Adrenergic regulation of catecholamine secretion from trout (*Oncorhynchus mykiss*) chromaffin cells

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Abstract

The interaction between extracellular catecholamines and catecholamine secretion from chromaffin cells was assessed in rainbow trout (*Oncorhynchus mykiss*) using an *in situ* saline-perfused posterior cardinal vein preparation. This was accomplished by comparing the effects of adrenergic receptor agonists and antagonists on stimulus-evoked secretion. An acute bolus injection or extended perfusion with saline containing high levels of either noradrenaline or adrenaline did not affect the baseline secretion of catecholamines. However, catecholamine secretion in response to a bolus injection of the general cholinergic receptor agonist carbachol or electrical stimulation of the nerves innervating the chromaffin cells was abolished or reduced respectively, in preparations perfused with saline containing either catecholamine.

To characterize the catecholaminergic inhibition of catecholamine release, secretion in response to carbachol and electrical stimulation was compared in preparations perfused with the adrenergic receptor agonists dobutamine (β_1), salbutamol (β_2), phenylephrine (α_1) or clonidine (α_2). Prior treatment with dobutamine or phenylephrine was

without effect on baseline catecholamine secretion or stimulus-evoked secretion. In contrast, pre-treatment with salbutamol significantly inhibited catecholamine secretion in response to carbachol or electrical stimulation. Pre-treatment with clonidine did not affect carbachol-evoked secretion but did reduce catecholamine secretion during electrical stimulation. The significance of this adrenergic mechanism of regulating stimulus-evoked catecholamine secretion was further established using the adrenergic receptor antagonists nadolol (β) or phentolamine (α). Catecholamine secretion in response to cholinergic stimulation was significantly enhanced in preparations perfused with saline containing nadolol. Furthermore, pre-treatment with phentolamine significantly enhanced adrenaline secretion in response to neuronal stimulation.

These results suggest that the mechanisms of adrenergic inhibition of catecholamine secretion from trout chromaffin cells include activation of chromaffin cell membrane β_2 -receptors and presynaptic α_2 -adrenergic receptors.

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Introduction

In teleost fish, the catecholamines (adrenaline and noradrenaline) that enter the circulation are synthesized, stored and released from chromaffin tissue that lines the walls of the posterior cardinal vein (PCV) (Reid *et al.* 1998). Under adverse conditions including exhaustive exercise, hypoxia, hypercarbia and physical disturbances (Randall & 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 & 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 pre-synaptic 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 & Boonyaviroj 1977, Boonyaviroj *et al.*

1979, Cohen *et al.* 1980, Greenberg & Zinder 1982, Wada *et al.* 1982, Powis & 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 & Eppele 1983, Hathaway *et al.* 1989). Furthermore, pre-treatment with α - or β -adrenergic receptor antagonists decreases 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*) does not affect the levels of plasma noradrenaline (Perry & 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 inhibits the cholinergic-elicited release of that particular catecholamine (Perry *et al.* 1991). Thus, there appear 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, secondly, to use a pharmacological approach to identify the underlying mechanisms.

Materials and Methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and held in large fiberglass tanks supplied with dechlorinated city of Ottawa tap water maintained at 13 °C. They were allowed to acclimate to the aquaria for at least 3 weeks before experimentation. Fish were maintained on a 12 h light:12 h darkness photoperiod and fed daily to satiation with a commercial fish diet.

In situ saline-perfused PCV preparation

Fish (256–365 g) were killed by a sharp blow to the head, weighed and placed on ice. A ventral incision was made along the length of the animal beginning at the anus and ending just anterior to the pectoral girdle. The tissue overlying the heart was removed by blunt dissection to expose the ventricle and the bulbus arteriosus. The

ventricle was cannulated (Clay-Adams PE160 polyethylene tubing; internal diameter=1.14 mm, outer diameter=1.57 mm; VWR, CanLab, Mississauga, ON, Canada) by making an incision in the bulbus and inserting the tube through the bulbus into the ventricle. The cannula was secured with a ligature between the two chambers. This cannula served as the outflow in the perfusion system. The PCV was also cannulated (PE160 tubing) in the anterograde direction and served as the inflow cannula (Fritsche *et al.* 1993). Each preparation was perfused for 20 min with aerated control saline (125 mmol/l NaCl, 2.0 mmol/l KCl, 2.0 mmol/l MgSO₄, 5.0 mmol/l NaHCO₃, 7.5 mmol/l glucose, 2.0 mmol/l CaCl₂ and 1.25 mmol/l KH₂PO₄, final pH 7.8) to allow stabilization of catecholamine secretion. Perfusion was accomplished by siphon resulting from a positive pressure difference (14.7 mmHg; 1.96 kPa) between the surface of the saline and the outflow cannula, which resulted in a flow rate of approximately 1.5 ml/min.

