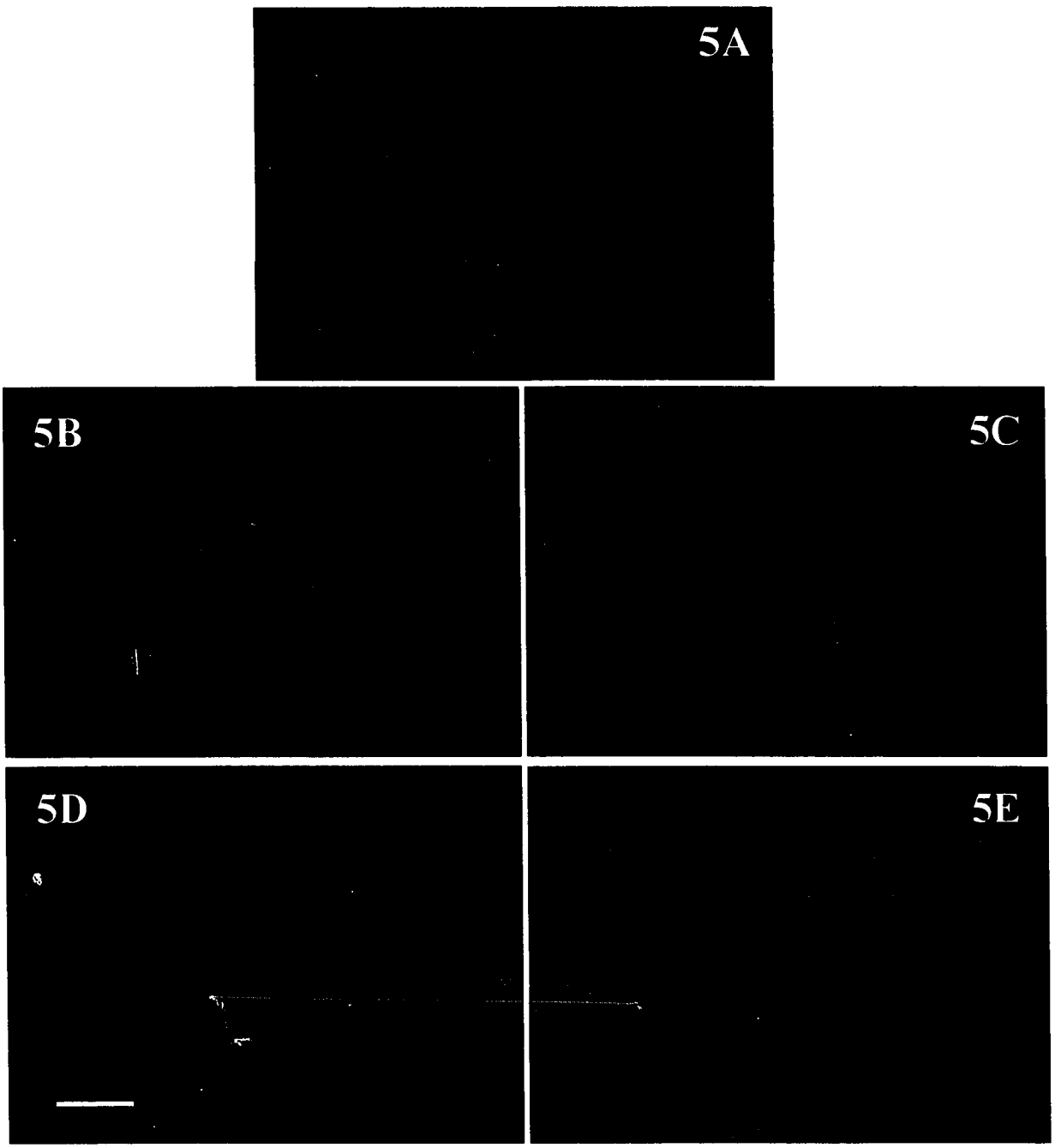


Figure 5.5

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 have 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 this study, different forms of VIP were used to visualise and characterise receptors. The primary sequences of VIPs throughout vertebrates share considerable sequence similarity 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 4 to 5 positions (Nilsson, 1975; Dimaline et al., 1987; Thwaites et al., 1989; Holmgren and Jensen, 1994; Wang and Conlon, 1995). 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; Wong et al., 1998; Montpetit and Perry, 2000).

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 suggest that the majority of chromaffin cells form aggregates adjacent to the lumen of the PCV although individual cells can also be observed outside of 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).

The concentration of catecholamines within the chromaffin tissue and the extent of their release differ to a large extent within and between fish species (Reid et al., 1998). Yet, given 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). In 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 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. Several approaches can be used to classify these receptors including ligand binding studies, assessing the relative potencies of the peptides in different tissue preparations, and evaluating differences between antagonist efficacy on VIP- and PACAP-elicited effects (Bodanszky et al., 1973; Robberecht et al., 1991; Fishbein et al., 1994; Gaspo et al., 1997; Robberecht and Waelbroeck, 1998; Wong et al., 1998). The

results of the present study, employing histochemistry in conjunction with prior application of receptor agonists and antagonists, clearly identified the chromaffin cell VIP binding sites as VPAC receptors. This finding is in marked contrast to the situation in mammals where PAC_1 receptors are believed to mediate the VIP/PACAP elicited secretion of catecholamines (Watanabe et al., 1992; Spengler et al., 1993; Gaspo et al., 1997; Vaudry et al., 2000; Lamouche and Yamaguchi, 2001).

Future research should be directed at further characterisation of the VPAC receptors of trout chromaffin cells. Recently, partial clones for $VPAC_1$ and $VPAC_2$ receptors from trout brain cDNA were obtained. Tissue distribution experiments using RT-PCR demonstrated the presence of $VPAC_1$ receptors, but not $VPAC_2$, in the PCV tissue (unpublished data, Montpetit and Perry, 2002). These preliminary results suggest that in trout, the chromaffin cell VIP receptors mediating adrenaline secretion are of the $VPAC_1$ subtype.

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 Ach, 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.

CHAPTER 6

MOLECULAR CLONING AND TISSUE EXPRESSION OF TROUT (*Oncorhynchus mykiss*) $VPAC_1$, $VPAC_2$, AND PAC_1 RECEPTORS

INTRODUCTION

The presence of multiple receptors mediating the effects of VIP and PACAP was confirmed by the cloning of at least three VIP/PACAP interacting receptors ($VPAC_1$, $VPAC_2$ and PAC_1 receptors) (Harmar et al., 1998; Vaudry et al., 2000). Sequence analysis of the VPAC and PAC_1 receptors show that they are members of a novel family of G-protein-coupled receptors first identified by cloning of secretin, calcitonin, and parathyroid hormone receptors, and which now includes receptors for PACAP, VIP, glucagon, and growth hormone releasing hormone (Harmar et al., 1998; Vaudry et al., 2000). These receptors contain a large N-terminal extracellular domain essential for ligand interactions, followed by seven highly conserved hydrophobic transmembrane helices. The typical VPAC and PAC_1 receptor transduction pathway results in the increased intracellular cAMP levels caused by G-protein mediated stimulation of adenylyl cyclase activity (Harmar et al., 1998; Vaudry et al., 2000). Since the initial cloning of the $VPAC_1$, $VPAC_2$, and PAC_1 receptors in human and rat (Ishihara et al., 1992; Pisegna and Wank, 1993; Lutz et al., 1993), molecular cloning and functional characterization of these receptors have been achieved in a variety of vertebrates including fish, amphibians, reptiles, birds and mammals (Chow, 1997; Chow et al., 1997; Wong et al, 1998; Alexandre et al., 1999; Hoo et al., 2001; Kansaku et al., 2001; You et al., 2001).

In vertebrates, VIP and PACAP immunoreactive nerves have been identified in both the central and peripheral nervous systems (Vaudry et al., 2000). Indeed, VIP and PACAP are known to influence the circulation, gastrointestinal function, glandular secretions, the immune system and the central nervous system. Physiology based studies

using several species of fish reveal that some of the typical mammalian VIP and PACAP responses also occur in fish. Some of these include vasodilation in pulmonary/gill and peripheral blood vessels, pituitary secretions (Holmgren 1995; Wong et al., 1998), relaxation of gastrointestinal smooth muscles (Holmgren 1985), and catecholamine secretion from chromaffin cells (Montpetit and Perry, 2000). The neuropeptides are also involved in many other physiological events in fish, such as osmoregulation by gill in tilapia (Mainoya and Bern, 1984), relaxation of the gall bladder in trout (Aldman and Holmgren 1992), and the regulation of the digestive system and swim bladder gas absorption in the cod (Lundin and Holmgren 1984).

Previous studies on the molecular biology of the VIP and PACAP receptors have focused almost exclusively on mammalian species. Despite the rapid progress in cDNA cloning, molecular information on these receptors from fish still remains in its infancy. With the exception of goldfish, no studies have dealt with the molecular biology of fish $VPAC_1$, $VPAC_2$, and PAC_1 receptors. Full-length sequences for $VPAC_1$ and PAC_1 have been obtained only from goldfish brain tissue (Chow, 1997; Chow et al., 1997; Wong et al, 1998). Moreover, no fish $VPAC_2$ receptor cDNA has yet been isolated. Owing to increasing comparative research on the VIP/PACAP neuropeptide system, it is the ultimate goal of this study to provide additional information on PAC_1 and VPAC receptors at the molecular level in fish. In this thesis, I describe the cloning and tissue expression of partial cDNAs for the trout PAC_1, $VPAC_1$, and $VPAC_2$ receptors.

MATERIALS AND METHODS

Tissue collection and RNA isolation

Rainbow trout, weighing between 250 and 350g were obtained and maintained under conditions outlined in Chapter 2. For tissue collection, fish were killed by a blow to the head, and tissues (brain, posterior cardinal vein, kidney, gill, spleen, heart, liver, intestine, and blood) were harvested and immediately frozen in liquid nitrogen prior to storage at –80°C. Total RNA was isolated using Trizol reagent (GIBCO BRL, Life Technologies, Rockville, MD) according to the manufacturers instructions. RNA concentrations and quality were verified using spectrophotometry (Eppendorf BioPhotometer) and gel electrophoresis.

Cloning and sequencing of VPAC and PAC receptor cDNA

Polymerase chain reaction (PCR) using brain cDNA as template

Degenerate primers were designed for semi-nested PCR on the basis of sequence comparisons of selected vertebrate $VPAC_1$ and PAC_1 receptor nucleotide sequences available in GenBank. Briefly, degenerate primers were designed (see Table 6.1) according to conserved regions within the third and seventh transmembrane domain (TMD) of the VPAC receptors and second and seventh TMD of the PAC_1 receptors of various vertebrates. Approximately 5µg 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) in a 20-µl mixture. PCR amplification of first strand cDNA (1µl) was performed in 50-µl reaction mixtures containing 0.2 µM of each primer (forward and reverse), 2 mM $MgCl_2$, 0.2 mM of each

dNTP, and 2.5 units of *Taq* DNA polymerase (GIBCO-BRL, Life Technologies) in 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 sec, 55°C (PAC_1 degenerate primers) or 60.5°C (VPAC degenerate primers) for 40 sec, 72°C for 40 sec, with a final extension for 10 min at 72°C in a thermal cycler (Eppendorf Mastercycler).

For the first round of amplification, 1µl of brain cDNA was used as template with the following primer pairs: VPAC F2 and VPAC R7 for VPAC receptors; PAC F2 and PAC R7 for PAC_1 receptors. A description of the primers is shown in Table 6.1. These yielded PCR products of 490-bp with the VPAC primers and 700-bp with the PAC primers (Fig. 6.1). Validity of the PCR products were assessed on the basis of a semi-nested PCR amplification of the products obtained in the initial PCRs. For the second round, 1µl of the first PCR reaction was used as template with the following primer pairs: VPAC F3 and VPAC R7 for VPAC receptors; PAC F3 and PAC R7 for PAC_1 receptors (see Table 6.1). Amplification profiles were performed as described above. Following PCR, 6 µl of 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 a commercial software (Quantity One software, version 4.1.1).

PCR products of interest were agarose gel purified (GenElute Gel purification kit, Sigma) and cloned into pCR2.1 vector (TOPO TA Cloning kit; Invitrogen). Plasmids from selected positive clones were purified (GeneElute Plasmid purification kit, Sigma; or Wizard kit, Promega) and the DNA was sequenced on both strands using Li-Cor 4200L DNA sequencer technology (Canadian Molecular Research Services, CMRSinc).

Of the different positive clones that were analysed, 3 groups could be identified after sequencing and determination of the highest nucleotide sequence identity with other known $VPAC_1$, $VPAC_2$, and PAC_1 receptors, respectively. As such, they were termed trout $VPAC_1$, trout $VPAC_2$ and trout PAC_1 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., 1990). Sequence alignments were carried out on derived protein sequences using the default settings in CLUSTAL W with DNAMAN software (Lynnon Biosoft, Quebec). The same software was used for sequence editing and translation as well as to construct phylogenetic trees. Phylogenetic trees were constructed using the Neighbour joining tree method. All sequences used for BLAST searches, alignments, and phylogenetic tree construction were edited to include only the area of the trout $VPAC_1$, $VPAC_2$, and PAC_1 receptors that was amplified in between the primer sites used to obtain the clones.

$VPAC_1$, $VPAC_2$, and PAC_1 receptor tissue distribution

Tissue distribution using Northern blot analysis

RNA samples (20μg) were incubated in loading buffer at 65°C for 5 min and electrophoresed through 1.5% (w/v) agarose gels in MOPS buffer containing 5% formaldehyde, and then transferred and UV-cross linked to GeneScreen+ membranes (NEN Life Sciences) by capillary action. Membranes were prehybridised at 65°C for 2-4

h in a buffer containing 6X SSC (0.9 M NaCl, 0.09 M sodium citrate, pH7.0), 5X Denhardt's (1X Denhardt's is 0.1% Ficoll 400 000, 0.1% polyvinylpyrrolidone, 0.1 % bovine serum albumin), 100 mg/ml denatured salmon sperm DNA, 1% sodium dodecylsulphate (SDS) and in 10% dextran sulfate (Amersham Pharmacia Biotech). After addition of probe to a concentration of 10^6 - 2 X 10^6 CPM ml^{-1}, hybridisation proceeded overnight at 65°C in the same solution. Following hybridisation, the membranes were washed several times at 65°C under conditions of increasing stringency to a final wash solution of 0.1X SSC, 0.1% SDS. Membranes were then exposed to BioMax film plus intensifying screen (Kodak) at –80°C for up to a week. Probes were prepared by ^{32}P random primer labeling of PCR amplified fragments of the trout $VPAC_1$, $VPAC_2$, and PAC_1 receptor partial cDNA clones. Unincorporated nucleotides were removed by spin columns. Prior to use, the labelled DNA was denatured by boiling for 5 min followed by rapid chilling in ice.

Tissue distribution using RT-PCR

Total RNA was isolated from various trout tissues (brain, posterior cardinal vein, kidney, gill, spleen, heart, liver, intestine, and blood) and reverse-transcribed as described above. 1µl of first strand product was then used as template for PCR analysis. Gene specific primers (GSP; Table 6.1), forward and reverse, based on the sequences obtained for trout $VPAC_1$, $VPAC_2$, PAC_1 receptors partial cDNAs, amplified a 313-bp (trout $VPAC_1$ receptor), 363-bp (trout $VPAC_2$ receptor), and a 700-bp (trout PAC_1 receptor) products, respectively. The reaction conditions consisted of 5 min of denaturation at 94°C, followed by 40 sec at 94°C, 40 sec at 55°C (PAC_1 primers) or 62°C ($VPAC_1$ and

$VPAC_2$ receptor primers), and 40 sec extension at 72°C 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 sec annealing at 55°C. Moreover, the specificity of the GSP primers were tested in PCR amplification (as described above) using the different GSPs in combination with the plasmids of the three clones. Minus template and single primer control PCRs were also performed.

Table 6.1. Forward and reverse primer sequences used in PCR amplification of VPAC and PAC_1 receptor cDNAs. The VPAC and PAC degenerate primers were deduced from conserved regions of nucleotide sequences within the third and seventh transmembrane domain (TMD) of VPAC receptors and second and seventh TMD of PAC_1 receptors of various vertebrates. Gene specific primers (GSP) were designed according to the partial cDNA sequences obtained for the three receptors. β-actin forward and reverse primers are also described. Expected band sizes obtained with different primer pairs are shown. *Abbreviations*, forward (FOR); reverse (REV); band size in base pairs (bp).

Primers	Sequence	Primer pair /expected size
VPAC degenerate primers		
VPAC F2 (FOR)	5' TTCTGGCTKCTRGTGGAAGG 3'	**F2-R7→ 490 bp**
VPAC F3 (FOR)	5' GAGMGGAARTAYTTCTGGKGGTACAT 3'	**F3-R7→ 410 bp**
VPAC R7 (REV)	5' ACCACAAADCCCTGRAADGABCC 3'	
$VPAC_1$ GSP		
$VPAC_1$ 3.1 (FOR)	5' AGGGCCAACAATCTTCATCACA 3'	**$VPAC_1$ 3.1 – 5.1 313 bp**
$VPAC_1$ 5.1 (REV)	5' CTGTGTTCACCTGCTCTGGG 3'	
$VPAC_2$ GSP		
$VPAC_2$ 3.1 (FOR)	5' GCATACACACACCTCGCTGT 3'	**$VPAC_2$ 3.1 – 5.1 363 bp**
$VPAC_2$ 5.1 (REV)	5' CTTGTACTCCTCCATCCCGTC 3'	

Continued on next page...

Table 6.1. Continued....

Primers	Sequence	Primer pair /expected size
PAC_1 degenerate primers		
PAC_1 F2 (FOR)	5' MGNAACTTCATCCACATGAAYC 3'	**F2-R7→ 700 bp**
PAC_1 F3 (FOR)	5' YGTBRTGTCMAACTACTTCTGGCT 3'	**F3-R7→ 540 bp**
PAC_1 R7 (REV)	5' GMYTGYACCTCBCCATTCA 3'	
PAC_1 GSP		
PAC_1 3.1 (FOR)	5' GTCGTGTCAAACTACTTCTGGC 3'	**PAC_1 3.1 – 5.1 540 bp**
PAC_1 5.1 (REV)	5' GACTGTACCTCACCATTCAGAA 3'	
β-actin		
(FOR)	5' TGTGATGGTGGGAATGGGTCAG 3'	**β-actin (FOR) and (REV) 530 bp**
(REV)	5' TTTGATGTCACGCACGATTTCC 3'	

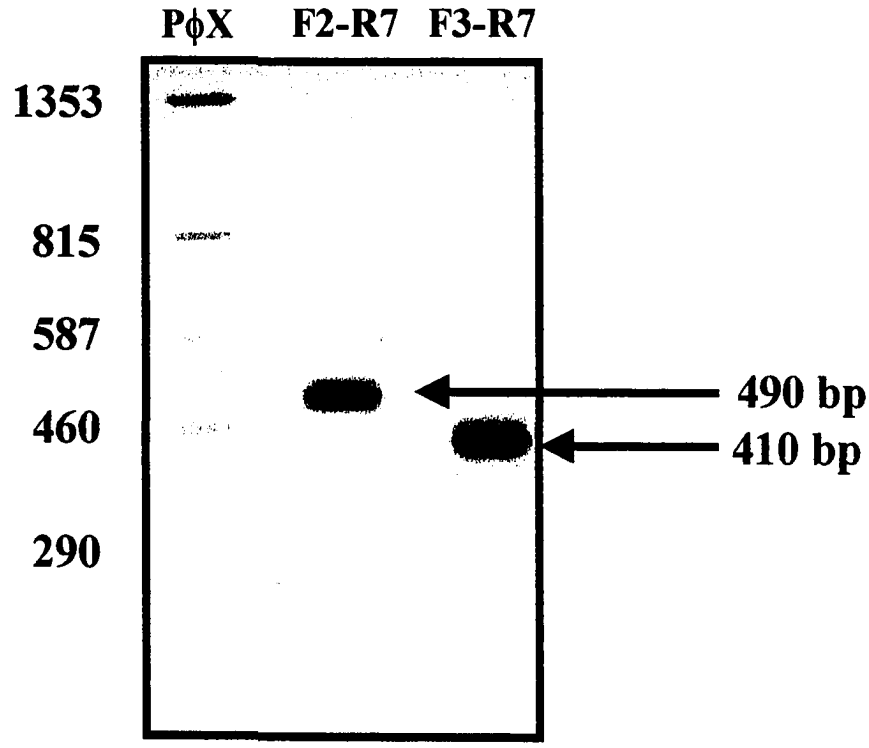
RESULTS

The available sequence information (GenBank) from cloned $VPAC_1$ and PAC_1 receptors was used to design degenerate primers on the basis of conserved regions within transmembrane domains (TMD) for semi-nested PCR of trout brain cDNA. Using degenerate primers designed against $VPAC_1$ receptors, a 490 base pair (bp) cDNA fragment was amplified (Fig. 6.1A). Likewise, a 700-bp cDNA fragment was amplified using degenerate primers designed against PAC_1 receptors (Fig.6.1B). Subsequently, these fragments were cloned into pCR2.1 vectors and sequenced. Although a 490-bp cDNA fragment was amplified following PCR with the degenerate primers designed against the $VPAC_1$ receptors, two different clones of similar sizes were isolated (Figs. 6.2 and 6.3). The sequence obtained using the PAC degenerate primers is shown in figure 6.4. Sequence analyses of the DNA inserts revealed several lines of evidence that the partial sequences belong to $VPAC_1$, $VPAC_2$, and PAC_1 receptors. As such, the partial cDNA clones were subsequently termed trout $VPAC_1$, trout $VPAC_2$, and trout PAC_1 receptors.

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, 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

Figure 6.1 PCR amplification of the putative trout VPAC (A) and PAC_1 (B) receptors from brain cDNA. PCR reactions were subjected to electrophoresis on agarose gels stained with ethidium bromide. In each case, PCR using primers deduced from conserved regions of the VPAC and PAC_1 receptor cDNAs of various vertebrates amplified an expected 490-bp (A) and 700-bp (B) fragment using the VPAC F2-R7 and PAC_1 F2-R7 primer pairs, respectively. Validity of the PCR products was assessed on the basis of a semi-nested PCR amplification of the products obtained in the initial PCR. PCR amplification using the VPAC F3-R7 and PAC_1 F3-R7 primer pairs amplified expected 410-bp and 540-bp products, respectively. PϕX, DNA molecular size ladder. The 490-bp and 540-bp products obtained in (A) and (B) were subsequently cloned and sequenced.

