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CATECHOLAMINE SECRETION DURING HYPOXIA IN NICOTINIC RECEPTOR - DESENSITISED RAINBOW TROUT, *Oncorhynchus mykiss*

By

Katherine N. Lapner B.Sc. (Hon.)

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
Ottawa-Carleton Institute of Biology
In partial fulfillment of the requirements for the
Degree Master of Science

0-612-67832-6

Master of Science (2001), University of Ottawa
Biology

Title: The Role of Angiotensin II in Regulating Catecholamine Secretion during Hypoxia in Rainbow Trout, *Oncorhynchus mykiss*

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Acknowledgements

First and foremost I would like to thank my supervisor Steve Perry for allowing me to do Master's research in his lab. His guidance and involvement in the research was much appreciated. I would also like to thank Dr. Tom Moon, Dr. Jim Fenwick, and Dr. Jim Cheetham for serving on my advisory committee.

I would like to thank Marwan Samia and Dr. Vance Trudeau for their valuable guidance with RIA. My lab colleagues have made the past few years an enjoyable learning experience, and I would particularly like to thank Doogie Doogan for playing pool with me, and Andrea Katynski, Emilie Lariviere, and Katherine Larivere for all the chats and dancing; I would like to thank John McKendry for all his support, especially during those last few hectic months, and also for not buying that minivan.

Finally, I would like to thank my family for their much appreciated support.

CATECHOLAMINE SECRETION DURING HYPOXIA IN NICOTINIC RECEPTOR-DESENSITISED RAINBOW TROUT, *Oncorhynchus mykiss*

Abstract

Experiments were performed *in vivo* using an extra-corporeal blood loop (EC-loop), on chronically cannulated adult rainbow trout (*Oncorhynchus mykiss*) to determine whether the ability of fish to respond to acute hypoxia was impaired by an intravenous nicotine infusion (1.3 x 10^{-5} mol kg^{-1} h^{-1}; designed to desensitise chromaffin nicotinic receptors). Cardiovascular and respiratory variables were measured during the 60 min nicotine infusion and throughout the ensuing 10 min period of acute hypoxia (45 – 45 mm Hg; 5.3 – 6.0 kPa) while the infusion continued. The next set of experiments was also performed *in vivo* on chronically cannulated rainbow trout and focused on determining the involvement of serotonergic and muscarinic receptor stimulation, or activation of the renin angiotensin system (RAS) in eliciting catecholamine release during acute hypoxia during periods of nicotinic receptor desensitisation.

Respiratory and cardiovascular measurements taken throughout the nicotine infusion of the desensitisation protocol indicate that non-chromaffin, neuronal nicotinic receptors also appear to be stimulated and to desensitise. During the nicotine infusion, increases in ventilation amplitude and frequency resulting from peripheral chemoreceptor stimulation, and increases in blood pressure resulting from stimulation to autonomic sympathetic nerve synapses innervating the systemic vasculature, demonstrate neuronal nicotinic receptor stimulation. Indeed, during hypoxia, ventilation amplitude and frequency, as well as blood pressure, show a significantly decreased response caused by desensitisation prior to hypoxia, however no evidence was found to suggest that recruitment of catecholamine secretagogues was impaired during acute hypoxia.

Plasma levels of arterial pH were also significantly lowered during the nicotine infusion resulting from the activation of the red blood cell membrane-bound β_1 adrenoceptor-mediated Na^+/H^+ exchanger by circulating catecholamines. The absence of a hyperventilatory response during hypoxia resulted in a respiratory acidosis, however, this did not affect the recruitment of catecholamine secretagogues. Finally, a barostatic reflex was observed during hypoxia in nicotinic receptor-desensitised fish that did not affect the acute stress response to hypoxia.

These results indicate that despite the stimulation and possible desensitisation of neuronal nicotinic receptors, and the secretion of catecholamines caused by the nicotine receptor desensitisation protocol, the ability to secrete catecholamines as part of an acute stress response to hypoxia was not impaired.