After the 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, fish were electrically stimulated using a previously validated field stimulation technique (Montpetit & Perry 1999). The specificity of the field stimulation technique was confirmed (Montpetit & Perry 1999) by demonstrating that the electrically evoked secretion of catecholamines was reduced in the presence of the nicotinic receptor antagonist hexamethonium, and prolonged in the presence of the cholinesterase inhibitor neostigmine. Further, the absence of a response during field stimulation in preparations perfused with Na⁺-free saline or from which the spinal cord was removed indicated that the electrical current was unable to elicit sufficient chromaffin cell membrane depolarization to initiate catecholamine secretion. Together, these results indicated that catecholamine secretion elicited by field stimulation was neurally mediated. As such, a pair of electrodes, connected to a Grass SD-9 stimulator (Quincy, MA, USA), was sutured to the body wall on either side of the fish in the anterior region of the PCV. Electrical stimulation was carried out at 60 V, 1 ms in duration for a period of 30 s and collection of post-stimulation samples began immediately at the onset of stimulation.

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 nmol/l, $n=6$; adrenaline bitartrate salt; Sigma Chemical Co., St Louis, MO, USA) or noradrenaline (124 nmol/l, $n=6$; 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). 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 nmol/l, $n=6$); in the third, only the noradrenaline concentration was elevated (124 nmol/l, $n=6$). A period of 1 min was allowed for the catecholamine to be delivered to the chromaffin tissue before post-stimulation samples were collected in pre-weighed tubes. In total, five post-stimulation 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 carbachol (mixed cholinergic receptor agonist; Sigma) or electrical/neuronal stimulation was compared in preparations perfused for 5 min with control saline ($n=6$), or saline containing adrenaline (330 nmol/l, $n=6$) or noradrenaline (124 nmol/l, $n=6$). Prior to stimulation, a 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-stimulation samples were collected in pre-weighed tubes. For neuronal stimulation, post-stimulation samples were collected immediately after stimulation. In total, five post-stimulation 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; 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} mol/l phenylephrine hydrochloride, α_1 ; Sigma; $n=6$) or clonidine (10^{-6} mol/l 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} mol/l dobutamine hydrochloride, β_1 ; Sigma; $n=6$) or salbutamol (10^{-6} mol/l 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} mol/l phentolamine hydrochloride, α -receptor antagonist; Sigma; $n=6$) or nadolol (10^{-6} mol/l, β -receptor antagonist; Sigma; $n=6$) during cholinergic or neuronal stimulation (as in Series 2).

Series 4. The effects of β_2 -receptor stimulation on catecholamine secretion elicited by vasoactive intestinal polypeptide (VIP) or angiotensin II Catecholamine secretion in response to a bolus injection of 10^{-9} mol/kg chicken VIP (Peninsula Laboratories Inc., San Carlos, CA, USA) or 10^{-9} mol/kg angiotensin II ([Asn1-Val5]-angiotensin II; Sigma) was compared in fish perfused with control saline ($n=6$) or saline containing salbutamol (10^{-6} mol/l; $n=6$). A period of 1 min was allowed for the drug to be delivered to the chromaffin tissue before post-stimulation samples were collected in pre-weighed tubes (as in Series 2).

Analytical procedures

All perfusate samples were frozen in liquid N_2 after collection and stored at $-80^\circ C$ until determination of catecholamine levels. Samples were re-weighed before catecholamine analysis to permit an estimation of perfusion flow rates and thus allow the calculation of catecholamine secretion rates. Perfusate adrenaline and noradrenaline concentrations were determined on alumina-extracted samples (200 μ l) 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, USA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments, Princeton, NJ, USA). The extracted samples were passed through an Ultratechsphere ODS- C_{18} 5 μ m column (HPLC Technology Ltd, Macclesfield, Cheshire, UK) 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 as an internal standard in all determinations. 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 time. 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 means $\pm$ 1 S.E.M. 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, St Louis, MO, USA).

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. 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-stimulation value (Fig. 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) caused a rapid increase in adrenaline and noradrenaline secretion (Fig. 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. 2B) or reduced (using adrenaline; Fig. 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. 3); noradrenaline secretion was unaffected.

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 catechol-

Figure 1 The effects of elevated concentrations of (B) noradrenaline or (C) adrenaline on basal catecholamine secretion (noradrenaline, open bars; adrenaline, solid bars) in *in situ* saline-perfused posterior cardinal vein (PCV) preparations of rainbow trout, *Oncorhynchus mykiss*. Catecholamines or saline (control experiments; A) were administered to the preparation by rapid bolus injection. Values are means $\pm$ 1 S.E.M.

amines, a bolus injection of carbachol (Figs 4 and 6) or electrical stimulation of the nerves innervating the chromaffin cells (Figs 5 and 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

Figure 2 The effects of elevated catecholamine concentrations on carbachol (Cch)-evoked (10^{-6} mol/kg, final volume 0.3 ml) stimulation of catecholamine secretion (noradrenaline, open bars; adrenaline, solid bars) in *in situ* saline-perfused PCV preparations of rainbow trout, *Oncorhynchus mykiss*. Carbachol was administered to preparations perfused with (A) control saline or with saline containing either (B) noradrenaline (124 nmol/l; $n=6$) or adrenaline (330 nmol/l; $n=6$). 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 (A); $P<0.05$). A single dagger denotes a significant difference of adrenaline only from the corresponding control value (control (A); $P<0.05$). Values are means ± 1 S.E.M.