A. VPAC



B. PAC_1

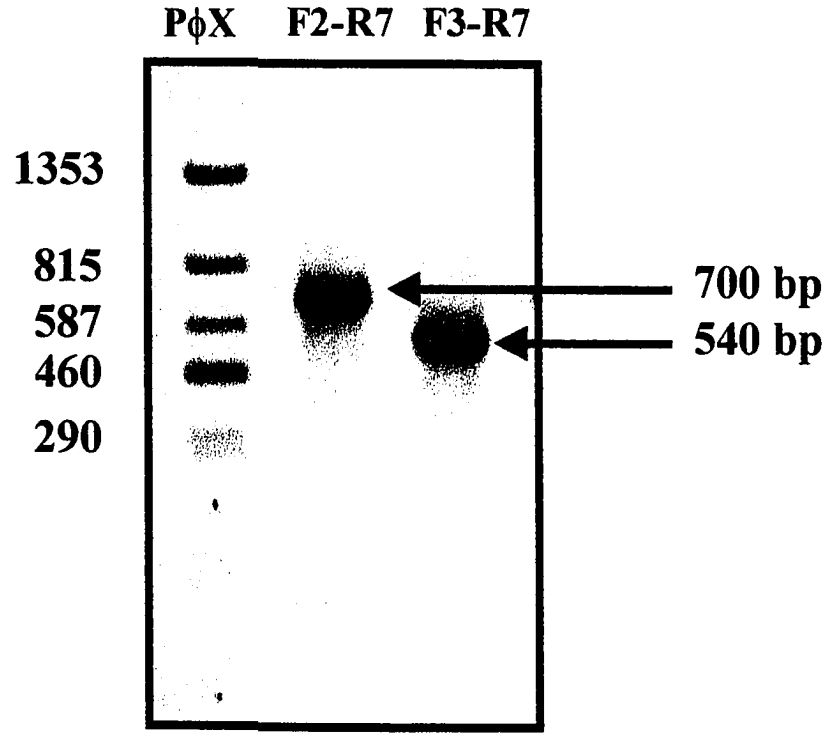


Figure 6.1

Figure 6.2 The partial nucleotide and deduced amino acid sequences of the trout (*Oncorhynchus mykiss*) $VPAC_1$ receptor. The black boxes indicate the positions of the degenerate primers (forward primer, VPAC F2; reverse primer, VPAC R7) used in the initial PCR to obtain the partial clone. The red boxes indicate the positions of the gene specific primers used in RT-PCR tissue distribution analysis (forward primer, $VPAC_1$ 3.1; reverse primer, $VPAC_1$ 5.1). Nucleotides shown in bold character were excluded from sequence alignments (see Materials and Methods for details).

Trout $VPAC_1$ receptor

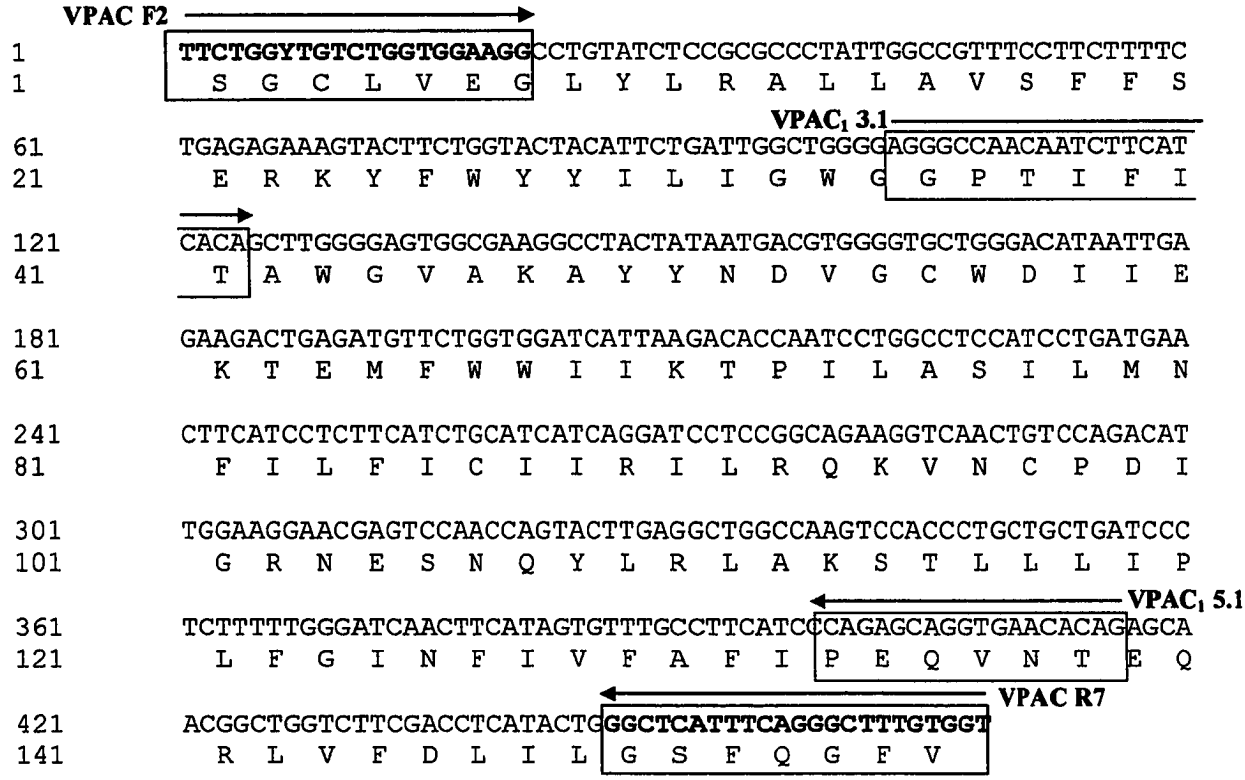


Figure 6.2

Figure 6.3 The partial nucleotide and deduced amino acid sequences of the trout (*Oncorhynchus mykiss*) $VPAC_2$ receptor. The black boxes indicate the positions of the degenerate primers (forward primer, VPAC F2; reverse primer, VPAC R7) used in the initial PCR to obtain the partial clone. The red boxes indicate the positions of the gene specific primers used in RT-PCR tissue distribution analysis (forward primer, $VPAC_2$ 3.1; reverse primer, $VPAC_2$ 5.1). Nucleotides shown in bold character were excluded from sequence alignments (see Materials and Methods for details).

Trout $VPAC_2$ receptor

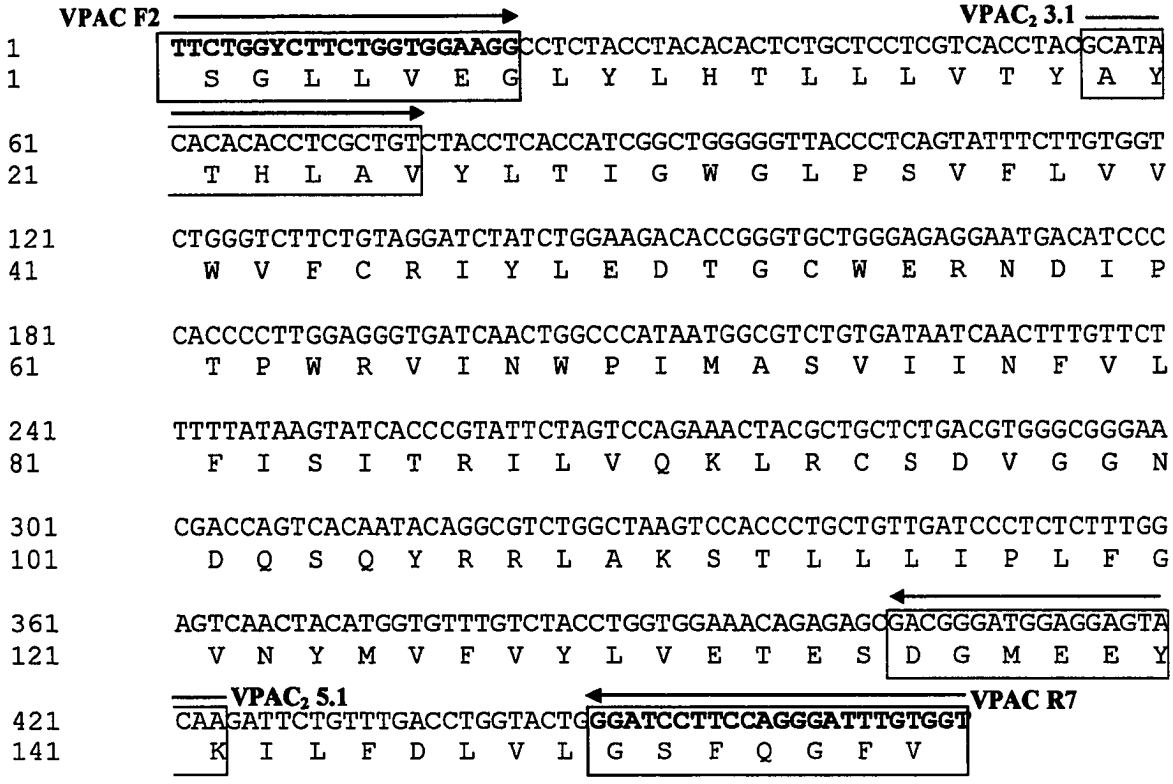


Figure 6.3

Figure 6.4 The partial nucleotide and deduced amino acid sequences of the trout (*Oncorhynchus mykiss*) PAC_1 receptor. The black boxes indicate the positions of the degenerate primers (forward primer, PAC F2; reverse primer, PAC R7) used in the initial PCR to obtain the partial clone. The red boxes indicate the positions of the gene specific primers used in RT-PCR tissue distribution analysis (forward primer, PAC_1 3.1; reverse primer, PAC_1 5.1). Nucleotides shown in bold character were excluded from sequence alignments (see Materials and Methods for details).

Trout PAC_1 receptor

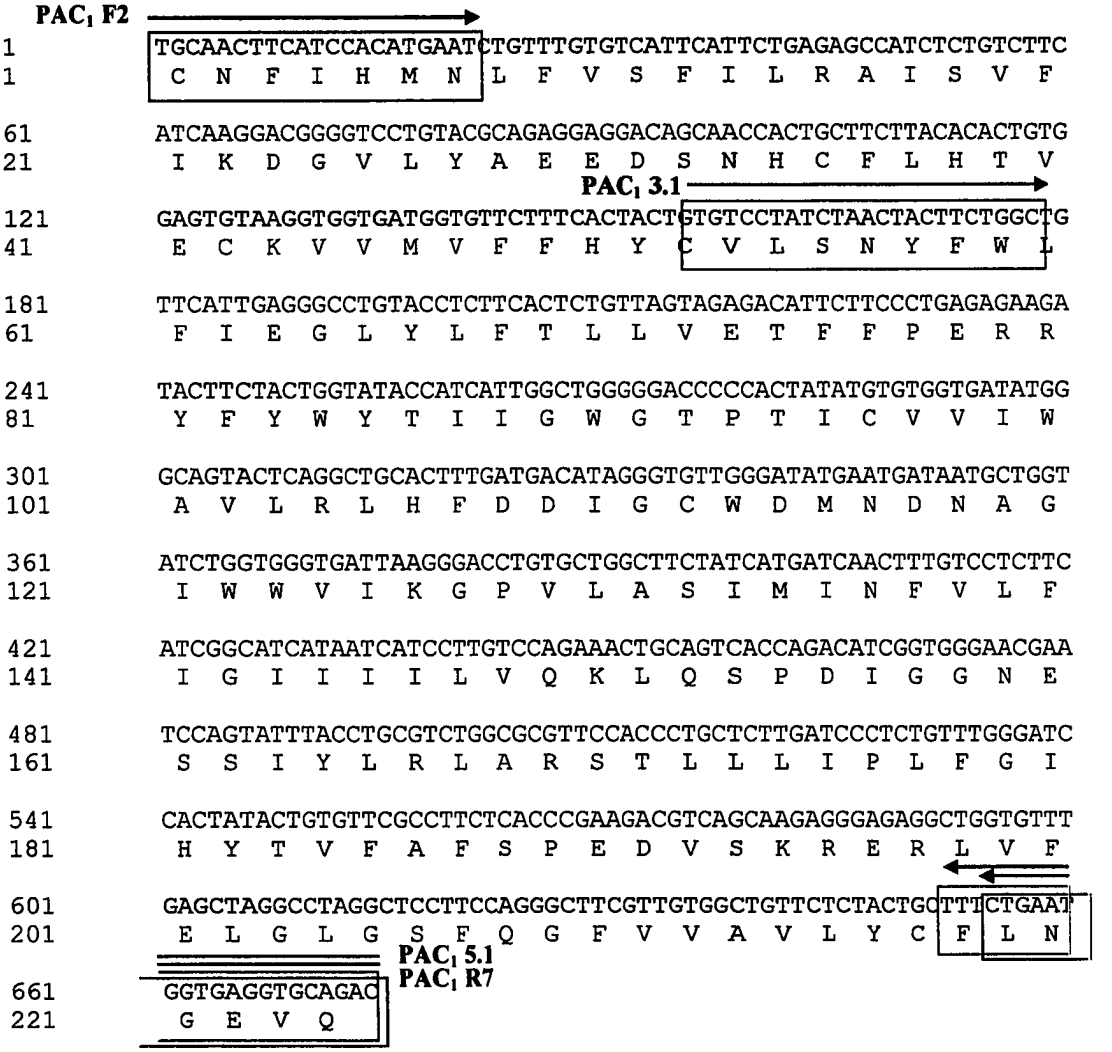


Figure 6.4

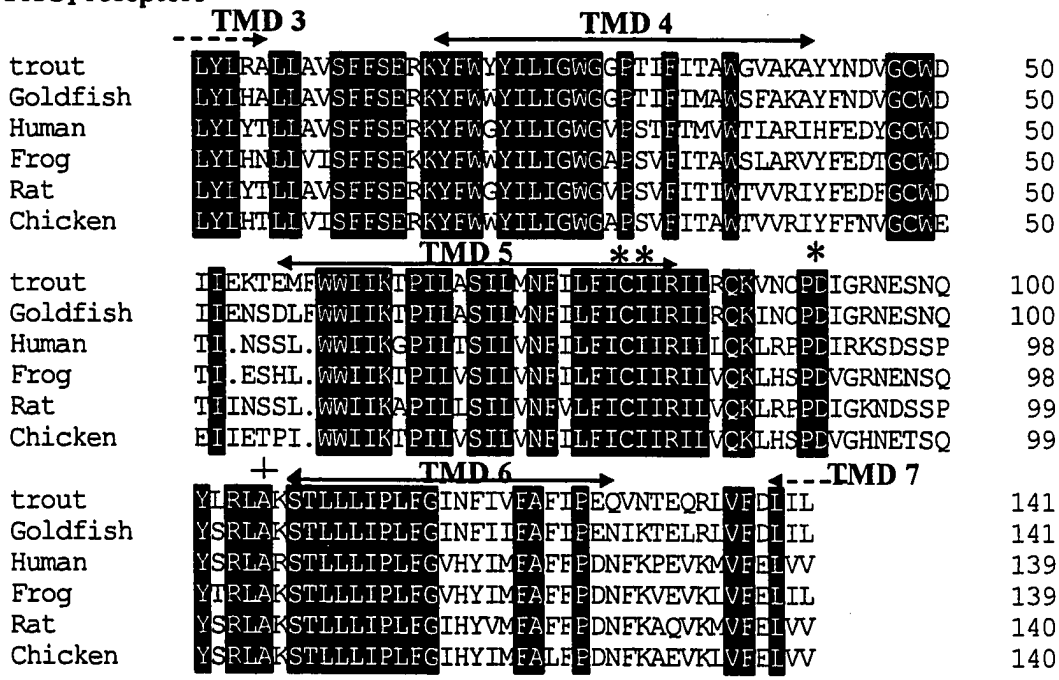
with the trout $VPAC_2$ sequence. Finally, for the trout PAC_1 partial cDNA clone, the best match was to the goldfish PAC_1 receptor (E value, 10^{-122}). At the protein level, the trout sequence also displayed highest amino acid identity (94%) with the goldfish PAC_1 receptor.

Sequence alignments of the trout sequences indicated that they are highly homologous to their vertebrate counterparts (Figs. 6.5). Visual inspection of the deduced amino sequences of the trout partial clones revealed motifs that are common to the $VPAC_1$, $VPAC_2$, and PAC_1 receptors. A signature motif for the $VPAC_1$ receptors was identified at a unique cysteine residue within the TMD5, just in front of the "IIRL". This cysteine residue is believed important for disulfide linkages for proper receptor function. 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 serine residue may be important for receptor activation/inactivation through secondary messenger systems. This conserved "SE-R/K" motif is also found in several other members in the same receptor family including secretin, GHRH, glucagon and PTH receptors. Another motif, the "P D I I/V" found only in $VPAC_1$, $VPAC_2$, and PAC_1 receptors between the putative TMD5 and TMD6 (Chow et al., 1997), was also present in the trout receptors. The "RLA K/R" motif immediately in front of TMD6 also matches the consensus found in other members of G-protein coupled receptors. This motif (basic-L/A L/A/V/S-basic) containing two basic amino acids separated by two hydrophobic residues has been indicated to be essential to $G_{s\alpha}$ protein coupling. The most conserved regions of the receptors appear to be the putative TMDs. Furthermore, phylogenetic

analysis indicates that the rainbow trout sequences are most related to their vertebrate counterparts (Fig. 6.6).

Figure 6.5 Comparison of partial amino acids sequences of the putative trout $VPAC_1$ (A), $VPAC_2$ (B), and PAC_1 (C) 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 (Lynnon Biosoft). The putative trans membrane domains (TMD) are labelled with arrows, and were deduced by visual inspection of published reports and hydrophobocity analysis. The $G_{s\alpha}$ -protein coupling motif (basic- L/A L/A/V/S-basic) is shown with a +. The RLA K/R motif common to members of the G-protein coupled receptor family. The PD I/V motif common to VIP binding receptors is shown with a *. Finally, the conserved cysteines in front of the "IIRIL motif, common only to $VPAC_1$ receptors is shown with a **. Sequences were edited to include only the region spanning the primer sites used to obtain the trout partial cDNA clones.

A. $VPAC_1$ receptors



B. $VPAC_2$ receptors

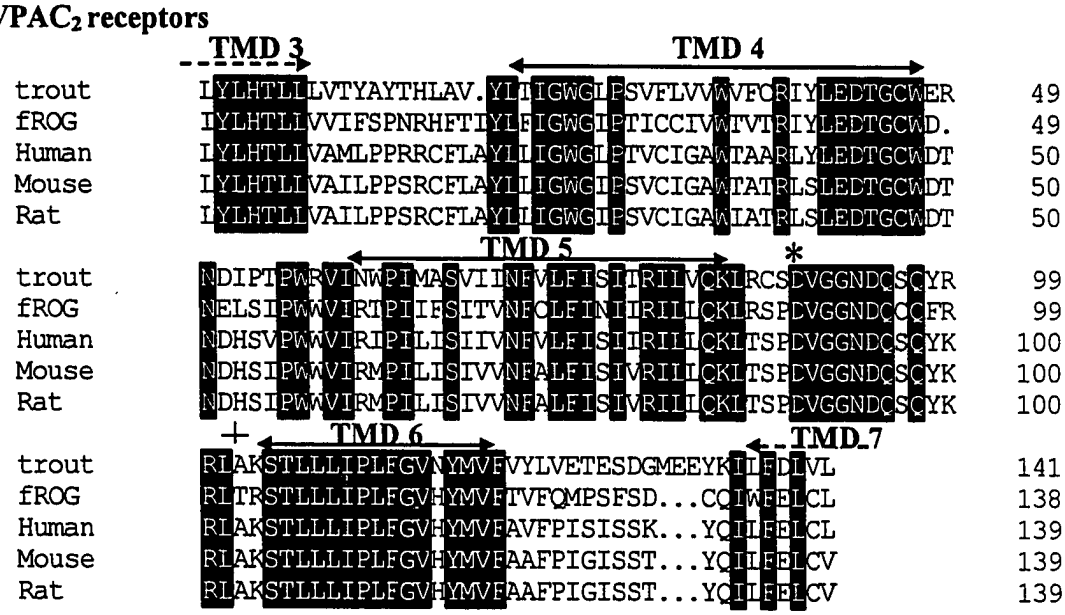


Figure 6.5

Continued on next page.....

Figure 6.5 continued....

C. PAC_1 receptors

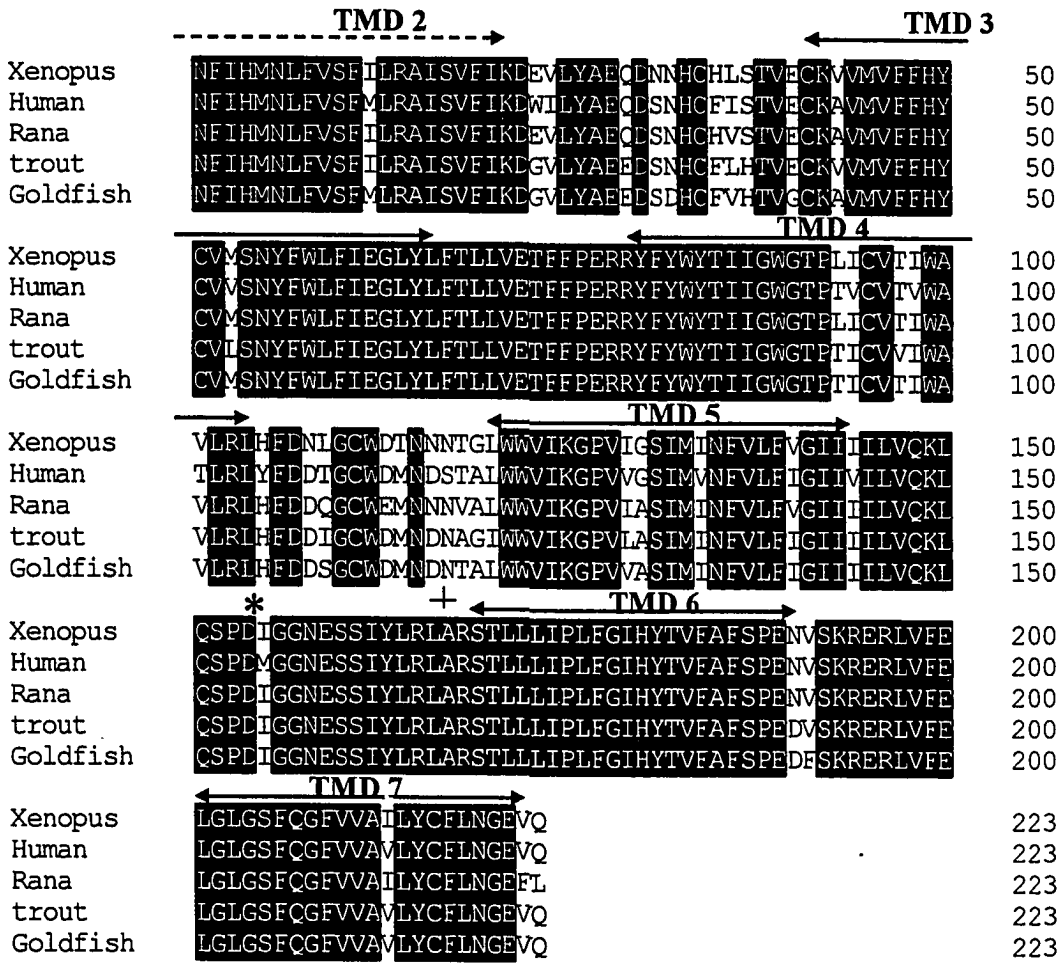


Figure 6.5

Figure 6.6 Phylogenetic tree of the $VPAC_1$, $VPAC_2$, and PAC_1 receptors using available vertebrate amino acid sequences from GenBank. Bootstrap values are indicated at the nodes. The scale bar indicates distance in units of fraction of amino acids differing between two sequences.