The next set of experiments showed that despite nicotinic receptor desensitisation induced by intravenous infusion of nicotine, plasma catecholamine levels were increased to similar levels (adrenaline plus noradrenaline = 125 – 200 nmol l^{-1}) as in control fish during severe hypoxia (40 - 45 mm Hg). Blockade of serotonergic receptors using methysergide or muscarinic receptors using atropine did not affect the ability of fish to elevate circulating catecholamine levels during hypoxia. However, selective blockade of the renin angiotensin system (RAS) using lisinopril to inhibit angiotensin converting enzyme (ACE), prevented the elevation of both angiotensin II and circulating catecholamines in acutely hypoxic fish experiencing nicotinic receptor desensitisation. In fish possessing functional nicotinic receptors, ACE blockade attenuated but did not prevent the elevation of plasma catecholamine levels during hypoxia. The results of this study indicate that the RAS is activated during hypoxia and plays a role in eliciting catecholamine release that is secondary to activation of nicotinic receptors. However, under conditions of nicotinic receptor desensitisation, activation of the RAS during hypoxia is a prerequisite for catecholamine release.

Résumé

Les expériences ont été performées *in vivo* sur une chute-de-sang extracorporelle (EC-blood loop) sur des truites arc-en-ciel (*Oncorhynchus mykiss*) cannulées, afin de déterminer si la réaction des poissons à l'hypoxie aigüe est comprimer par une infusion de nicotine (1.3 x 10^{-5} mol kg^{-1} h^{-1}; afin de désensibiliser les récepteurs nicotinique de la tissue chromaffin) qui dure 60 minutes. Les variables cardiovasculaire et respiratoire ont été mesurés pendant les 60 minutes de l'infusion nicotinique et aussi pendant les prochaines 10 minutes hypoxique (40 – 45 mm Hg; 5.3 – 6.0 kPa), pendant que l'infusion continuait. Les prochaines expériences ont aussi été performées *in vivo* sur des truites arc-en-ciel cannulées afin de déterminer si les récepteurs serotonique ou muscarinique, ou le système renin-angiotensin, sont impliquer dans la sécretion des catécholamines pendant l'hypoxie aigüe dans les poissons avec les récepteurs nicotinique desensibilisés.

Les mesures cardiovasculaires et respiratoires prises durant l'infusion de la nicotine indique que les récepteurs nicotiniques des nevronnes sont aussi stimulées et desensibilisées. Pendant l'infusion, l'amplitude et la fréquence de la respiration se sont elevées, causé par la stimulation des chémo-récepteur périphéraux. Des augmentations dans la tension artériel ont resulté par la stimulation des nevronnes autonomes sympatiques énnervé par la vasculature systémique, démontrant la stimulation des récepteurs nicotiniques des nevronnes. Pourtant, ces variables physiologiques ont démontré une réaction diminuées, causée par la désensibilisation des récepteurs nicotiniques, pendant l'hypoxie aigüe. Aucune évidence a suggérée que la sécretion des catécholamines était comprimer par la désensibilisation.

Les niveaux de pH de la plasme artériel sont aussi diminués significamment durant l'infusion de la nicotine, ce qui est un résultat de l'activation de la ßNHE par les catécholamines circulantes. L'absence d'une réaction hyperventilatoire durant l'hypoxie a résulté dans de la plasme acide, pourtant ceci n'a pas affecté le récrutement des agents qui stimulent la sécretion des catécholamines. Finalement, une réflexe barostatique a été observé dans les poissons avec les récepteurs désensibilisées pendant l'hypoxie aigüe qui n'a pas comprimé la réaction à l'hypoxie.

Ces résultants indique qu'en dépit de la stimulation et désensibilisation des récepteurs nicotinique des nevronnes, et de la sécretion des catécholamines circulantes, la capacité de la tissue chromaffin à sécréter les catécholamines pendant l'hypoxie aigüe n'a pas été comprimé.

Les prochaines expériences ont montré qu'en dépit de la désensibilisation des récepteurs nicotiniques causé par l'infusion de la nicotine, les niveaux de catécholamines dans la plasme se sont élevée pendant l'hypoxie aigüe (adrenaline plus noradrenaline = 125 – 200 nmol l-1), tout comme les poissons sans désensibilisation. Bloquant les récepteurs sérotonique avec de la methysergide, ou les récepteurs muscarinique avec l'atropine n'a pas affecté la capacité des poissons à élever les niveaux de catécholamines circulantes durant l'hypoxie. Pourtant, bloquant le SRA avec le lisinopril afin d'inactivé ACE a empêché l'élévation de l'angiotensin II et des catécholamines circulantes durant l'hypoxie aigüe dans les poissons désensibilisées. Pourtant, dans les poissons possédant des récepteurs fonctionales, bloquant le ACE n'a pas empêcher l'élévation des niveaux de catécholamines circulantes. Ces résultats indiquent que le SRA est activé durant l'hypoxie et joue un rôle dans la sécretion des catécholamines qui est secondaire à l'activation des récepteurs nicotiniques. Pourtant, durant la désensibilisation des récepteurs nicotiniques, le SRA doit être activer pendant l'hypoxie pour atteindre la sécretion des catécholamines circulantes.