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

Figure 3 The effects of elevated catecholamine concentrations on neuronal (60 V, 20 pps, 0.1 ms, 30 s duration) stimulation of catecholamine secretion (noradrenaline, open bars; adrenaline, solid bars) in *in situ* saline-perfused PCV 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 nmol/l; $n=6$) or adrenaline (330 nmol/l; $n=6$). An asterisk denotes a significant difference ($P<0.05$) from the pre-stimulation (pre) value. A single dagger denotes a significant difference of adrenaline only from the corresponding control value (control (A); $P<0.05$). Values are means ± 1 S.E.M.

not affect the release of catecholamines in response to cholinergic stimulation (Fig. 4). However, adrenaline release in response to neuronal stimulation was significantly reduced in the presence of clonidine (Fig. 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. 5B).

Figure 4 The effects of (A) α -adrenergic receptor agonists or (B) antagonists on total noradrenaline (open bars) and adrenaline (solid bars) release evoked by cholinergic stimulation (carbachol (Cch); 10^{-6} mol/kg, 0.3 ml) in *in situ* saline-perfused PCV 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.

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 6 and 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. 6B) but not neuronal

Figure 5 The effects of (A) α -adrenergic receptor agonists or (B) antagonists on total noradrenaline (open bars) and adrenaline (solid bars) release evoked by electrical neuronal stimulation (60 V, 20 pps, 0.1 ms, 30 s duration) in *in situ* saline-perfused PCV 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.

stimulation (Fig. 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; adrenaline 0.06 ± 0.02 nmol/min).

Series 4. The effects of 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

Figure 6 The effects of (A) β -adrenergic receptor agonists or (B) antagonists on total noradrenaline (open bars) and adrenaline (solid bars) release evoked by cholinergic stimulation (carbachol (Cch); 10^{-6} mol/kg, 0.3 ml) in *in situ* saline-perfused PCV 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.

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. 8).

Discussion

In situ saline-perfused PCV preparation

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 *et al.* 1999a,b), *in vitro* perfusion techniques (e.g. Gfell *et al.* 1997, Abele *et al.* 1998) and *in situ* saline-perfused PCV preparations (e.g. Perry *et al.* 1991, Fritsche

Figure 7 The effects of (A) β -adrenergic receptor agonists or (B) antagonists on total noradrenaline (open bars) and adrenaline (solid bars) release evoked by electrical neuronal stimulation (60 V, 20 pps, 0.1 ms, 30 s duration) in *in situ* saline-perfused PCV 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.

et al. 1993, Al-Kharat *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 PCV 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).

Although high concentrations of adrenergic agonists and antagonists were used in the present study, their effective levels in the vicinity of the chromaffin cells may have been considerably lower because of diffusion limitations of the perfused PCV preparation. The actual concentration of agonist/antagonist achieved in the extracellular fluid

Figure 8 The effects of the β_2 -adrenergic receptor agonist salbutamol on total noradrenaline (open bars) and adrenaline (solid bars) release in response to (A) angiotensin II (ANG II; 10^{-9} mol/kg, 0.3 ml) or (B) vasoactive intestinal polypeptide (VIP; 10^{-9} mol/kg, 0.3 ml) in *in situ* saline-perfused PCV preparations of rainbow trout, *Oncorhynchus mykiss*. An asterisk denotes a significant difference ($P < 0.05$) from the control (no salbutamol) value.

bathing the chromaffin cells is unknown. Nevertheless, the drug concentrations chosen for this study are similar to those of previous investigations conducted using mammalian chromaffin cells and perfused adrenal glands (Starke *et al.* 1974a, Boonyaviroj & Gutman 1979, Cohen *et al.* 1980, Collett & Story 1982, Greenberg & Zinder 1982, Wada *et al.* 1982, 1985, Hernandez-Guijo *et al.* 1999), as well as in other physiological systems studied in fish (Chang & Peter 1984, Sebert & Barthelemy 1985, Tilzey *et al.* 1985, Zhang *et al.* 1992).

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 & Eppler 1983), no such interactions were observed in this study. In agreement with former studies conducted *in vivo* in trout (Perry & 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 have clearly demonstrated 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 neurally 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 with 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. 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 & 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 & Story 1984), others have suggested a stimulatory role (Boonyaviroj & Gutman 1979, Greenberg & 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 & Gutman 1979, Greenberg & Zinder 1982). In agreement with those studies, a β -stimulatory pathway has also been suggested to operate in eels (Al-Kharat *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 & 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 & Gutman 1979, Cohen *et al.* 1980, Greenberg & 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 presynaptic 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 presynaptically. 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 (acetylcholine) neurotransmitters (Reid *et al.* 1995, Montpetit & 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 & Perry 2000), while angiotensin II elicited the secretion of both adrenaline and noradrenaline (Bernier & Perry 1997, Bernier *et al.* 1999b). Subsequent experiments were designed to determine whether the catecholaminergic negative feedback mechanism observed for cholinergic and neuronally evoked secretion was also 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 & 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 diacylglycerol (DAG) 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 & 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.

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