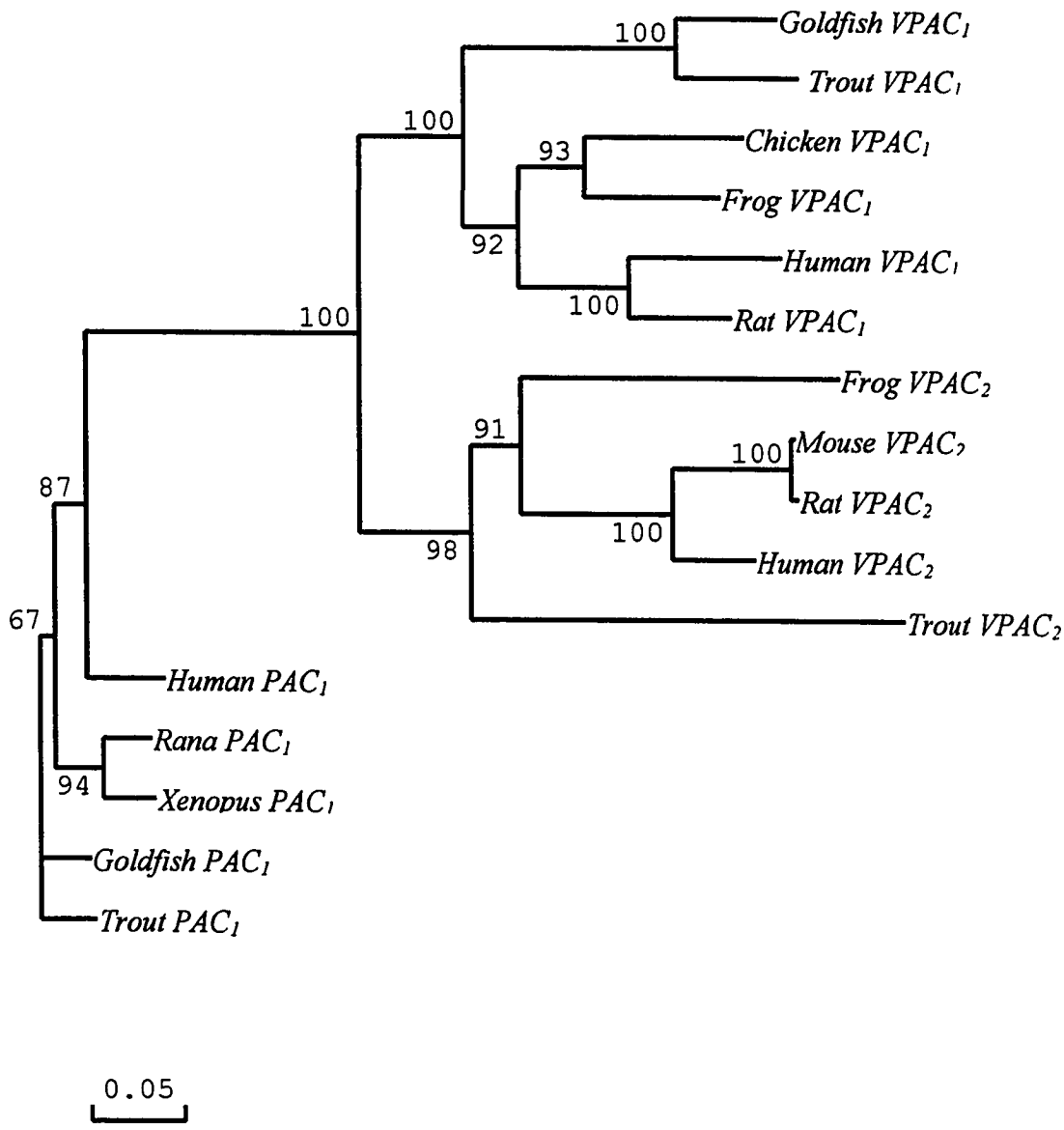

Goldfish VPAC1
Trout VPAC1
Chicken VPAC1
Frog VPAC1
Human VPAC1
Rat VPAC1
Frog VPAC2
Mouse VPAC2
Rat VPAC2
Human VPAC2
Trout VPAC2
Human PAC1
Rana PAC1
Xenopus PAC1
Goldfish PAC1
Trout PAC1
100
100
93
92
100
100
91
100
100
98
87
67
94
0.05

Figure 6.6

Tissue specific expression of trout $VPAC_1$, $VPAC_2$, and PAC_1 receptors

Northern blot analyses to study the tissue distribution of each receptor were performed but no transcript was detected in any of the tissues even after prolonged exposure. The lack of a hybridization signal was likely due to the sensitivity limit of Northern blot as neuroreceptors are usually expressed at low levels. A control β-actin hybridisation, however, was not performed; therefore the quality of the blot was not confirmed. Regardless, a more sensitive RT-PCR tissue distribution analyses of the trout $VPAC_1$, $VPAC_2$, and PAC_1 receptor were performed.

Gene specific primers were designed for each receptor according to the partial nucleotide sequences (Figs. 6.2, 6.3, and 6.4). The specificity of the gene specific primers were tested in PCR amplification using the putative trout $VPAC_1$, $VPAC_2$ and PAC_1 receptor partial clones as template. Trout $VPAC_1$ receptor mRNA was found in brain, liver, and intestine (Fig. 6.7). Additionally, while less prominent products were observed in the PCV, kidney, gill, spleen, and heart, no product was observed in blood. For trout $VPAC_2$ receptor mRNA, products were observed in the brain, spleen, heart, liver, and intestine (Fig. 6.7). No products were evident in the PCV, kidney, gill or blood. Finally, PAC_1 receptor mRNA was detected in every tissue with the exception of blood and kidney (Fig. 6.8).

Figure 6.7 Tissue distributions of the putative trout $VPAC_1$ and $VPAC_2$ receptors as revealed by RT-PCR. PCR was performed using the gene specific primers (GSP) deduced from the partial cDNA clones for each receptor. Using the GSP for trout $VPAC_1$ receptors, a 313-bp product was observed in all tissues with the exception of blood. Prominent products were observed in the liver and intestine. On the other hand, using GSP for trout $VPAC_2$ receptors, a 363-bp product was observed in brain, spleen, heart, liver and intestine. No products were observed in 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.

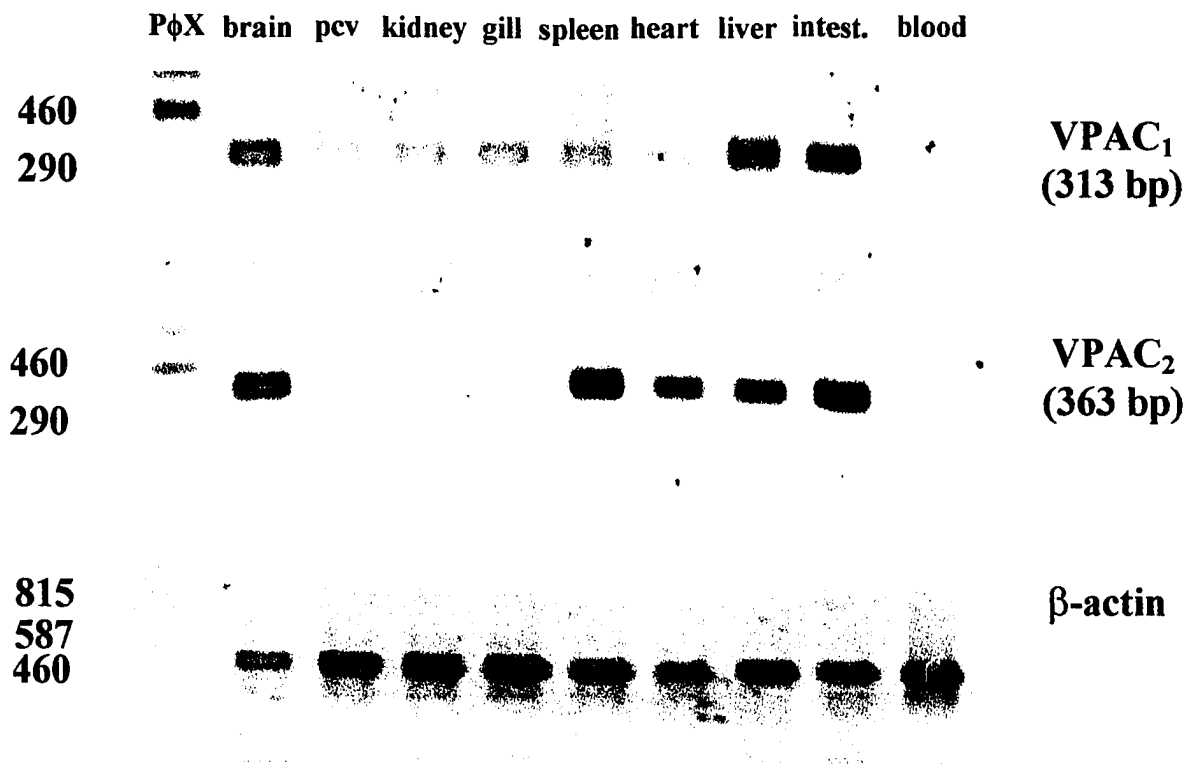

PϕX
brain
pcv
kidney
gill
spleen
heart
liver
intest.
blood
460
290
VPAC1
(313 bp)
460
290
VPAC2
(363 bp)
815
587
460
β-actin

Figure 6.7

Figure 6.8 Tissue distribution of the putative trout PAC_1 receptor as revealed by RT-PCR. PCR was performed using the gene specific primers (GSP) deduced from the partial cDNA clones for the PAC_1 receptor. Using the GSP for trout PAC_1 receptors, a 540-bp product was observed in all tissues with the exception of 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.

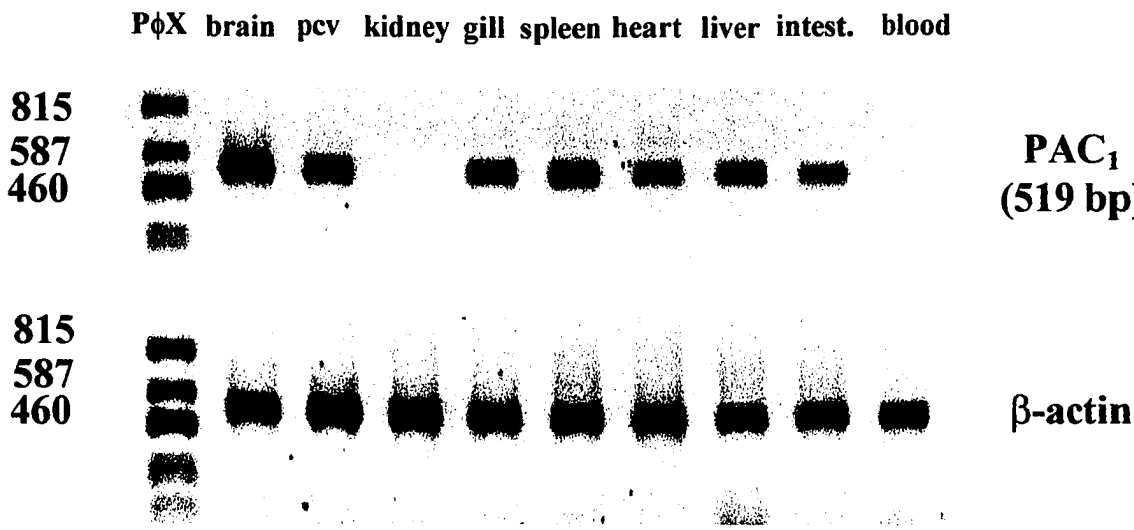

PφX brain pcv kidney gill spleen heart liver intest. blood
815
587
460
PAC1
(519 bp)
815
587
460
β-actin

Figure 6.8

DISCUSSION

The VPAC and PAC_1 receptors of mammals have been well characterized (Harmar et al., 1998; Robberecht and Waelbroeck, 1998; Vaudry et al., 2000). These receptors have been studied using binding assays that employ agonists and antagonists shown to have specific affinities for each receptor, and also on the affinity profiles of VIP and PACAP for given binding sites (Harmar et al., 1998). However, identification of specific receptors mediating the effects of VIP and PACAP based solely on the use of pharmacological agents may be at times problematic owing to the existence of multiple receptors and peptides within a given tissue. Hopefully, the classification of the sequences reported in this study, as being $VPAC_1$, $VPAC_2$, and PAC_1 receptor partial cDNA clones will provide additional tools to confirm and support the results of previous and future pharmacological results on the roles of these neuropeptides and receptors in various physiological systems.

The approach used to obtain the clones was based on the amplification of G-protein-coupled receptor cDNAs using degenerate primers designed against conserved regions within transmembrane domains of VPAC and PAC_1 receptors of selected vertebrates. At the molecular level, the trout $VPAC_1$, $VPAC_2$, and PAC_1 deduced partial amino acid sequences shows high degree of overall sequence conservation with mammalian and amphibian receptors, respectively. The highest level of sequence conservation occurs within the putative transmembrane domain regions. Furthermore, visual inspection for signature motifs reveal that each clone probably belongs to a G-protein coupled receptor most likely of the $VPAC_1$, $VPAC_2$, and PAC_1 receptors. Phylogenetic analysis indicates that the rainbow trout sequences are most related to their

vertebrate counterparts. Taken together, the sequence analyses provide evidence for the likelihood that the sequences reported here are rainbow trout $VPAC_1$, $VPAC_2$, and PAC_1 receptor partial cDNA clones.

The isolation of a clone exhibiting identity to the $VPAC_2$ receptor was admittedly, a surprise. Initially the purpose to this study was to clone the trout $VPAC_1$ receptor. As such, primers were designed based on nucleotide sequences of $VPAC_1$ receptors from selected vertebrates. Upon review, the primer sites used to obtain the clones were very similar in both $VPAC_1$ and $VPAC_2$ receptors. Therefore, it is likely that the primers amplified both receptors during PCR with the VPAC degenerate primers. To further appreciate the finding, the primer site used to design the semi-nested forward primer is not present within the trout $VPAC_2$ receptor sequence. As such, the semi-nested approach was in fact amplifying the $VPAC_1$ receptors and not the $VPAC_2$ receptors. In a previous study, two VPAC receptor sequences from goldfish brain tissue were isolated that shared approximately 70% sequence similarity (Chow et al., 1997). The authors suggested that this was likely due to the fact that goldfish are tetraploids, and it is not uncommon that these fishes express two different mRNAs from duplicate gene loci. In this study, however, the VPAC receptor sequences exhibited approximately 50% sequence similarity, and owing to the phylogenetic analysis, it is unlikely that the two sequences are variants of the $VPAC_1$ receptors.

Previously, full gene sequences have been isolated for goldfish $VPAC_1$ and PAC_1 receptors (Chow 1997; Chow et al., 1997; Wong et al., 1998). Also, partial sequences have been obtained for $VPAC_1$ receptors in salmon (Chow et al., 1997) and PAC_1 receptors in zebrafish. In this study, I report the first rainbow trout $VPAC_1$, $VPAC_2$, and

PAC_1 receptor partial sequences. To my knowledge, the putative trout $VPAC_2$ receptor sequence is the first ever reported for any fish studied to date. Although several lines of evidence indicate the likelihood that these receptors are of the VPAC and PAC_1 receptor types, definite identification of these receptors at the molecular level will only be possible by isolating full gene sequences and functional characterization of the encoded proteins.

Tissue specific expression of trout $VPAC_1$, $VPAC_2$, and PAC_1 receptors

Primers designed to amplify regions specific to the trout $VPAC_1$, $VPAC_2$, and PAC_1 receptors were used to investigate tissue distributions of each receptor. RT-PCR demonstrated that the receptors are expressed in several tissues. For $VPAC_1$ receptors, strong signals were detected in brain, liver and intestine, while less prominent products were observed in posterior cardinal vein, kidney, gill, spleen, and heart. As for PAC_1 receptors, strong signals were detected in brain, PCV, gill, spleen, heart, liver, and intestine. The kidney did not exhibit any signal for this receptor. Indeed, the tissue distributions of the $VPAC_1$ and PAC_1 receptors were consistent with previous investigations conducted in goldfish (Chow 1997; Wong et al., 1998). Because this study is the first to report a $VPAC_2$ receptor at the molecular biology level for any fish, this thesis also presents the first account of a tissue distribution for this receptor in fish. Recently, a $VPAC_2$ receptor was cloned and characterized in frogs (Hoo et al., 2001). Consistent with the results of the study, $VPAC_2$ receptors were detected in brain, heart, spleen, liver and intestine. In contrast to frogs, however, $VPAC_2$ receptors were not detected in the PCV or kidney.

Expression of the rainbow trout receptors in some of these tissues is consistent with previous publications that showed, by pharmacology, the presence of VPAC and PAC_1 receptors in fish tissues. VIP-immunoreactive nerve fibers are widespread in the arterial and venous systems of the trout and cod (Johnsson et al., 2001). In vitro, VIP induces relaxation of pre-contracted arteries of rainbow trout (Kagstrom and Holmgren, 1997). VIP appears to have a similar effect in cod where *in vivo* and *in vitro* experiments show a decrease in vascular resistance in response to VIP (Lundin and Holmgren 1984). The co-localization of VIP and PACAP-immunoreactive nerves in the posterior cardinal vein is consistent with the findings of this study (Johnsson et al., 2001). Also, VIP and PACAP have been demonstrated in nerve fibers in the vicinity of the chromaffin cells of several species of fish (Reid et al., 1995). Recently, a role for VIP and PACAP has been demonstrated in the secretion of catecholamine release from trout chromaffin cells (Montpetit and Perry, 2000). Evidence indicated that a VPAC type receptor mediated the effects of VIP and PACAP on catecholamine secretion in trout. The detection of $VPAC_1$ receptors in the PCV is consistent with these findings.

Extensive VIP neuronal networks have also been demonstrated in the gastrointestinal system in several species of cyprinidae (Langer et al., 1979). The presence of $VPAC_1$, $VPAC_2$, and PAC_1 receptors in the rainbow trout intestine supports a role of VIP and/or PACAP in aspects of gut motility and secretions (Jensen and Holmgren, 1985; Holmgren 1985). VIP also is involved in several other physiological functions in fish such as osmoregulation (Mainoya and Bern 1984), relaxation of the gall bladder (Aldman and Holmgren, 1984) and the regulation of swim bladder gas absorption (Lundin and Holmgren, 1984). The presence of the three receptors in brain is consistent

with the roles of VIP and PACAP as a neurotransmitter and neuromodulator. Consistent with these findings, PACAP has been shown to stimulate the release of growth hormone from goldfish pituitary (Wong et al., 1998).

In summary, using molecular cloning techniques, partial sequences for the trout $VPAC_1$, $VPAC_2$, and PAC_1 receptors were obtained. The presence of multiple receptors for VIP and PACAP is consistent with previous pharmacological characterizations that showed three types of receptors that could mediate the effects of VIP and PACAP. The isolation and sequencing of the rainbow trout partial $VPAC_1$, $VPAC_2$, and PAC_1 receptor clones are initial steps toward a broader understanding of the VIP/PACAP system in fish and the functioning of vertebrate VIP and PACAP receptors in general. Future work will focus on several areas including the isolation of full gene sequences, pharmacological characterization of the encoded proteins and development and use of homologous antibodies.

CHAPTER 7

ADRENERGIC REGULATION OF CATECHOLAMINE SECRETION FROM TROUT (*Oncorhynchus mykiss*) CHROMAFFIN CELLS

(Based in part on Montpetit CJ and Perry SF. 2002.
Journal of Endocrinology 173, 187-197)

INTRODUCTION

In teleost fish, the catecholamines (adrenaline and noradrenaline) that enter the circulation are synthesized, stored, and released from chromaffin tissue that line the walls of the posterior cardinal vein (PCV; Reid et al., 1998). Under adverse conditions including exhaustive exercise, hypoxia, hypercarbia and physical disturbances (Randall and Perry, 1992), the rise in plasma catecholamine levels modulates various physiological responses that serve to enhance cardio-respiratory and metabolic functions (Wendelaar Bonga, 1997; Reid et al., 1998; Perry and Gilmour, 1999). The control of chromaffin cell activity and catecholamine secretion involves neuronal signals derived from cholinergic and non-cholinergic neurotransmitters and humoral signals of endocrine origin (Reid et al., 1998). While numerous investigations have studied the regulation of the adrenergic stress response in fish, few studies have investigated the impact of catecholamines, themselves, on the release of catecholamines from chromaffin cells.

Chromaffin cells like adrenergic neurons, are derived from the neural crest during development and share several properties including synthesis, storage and release of catecholamines (Nilsson 1983). In the adrenergic neurons of peripheral and central nerves, specific presynaptic inhibition of neurotransmitter release has been well-documented in mammals. For example, it has been reported frequently that α-adrenoceptor agonists and antagonists exert powerful influences on stimulus-evoked release of noradrenaline from nerve terminals (Starke et al., 1974b; Langer et al., 1977; Nilsson 1983). Although controversial, similar interactions have been described in mammalian chromaffin cells leading investigators to suggest an autocrine/paracrine regulation of catecholamine release via activation of chromaffin cell adrenergic receptors

(Gutman and Boonyariviroj, 1977; Boonyariviroj et al., 1979; Cohen et al., 1980; Greenberg and Zinder, 1982; Wada et al., 1982; Powis and Baker, 1985; Sharma et al., 1986; Orts et al., 1987).

Studies designed to investigate the effects of catecholamines on catecholamine release from piscine chromaffin cells have produced conflicting results. In lamprey (*Petromyzon marinus*) and American eel (*Anguilla anguilla*), administration of adrenaline or noradrenaline *in vivo* causes the elevation of plasma catecholamines (both adrenaline and noradrenaline; Dashow and Epple, 1983; Hathaway et al., 1989). Furthermore, pre-treatment with α- or β-adrenergic receptor antagonists decreased catecholamine release in the eel in response to neuronal or cholinergic stimulation of the chromaffin tissue *in situ* or *in vitro* (Al-Kharrat et al., 1997; Abele et al., 1998). In contrast to eels and lampreys, continuous infusion of adrenaline *in vivo* in rainbow trout (*Oncorhynchus mykiss*) did not affect the levels of plasma noradrenaline (Perry and Vermette, 1987). Moreover, in Atlantic cod (*Gadus morhua*), the addition of high levels of a particular catecholamine to the inflowing perfusion fluid of an *in situ* preparation inhibited the cholinergic-elicited release of that particular catecholamine (Perry et al., 1991). Thus, there appears to be species-dependent differences in the response of piscine chromaffin cells to extracellular catecholamines. To date, a thorough analysis of the response of trout chromaffin cells to catecholamines has not yet been performed. Thus, the goal of this investigation was to first assess the impact of extracellular catecholamines on catecholamine secretion from rainbow trout chromaffin cells, and second, use a pharmacological approach to identify the underlying mechanisms.