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List of Abbreviations

3,4-Dihydroxybenzylamine Hydrobromide	DHBA
Adrenocorticotropic Hormone	ACTH
Analysis of Variance	ANOVA
Angiotensin Converting Enzyme	ACE
Angiotensin II	Ang II
Arterial CO_2 Tension	$PaCO_2$
Arterial O_2 Tension	PaO_2
Arterial pH	pHa
Atrial Natriuretic Peptide	ANP
Blood Pressure	P_{DA}
Catechol-O-Methyl Transferase	COMT
Extra-corporeal Blood Loop	EC-loop
Haemoglobin	Hb
Heart Frequency	Hf
High-Pressure Liquid Chromatography	HPLC
Intracellular pH	pHi
L-Aromatic Amino Acid Decarboxylase	AADC, or DOPA-decarboxylase
L-Dihydroxyphenylalanine	L-DOPA
Monoaminoxidase	MAO
Oncorhynchus mykiss	*O. mykiss*
Phenylethanolamine-N-Methyltransferase	PNMT
Pituitary Adenylate-Cyclase Activating Polypeptide	VACAP
Posterior Cardinal Vein	PCV
Red Blood Cell	RBC
Renin Angiotensin System	RAS
β_1 Adrenoceptor-Mediated Na^+/H^+ Exchanger	βNHE
Standard Error of the Mean	SEM
Tyrosine Hydroxylase	TH
Vasoactive Intestinal Polypeptide	VIP
Ventilation Amplitude	Vamp
Ventilation Frequency	Vf
Water PO_2	PwO_2

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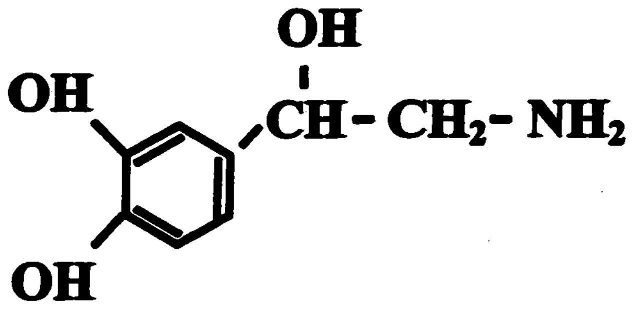
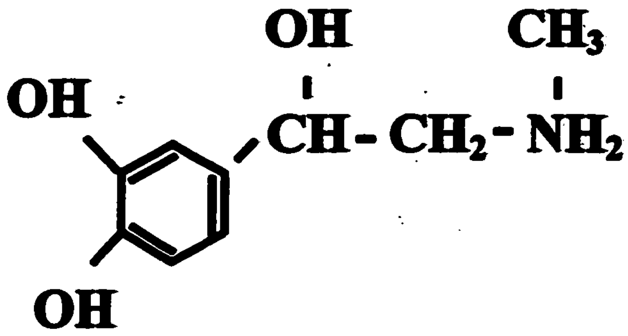
Chapter 1

GENERAL INTRODUCTION

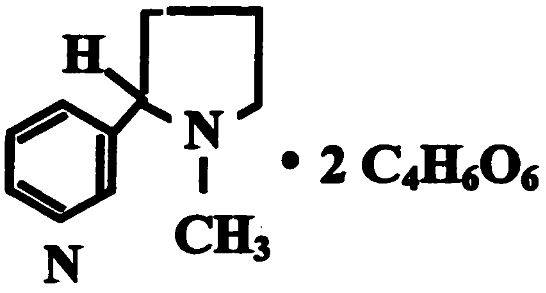
Figure 1-1: Diagram depicting the amino acid structure of the angiotensin II peptide and the molecular structure of the catecholamines noradrenaline and adrenaline, the nicotinic cholinergic agonist nicotine, and the indolamine serotonin.



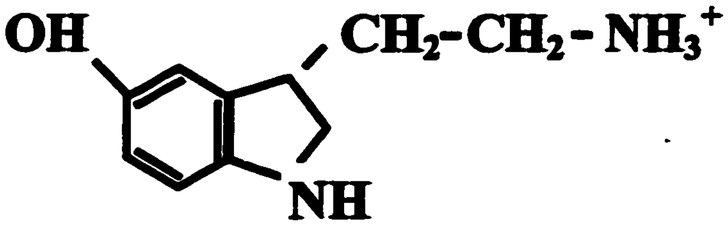
Noradrenaline



Adrenaline



Nicotine



Serotonin

H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH

Angiotensin II

in the formation of noradrenaline. Tyrosine hydroxylase requires Fe^{2+} and molecular oxygen, and the cofactor tetrahydropteridine. The next step in the synthesis of noradrenaline also occurs in the cytosol and involves a decarboxylation reaction by the enzyme L-aromatic amino acid decarboxylase (AADC, or DOPA-decarboxylase). DOPA-decarboxylase serves to decarboxylate L-DOPA to form dopamine, which is then transported into a secretory vesicle (granule). Once in the granule, dopamine is converted to noradrenaline *via* a side-chain hydroxylation reaction by the enzyme dopamine-ß-hydroxylase (DßH), which is the last step in noradrenaline-containing chromaffin cells. However, in the adrenaline-containing cells, noradrenaline is transported out of the granule into the cytoplasm, where a side-chain of noradrenaline is methylated by the enzyme phenylethanolamine-N-methyltransferase (PNMT) to form adrenaline. The method used to transport noradrenaline into the cytoplasm and the subsequent uptake of adrenaline into secretory vesicles is unknown. The methylation of noradrenaline is the rate-limiting step in the synthesis of adrenaline (Jönsson, 1982), which may then feed back to inhibit PNMT (Fuller and Hunt, 1965, 1967; Fuller and Roush, 1972).

In trout, nicotinic receptor stimulation is considered to be the primary mediator of cholinergic-induced catecholamine secretion during acute stress (Reid *et al.*, 1998), although muscarinic receptors may enhance the nicotinic-evoked response in rainbow trout (Julio *et al.*, 1998; Montpetit and Perry, 1999), and in other teleosts (Gfell *et al.*, 1997; Abele *et al.*, 1998). Following nicotinic cholinergic stimulation, the sequence of intracellular events leading to catecholamine secretion involves the opening of calcium channels and the entry of Ca^{2+} ions into the cell from the extracellular fluid (Burgoyne, 1991; Burgoyne and Morgan, 1995; Burgoyne *et al.*, 1993; Furimsky *et al.*, 1997; Ungar and Phillips, 1983). Studies in mammalian systems have shown that the opening of calcium channels is preceded by the conductance of Na^+ into the cell through a nicotinic receptor-linked ion channel. The resultant membrane depolarization activates two main voltage-sensitive ion channels: fast Na^+ channels and slow Ca^{2+} channels (as aforementioned). The elevated intracellular Ca^{2+} levels elicit a series of events leading to the rearrangement of the cytoskeleton to allow the movement of catecholamine-containing granules toward the cell membrane where they fuse and release their contents into the extracellular fluid *via* exocytosis (Burgoyne, 1991).

Actions of Catecholamines

Once in the circulation, catecholamines bind to adrenergic receptors (adrenoceptors) and ultimately serve to maintain or enhance cardiovascular function (Perry and Gilmour, 1999), and blood oxygen transport (Nikinmaa, 1992) during acute, stressful situations. Stressors capable of eliciting catecholamine release include hypoxia (Ristori and Laurent, 1989), hypercapnia (Perry and Gilmour, 1996), intensive exercise (Primmett *et al.*, 1986), and air exposure (Walhqvist and Nilsson, 1980). The adrenoceptors to which circulating catecholamines, or catecholamines released from adrenergic nerve terminals bind, are classified as either alpha (α) or beta (ß) receptors. The types of α-receptors include $\alpha_{1a,\ b,\ c,}$ and $\alpha_{2a,\ b,\ c,}$ and ß-receptors include $ß_{1,\ 2,\ 3}$ according to the agonists and antagonists to which they bind and respond (Ahlquist, 1948; Lefkowitz, 1978; Collins *et al.*, 1991; Jacobs *et al.*, 1991; Nichols and Ruffolo, 1991; Strosberg, 1991; Wang *et al.*, 1991; Minneman and Esbenshade, 1994; Ruffolo and Hieble, 1994).