MATERIALS AND METHODS

Experimental animals

Rainbow trout weighing between 250 and 350g were maintained under conditions outlined in Chapter 2.

In situ saline perfused posterior cardinal vein preparation

The posterior cardinal vein tissue was perfused as outlined in Chapter 2. After the 20-min stabilization period, preparations were processed according to the protocols described below. Agonists were administered by bolus injection through a three-way valve connected to the inflow catheter. Alternatively, agonists and antagonists were delivered continuously via the perfusion fluid by acutely switching the perfusion media from one container (control saline) to another container (control saline or saline containing adrenergic agents) using a three-way valve. For neuronal stimulation, whole body field electrical stimulation was administered as outlined in Chapter 2.

Series 1: The effects of catecholamines on catecholamine secretion. Two approaches were used to assess the effects of exogenous adrenaline and noradrenaline on catecholamine secretion. To study the acute effects, preparations were given a single bolus injection (final volume 0.3 ml) of adrenaline (330 nM, N = 6; adrenaline bitartrate salt; Sigma) or noradrenaline (124 nM, N = 6; arterenol bitartrate salt; Sigma). These levels of catecholamines were chosen to simulate the peak levels of perfusate catecholamine concentration following a bolus injection of carbachol (10^{-6} mol kg^{-1}). A bolus injection of saline (0.3 ml) was used in control experiments (N = 6). To study the potential longer term effects, three separate experimental series were performed in which the concentration of catecholamines in the inflowing perfusion media was varied. In the

first (control, N = 6), both adrenaline and noradrenaline were nominally zero. In the second, only the adrenaline concentration was elevated (330 nM, N = 6); in the third, only the noradrenaline concentration was elevated (124 nM, N = 6). A period of 1 min was allowed for the catecholamine to be delivered to the chromaffin tissue before post-samples were collected in pre-weighed tubes. In total, five post-samples were collected 1,2,3,4 and 5 min after intervention.

Series 2: The effects of catecholamines on stimulus evoked secretion of catecholamines Experiments were performed to evaluate the effects of adrenaline and noradrenaline on the secretion of catecholamines elicited by carbachol or electrical stimulation of the nerves innervating the chromaffin tissue. Catecholamine secretion in response to a bolus injection of 10^{-6} mol kg^{-1} carbachol (mixed cholinergic receptor agonist; Sigma) or electrical/neuronal stimulation was compared in preparations pre-perfused for 5 min with control saline (N = 6), or saline containing adrenaline (330 nM, N = 6) or noradrenaline (124 nM, N = 6). Prior to stimulation, a pre-sample was collected in pre-weighed tubes. For a bolus injection, 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. For neuronal stimulation, post-samples were collected immediately after stimulation. In total, five post-samples were collected at 1 min intervals after the intervention.

Series 3: The effects of adrenergic receptor agonists/antagonists on stimulus-evoked secretion of catecholamines. In a first set of experiments, a bolus injection of carbachol (10^{-6} mol kg^{-1}; 0.3 ml) or neuronal stimulation was administered to preparations perfused with control saline (N = 6), or saline containing the α-adrenergic

receptor agonists phenylephrine (10^{-6} M phenylephrine hydrochloride, α_1; Sigma; N = 6) or clonidine (10^{-6} M clonidine hydrochloride, α_2; Sigma; N = 6). In other experiments, catecholamine secretion was compared in preparations perfused with control saline or saline containing the β-adrenergic receptor agonists dobutamine (10^{-6} M dobutamine hydrochloride, β_1; Sigma; N = 6) or salbutamol (10^{-6} M salbutamol hemisulfate salt, β_2; Sigma; N = 6). In a third set of experiments, catecholamine secretion was measured in preparations perfused with saline containing the adrenergic receptor antagonists phentolamine (10^{-6} M phentolamine hydrochloride, α receptor antagonist; Sigma; N = 6) or nadolol (10^{-6} M, β receptor antagonist; Sigma; N = 6) during cholinergic or neuronal stimulation (as in Series 2).

Series 4. The effects of cAMP on stimulus-evoked secretion of catecholamines. Catecholamine secretion in response to a bolus injection of 10^{-9} mol kg^{-1} ckVIP (chicken VIP, vasoactive intestinal polypeptide; Peninsula Laboratories Inc., San Carlos, California, USA) or 10^{-6} mol kg^{-1} carbachol was compared in fish perfused with control saline (N = 6) or saline containing dibutyryl-cAMP (10^{-6} M; N = 6). 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 (as in Series 2).

Series 5: The effects of β_2 receptor stimulation on catecholamine secretion elicited by VIP or angiotensin II. Catecholamine secretion in response to a bolus injection of 10^{-9} mol kg^{-1} ckVIP or 10^{-9} mol kg^{-1} angiotensin II ([Asn1-Val5]-Angiotensin II, Sigma) was compared in fish perfused with control saline (N = 6) or saline containing salbutamol (10^{-6} M; N = 6). 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 (as in Series 2).

Analytical procedures

Determination of perfusate catecholamine levels was performed as outlined in Chapter 2. In experiments performed using saline containing adrenaline or noradrenaline, catecholamine release into the perfusate from the chromaffin tissue was determined from the difference in catecholamine concentrations between the inflowing and outflowing perfusion media at each sample times. The total quantity of catecholamines secreted over the duration of the experiment was calculated by summing the amounts of adrenaline or noradrenaline (nmol) calculated in the perfusate for each time point.

Statistical Analysis

The data are presented as the mean ± 1 standard error of the mean (SEM). Where appropriate, the data were statistically analyzed by a one-way repeated measures ANOVA followed by Dunnett's multiple comparison test; if the normality test failed, an ANOVA on ranks was performed followed by Dunnett's multiple comparison. In other instances, the data were statistically analyzed by a one or two-way ANOVA followed by Dunnett's test for comparison with control 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; SPSS, Sigma).

RESULTS

Series 1: The effects of catecholamines on catecholamine release. This experimental series was designed to investigate the effects of locally elevated catecholamines on the basal secretion of catecholamines from the chromaffin tissue. Regardless of the mode of administration, delivery of exogenous catecholamines did not affect the basal secretion of either adrenaline or noradrenaline. Neither a bolus injection (Fig 7.1) nor continuous perfusion (data not shown) with saline containing high levels of adrenaline or noradrenaline altered the rate of catecholamine secretion. Although noradrenaline secretion appeared to increase initially in response to a bolus injection of noradrenaline, the rate of secretion was not statistically significant from the pre-value (Fig 7.1B).

Series 2: The effects of high concentrations of catecholamines on cholinergic and neuronally induced secretion of adrenaline and noradrenaline. In control preparations (perfused with saline containing no added catecholamine), a bolus injection of carbachol (10^{-6} mol kg^{-1}) caused a rapid increase in adrenaline and noradrenaline secretion (Fig 7.2A). Secretion was greatest in the first few minutes after carbachol addition, after which catecholamine secretion returned to baseline levels. In contrast, the addition of catecholamines to the perfusate abolished (using noradrenaline; Fig 7.2B) or reduced (using adrenaline; Fig 7.2C) the carbachol-induced increase in catecholamine secretion. Similarly, although neuronal stimulation of the chromaffin cells elicited significant increases in the rate of secretion of both catecholamines under all treatments, the presence of adrenaline or noradrenaline in the perfusion fluid significantly reduced the secretion of adrenaline (Fig 7.3); noradrenaline secretion was unaffected.

Figure 7.1 The effects of elevated concentrations of B) noradrenaline or C) adrenaline on basal catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Catecholamines or saline (control experiments; panel A) were administered to the preparation by rapid bolus injection. Values are mean ± 1 SEM.

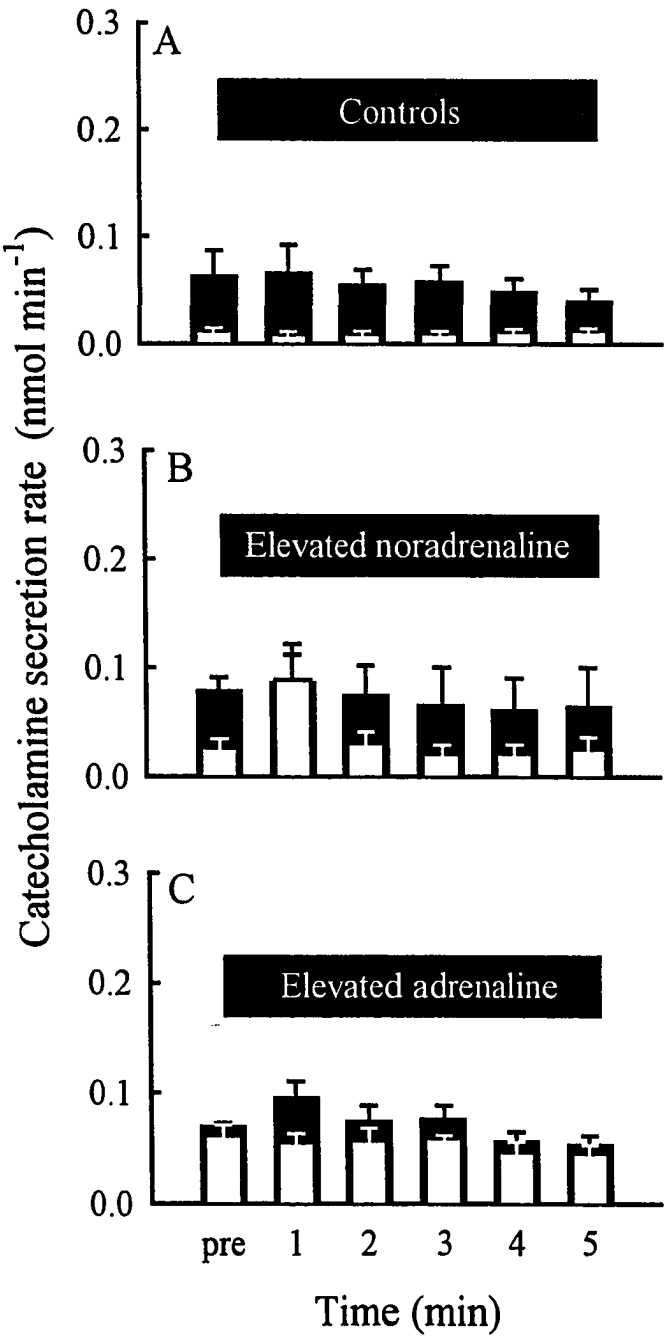

A
Controls
B
Elevated noradrenaline
C
Elevated adrenaline
0.3
0.2
0.1
0.0
Catecholamine secretion rate (nmol min-1)
pre
1
2
3
4
5
Time (min)

FIGURE 7.1

Figure 7.2 The effects of elevated catecholamine concentrations on carbachol (Cch)-evoked (10^{-6} mol kg^{-1}, final volume 0.3 ml) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Carbachol was administered to preparations perfused with A) control saline or with saline containing either B) noradrenaline (124 nM; N = 6) or adrenaline (330 nM; N = 6). An asterisk denotes a significant difference ($P < 0.05$) from the pre-stimulation (pre) value. A double dagger denotes a significant difference of both catecholamines (noradrenaline and adrenaline) from the corresponding control value (control, panel A; $P < 0.05$). A single dagger denotes a significant difference of adrenaline only from the corresponding control value (control, panel A; $P < 0.05$). Values are mean ± 1 SEM.

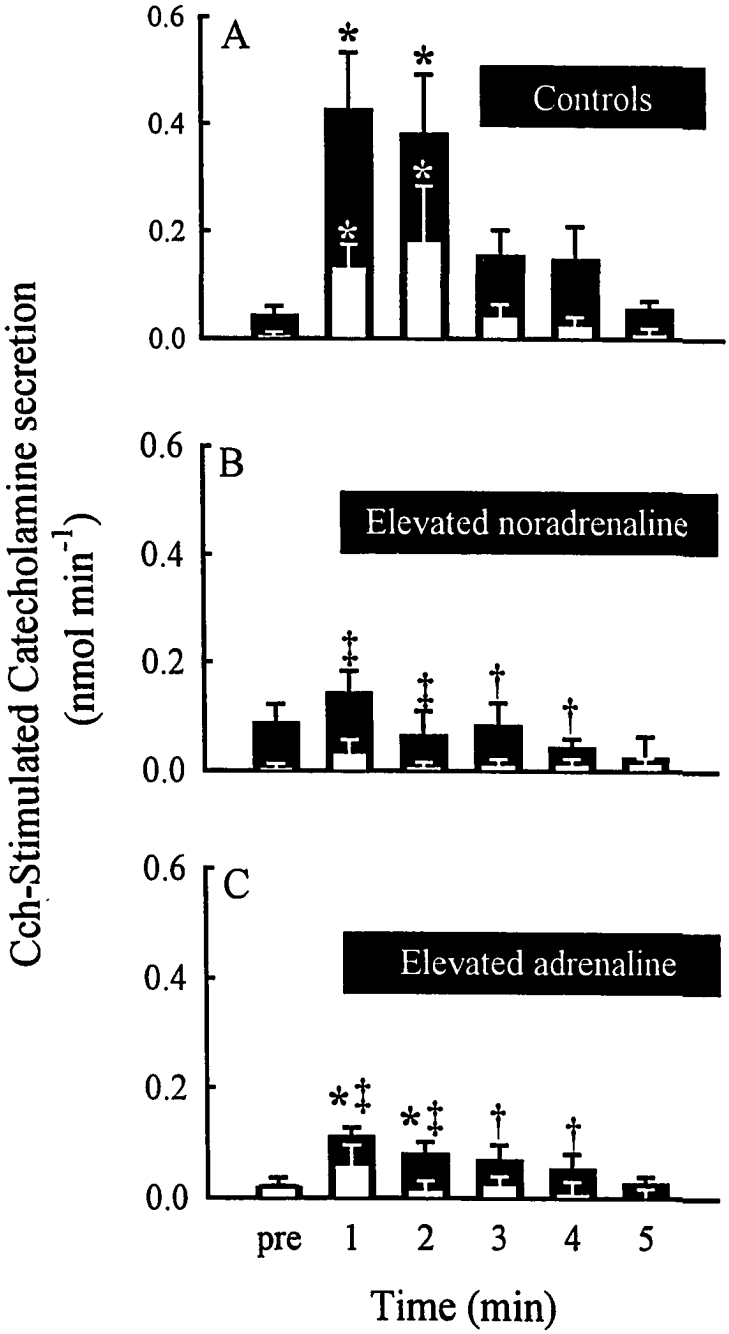

A
Controls
B
Elevated noradrenaline
C
Elevated adrenaline
0.6
0.4
0.2
0.0
pre
1
2
3
4
5
Time (min)
Cch-Stimulated Catecholamine secretion
(nmol min-1)

FIGURE 7.2

Figure 7.3 The effects of elevated catecholamine concentrations on neuronal (60 V, 20 pps, 0.1 msec, 30 sec duration) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Electrical stimulation was applied to preparations perfused with A) control saline or with saline containing either B) noradrenaline (124 nM; N = 6) or adrenaline (330 nM; N = 6). An asterisk denotes a significant difference ($P < 0.05$) from the pre-stimulation (pre) value. A double dagger denotes a significant difference of both catecholamines (noradrenaline and adrenaline) from the corresponding control value (control, panel A; $P < 0.05$). A single dagger denotes a significant difference of adrenaline only from the corresponding control value (control, panel A; $P < 0.05$). Values are mean ± 1 SEM.

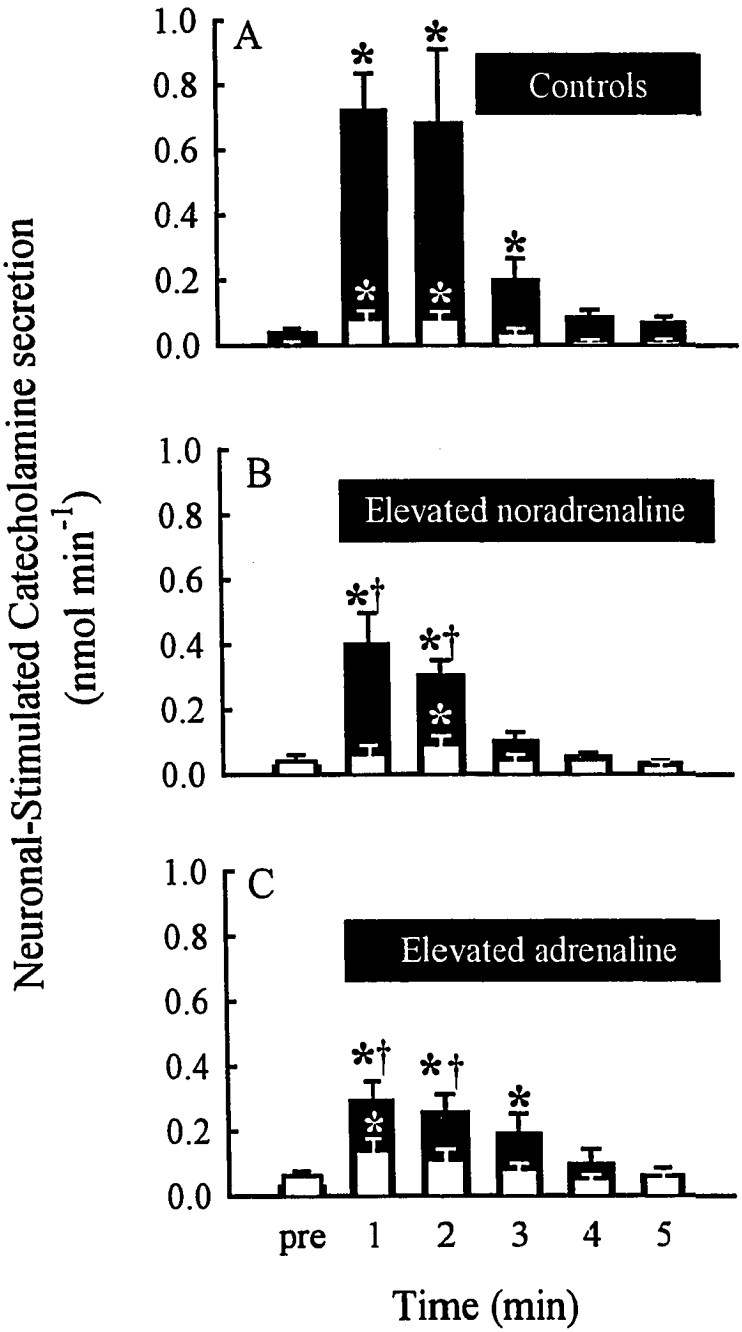

Neuronal-Stimulated Catecholamine secretion
(nmol min-1)
A
Controls
B
Elevated noradrenaline
C
Elevated adrenaline
1.0
0.8
0.6
0.4
0.2
0.0
pre
1
2
3
4
5
Time (min)

FIGURE 7.3

Series 3: The effects of adrenergic receptor agonists/antagonists on stimulus-evoked secretion of catecholamines. To characterize the effects of catecholamines on the cholinergic and neuronally evoked secretion of catecholamines, a bolus injection of carbachol (Figs. 7.4 & 7.6) or electrical stimulation of the nerves innervating the chromaffin cells (Figs. 7.5 & 7.7) was administered to preparations perfused with adrenergic receptor agonists or antagonists. In one group of experiments, total catecholamine secretion was compared in fish perfused with control saline or with saline containing the α-adrenergic receptor agonists phenylephrine (α_1), clonidine (α_2) or the α-adrenergic receptor antagonist phentolamine. In addition to having no effect on basal catecholamine secretion (data not shown), the presence of α-adrenergic receptor agonists or antagonists in the perfusion media did not affect the release of catecholamines in response to cholinergic stimulation (Fig. 7.4). However, adrenaline release in response to neuronal stimulation was significantly reduced in the presence of clonidine (Fig. 7.5A). An α-receptor mediated inhibition of neuronally evoked adrenaline release was confirmed in preparations perfused with phentolamine in which secretion was significantly enhanced (Fig. 7.5B).

In a separate group of experiments, preparations were pre-treated with β-adrenergic receptor agonists dobutamine (β_1) or salbutamol (β_2), or the β-adrenergic receptor antagonist, nadolol. While these treatments were without effects on basal catecholamine release (data not shown), the addition of salbutamol caused a significant reduction in the release of both catecholamines in response to cholinergic stimulation (carbachol), and reductions in adrenaline release only, during neuronal (electrical) stimulation (Figs. 7.6 & 7.7); pre-treatment with saline containing dobutamine did not

affect secretion. To confirm the β-adrenergic receptor mediated inhibition of stimulus-evoked catecholamine secretion, preparations were perfused with the β-receptor antagonist, nadolol. Blockade of β-receptors caused a significant increase in adrenaline release in response to carbachol (Fig. 7.6B) but not neuronal stimulation (Fig. 7.7B). Under all treatments, catecholamine secretion in response to carbachol or neuronal stimulation was significantly elevated over baseline levels (mean pre-stimulation value, N = 120, noradrenaline 0.02 ± 0.01 nmol min^{-1}; adrenaline 0.06 ± 0.02 nmol min^{-1}).

Series 4: The effects of cAMP on stimulus-evoked secretion of catecholamines. Bolus injection of carbachol elicited the secretion of both adrenaline and noradrenaline while ckVIP caused the secretion of adrenaline (Fig. 7.8A & 7.9A, respectively). Moreover, while pre-treatment with dibutyryl-cAMP reduced the secretion of adrenaline and noradrenaline in response to cholinergic stimulation (Fig. 7.8B), the ckVIP elicited secretion of adrenaline was unaffected by the compound (Fig. 7.9B)

Series 5. The effects β_2 receptor stimulation on catecholamine secretion elicited by VIP or angiotensin II. Bolus injection of angiotensin II elicited significant increases in the rate of secretion of both adrenaline and noradrenaline while VIP caused the secretion of adrenaline only. Furthermore, while pre-treatment with salbutamol had no effect on the VIP-induced secretion of adrenaline, the presence of the β_2-receptor agonist reduced the secretion of both adrenaline and noradrenaline in response to a bolus injection of angiotensin II (Fig 7.10).

Figure 7.4 The effects α-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by cholinergic stimulation (carbachol (Cch); 10^{-6} mol kg^{-1}, 0.3 ml) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Carbachol was administered to preparations (N = 6 for each group) that were perfused with control saline or saline containing the α-receptor agonists clonidine (α_2) or phenylephrine (α_1) or the general α-receptor antagonist phentolamine.