Some adrenergically-mediated responses caused by circulating catecholamines include the α_1 receptor-mediated contraction of the spleen that causes the release of stored red blood cells (Nilsson and Grove, 1974; Perry and Kinkead, 1989) into the circulation. Catecholamines may also increase the blood oxygen carrying capacity by acting on the β_1 adrenoceptor-mediated Na^+/H^+ exchanger (ßNHE; Borgese *et al.*, 1992) that is present on the red blood cell (RBC) plasma membrane. The ßNHE extrudes intracellular RBC protons in exchange for extracellular sodium ions, leading to an increase in intracellular RBC pH (pHi; Nikinmaa 1983, 1992; Holk and Lykkeboe, 1995). Protons present in the RBC may have a modulatory effect on Hb-O_2 binding, where a decrease in RBC pHi can decrease the affinity of Hb for molecular oxygen (Bohr effect), which is reflected as a right-shift in the hemoglobin-oxygen dissociation curve and an increase in the P_{50} (the partial pressure of oxygen at which 50% of the hemoglobin is bound with oxygen). Furthermore, a decrease in blood pH can lower the capacity of hemoglobin to bind with oxygen (Root effect; Root, 1931), which further confirms a direct role of catecholamines on blood oxygen content.

Although the ß-adrenergic stimulation of the red blood cell NHE has an effect on the transport of oxygen in the blood, it also has significant effects on the CO_2 transport and its excretion at the gills (Perry, 1986; Perry and Wood, 1989). Under normal conditions, CO_2 from the tissues enters the RBC where it is hydrated by the enzyme carbonic anhydrase (CA) to form bicarbonate (HCO_3^-) and H^+. The newly formed HCO_3^- is then extruded from the RBC to the plasma in exchange for a Cl^- ion (chloride shift) *via* a band 3 anionic exchanger (Romano and Passow, 1984), and is thus transported until it reaches the gills. At the gills, the reverse reaction takes place within the RBCs to form CO_2 and H_2O. The CO_2 may then diffuse out of the RBC and across the lamellar epithelium into the surrounding water. The activation of the ß-adrenergic NHE on the RBC membrane interferes with this process, however, and impairs CO_2 transport and excretion at the gills by rendering the extruded protons unavailable for the hydration reactions within the RBC. Thus, intracellular PCO_2 levels would decline and reverse the PCO_2 gradient between the plasma and the RBC (Thomas and Perry, 1991). CO_2 that diffuses into the RBC would be hydrated to form HCO_3^- and H^+, resulting in increased levels of HCO_3^- in the RBC that is released into the plasma *via* a chloride shift. Finally, because CO_2 cannot be excreted from the gills in the form of HCO_3^-, the increased HCO_3^- levels in the plasma impairs CO_2 excretion at the gills until RBC intracellular pH is restored.

Cardiovascular control in teleosts is predominantly controlled by adrenergic innervation to the heart and systemic vasculature, however circulating catecholamines may also bind to these receptors to elicit positive inotropic and chronotropic effects (Nilsson 1983, 1984; Wahlqvist, 1980). Systemic vascular resistance is mediated by constrictory α- and dilatory ß-receptors, however the constrictory α-adrenoceptor effect predominates (Nilsson, 1983). An increase in systemic vascular resistance can increase the systemic blood pressure, which in turn can recruit distal lamellae in the gills (Randall and Daxboeck, 1984) and result in a more even distribution of blood flow through the lamellae (Farrell *et al.*, 1980). Furthermore, ß adrenoceptor-mediated vasodilation of afferent arterioles in the lamellae may increase the gill surface area and enhance gill diffusion (Randall and Daxboeck, 1984).

The ability of circulating catecholamines to affect gill ventilation is controversial. There is evidence supporting a role for (Randall and Taylor, 1992), and against (Perry *et al.*, 1992) a role for circulating catecholamines in controlling ventilation. There is, however, general agreement that circulating catecholamines may act on a central respiratory control center to regulate gill ventilation (Randall and Perry, 1992).

Circulating catecholamines can also serve to mobilize energy stores by activating ß-adrenoceptors (Wright *et al.*, 1989) that activate liver glycogenolysis, gluconeogenesis, and inhibit glycolysis (Moon *et al.*, 1985; Mommsen *et al.*, 1988; Moon and Mommsen, 1990).

Metabolism of Catecholamines

In rainbow trout, the biological half-time of circulating catecholamines in less than 10 min (Nekvasil and Olson, 1986). Factors that influence the levels of circulating catecholamines during stress include accumulation by neuronal and extraneural tissues, overflow from adrenergic neurons, and the rate of metabolic degradation (see Nekvasil and Olson, 1986a; Randall and Perry, 1992).

The uptake of catecholamines into the tissues may be either neuronal (Type 1) or extraneuronal (Type 2; see Nilsson, 1983). Neuronal accumulation refers to the reuptake of catecholamines into adrenergic nerve terminals where they may be repackaged into secretory vesicles to be re-secreted, or they may be metabolically broken down. Extraneuronal accumulation, however, refers to the non-neuronal uptake of catecholamines that are subsequently metabolically broken down. The proportion of catecholamines that are re-absorbed by adrenergic nerve terminals is 70% (Langer, 1970; Bennet, 1972), while 23% are taken up by the target organ (Langer, 1970; Bennet, 1972). The remaining neurotransmitter and circulating catecholamines are metabolically broken down by extraneuronal mechanisms.

The ability of tissues to accumulate circulating catecholamines depends on their relative mass, the blood flow to the tissue, the availability of catabolic enzymes, the endogenous catecholamine levels, and the density of adrenergic neurons (for neuronal uptake; see Randall and Perry, 1992). Thus, although most tissues accumulate both catecholamines to varying degrees (Busacker and Chavin, 1987; Ungell, 1985a,b; Nekvasil and Olson, 1986a), the gills are a highly efficient tissue for the uptake of both neuronal and extraneuronal catecholamines.

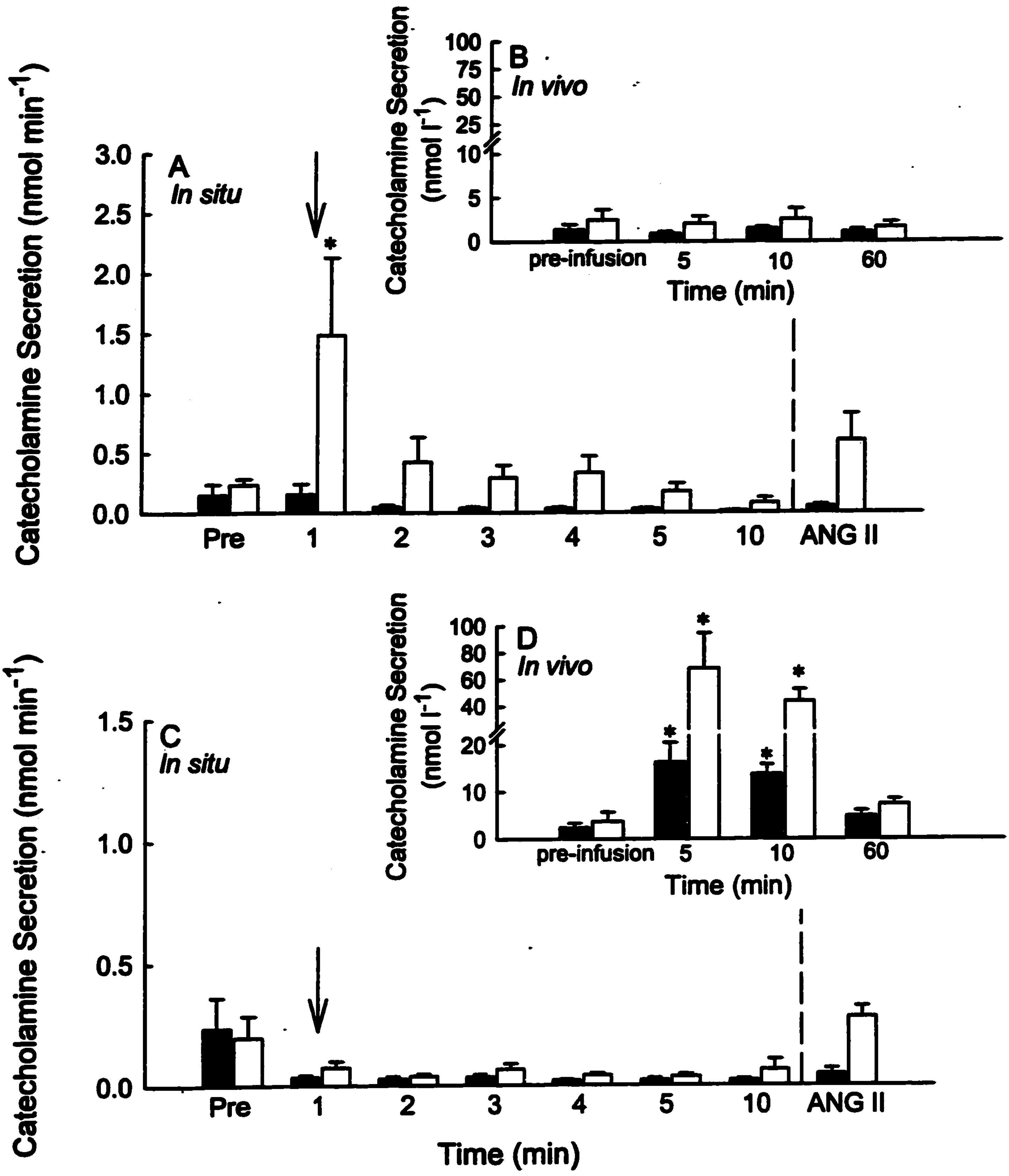
The enzyme that is predominantly responsible for the breakdown of neuronal catecholamines is mitochondrial monoaminoxidase (MAO), which removes the amino group on the side-chain of adrenaline and noradrenaline (see Figure 1-1). The enzyme that catabolizes the circulating catecholamines, however, is catechol-O-methyl transferase (COMT; Kopin, 1964; Fänge and Hanson, 1973), which methylates the hydroxyl group on the 3-position (see Figure 1-1; see Nilsson, 1993). The individual catabolites are then excreted in the urine.