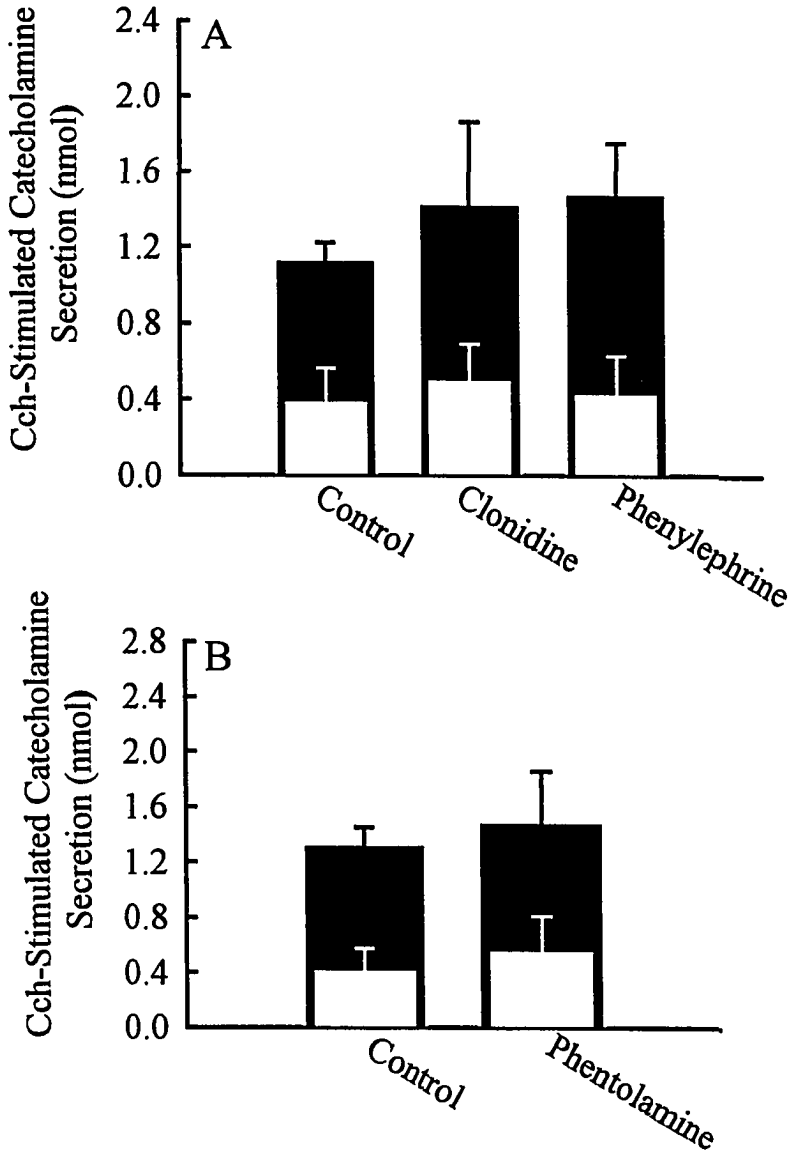

A
Cch-Stimulated Catecholamine Secretion (nmol)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Control
Clonidine
Phenylephrine
B
Cch-Stimulated Catecholamine Secretion (nmol)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Control
Phentolamine

FIGURE 7.4

Figure 7.5 The effects α-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by electrical neuronal stimulation (60 V, 20 pps, 0.1 msec, 30 sec duration) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Electrical stimulation was applied to preparations (N = 6 for each group) that were perfused with control saline or saline containing the α-receptor agonists clonidine (α_2) or phenylephrine (α_1) or the general α-receptor antagonist phentolamine. An asterisk denotes a significant difference ($P < 0.05$) from the control value.

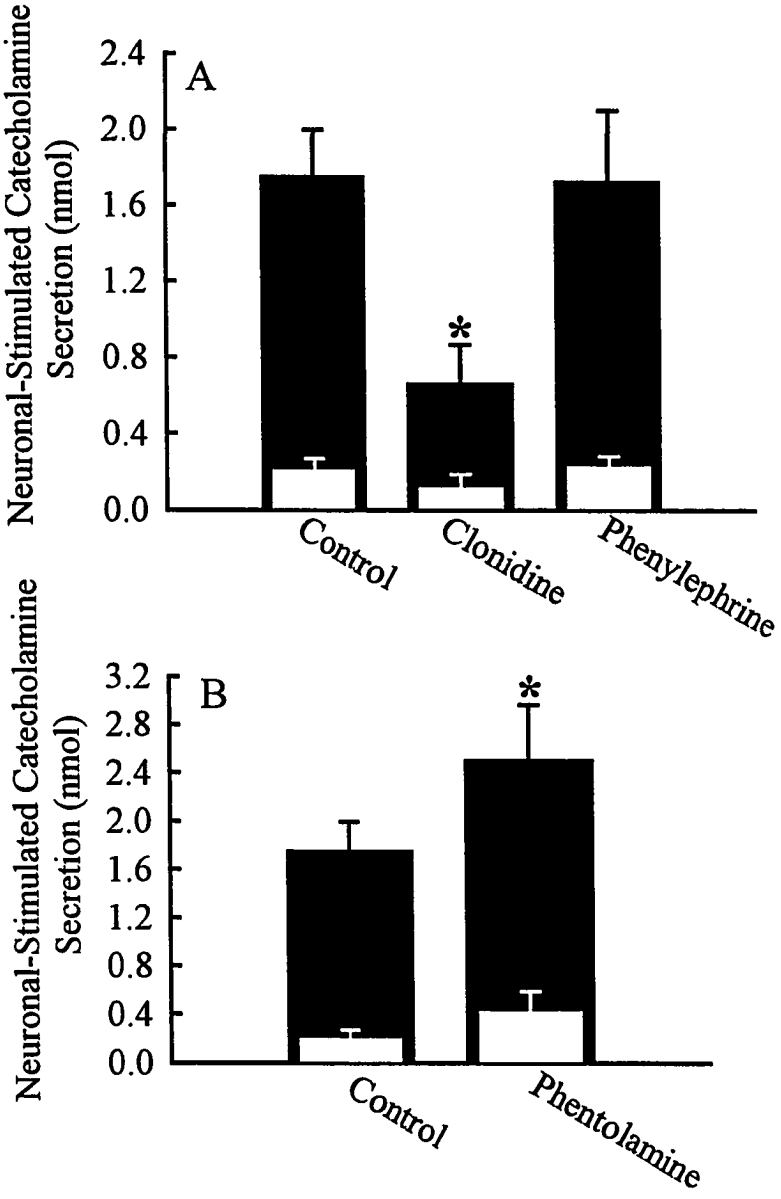

A
Neuronal-Stimulated Catecholamine Secretion (nmol)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
Control
Clonidine
Phenylephrine
B
Neuronal-Stimulated Catecholamine Secretion (nmol)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
Control
Phentolamine

FIGURE 7.5

Figure 7.6 The effects of β-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by cholinergic stimulation (carbachol (Cch); 10^{-6} mol kg^{-1}, 0.3 ml) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Carbachol was administered to preparations (N = 6 for each group) that were perfused with control saline or saline containing the β-receptor agonists salbutamol (β_2) or dobutamine (β_1) or the general β-receptor antagonist nadolol. An asterisk denotes a significant difference ($P < 0.05$) from the control value.

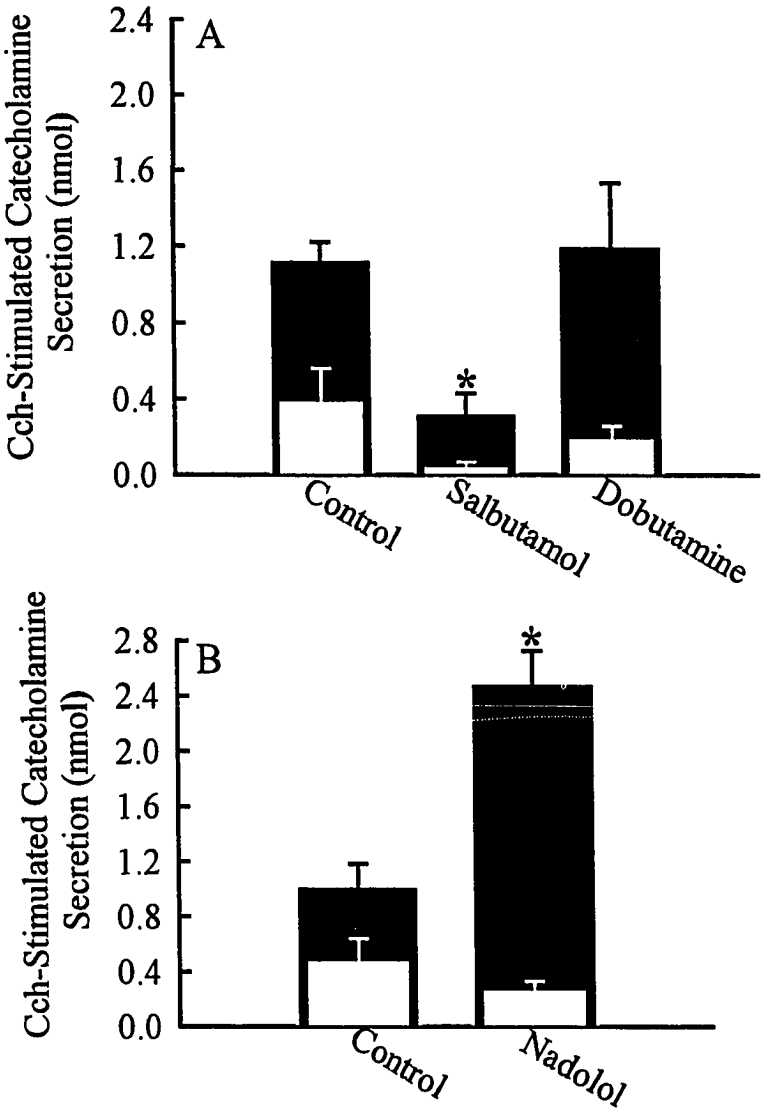

A
Cch-Stimulated Catecholamine Secretion (nmol)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
Control
Salbutamol
Dobutamine
B
Cch-Stimulated Catecholamine Secretion (nmol)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
Control
Nadolol

FIGURE 7.6

Figure 7.7 The effects of β-adrenergic receptor agonists (A) or antagonists (B) on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release evoked by electrical neuronal stimulation (60 V, 20 pps, 0.1 msec, 30 sec duration) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Electrical stimulation was applied to preparations (N = 6 for each group) that were perfused with control saline or saline containing the β-receptor agonists salbutamol (β_2) or dobutamine (β_1) or the general β-receptor antagonist nadolol. An asterisk denotes a significant difference ($P < 0.05$) from the control value.

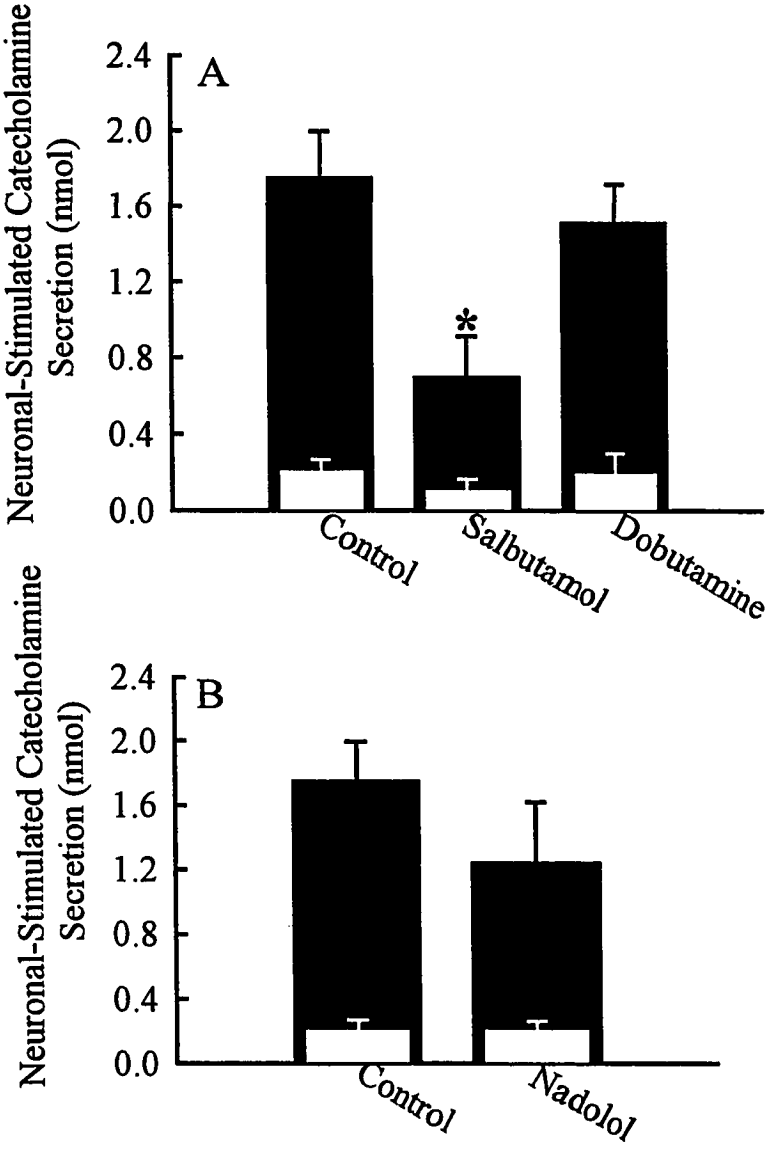

Neuronal-Stimulated Catecholamine Secretion (nmol)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
A
*
Control
Salbutamol
Dobutamine
Neuronal-Stimulated Catecholamine Secretion (nmol)
2.4
2.0
1.6
1.2
0.8
0.4
0.0
B
Control
Nadolol

FIGURE 7.7

Figure 7.8 The effects of elevated dibutyryl-cAMP concentrations on carbachol (Cch)-evoked (10^{-6} mol kg^{-1}, final volume 0.3 ml) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. Carbachol was administered to preparations perfused with A) control saline or B) with saline containing dibutyryl-cAMP (10^{-6} M; N = 6). An asterisk denotes a significant difference ($P < 0.05$) from the pre-stimulation (pre) value. A double dagger denotes a significant difference of both catecholamines (noradrenaline and adrenaline) from the corresponding control value (control, panel A; $P < 0.05$). Values are mean ± 1 SEM.

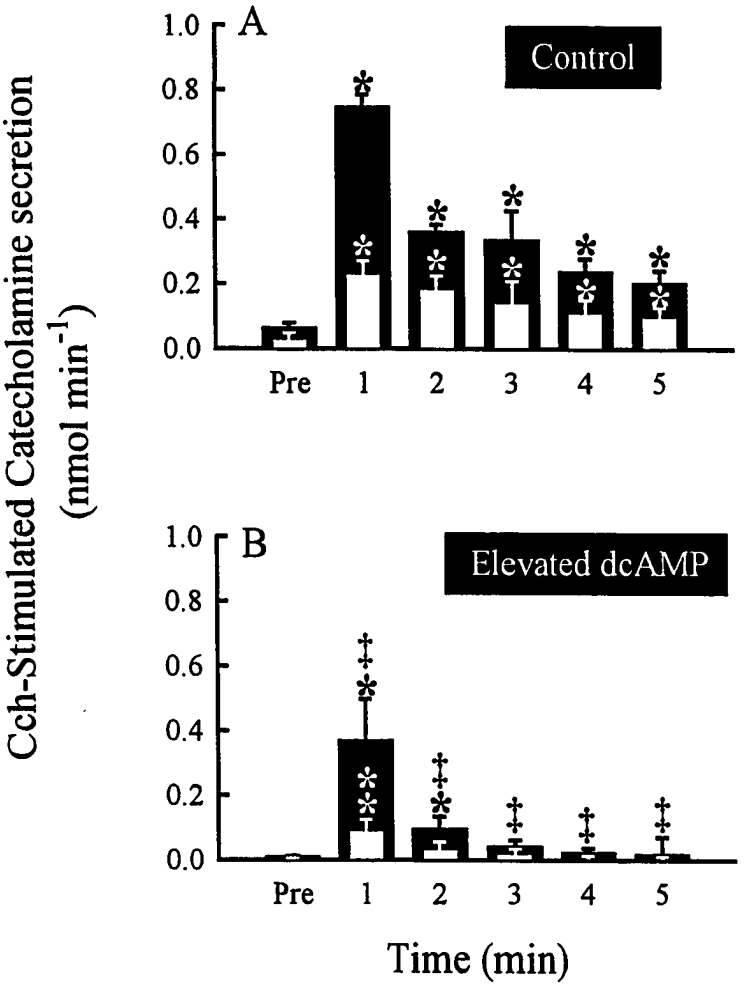

A
Control
B
Elevated dcAMP
1.0
0.8
0.6
0.4
0.2
0.0
Pre
1
2
3
4
5
Cch-Stimulated Catecholamine secretion
(nmol min-1)
Time (min)

FIGURE 7.8

Figure 7.9 The effects of elevated dibutyryl-cAMP concentrations on VIP-evoked (ckVIP; 10^{-9} mol kg^{-1}, final volume 0.3 ml) stimulation of catecholamine secretion (noradrenaline, *unfilled bars*; adrenaline, *filled bars*) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. ckVIP was administered to preparations perfused with A) control saline or B) with saline containing dibutyryl-cAMP (10^{-6} M; N = 6). An asterisk denotes a significant difference ($P < 0.05$) from the pre-stimulation (pre) value. Values are mean ± 1 SEM.

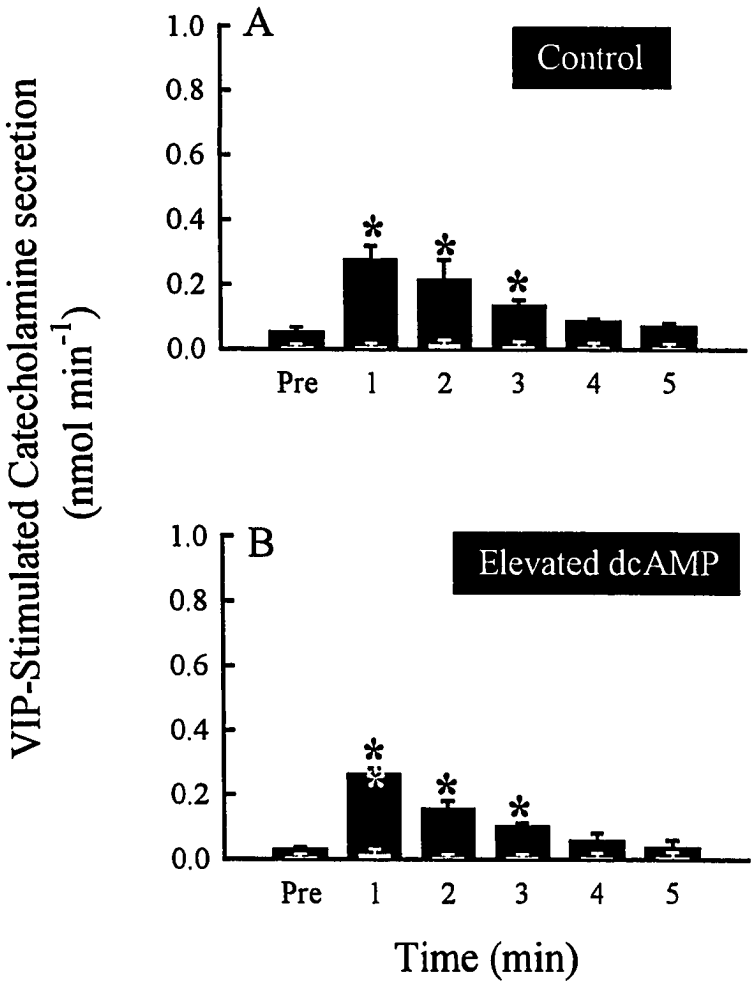

VIP-Stimulated Catecholamine secretion
(nmol min-1)
A
Control
1.0
0.8
0.6
0.4
0.2
0.0
Pre
1
2
3
4
5
B
Elevated dcAMP
Time (min)

FIGURE 7.9

Figure 7.10 The effects of the β_2-adrenergic receptor agonist salbutamol on total noradrenaline (*unfilled bars*) and adrenaline (*filled bars*) release in response to A) angiotensin II (ANG II; 10^{-9} mol kg^{-1}, 0.3 ml) or B) vasoactive intestinal polypeptide (VIP; 10^{-9} mol kg^{-1}, 0.3 ml) in *in situ* saline perfused posterior cardinal vein preparations of rainbow trout, *Oncorhynchus mykiss*. An asterisk denotes a significant difference ($P < 0.05$) from the control (no salbutamol) value.