Control of Catecholamine Release: Desensitisation of Nicotinic Cholinergic Receptors

The successful adaptation of many fish species to stressors depends, in part, upon the proper control of catecholamine secretion into the circulation. In certain tissues, excessive stimulation by catecholamines can lead to reduced sensitivity and attenuate responses to subsequent adrenergic stimulation (Thomas *et al.*, 1991). In mammals, excessive secretion of catecholamines may be prevented by rapid desensitisation of nicotinic receptors (Boska and Livett, 1984; Malhotra *et al.*, 1988), a process whereby receptors are inactivated after prolonged or repeated application of agonist. Results from numerous studies utilizing mammalian systems suggest that there are probably several processes that contribute simultaneously to desensitisation of the nicotinic receptors. When desensitised, the nicotinic receptor-linked ion channel is inactivated and membrane depolarization is prevented (for reviews, see Marley, 1988; Ochoa *et al.*, 1989). Inactivation of the ion channel is thought to involve phosphorylation of the nicotinic receptor following activation of several protein kinases (for a review, see Swope *et al.*, 1999). In addition to nicotinic receptor desensitisation, the decreased responsiveness of chromaffin cells may be caused by post-receptor modifications of the intracellular reactions leading to catecholamine release. In mammalian systems, the time course of desensitisation is highly variable and related to the duration and strength of prior receptor stimulation (Rowell and Duggan, 1998), and to the particular alpha (α) and beta (ß) molecular subunits that make up the chromaffin cell nicotinic receptor (Cachelin and Jaggi, 1991; Juetje and Patrick, 1991). Furthermore, mammalian systems also display an up-regulation of the nicotinic receptors upon desensitisation (Schwartz and Kellar, 1983) and an increased affinity for agonist (Higgins and Berg, 1988).

In rainbow trout (*Oncorhynchus mykiss*), Lapner *et al.* (2000) developed and validated a protocol to elicit nicotinic receptor desensitisation *in vivo*, *via* nicotine infusion (for a diagram of nicotine, see Figure 1-1). Figure 1-2 depicts the results from a

Figure 1-2. The effects of nicotine perfusion (10^{-5} mol l^{-1}; beginning at the arrow) on noradrenaline (filled columns) or adrenaline (open columns) secretion in *in situ* perfused posterior vein preparations of rainbow trout (*Oncorhynchus mykiss*) obtained from fish pre-infused (0.2 ml min^{-1}) *in vivo* for 60 min with (A) saline (N=8) or (C) nicotine (1.3 x 10^{-5} mol kg^{-1}; N=7). The vertical dashed line indicates a bolus injection of trout angiotensin II (ANG II). B and D illustrate the effects of the prior saline and nicotine infusions, respectively, *in vivo* on plasma noradrenaline (filled columns) and adrenaline (open columns) levels. Values are shown as means + SEM. * denotes a statistically significant difference ($P<0.05$) from the appropriate pre-injection (Pre) value *in vivo* or *in situ*; ‡ denotes a significant difference ($P<0.05$) from the 10 min *in situ* values.