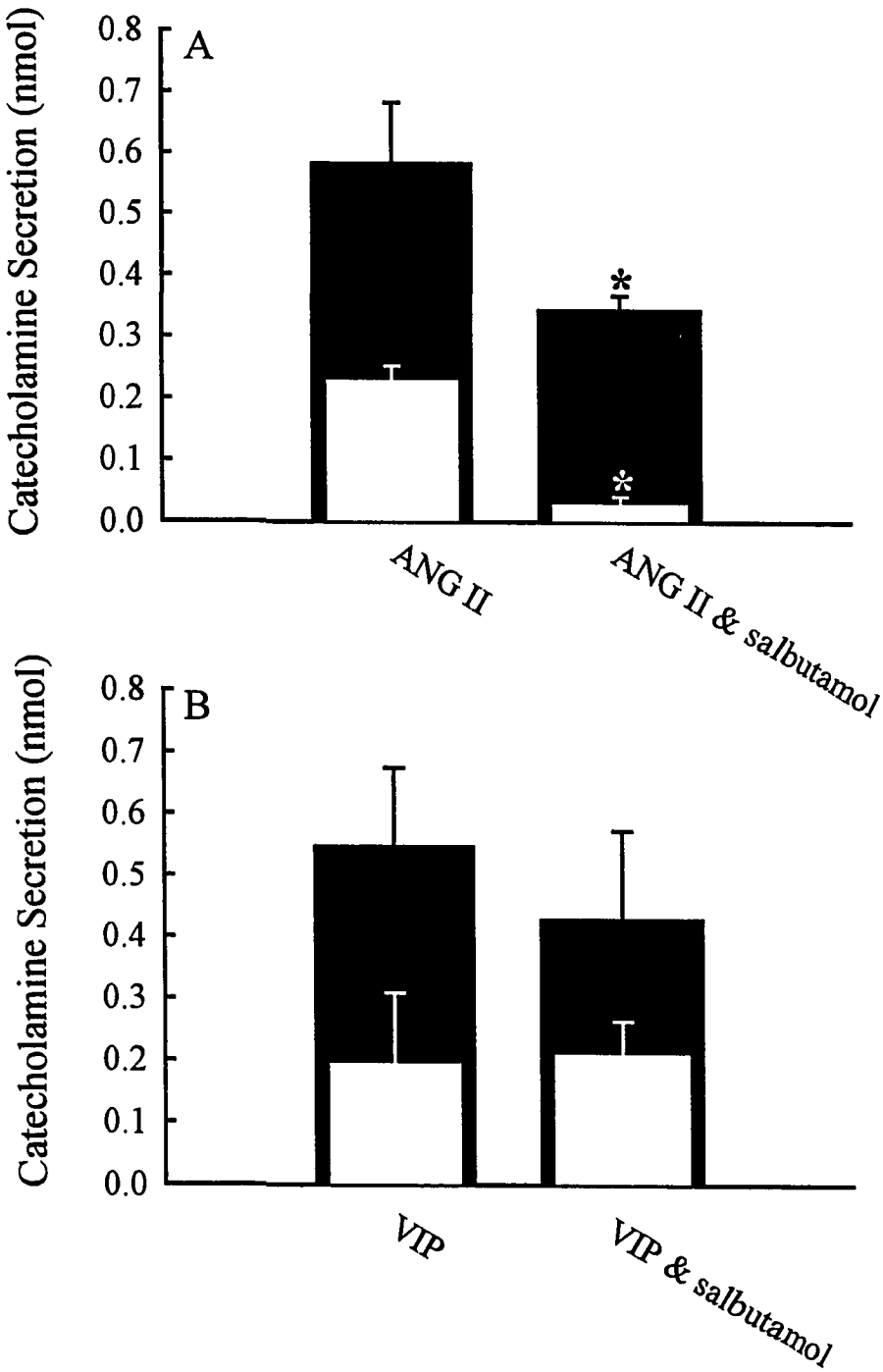

Catecholamine Secretion (nmol)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A
*
*
ANG II
ANG II & salbutamol
Catecholamine Secretion (nmol)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B
VIP
VIP & salbutamol

FIGURE 7.10

DISCUSSION

Effects of catecholamines on catecholamine release

Although previous studies have reported catecholaminotropic activity of catecholamines in the eel (*Anguilla rostrata*, Hathaway et al., 1989) and lamprey (*Petromyzon marinus*; Dashow and Epple, 1983), no such interactions were observed in this study. In agreement with former studies conducted *in vivo* in trout (Perry and Vermette, 1987) and *in situ* in Atlantic cod (*Gadus morhua;* Perry et al., 1991), the presence of high levels of adrenaline and noradrenaline did not affect the basal secretion of catecholamines from trout chromaffin cells. On the other hand, the results of the present study clearly demonstrate that stimulus-evoked secretion of adrenaline is highly sensitive to extracellular catecholamine levels. Specifically, in the presence of high concentrations of adrenaline or noradrenaline in the perfusion fluid, the cholinergic and neuronally evoked secretion of adrenaline was reduced or abolished. On the other hand, while the secretion of noradrenaline in response to carbachol was sensitive to extracellular catecholamine levels, elevated concentrations of noradrenaline appear to have little, if any, effect on noradrenaline secretion during neuronal stimulation. These findings reveal the presence of a negative feedback system whereby secreted catecholamines serve to inhibit additional catecholamine release. This adrenergic negative feedback regulation of catecholamine release was subsequently characterized using standard pharmacological techniques that employed classic α- and β-receptor agonists and antagonists.

The role of β-adrenergic receptors

The results of the pharmacological study support a physiological role for a β-adrenergic receptor system on trout chromaffin cells. The adrenergic inhibition of catecholamine release was mimicked by the β_2-adrenergic receptor agonist salbutamol. Indeed, adrenaline secretion in response to carbachol and neuronal stimulation was reduced in fish perfused with saline containing salbutamol. The significance of this system in regulating stimulus-evoked adrenaline secretion was further established using the β-receptor antagonist nadolol. Adrenaline secretion in response to cholinergic stimulation in preparations perfused with saline containing nadolol was significantly elevated in comparison to controls. This finding indicates that adrenaline secretion in trout may normally be inhibited in an autocrine/paracrine fashion by the action of the secreted catecholamines on chromaffin cell β_2 receptors. Typically, β_2 adrenergic receptor stimulation is associated with activation of adenylyl cyclase and increases in intracellular cAMP. Owing to the ability of cAMP to reduce catecholamine secretion in response to cholinergic stimulation, the β_2 adrenergic inhibitory mechanisms may involve cAMP dependent post-receptor events that affect exocytosis.

The lack of an effect of β-receptor blockade on catecholamine secretion following neuronal stimulation was unexpected, but may reflect the activation of other pathways during neuronal stimulation that are insensitive to adrenergic negative feedback. Reid et al. (1995) demonstrated the presence of several neurotransmitters and neuropeptides capable of regulating chromaffin cell activity during neuronal stimulation. Indeed, a possible candidate is VIP, a potent activator of catecholamine release in trout (Montpetit

and Perry, 2000) that is insensitive to autocrine/paracrine stimulation of chromaffin cell β_2-receptors (see below).

Attempts to demonstrate a role for a β-adrenergic receptor mediated regulation of catecholamine secretion from mammalian chromaffin cells have yielded mixed results. While a number of studies were unable to demonstrate an involvement of β-adrenergic receptors using the β-receptor blocker propranolol (Wakade 1981: Collett and Story, 1984), others have suggested a stimulatory role (Boonyaviroj and Gutman, 1979; Greenberg and Zinder, 1982). In the latter studies, catecholaminotropic activity of catecholamines via activation of β-receptors was established using isoproterenol (non-specific β-agonist) and salbutamol (β_2-agonist). Moreover, treatment with β-receptor antagonists (propranolol or H35/25) caused a reduction in the cholinergic evoked secretion of catecholamines (Boonyaviroj and Gutman, 1979; Greenberg and Zinder, 1982). In agreement with those studies, a β-stimulatory pathway also has been suggested to operate in eels (Al-Kharrat et al., 1997; Abele et al., 1998). Thus, to our knowledge, this is the first study to report a β_2-receptor mediated inhibition of catecholamine secretion from chromaffin cells of any vertebrate species.

The role of α-adrenergic receptors

An inhibitory role for presynaptic α_2-adrenergic receptors that regulate neurotransmitter release is well established in the central and peripheral nervous systems (Holmgren and Nilsson, 1982). Given their relatedness to adrenergic neurons, investigators have suggested that adrenomedullary chromaffin cells may be regulated in a similar fashion. Studies conducted on mammalian adrenal glands and isolated chromaffin cells have indeed shown that α-receptors are able to regulate secretion during cholinergic

stimulation (Boonyaviroj and Gutman, 1979; Cohen et al., 1980; Greenberg and Zinder, 1982; Wada et al., 1982).

In the present study, the addition of α_2-adrenergic receptor agonists or antagonist to the perfusate did not modify catecholamine secretion in response to a bolus injection of carbachol. These results suggest that chromaffin cell membrane α-adrenergic receptors, if present in trout, do not play a role in the regulation of catecholamine release. In contrast, adrenaline secretion in response to neuronal stimulation was significantly reduced in the presence of the α_2-receptor antagonist clonidine. Given that clonidine was without effect on carbachol-evoked secretion, these results suggest that the inhibitory action of clonidine on neuronally-evoked adrenaline secretion results from pre-synaptic adrenergic regulation of neurotransmitter release from the preganglionic nerves innervating the chromaffin cells. The physiological significance of this mechanism was demonstrated by showing that pre-treatment with the α-adrenergic receptor antagonist phentolamine significantly enhanced adrenaline secretion in response to neuronal stimulation. Therefore, in addition to β_2-receptors, the adrenergic control of catecholamine secretion from trout chromaffin cells includes activation of inhibitory α_2-adrenoceptors located pre-synaptically. While the results demonstrate profound effects of adrenergic agonists and antagonists on adrenaline secretion, changes in adrenaline secretion were not always associated with changes in noradrenaline secretion. Thus, while both catecholamines have the ability to influence secretion, the system appears to be geared towards the regulation of adrenaline secretion.

Adrenergic regulation of non-cholinergic secretagogues

In teleosts, the control of catecholamine secretion from chromaffin cells involves numerous physiological stimuli including stimulation by preganglionic sympathetic nerve fibers through non-cholinergic (VIP) and cholinergic (Ach) neurotransmitters (Reid et al., 1995; Montpetit and Perry, 1999, 2000), and activation of the renin-angiotensin system (Bernier et al., 1999b). In agreement with previous studies, a bolus injection of VIP caused a significant elevation of adrenaline secretion (Montpetit and Perry, 2000), while angiotensin II elicited the secretion of both adrenaline and noradrenaline (Bernier et al., 1997, 1999a,b). Subsequent experiments were designed to determine whether the catecholaminergic negative feedback mechanism observed for cholinergic and neuronally evoked secretion also was operative for secretion elicited by non-cholinergic substances. The results clearly revealed that catecholamine secretion elicited by angiotensin II was subject to negative feedback control whereas VIP-elicited secretion was not.

The precise mechanisms underlying the adrenergic inhibition of catecholamine release from chromaffin cells in response to carbachol, neuronal stimulation, and angiotensin II (but not VIP) are unknown but may reflect differences in calcium signaling pathways. An elevation in intracellular Ca^{2+} is a prerequisite for catecholamine secretion (Burgoyne et al., 1993; Furimsky et al., 1996) but the source of this elevation may differ depending on the specific secretagogue. In rainbow trout, external Ca^{2+} is required to initiate catecholamine secretion in response to carbachol (Furimsky et al., 1996), and angiotensin II (Bernier and Perry, 1997). Although similar studies have not been performed in fish, VIP receptor stimulation in mammalian chromaffin cells is associated with an elevation of IP_3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol) leading to

an increase in intracellular Ca^{2+} predominantly from intracellular stores (Malhotra et al., 1989; Isobe et al., 1993; Przywara et al., 1996; Tanaka et al., 1996). Therefore, the link between the β_2-adrenergic receptor activation and catecholamine release may involve specific regulation of Ca^{2+} signaling from external sources. Indeed, a recent study performed using rat chromaffin cells has demonstrated an autocrine negative modulation of L-type Ca^{2+} channels via adrenergic receptors, representing a possible mechanism regulating Ca^{2+}-dependent exocytosis through voltage-gated channels (Hernandez-Guijo et al., 1999). Moreover, other studies have demonstrated an inhibitory adrenergic regulation of Na^{+} influx thereby affecting the extent of cell depolarization and catecholamine release (Gutman and Boonyaviroj, 1977; Wada et al., 1985). Because L-type channels (Ca^{+2} voltage-gated channels) contribute over 50% of the total Ca^{2+} current, these mechanisms may form the basis of the adrenergic autocrine/paracrine control of catecholamine secretion.

CHAPTER 8

GENERAL DISCUSSION

SUMMARY OF PRINCIPLE FINDINGS

CHAPTER 2: A whole body field stimulation protocol, able to specifically activate nerve fibres innervating the chromaffin cells to elicit secretion of catecholamines, was developed and validated.

CHAPTER 3: Cholinergic stimulation of catecholamine release involves both muscarinic and nicotinic cholinergic receptors. Muscarinic cholinergic stimulation indirectly contributes by enhancing the nicotinic-evoked secretion of catecholamines. Under intense stimulation, muscarinic receptors may directly cause secretion.

CHAPTER 4: VIP and PACAP can elicit the release of adrenaline (but not noradrenaline) from the chromaffin tissue of the rainbow trout, via activation of VPAC type receptors. During neuronal excitation of catecholamine release, the contribution of cholinergic stimulation predominates during high levels of neuronal activity, while the non-cholinergic component (VIP/PACAP) participates at low levels of activity.

CHAPTER 5: VIP and PACAP can elicit catecholamine release from the chromaffin tissue via specific VIP binding sites that exhibit properties of VPAC type receptors. Although VIP binding sites are observed on both noradrenaline- and adrenaline-chromaffin cell types, the effects of VIP and PACAP appear to be targeted towards the secretion of adrenaline via post-receptor events.

CHAPTER 6: Three putative cDNAs corresponding to $VPAC_1$, $VPAC_2$, and PAC_1 were cloned and sequenced from trout brain cDNA. Sequence alignments indicate that they are highly homologous to other vertebrate $VPAC_1$, $VPAC_2$, and PAC_1

receptors. Tissue distributions implicate roles for these receptors in the brain and various peripheral organs.

CHAPTER 7: High levels of catecholamines negatively impact the ability of chromaffin cells to secrete additional catecholamines in response to cholinergic stimulation but not VIP. Mechanisms of this adrenergic inhibition of catecholamine secretion include activation of chromaffin cell membrane β_2-receptors and presynaptic α_2-adrenergic receptors.

DISCUSSION

Part I. *In situ* saline perfused posterior cardinal vein preparation

Although a role for the neuronal control of catecholamine release in fish was previously established *in vivo*, the identification of the participating neurotransmitters and receptors has largely been accomplished using *in situ* perfused (e.g. Perry et al., 1991; Fritsche et al., 1993; Al-Kharrat et al., 1997) or perifused (e.g.; Gfell et al., 1997; Abele et al. 1998) PCV preparations. *In situ* saline perfused PCV preparations have been used extensively to study the regulation of catecholamine secretion from chromaffin tissue in agnathans (e.g. Bernier and Perry, 1996), elasmobranchs (Abrahamsson 1979; Montpetit et al., 2001), and teleosts (e.g. Nilsson et al., 1976; Reid et al., 1996; Abele et al., 1998; McKendry et al., 1999; Montpetit and Perry, 1999 & 2000). The results of validation experiments demonstrate that the chromaffin tissue that line the walls of the PCV respond to pharmacological and neuronal manipulations (Chapter 2) in a manner expected based on available information regarding catecholamine secretion from chromaffin cells in higher vertebrates. Indeed, this preparation has become an important tool with which to examine various aspects (neuronal and hormonal) of catecholamine release from chromaffin cells in fish.

Part II. Neuronal Control of Catecholamine Release

Cholinergic control-Acetylcholine

Previously, the cholinergic regulation of catecholamine release from trout chromaffin cells was considered to be mediated exclusively through nicotinic receptors. The results of recent investigations, however, have provided indirect evidence that muscarinic receptors may also be involved in the cholinergic control of catecholamine

release in fish (Fritsche et al., 1993; Furimsky et al., 1996). Therefore, the focus of Chapter 3 was to examine whether or not muscarinic receptors participated in the cholinergic control of catecholamine secretion from the chromaffin cells in rainbow trout. This was accomplished using nicotinic and muscarinic receptor agonists, in addition to the mixed nicotinic/muscarinic receptor agonist carbachol.

A role for muscarinic receptors during neuronal stimulation was demonstrated by the ability of atropine (muscarinic receptor antagonist) to reduce catecholamine secretion in response to electrical excitation of the preganglionic nerves innervating the chromaffin cells. On the other hand, the ability of atropine to specifically inhibit the carbachol-elicited secretion and hexamethonium to prevent it, suggests a positive modulatory role for muscarinic receptors. A modulatory role for muscarinic receptors is further supported with the finding that catecholamine secretion was enhanced following a bolus injection of nicotine in combination with oxotremorine, when compared to the secretion elicited by either agonist when administered independently.

An involvement of muscarinic receptors in the cholinergic regulation of catecholamine secretion from piscine chromaffin cells is not without precedent. As in trout (Chapter 3), muscarinic receptor agonists (muscarine and pilocarpine) elicited catecholamine secretion from perifused head kidney preparations of the common carp (*Cyprinus carpio*) (Gfell et al. 1997). While Reid and Perry (1995) were unable to demonstrate any involvement of muscarinic receptors in eels, other studies have documented an inhibitory role on basal secretion (Al-Kharrat et al. 1997), or a positive modulatory role during electrical stimulation of the sympathetic fibres (Abele et al., 1998). Despite the variable responses elicited through muscarinic cholinergic stimulation,

overall, muscarinic receptors appear to play either a direct or positive modulatory role during neuronal stimulation of catecholamine release in fish.

The precise mechanisms underlying the initiation of catecholamine secretion by muscarinic receptor stimulation of trout chromaffin cells are not known. However, owing to the specific requirement of Ca^{2+} in the secretory process (Burgoyne et al. 1993; Furimsky et al. 1996), pathways for increasing intracellular Ca^{2+} are likely responsible. Activation of muscarinic receptors is generally considered to be associated with increases in the intracellular concentration of calcium through the liberation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). However, the rise in intracellular calcium following muscarinic receptor stimulation might not be sufficient, in itself, to trigger exocytosis. Owing to the positive modulatory influences of muscarinic receptor activation, the rise in intracellular calcium may, however, be sufficient to augment catecholamine secretion in response to other stimuli.

In summary, the results of several experimental approaches support a role for muscarinic receptors (in addition to nicotinic receptors) in the overall cholinergic regulation of catecholamine release from trout chromaffin cells. Given the presence of different cholinergic pathways (muscarinic and nicotinic receptors) regulating the process of catecholamine release, one may speculate that chromaffin cells of trout exhibit a degree of flexibility with respect to their responses to acetylcholine released from the preganglionic nerves. Perhaps the presence of two types of cholinergic receptors capable of eliciting catecholamine release may allow chromaffin cells to continue to release catecholamines in the event of impairment (e.g. down-regulation, desensitization) of one type (e.g. nicotinic receptors) of cholinergic receptor.

Non-cholinergic control- VIP and PACAP

An overview of the principle findings from this thesis suggests that the neuronal control of catecholamine secretion via the sympathetic preganglionic innervation of the chromaffin tissue is not confined to cholinergic evoked events. If acetylcholine were the only neurotransmitter released from the preganglionic nerves that innervate the chromaffin tissue, one would expect a complete inhibition of the secretion of catecholamines after blockade of cholinergic receptors. However, unlike the situation with carbachol, the neuronally evoked secretion of catecholamines was not totally prevented by a combination of hexamethonium and atropine. Indeed, the results of Chapter 4 indicate that the neuropeptides VIP and/or PACAP may act as neurotransmitters, along with acetylcholine, in the neuronal control of chromaffin cell activity in fish. Several lines of evidence argue for a neurotransmitter role of these peptides.

First, VIP and PACAP immunoreactive nerve fibres have been demonstrated in the vicinity of chromaffin cells that line the walls of the posterior cardinal vein of various species of fish including rainbow trout (Reid et al., 1995). Similar histological evidence has been demonstrated in adrenal medullary synapses of higher vertebrates (Wakade et al., 1991). Second, VIP and PACAP exert direct stimulatory effects on the chromaffin cells of trout. Third, the stimulatory effects of VIP, PACAP, and neuronal stimulation could be antagonized by the VPAC receptor antagonist, VIP6-28. Fourth, although Ach is believed to be the main neurotransmitter released from the preganglionic nerves, the effect of neuronal stimulation on catecholamine release could not be abolished by treatment with atropine and hexamethonium. The non-cholinergic portion of the secretion

was not due to inadequate antagonism of the cholinergic receptors because catecholamine secretion in response to carbachol was totally prevented by atropine and hexamethonium (Chapter 3). It is clear from the results of **Chapter 4** that the stimulatory effects of VIP and PACAP occur independently of cholinergic receptors. Indeed, specific VIP binding sites exhibiting properties of VPAC receptors were detected on the chromaffin cells that line the walls of the posterior cardinal vein in trout. Accordingly, VIP and/or PACAP can be regarded as neurotransmitters released upon activation of the preganglionic fibres and are likely to play a key role in the secretion of catecholamines from the chromaffin tissue of trout. Given that VIP and PACAP immunoreactive nerve fibres have been demonstrated in the vicinity of the chromaffin cells of other fish, including cod (*Gadus morhua*), European eel (*Anguilla anguilla*), and spiny dogfish (*Squalus acanthias*) (Reid et al., 1995), it is reasonable to speculate a neurotransmitter role for the peptides in the neuronal regulation of catecholamine release in these fish, if not all fish.

In mammals, VIP and PACAP activate two separate signaling pathways, including the adenylyl cyclase-cAMP pathway and the phosphoinositide-phospholipase C pathway (Ishihara et al., 1992; Lutz et al., 1993; Spengler et al., 1993). It is believed that the direct stimulatory actions of VIP and PACAP are mediated by increases of intracellular concentration of Ca^{2+} through the liberation of IP_3 (Malhotra et al., 1989; Isobe et al., 1993; Przywara et al., 1996; Tanaka et al., 1996). The demonstration that VIP elicited the secretion of adrenaline from *in situ* preparations perfused with Ca^{2+} -free saline (**Chapter 4**) is consistent with this hypothesis.

VPAC and PAC_1 receptors

To characterize the receptors that mediate the stimulatory effects of VIP and PACAP on catecholamine release from trout chromaffin cells, several pharmacological experiments were designed that relied on receptor antagonism and affinity parameters. Exogenous administration of a range of doses of VIP and PACAP to *in situ* perfused PCV preparations of rainbow trout 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, adrenaline secretion in response to VIP and PACAP, or neuronal stimulation (low frequency) was inhibited in the presence of VPAC receptor antagonist. Moreover, labeling of VIP-binding sites was prevented in tissues pre-treated with unlabelled VIP, VPAC receptor antagonist, and PACAP, but not PAC_1 receptor antagonist. Together, these results argue for a role of VPAC receptors regulating chromaffin cell activity in the rainbow trout.

An involvement of VPAC receptors in the control of catecholamine release in trout is in marked contrast to observations reported for amphibian and mammalian species. 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 characterized (Chow 1997; Chow et al., 1997; Wong et al., 1998). In this thesis, three partial clones exhibiting properties of the $VPAC_1$, $VPAC_2$, and PAC_1 receptors were obtained from trout brain. It would appear then, like higher vertebrates, trout possess all three receptors. Although pharmacological characterizations of the encoded proteins have not been performed, it is highly likely that they would

respond, like the goldfish receptors, in a typical fashion based on the similar pharmacological characteristics displayed by each receptor, respectively, in different vertebrate species.

Tissue distribution experiments using RT-PCR demonstrated the presence of $VPAC_1$ receptors, but not $VPAC_2$, in the PCV tissue (unpublished data, Montpetit and Perry, 2002). These preliminary results suggest that in trout, the chromaffin cell VIP receptors mediating adrenaline secretion are of the $VPAC_1$ subtype. However, the results of the RT-PCR experiments cannot confirm with certainty the source of cells expressing the $VPAC_1$ mRNA. Unlike mammals, fish chromaffin cells are not organized into a distinct gland. Indeed, the heterogeneous populations of cells within the posterior cardinal vein has made it difficult to isolate pure populations of chromaffin cells from the tissue. Therefore, detection of these receptors specifically on chromaffin cells at the PCR or Northern levels could not be performed. As such, results of the RT-PCR analysis can only provide hypotheses concerning its presence in chromaffin cells. By isolating partial clones for the trout $VPAC_1$, $VPAC_2$, and PAC_1 receptors, however, I have provided direction for future investigations into the VIP and PACAP system and the neuronal control of catecholamine release in fish.

Relative contribution of cholinergic and non-cholinergic neurotransmitters

Overall, the neuronal regulation of catecholamine release from trout chromaffin cells appears to be mediated by **increased stimulation** via preganglionic nerves. It would seem that repetitive stimulation above a certain threshold is necessary to yield a sufficient total output to trigger post-synaptic responses. Moreover, the relative rates of secretion of

cholinergic and non-cholinergic neurotransmitters are partially dependent on the frequency of neuronal action potentials. Specifically, cholinergic stimulation, although participating at low neuronal activities, predominated during high levels of stimulation frequencies. In contrast, the secretion of non-cholinergic neurotransmitters is favored during low frequency nerve activity.

If more than one type of neurotransmitter is present within the neurons of the preganglionic nerve fibres, then the release of each type seems to be controlled by the rate of impulse traffic. The present observation raises a question of considerable interest regarding the storage and release of different neurotransmitters in the presynaptic neurons. One possibility is that each neurotransmitter (Ach, VIP and/or PACAP) is stored within its own vesicles that exhibit different kinetics of release. There is evidence that the magnitude of entry of calcium into nerve terminals is related to the frequency of stimulation (Kirpekar et al., 1975). Thus, at low frequency the amount of calcium entering the nerve would be low and only a certain population of vesicles with high affinity for calcium would be released, presumably VIP/PACAP. At high rates of stimulation, other vesicles requiring higher levels of calcium would enter the exocytotic process, as such Ach. Alternately, the presynaptic fibres may contain populations of different neurons expressing different types of neurotransmitters. These neurons may have different thresholds for firing and conduction of action potentials, which may account for the differential release of neurotransmitters and consequently the secretion of adrenaline and noradrenaline. Whether or not VIP/PACAP are co-stored with acetylcholine and the exact pattern of their release is unknown. Furthermore, whether repetitive stimulation at different frequencies represents the true nature of the differential

regulation of catecholamine release during neuronal stimulation remains to be established. An additional consideration is that neuronal traffic may occur in bursts rather than fixed rates of impulses.

Because the actual release of the neurotransmitters was not measured in this thesis, the precise neuropeptide released during low frequency neuronal stimulation is not known. Given that catecholamine secretion in response to low neuronal activity is sensitive to VPAC receptor antagonism, it is safe to speculate the participation of VIP and/or PACAP. However, to conclusively determine the relative involvement of VIP and PACAP, additional work is needed to identify which peptides are secreted from the preganglionic nerve terminals during electrical stimulation of these nerves.

Part III. Adrenaline versus noradrenaline secretion

The secretory profiles in response to cholinergic and non-cholinergic stimulation differed substantially. While cholinergic stimulation was associated with the secretion of adrenaline and noradrenaline, the secretory profile following VIP/PACAP stimulation include only adrenaline. Originally, the ability of the chromaffin cells to secrete noradrenaline and adrenaline was believed to be due, in large part, to the differential regulation of adrenaline- versus noradrenaline- subtypes of chromaffin cells. While the chromaffin cell fraction of the posterior cardinal vein contains predominantly "adrenaline-containing chromaffin cells", mechanisms that regulate the secretion of catecholamines are more complex than simple stimulation of a single chromaffin cell subtype (adrenaline versus noradrenaline-containing chromaffin cells).

The ability of VIP and PACAP to stimulate the secretion of adrenaline, but not noradrenaline, has previously been reported in rats (Guo and Wakade, 1994). Thus far, there is no explanation for the preferential release of adrenaline in response to these peptides. With the observation of VIP binding sites on adrenaline and noradrenaline chromaffin cell subtypes, one would predict the ability of VIP and PACAP to elicit the secretion of both catecholamines despite the source of secretion. Therefore, post-receptor events capable of discriminating between releasable pools of adrenaline and noradrenaline granules from adrenaline-cells, and for that matter noradrenaline granules from noradrenaline-cells, must be responsible for the preferential release of adrenaline.

Although the exact mechanism by which exocytosis occurs is unknown, a rise in intracellular calcium is a necessary prerequisite for catecholamine release to occur. Rearrangement of the cytoskeleton is considered to be a key event in the process of exocytosis (Burgoyne 1991). Under normal conditions, a network of actin filaments separates catecholamine-containing granules from the plasma membrane preventing release from occurring. The influx of calcium ions, either from extracellular or intracellular sources, causes the actin network to disassemble allowing for secretory vesicles to move to the plasma membrane, fuse, and release their contents. Microtubules may also play a role in the translocation of secretory granules to the plasma membrane. As such, any of these steps may possibly be regulated to allow for the differential release of catecholamines. In addition to stimulating the liberation of IP_3 and subsequently intracellular calcium, VIP and PACAP stimulation also causes an increase in cAMP (Vaudry et al., 2000). To date, a role for cAMP in the regulation of catecholamine secretion, in trout, as in other vertebrates, has not been established.

In general, cholinergic stimulation elicits a larger secretion of catecholamines than VIP/PACAP. Although post-receptor events may explain some of the differences in the secreted catecholamines (adrenaline and/or noradrenaline), other factors clearly are involved to account for the differences in the levels of the secreted catecholamines. Possible explanations for this phenomenon could include a greater number of cholinergic receptors on the chromaffin cells. Owing to the large distribution of VIP-binding sites on the chromaffin cells lining the walls of the PCV, this may not be the case. Possibly, the differences may be due in large part to the recruited pathways that are responsible for the increase in intracellular calcium and exocytosis. Unlike nicotinic cholinergic stimulation, activation of VIP and PACAP receptor does not appear to recruit calcium from extracellular sources through cell depolarization events. Therefore, like muscarinic receptor stimulation, the ensuing increase of intracellular calcium levels may not reach the same levels as achieved with nicotinic receptor stimulation, and thus not allow for similar secretion levels.

Part IV. Adrenergic regulation of catecholamine release

In general, an elevation of plasma catecholamine levels is considered to be a response to acute stress with catecholamine release occurring almost immediately upon exposure to the stressful condition. In addition to the effects on several peripheral tissues and organs, high levels of catecholamines flowing over the chromaffin cells may serve to inhibit the release of additional catecholamine in trout. The results of Chapter 7 clearly demonstrate that **stimulus-evoked (neuronal and carbachol) secretion** of catecholamines is highly sensitive to extracellular catecholamine levels. The results of

several pharmacological approaches revealed the possible involvement of β_2-adrenergic receptors on the cell surface of chromaffin cells directly controlling the release of catecholamines, and a role for α_2-receptors controlling presynaptic release of the neurotransmitter acetylcholine, and in doing so, the release of catecholamines.

Upon stimulation, the nicotinic cholinergic receptor of trout chromaffin cells undergoes rapid desensitization (Lapner et al., 2000). While the re-sensitization process is gradual (as long as 40 min in trout), nicotinic receptor desensitization, if prolonged, could potentially impair catecholamine release in individuals experiencing extended and repeated bouts of acute stress (Lapner et al., 2000). As such, secreted catecholamines may protect against cholinergic receptor desensitization by negatively regulating the release of acetylcholine from the preganglionic nerve terminals and therefore interactions with cholinergic receptors thus allowing for a more rapid re-sensitization period. Or perhaps, the negative feedback pathways, by both the β_2-receptors on the chromaffin cells and α_2-receptors on the presynaptic nerve terminal may serve as catecholamine release termination factors. Indeed, in trout, adrenergic feedback mechanisms may prevent the secretion of catecholamines to levels that may become pathological.

Unlike in eels (Epple and Nibbio, 1985) and lampreys (Dashow and Epple, 1983), there is no evidence that catecholamines can elicit catecholamine release in trout (so-called catecholaminotropic effects). While adrenaline and noradrenaline could elicit the secretion of the other catecholamine in eels (Epple and Nibbio, 1985; Hathaway et al., 1989), they did not stimulate the secretion of adrenaline or noradrenaline from trout chromaffin cells. Adrenergic stress responses and elevation of plasma catecholamine levels are most likely of greater importance in the trout than the eel. Whereas trout

release catecholamines in the 150-200 nM ranges in response to bouts of acute stresses (air exposure, hypoxia) (Perry et al., 1991; Perry and Reid, 1992; Perry and Reid, 1994; Reid and Perry, 1994), catecholamine release in eels reaches approximately 10 nM following similar stresses (Perry and Reid, 1992; Reid and Perry, 1994). Therefore, such safeguard mechanisms may not be as important in the eel given the much-reduced release of catecholamines in eels compared to trout.

In contrast to the β_2-adrenergic receptor mediated inhibition of the cholinergic and angiotensin II induced release of catecholamines, adrenaline secretion in response to VIP appeared to be insensitive to this type of regulation. The precise mechanisms for these differences are not known. However, owing to the prerequisite elevation of intracellular calcium from external sources for both the cholinergic and angiotensin II elicited secretion of catecholamines, mechanisms regulating the entry of calcium may be responsible for the β_2-adrenergic inhibition. If so, then the ability of VIP to cause catecholamine release, even in the absence of external calcium, would explain the lack of an adrenergic negative feedback signals on the VIP induced secretion of adrenaline. Possibly, the ability of VIP to elicit the secretion of adrenaline, even in the presence of high levels of circulating levels of catecholamines, would allow for secretion to continue unabated in response to prolonged and/or repeated bouts of acute stress. Whether or not the release of VIP from the nerve terminals is subject to an adrenergic control was not examined in this thesis. Owing to the low levels of catecholamines that are released from the chromaffin tissue in response to VIP or neuronal stimulation (low frequency stimulation), one might speculate that they would not exert a large impact on catecholamine secretion. Overall, catecholamines released from "adrenal" chromaffin

cells have the ability to function in an autocrine and paracrine fashion to regulate the secretion of additional catecholamines. Given the marked inter-species differences observed with respect to the adrenergic regulation of catecholamine release from chromaffin cells, however, it is difficult to generalize on what the overall significance of catecholamines as a modulator of catecholamine release in fish might be. Owing to the reliance of circulating catecholamines for adrenergic cardiovascular control and gas transfer during conditions of acute stress, the adrenergic inhibition of catecholamines may possibly play a key role in controlling the extent of expression of adrenergic stress responses in trout. In contrast, a catecholaminotropic effect of catecholamines may be necessary to elevate circulating catecholamines to physiologically relevant levels in eels.

Part V. Significance of the neuronal control of catecholamine release.

In the cyclostome hagfishes, unlike teleosts, the chromaffin tissue is not innervated and thus catecholamine release in these fish presumably is mediated exclusively by the endocrine system via the bloodstream (Perry et al., 1993; Epple et al., 1995; Bernier and Perry, 1996). Possibly, the emergence of a nervous control of catecholamine release was induced by a necessity to enable organisms to elicit different physiological responses depending on the nature and severity of stresses encountered. Although the cholinergic induced release of catecholamines is the predominant mechanisms leading to the secretion of catecholamines in teleosts, the presence of non-cholinergic neurotransmitters such as VIP and PACAP provides a greater degree of flexibility with respect to the neuronal control of catecholamine release from the chromaffin cells.

The results of this thesis 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 Ach, 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.

CONCLUSION

This thesis primarily investigated aspects of the neuronal control of catecholamine release from the chromaffin cells of the rainbow trout. In addition to cholinergic stimulation, experimental evidence suggests that the peptides VIP and PACAP may function as neurotransmitters in the overall control of catecholamine release in this species. Owing to the presence of VIP and PACAP immunoreactive nerve fibres in the vicinity of the chromaffin cells in several other species of fish, a neurotransmitter role for these peptides in the secretion of catecholamines may also be extended to fish in general. Whether or not this is representative of all fish points to the need for a more comparative approach in order to further our understanding of this system.

REFERENCES

Abad F, Garrido B, Lopez MG and Garcia AG 1992 The source of calcium for muscarinic mediated catecholamine release from cat adrenals. Journal of Physiology London 445: 725-740.

Abele B, Hathaway CB, Nibbio B and Epple A 1998 Electrostimulation of catecholamine release in the eel: modulation by antagonists and autocrine agonists. General and Comparative Endocrinology 109: 366-374.

Abelli L, Gallo VP, Civinni A and Mastrolia L 1996 Immunohistochemical and ultrastructural evidence of adrenal chromaffin cell subtypes in sea bass, *Dicentrarchus labrax* (L.). General and Comparative Endocrinology 102: 113-122.

Abrahamsson T 1979 Phenylethanolamine-N-methyl transferase (PNMT) activity and catecholamine storage and release from chromaffin tissue of the spiny dogfish, *Squalus acanthias*. Comparative Biochemistry and Physiology 64C: 169-172.

Accordi F 1991 The chromaffin cells of urodele amphibiams. Journal of Anatomy 179: 1-8.

Aldman G and Holmgren S 1992 VIP inhibits CCK-induced gall bladder contraction involving a beta-adrenoceptor mediated pathway in the rainbow trout, *Oncorhynchus mykiss, in vivo*. General and Comparative Endocrinology 88: 287-291.

Alexandre D, Anouar Y, Jegou S, Fournier A and Vaudry H 1999 A cloned frog vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating polypeptide receptor exhibits pharmacological and tissue distribution characteristics of both $VPAC_1$ and $VPAC_2$ receptors in mammals. Endocrinology 140: 1285-1293.

Al-Kharrat H, Weiss U, Tran Q, Nibbio B, Scholz S and Epple A 1997 Cholinergic control of catecholamine release in the eel General and Comparative Endocrinology 108: 102-108.

Altschul SF, Gish W, Miller W, Myers EW and Lipmann DJ 1990 Basic alignment search tool. Journal of Molecular Biology 215: 403-410.

Augustinsson KB, Fange R, Johnels A, & Ostlund E 1956 Histological, physiological and biochemical studies on the heart of two cyclostomes, hagfish (*Myxine*) and lamprey (*Lampetra*). Journal of Physiology (London) 131: 257-276.

Ballesta JJ, Borges R, Garcia AG, and Hidalgo MJ 1989 Secretory and radioligand binding studies on muscarinic receptors in bovine and feline chromaffin cells. Journal Physiology London 418: 411-426.

Becker A, Hessenius C, Bhargava S, Grotzinger C, Licha K, Schneider-Mergener J, Wiedenmann B and Semmler W 2000 Cyanine dye labeled vasoactive intestinal peptide and somatostatin analogue for optical detection of gastroenteropancreatic tumors. Annals of the New York Academy if Sciences 921: 275-278.

Bernier NJ, Gilmour KM, Takei Y and Perry SF 1999a Cardiovascular control via angiotensin II and circulating catecholamines in the spiny dogfish, *Squalus acanthias*. Journal of Comparative Physiology B 169: 237-248.

Bernier NJ, McKendry JE and Perry SF 1999b Blood pressure regulation during hypotension in two teleost species: differential involvement of the renin-angiotensin and adrenergic systems. Journal of Experimental Biology 202: 1677-1690.

Bernier NJ and Perry SF 1996 Control of catecholamine and serotonin release from chromaffin tissue of the Atlantic hagfish. Journal of Experimental Biology 199: 2485-2497.

Bernier NJ and Perry SF 1997 Angiotensins stimulate catecholamine release from the chromaffin tissue of the rainbow trout. American Journal of Physiology 273: R49-R57.

Bernier NJ and Perry SF 1999 Cardiovascular effects of angiotensin II- mediated adrenaline release in rainbow trout *Oncorhynchus mykiss*. Journal of Experimental Biology 202: 55-66.

Bodanszky M, Klausner YS and Said SI 1973 Biological activities of synthetic peptides corresponding to fragments of and to the entire sequence of the vasoactive intestinal peptide. Proceedings of National Academy of Science USA 70 (2): 382-384.

Boksa P and Livett BG 1984 Desensitization to nicotinic cholinergic agonists and K^+, agents that stimulate catecholamine secretion, in isolated adrenal chromaffin cells. Journal of Neurochemistry 42: 607-617.

Boonyaviroj P & Gutman Y 1979 α- and β- Adrenoceptors and PGE_2 in the modulation of catecholamine secretion from bovine adrenal medulla in vitro. Journal of Pharmacy and Pharmacology 31: 716-717.

Brandt BL, Hagiwara Y, Kidokoro Y and Miyasaki S 1976 Action potentials in the rat chromaffin cell and effects of acetylcholine. Journal of Physiology London. 263: 417-439.

Burgoyne RD 1991 Control of exocytosis in adrenal chromaffin cells. Biochemica Biophysica Acta 1071: 174-202.

Burgoyne RD, Morgan A, Robinson I, Pender N and Cheek T 1993 Exocytosis in adrenal chromaffin cells. Journal Anatomy 183: 309-314.

Capra MF and Satchell GH 1977 The differential haemodynamic responses of the elasmobranch, *Squalus acanthias*, to the naturally occurring catecholamines, adrenaline and noradrenaline. Comparative Biochemistry and Physiology 58C: 41-47.

Cheek TR and Burgoyne RD 1985 Effect of activation of muscarinic receptors on intracellular free calcium and secretion in bovine adrenal chromaffin cells. Biochemica and Biophysica Acta 846: 167-173.

Chow BKC 1997 The goldfish vasoactive intestinal polypeptide receptor: functional studies and tissue distribution. Fish Physiology and Biochemistry 17: 213-222.

Chow BKC, Tat-Hung Yuen T, Chan KW 1997 Molecular cloning of vertebrate VIP receptors and functional characterization of a VIP receptor from Goldfish *Carassius auratus*. General and Comparative Endocrinology 105: 176-185.

Chowdhury PS, Guo S, Wakade TD, Przywara DA and Wakade AR 1994 Exocytosis from a single rat chromaffin cell by cholinergic and peptidergic neurotransmitters. Neuroscience 59: 1-5.

Cohen J, Eckstein L and Gutman Y 1980 The mechanism of α-adrenergic inhibition of catecholamine release. British Journal of Pharmacology 71: 135-142.

Collett AR and Story DF 1984 Effects of adrenoceptor antagonists and neuronal uptake inhibitors on dimethylphenylpiperazinium-induced release of catecholamines from rabbit isolated adrenal gland and guinea-pig atria. Journal of Pharmacology and Experimental Therapeutics 231: 379-386

Coupland RE 1963 The innervation of the rat adrenal medulla. An electron microscopic study. Journal of Anatomy 97: 141-142.

Dashow L and Epple A 1983 Effects of exogenous catecholamines on plasma catecholamines and glucose in the sea lamprey, *Petromyzon marinus*. Journal of Comparative Physiology 152: 35-41.

Dashow L and Epple A 1985 Plasma catecholamines in the lamprey: intrinsic cardiovascular messengers? Comparative Biochemistry and Physiology 82C: 119-122.

Dimaline R, Young J, Thwaites, DT, Lee CM, Shuttleworth TJ and Thorndyke MC 1987 A novel vasoactive intestinal peptide (VIP) from elasmobranch intestine has full affinity for mammalian pancreatic VIP receptors. Biochemica Biophysica Acta 930: 97-100.

Eberhard DA and Holtz RW 1987 Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. Journal of Neurochemistry 49: 1634-1643.

Edwards AV and Jones CT 1993 Autonomic control of adrenal function. Journal of Anatomy 183: 291-307.

Epple A 1993 Adrenomedullary Catecholamines. In *The Endocrinology of Growth, Development and Metabolism in Vertebrates,* (ed. M.P. Schreibman, C.G. Scanes, and P.K.T. Pang), pp. 327-343. San Diego, Academic Press.

Epple A, Brinn JE and Gill TS 1995 The evolution of the adrenal medulla. In *Embryos, Endocrine Cells and the Neural Crest,* pp.125-140. Eds B Kramer & B Rawdon. Johannesburg, South Africa: Witwatersand University Press.

Epple A and Nibbio B 1985 Catecholaminotropic effects of catecholamines in a teleost fish, *Anguilla rostrata*. Journal of Comparative Physiology B 155: 285-290.

Epple A, Porta S and Nibbio B 1995 Release of conjugated catecholamines from the sinus venosus of a lamprey, *Petromyzon marinus*. Comparative Biochemistry and Physiology 111C: 45-48.

Fabbri E, Capuzzo A, and Moon TW 1998 The role of circulating catecholamines in the regulation of fish metabolism: An overview. Comparative Biochemistry and Physiology 120C: 177-192.

Fishbein, VA, Coy, DH, Hocart, SJ, Jiang, NY, Mrozinski, JE, Mantey, SA and Jensen, RT 1994 A chimeric VIP-PACAP analogue but not VIP pseudopeptides function as VIP receptor antagonists. Peptides 15: 95-100.

Fritsche R, Reid SG, Thomas S and Perry SF 1993 Serotonin-mediated release of catecholamines in the rainbow trout *Oncorhynchus mykiss*. Journal of Experimental Biology 178: 191-204.

Furimsky M, Moon TW and Perry SF 1996 Calcium signaling in isolated single chromaffin cells of the rainbow trout (*Oncorhynchus mykiss*). Journal Comparative Physiology B 166: 396-404.

Furness JB, Costa M and Wilson AJ 1977 Water-stable fluorophores, produced by reaction with aldehyde solutions, for the histochemical localization of catechol- and indolethylamines. Histochemistry 52: 159-170.

Gallo VP, Civinini A, Mastrolia L, Leitner G and Porta S 1993 Cytological and biochemical studies on chromaffin cells in the head kidney of *Gasterosteus*

aculeatus (Teleostei, Gasterosteidae). General and Comparative Endocrinology 92: 133-142.

Gaspo R, Lamarche L, deChamplain J and Yamaguchi N 1997 Canine adrenal catecholamine response to VIP is blocked by PACAP-(6-27) in vivo. American Journal of Physiology 272: R1606-R1612.

Gfell B, Kloas W and Hanke W 1997 Neuroendocrine effects on adrenal hormone secretion in carp (*Cyprinus carpio*). General and Comparative Endocrinology 102: 310-319.

Green CW 1902 Contribution to the physiology of the California *hagfish Polistrotremas stoutii.* II. The absence of regulative nerves for the systemic heart. American Journal of Physiology 6: 318-324.

Greenberg A and Zinder O 1982 α- and β-receptor control of catecholamine secretion from isolated adrenal medulla cells. Cell and Tissue Research 226: 655-665.

Guo X & Wakade AR 1994 Differential secretion of catecholamines in response to peptidergic and cholinergic transmitters in rat adrenals. Journal of Physiology 475: 539-545.

Gutman Y & Boonyaviroj P 1977 Inhibition of catecholamine release by alpha-adrenergic activation: Interaction with Na, K-ATPase. Journal of Neural Transmission 40: 245-252.

Harmar AJ, Arimura, A, Gozes, I, Journot, L, Laburth, M, Pisegna, JR, Rawlings, SR, Robberecht, P, Said, SI, Sreedharan, SP, Wank, SA and Waschek, JA 1998 International union of pharmacology. XVIII. Nomenclature of receptors for

vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacological Reviews 50: 265-270.

Hathaway CB, Brinn JE and Epple A 1989 Catecholamine release by catecholamines in the eel does not require the presence of the spinal cord. Journal of Experimental Zoology 249: 238-242.

Hernandez-Guijo JM, Carabelli V, Gandia L, Garcia AG and Carbone E 1999 Voltage-independent autocrine modulation of L-type channels mediated by ATP, opioids and catecholamines in rat chromaffin cells. European Journal of Neuroscience 11: 3574-3584.

Holmgren S 1985 Neuropeptides functions in the fish gut. Peptides 6, Suppl. 3: 363-368.

Holmgren S 1995 Neuropeptide control of the cardiovascular system in fish and reptiles. Brazilian Journal of Medical and Biological Research 28: 1207-1216.

Holmgren S and Jensen J 1994 Comparative Aspects on the biochemical identity of neurotransmitters of autonomic neurons. In *Comparative physiology and evolution of the autonomic nervous system,* pp. 69-95. Eds S Nilsson & S Holmgren. Switzerland: Harwood Academic Publishers.

Holmgren S and Nilsson S 1982 Neuropharmacology of adrenergic neurons in teleost fish. Comparative Biochemistry and Physiology 72C: 289-302.

Hoo RLC, Alexandre D, Chan SM, Anouar Y, Pang RTK, Vaudry H and Chow BKC 2001 Structural and functional identification of the pituitary adenylate cylase-activating polypeptide receptor $VPAC_2$ from the frog *Rana trigina rugulosa*. Journal of Molecular Endocrinology 27: 229-238.

Ishihara T, Shigemoto R, Mori K, Takahashi K and Nagata S 1992 Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. Neuron 8: 811-819.

Isobe K, Nakai T and Takuwa Y 1993 Ca^{2+}-dependent stimulatory effect of pituitary adenylate cyclase-activating polypeptide on catecholamine secretion from cultured porcine adrenal medullary chromaffin cells. Endocrinology 132: 1757-1765.

Isobe K, Yukimasa N, Nakai T, and Takuwa Y 1996 Pituitary adenylate cyclase-activating polypeptide induces gene expression of the catecholamine synthesizing enzymes, tyrosine hydroxylase, and dopamine β-hydroxylase in cultured porcine adrenal medullary chromaffin cells. New York Academy of Sciences 805: 406-469.

Jensen J and Holmgren S 1985 Neurotransmittres in the intestine of the Atlantic cod, *Gadus morhua*. Comparative Biochemistry and Physiology C82: 81-89.

Johnsson M, Axelsson M, and Holmgren S 2001 Large veins in the Atlantic *cod (Gadus morhua*) and the rainbow trout (*Oncorhynchus mykiss*) are. innervated by neuropeptide-containing nerves. Anat. Embryol. 204: 109-115.

Julio AE, Montpetit CJ and Perry SF 1998 Does blood acid-base status modulate catecholamine secretion in the rainbow trout (*Oncorhynchus mykiss*)? Journal of Experimental Biology 201: 3085-3095.

Kagstrom J and Holmgren S 1997 VIP-induced relaxation of small arteries of the rainbow trout, *Oncorhynchus mykiss*, involves prostaglandin synthesis but not nitric oxide. Journal of Autonomic Nervous System 63: 68-76.

Kansaku N, Shimada K, Ohkubo T, Saito N, Suzuki, Matsuda and Zadwormy D 2001 Molecular cloning of chicken vasoactive intestinal polypeptide receptor

complementary DNA, tissue distribution and chromosomal localization. Biology of Reproduction 64: 1575-1581.

Kirpekar SM, Prat JC and Wakade AR 1975 Effect of calcium on the relationship between frequency of stimulation and release of noradrenaline from the perfused spleen of the cat. Naunyn Schmiedebergs Archives of Pharmacology 287: 205-212.

Kloas W, Reinecke M and Hanke W 1994 Role of the atrial natriuretic peptide for adrenal regulation in the teleost fish Cyprinus carpio. American Journal of Physiology 267: R1034-1042.

Knight DE and Baker PF 1986 Observations on the muscarinic activation of catecholamine secretion in the chicken adrenal. Neuroscience 19: 357-366.

Lacoste A, Malham SK, Cueff A, Jalabert F, Gelebart F and Poulet SA 2001 Evidence for a form of adrenergic response to stress in the mollusc *Crassostrea gigas*. Journal of Experimental Biology 204: 1247-1255.

Lamouche S and Yamaguchi N 2001 Role of PAC_1 receptor in adrenal catecholamine secretion induced by PACAP and VIP in vivo. American Journal of Physiology (Regulatory, Integrative and Comparative Physiology) 280: R510-R518.

Langer SZ, Adler-Graschinsky E and Giorgi O 1977 Physiological significance of α-adrenoceptor-mediated negative feedback mechanism regulating noradrenaline release during nerve stimulation. Nature 265: 648-650.

Langer S, Van Noorden S, Polak JM, and Pearse AGE 1979 Peptide hormone-like immunoreactivity in the gastrointestinal tract and endocrine pancreas of eleven teleost species. Cell and Tissue Research 199: 493-508.

Lapner KN, Montpetit CJ and Perry SF 2000 Desensitisation of chromaffin cell nicotinic receptors does not impede catecholamine secretion during acute hypoxia in rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology 203: 1589-1597.

Liang BT and Perlman RL 1979 Catecholamine secretion by hamster adrenal cells. Journal of Neurochemistry 32: 927-933.

Liu PS and Kao LS 1990 Na^+-dependent Ca^{2+} influx in bovine adrenal chromaffin cells. Cell Calcium 11: 573-579.

Livett BG and Marley PD 1993 Noncholinergic control of adrenal catecholamine release. Journal of Anatomy 183: 277-289.

Lundin K and Holmgren S 1984 Vasoactive intestinal polypeptide-like immunoreactivity and effects of VIP in the swim bladder of the cod, *Gadus morhua*. Journal of Comparative Physiology B 154: 627-633.

Lutz, E-M, Sheward WJ, West KM, Morrow JA, Fink G and Harmar AJ 1993 The VIP_2 receptor: molecular characterization of a cDNA encoding a novel receptor for vasoactive intestinal peptide. FEBS Letters 334: 3-8.

Mainoya JR and Bern HA 1984 Influence of vasoactive intestinal polypeptide, urotensin II on the absorption of water and NaCl by the anterior intestine of the tilapia, *Sarotherodon mossambicus*. Zoological Science 1: 100-105.

Malhotra RK and Wakade AR 1987 Non-cholinergic component of rat splanchnic nerves predominates at low neuronal activity and is eliminated by naloxone. Journal of Physiology 383: 639-652.

Malhotra RK & Wakade AR 1987 Vasoactive intestinal polypeptide stimulates the secretion of catecholamines from the rat adrenal gland. Journal of Physiology 388: 285-294.

Malhotra, RK, Wakade TD and Wakade AR 1989 Cross-communication between acetylcholine and VIP in controlling catecholamine secretion by affecting cAMP, inositol triphosphate, protein kinase C, and calcium in rat adrenal medulla. Journal of Neuroscience 9 (12): 4150-4157.

Mastrolia L, Gallo VP and La Marca A 1984 The adrenal chromaffin cells of *Salmo Gairdneri* Richardson (Teleosteii, Salmonidae). Journal of Anatomy 138:503-511.

Mckay DB, Lopez I, Sanchez PA, English JL and Wallace LJ 1991 Characterization of muscarinic receptors of bovine adrenal chromaffin cells: binding, secretion and anti-microtubule drug effects. General Pharmacology 22: 1185-1189.

McKendry JE, Bernier NJ, Takei Y., Duff, DW, Olson KR and Perry SF 1999 Natriuretic peptides and the control of catecholamine release in two freshwater teleosts and a marine elasmobranch. Fish Physiology and Biochemistry 20: 61-77.

Misbahuddin M, Oka M, Nakanishi A and Morita K 1988 Stimulatory effect of vasoactive intestinal polypeptide on catecholamine secretion from isolated guinea pig adrenal chromaffin cells. Neuroscience Letters 92: 202-206.

Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, Culler MD and Coy DH 1989 Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochemical and Biophysical Research Communications 164: 567-574.

Miyata A, Jiang L, Dahl RD, Kitada C, Kubo K, Fujino M, Minamino N and Arimura A 1990 Isolation of neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase-activating polypeptide with 38 residues (PACAP38). Biochemical and Biophysical Research Communications 170: 643-648.

Montpetit CJ, McKendry JE and Perry SF. 2001 The effects of C-type natriuretic peptide on catecholamine release in the pacific spiny dogfish, *Squalus acanthias*. General and Comparative Physiology 123: 210-221.

Montpetit CJ and Perry SF 1998 The effects of chronic hypoxia on the acute adrenergic stress response in the rainbow trout (*Oncorhynchus mykiss*). Physiological Zoology 71:377-386.

Montpetit CJ and Perry SF 1999 Neuronal control of catecholamine secretion from chromaffin cells in the rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology 202: 2059-2069.

Montpetit CJ and Perry SF 2000 Vasoactive intestinal polypeptide- and pituitary adenylate cyclase activating polypeptide-mediated control of catecholamine release from chromaffin tissue in the rainbow trout, *Oncorhynchus mykiss*. Journal of Endocrinology 166: 705-714.

Nandi J 1961 New arrangement of interrenal and chromaffin tissues of teleost fishes. Science 134: 389-390.

Nassar-Gentina V, Catalan L and Luxoro M 1997 Nicotinic and muscarinic components in acetylcholine stimulation of porcine adrenal medullary cells. Molecular and Cellular Biochemistry 169: 107-113.

Nassar-Gentina V, Luxoro M and Urbina N 1991 Cholinergic receptors and catecholamine secretion from adrenal chromaffin cells of the toad. Comparative Biochemistry and Physiology C 100: 495-500.

Nekvasil NP and Olson KR 1986a Extraction and metabolism of circulating catecholamines by the trout gill. American Journal of Physiology 19: R526-531.

Nekvasil NP and Olson KR 1986b Plasma clearance, metabolism, and tissue accumulation of ^{3}H-labeled catecholamines in trout. American Journal of Physiology 19: 519-525.

Nilsson A 1975 Structure of the vasoactive intestinal octasopeptide from chicken intestine. The amino acid sequence. FEBS letters 60 (2): 322-326.

Nilsson S 1984 Adrenergic control systems in fish. Marine Biology Letters 5: 127-146.

Nilsson S 1983 Autonomic nerve function in vertebrates. In *Zoophysiology*, vol. 13. Berlin: Springer Verlag.

Nilsson S 1976 Fluorescent histochemistry and cholinesterase staining of sympathetic ganglia in a teleost, Gadus morhua. Acta Zoologica 57: 69-77.

Nilsson S, Abrahamsson T and Grove DJ 1976 Sympathetic nervous control of adrenaline release from the head kidney of the cod, *Gadus morhua*. Comparative Biochemistry and Physiology C 55: 123-127.

Nilsson S and Holmgren S 1989 Novel neurotransmitters in the autonomic nervous systems of nonmammalian vertebrates. Pharmac. Ther. 41: 257-287.

Nilsson S and Holmgren S 1994 Comparative physiology and evolution of the autonomic nervous system. Series Editor, G Burnstock, Harwood academic publishers, Switzerland, 376p.

Okamoto T, Murayama Y, Hayashi Y, Inagaki M, Ogata E and Nishimoto I 1991 Identification of a Gs activator region of the beta2-adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation. Cell 67: 723-730.

Olson KR, Conklin DJ, Conlon JM, Kellogg M, Smith MP, Weaver Jr L, Bushnell PG and Duff DW 1997 Physiological inactivation of vasoactive hormones in rainbow trout. Journal of Experimental Zoology 279: 254-264.

Opdyke DF, Bullock J, Keller NE and Holmes K 1983a Dual mechanism for catecholamine secretion in the dogfish shark, *Squalus acanthias*. American Journal of Physiology 244: R641-R645.

Opdyke DF, Bullock J, Keller NE and Holmes K 1983b Effect of ganglionic blockade on catecholamine secretion in exercised dogfish. American Journal of Physiology 245: R915-R919.

Opdyke DF, Carroll RG and Keller NE 1981 Angiotensin II releases catecholamine in dogfish. Comparative Biochemistry and Physiology 70C: 131-134.

Opdyke DF, Caroll RG, and Keller NE 1981 Systemic arterial pressor responses induced by potassium in dogfish, Squalus acanthias. American Journal of Physiology 241: R228-R232.

Orts A, Orellana C, Canto T, Cena V, Gonzalez-Garcia C and Garcia AG 1987 Inhibition of adrenomedullary catecholamine release by propranolol isomers and clonidine involving mechanisms unrelated to adrenoceptors. British Journal of Pharmacology 92: 795-801.

O'Sullivan AJ and Burgoyne RD 1989 A comparison of bradykinin, angiotensin II and muscarinic stimulation of cultured bovine adrenal chromaffin cells. Bioscience reports 9: 243-252.

Perry SF and Bernier NJ 1998 The acute humoral adrenergic stress response in fish: facts and fiction. Aquaculture 177: 285-295.

Perry SF, Fritsche R, Kinkead R and Nilsson S 1991 Control of catecholamine release in vivo and in situ in the Atlantic cod (*Gadus morhua*) during hypoxia. Journal of Experimental Biology 155: 549-566.

Perry SF, Fritsche R and Thomas S 1993 Storage and release of catecholamines from the chromaffin tissue of the Atlantic hagfish, *Myxine glutinosa*. Journal of Experimental Biology 183: 165-184.

Perry SF and Gilmour KM 1999 Respiratory and Cardiovascular Systems. In *Stress Physiology*, pp. 52-107. Ed. P. H. M. Balm. Sheffield: Sheffield Academic Press.

Perry SF and Reid SD 1992 Relationship between blood O_2 content and catecholamine levels during hypoxia in rainbow trout and American eel. American Journal of Physiology 263: R240-R249.

Perry SF and Reid SG 1994 The effects of acclimation temperature on the dynamics of catecholamine release during acute hypoxia in the rainbow trout Oncorhynchus mykiss. Journal of Experimental Biology 186: 289-307.

Perry SF and Vermette MG 1987 The effects of prolonged epinephrine infusion on the physiology of the rainbow trout, *Salmo gairdneri*. I. Blood respiratory, acid base and ionic states. Journal of Experimental Biology 128: 235-253.

Perry SF and Wood CM 1989 Control and coordination of gas transfer in fishes. Canadian Journal of Zoology 67: 2961-2970.

Pisegna JR and Wank SA 1993 Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. Proceedings of the National Academy of Sciences USA 90: 6345-6349.

Powis DA and Baker PF 1985 α_2-Adrenoceptors do not regulate catecholamine secretion by bovine adrenal medullary cells: a study with clonidine. Molecular Pharmacology 29: 134-141.

Przywara DA, Guo X, Lu Angelilli M., Wakade TD and Wakade AR 1996. A non-cholinergic transmitter, pituitary adenylate cyclase-activating polypeptide, utilizes a novel mechanism to evoke catecholamine secretion in rat adrenal chromaffin cells. Journal of Biological Chemistry 271: 10545-10550.

Raiteri M, Marchi M and Paudice P 1990 Presynaptic muscarinic receptors in the central nervous system. Annals of the New York Academy of Sciences 604: 113-129.

Randall DJ and Perry SF 1992 Catecholamines. In *Fish Physiology: The cardiovascular System,* vol. 12B, pp. 255-300. Eds DJ Randall & WS Hoar. New York: Academic Press.

Reid SG, Bernier N and Perry SF 1998 The adrenergic stress response in fish: control of catecholamine storage and release. Comparative Biochemistry and Physiology 120C: 1-27.

Reid SG, Fritsche R and Jonsson AC 1995 Immunohistochemical localization of bioactive peptides and amines associated with the chromaffin tissue of five species of fish. Cell Tissue Research 280: 499-512.

Reid SG and Perry SF 1995 Cholinoceptor-mediated control of catecholamine release from chromaffin cells in the American eel, *Anguilla rostrata*. Journal Comparative Physiology B 165: 464-470.

Reid SG and Perry SF 1994 Storage and differential release of catecholamines in rainbow trout (*Oncorhynchus mykiss*) and American eel (*Anguilla rostrata*). Physiological Zoology 67: 216-237.

Reid SG, Vijayan MM, and Perry SF 1996 Modulation of catecholamine storage and release by the pituitary-interrenal axis in the rainbow trout (*Oncorhynchus mykiss).* Journal of Comparative Physiology B 165: 665-676.

Robberecht P and Waelbroeck M 1998 A critical view of the methods for characterization of the VIP/PACAP receptor subclasses. New York Academy of Sciences 865: 157-163.

Robberecht P, Woussen-Colle M, DeNeef P, Gourlet P, Buscail L, Vandermeers A, Vandermeers-Piret MC and Christophe J 1991 The two forms of the pituitary adenylate cyclase activating polypeptide (PACAP (1-27) and PACAP (1-38)) interact with distinct receptors on rat pancreatic AR 4-2J cell membranes. FEBS 286 (1): 133-136.

Robitaille R, Jahromi BS and Charlton MP 1997 Muscarinic Ca^{2+} responses resistant to muscarinic antagonists at perisynaptic Schwann cells of the frog neuromuscular junction. Journal of Physiology 504: 337-347.

Role LW and Perlman RL 1983 Both nicotinic and muscarinic receptors mediate catecholamine secretion by isolated guinea-pig chromaffin cells. Neuroscience 10: 979-985.

Said SI and Mutt V 1970 Polypeptide with broad biological activity: Isolation from small intestine. Science (Wash. DC) 169: 1217-1218.

Said SI and Mutt V 1972 Isolation from porcine-intestinal wall of a vasoactive octacosapeptide related to secretin and glucagon. European Journal of Biochemistry 28: 199-204.

Scheuermann D 1993 Comparative morphology, cytochemistry and innervation of chromaffin tissue in vertebrates. Journal of Anatomy 183: 327-342.

Sharma TR, Wakade TD, Malhotra RK and Wakade AR 1986 Secretion of catecholamines from the perfused adrenal gland of the rat is not regulated by α-adrenoceptors. European Journal of Pharmacology 122: 167-172.

Sorimachi M, Yamagami K and Nishimura S 1992 A muscarinic receptor agonist mobilizes Ca^{2+} from caffeine and inositol-1,4,5- trisphosphate-sensitive Ca^{2+} stores in cat adrenal chromaffin cells. Brain Research 571: 154-158.

Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaer J, Seeburg PH and Journot L 1993 Differential signal transduction by five splice variants of the PACAP receptor. Nature London 365: 170-175.

Starke K, Gorlitz BD, Montel H and Shumann HJ 1974a Local α-adrenoceptor mediated feed-back inhibition of catecholamine release from the adrenal medulla? Experientia 30, 1170-1172.

Swilem AMF and Hawthorn JN 1983 Catecholamine secretion by perfused bovine adrenal medulla in response to nicotinic activation is inhibited by muscarinic receptors. Biochem. Pharmacol. 32: 3873-3874.

Tanaka K, Shibuya I, Nagatomo T, Yamashita H and Kanno T 1996 Pituitary adenylate cyclase-activating polypeptide causes rapid Ca^{2+} release from intracellular stores and long lasting Ca^{2+} influx mediated by Na^{+} influx-dependent membrane depolarization in bovine adrenal chromaffin cells. Endocrinology 137: 956-966.

Thomas S and Perry SF 1992 Control and consequences of adrenergic activation of red blood cell NA+/H+ exchange on blood oxygen and carbon dioxide trasnport in fish. Journal of Experimental Zoology 263: 160-175.

Thwaites DT, Young J, Thorndyke MC and Dimaline R 1989 The isolation and chemical characterization of a novel vasoactive intestinal peptide-related peptide from a teleost fish, the cod, *Gadus morhua*. Biochemica Biophysica Acta 999: 217-220.

Tobin JR, Breslow MJ and Traystman RJ 1992 Muscarinic cholinergic receptors in canine adrenal gland. American Journal of Physiology 263: H1208-H1212.

Ungar A & Phillips JH 1983 Regulation of adrenal medulla. Physiological Reviews 63: 787-843.

Usdin TB, Bonner TI and Mezey E 1994 Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. Endocrinology 135: 2662-2680.

Vaudry D, Gonzalez BJ, Basille M, Yon L., Fournier A and Vaudry H 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to function. Pharmacology Review 52: 269-324.

Vertongen P, Schiffman SN, Gourlet P and Robberecht P 1997 Autoradiographic for vasoactive intestinal polypeptide (VIP) in rat brain. Peptides 18: 1547-1554.

Wada A, Sakurai S, Kobayashi H, Yanagihara N and Izumi F 1982 α_2-Adrenergic receptors inhibit catecholamine secretion from bovine adrenal medulla. Brain Research 252: 189-191.

Wada A, Takara H, Izumi F, Kobayashi H and Yanagihara N 1985 Influx of ^{22}Na through receptor-associated Na channels: relationship between ^{22}Na influx, ^{45}Ca influx and secretion of catecholamines in cultured bovine adrenal medulla cells. Neuroscience 15: 283-292.

Wakade AR 1981 Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. Journal of Physiology 313: 463-480.

Wakade TD, Blank MA, Malhotra RK, Pourcho R and Wakade AR 1991 The peptide VIP is a neurotransmitter in rat adrenal medulla: Physiological role in controlling catecholamine secretion. Journal of Physiology 444: 349-362.

Wakade AR and Wakade TD 1983 Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and exogenous acetylcholine. Neuroscience 10: 973-978.

Wang Y and Conlon JM 1995 Purification and structural characterization of vasoactive intestinal polypeptide from the trout and bowfin. General and Comparative Endocrinology 98: 94-101.

Watanabe T, Masuo Y, Matsumoto H, Suzuki N, Ohtaki T, Masuda Y, Kitada C, Tsuda M and Fujino M 1992 Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline. Biochemical and Biophysical Research Communications 182 (1): 403-411.

Watanabe T, Shimamoto N, Takahashi A and Fujino M 1995 PACAP stimulates catecholamine release from the adrenal medulla: a novel noncholinergic secretagogue. American Journal of Physiology 269: E903-E909.

Wei Y, Martin SC, Heinrich G and Mojsov S 1998 Cloning and functional characterization of PACAP specific receptors in zebrafish. Annals of the New York Academy of Sciences 865: 45-48.

Wendelaar Bonga SE 1997 The stress response in fish. Physiological Reviews 77: 591-625.

Wolf K 1963 Physiological salines for freshwater teleosts. Progress in Fish Culture 25: 135-140.

Wong AOL, Leung MY, Shea WLC, Tse LY, Chang JP and Chow BKC 1998 Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. Endocrinology 139: 3465-3479.

Xu Y, Duarte EP and Forsberg EJ 1991 Calcium dependency of muscarinic and nicotinic agonist-induced ATP and catecholamine secretion from porcine adrenal chromaffin cells. Journal of Neurochemistry 56: 1889-1896.

Yamagami K, Nishimura S and Sorimachi M 1991 Internal Ca^{2+} mobilization by muscarinic stimulation increases secretion from adrenal chromaffin cells only in the presence of Ca^{2+} influx. Journal of Neurochemistry 57: 1681-1689.

Yamaguchi N 1993 In vivo evidence for adrenal catecholamine release mediated by nonnicotinic mechanisms: local medullary effect of VIP. American Journal of Physiology 265: R766-R771.

Yon L, Chartrel N, Feuilloley M, deMarchis S, Fournier A, deRijk E, Pelletier G, Roubos E and Vaudry H 1994 Pituitary adenylate cyclase-activating polypeptide stimulates both adrenocortical cells and chromaffin cells in the frog adrenal gland. Endocrinology 135: 2749-2758.

You S, Hsu CC, Kim H, Kho Y, Choi YJ, El-Halawani ME, Farris J and Foster DN 2001 Molecular cloning and expression analysis of the turkey vasoactive intestinal peptide receptor. General and Comparative Endocrinology 124: 53-65.

Young JZ 1939 Partial degeneration of the nerve supply of the adrenal. A study in autonomic innervation. Journal of Anatomy 73: 540-550.