A
In situ
Catecholamine Secretion (nmol min^{-1})
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Pre
1
2
3
4
5
10
ANG II
B
In vivo
Catecholamine Secretion (nmol l^{-1})
100
75
50
25
10
5
0
pre-infusion
5
10
60
Time (min)
C
In situ
Catecholamine Secretion (nmol min^{-1})
1.5
1.0
0.5
0.0
Pre
1
2
3
4
5
10
ANG II
Time (min)
D
In vivo
Catecholamine Secretion (nmol l^{-1})
100
80
60
40
20
10
0
*
*
*
*
pre-infusion
5
10
60
Time (min)

combination of *in vivo* and *in situ* techniques that confirmed nicotinic receptor desensitisation (Lapner *et al.*, 2000). Perfused posterior vein preparations derived from fish pre-infused for 60 min with saline demonstrated a pronounced secretion of adrenaline in response to 10^{-5} mol l^{-1} nicotine (Fig. 1-2A). The secretion of adrenaline was transient despite the continuing presence of nicotine in the perfusate. In contrast, perfused preparations derived from fish previously experiencing nicotine infusion did not secrete catecholamines during perfusion with 10^{-5} mol l^{-1} nicotine (Fig. 1-2C). Unlike the saline-infused group, in which plasma catecholamine levels were constant, the nicotine-infused fish displayed a transient elevation of circulating catecholamine levels (compare Fig. 1-2B and D). To ensure the lack of responsiveness of the preparations derived from nicotine-infused fish was not simply a consequence of non-viable chromaffin tissue, the potent non-cholinergic secretagogue angiotensin II was delivered as a bolus injection prior to terminating each experiment. The control preparations (Fig. 1-2A) and the unresponsive (desensitised) preparations (Fig. 1-2C) exhibited similar increases in rates of adrenaline secretion after angiotensin II injection. These data confirm that the rainbow trout chromaffin cell nicotinic receptors were, indeed, desensitised. A significant recovery period is likely required prior to the re-establishment of normal nicotinic receptor function (Lapner *et al.*, 2000).

Non-Cholinergic Catecholamine Secretagogues

There are numerous non-cholinergic mechanisms of evoking catecholamine secretion in fish. These include angiotensin II, the bioactive peptide of the renin angiotensin system (RAS; see Figure 1-1; Bernier and Perry, 1997), serotonin (see Figure

1-1; Fritsche *et al.*, 1993), adrenocorticotropic hormone (ACTH; Reid *et al.*, 1996) and a variety of autocrine/paracrine agents (Epple *et al.*, 1993, 1994; Reid *et al.*, 1996). Although in the Atlantic cod (*Gadus morhua*), local hypoxia in the vicinity of the chromaffin cells can directly evoke catecholamine secretion (Perry *et al.*, 1991), a similar mechanism does not appear to exist in trout (Perry *et al.*, 2000). Other factors include non-cholinergic neurotransmitters such as vasoactive intestinal polypeptide (VIP) and pituitary adenylate-cyclase activating polypeptide (VACAP; Reid *et al.*, 1995; Montpetit and Perry, 2000).

Using the information provided in the preceding pages, the following goals and hypotheses were established for my study.

Goals:

Record *in vivo* respiratory, blood gas, and cardiac parameters during 60 min nicotine infusion followed by acute hypoxia (40 – 45 mm Hg) and compare these results with saline-infused (control) fish.

Measure plasma catecholamine levels in nicotinic receptor-desensitised fish exposed to acute hypoxia after selective blocking of either the serotonergic or muscarinic receptors, or the RAS, to determine whether fish retain the ability to secrete catecholamines.

Test hypotheses: 1) that nicotine infusion and possible side-effects elicited by nicotine stimulation of sympathetic neuronal nicotinic receptors will not undermine the ability of the fish to recruit other catecholamine secretagogues during chromaffin cell nicotinic receptor desensitisation, 2) that the secretion of catecholamines from chromaffin cells is reliant on the serotonergic or muscarinic receptors, or the RAS in eliciting catecholamine release during nicotinic receptor desensitisation, and hence selectively blocking these secretagogues prior to acute hypoxia in nicotinic receptor-desensitised fish will prevent catecholamine release.

Chapter 2

VALIDATION OF *IN VIVO* NICOTINIC RECEPTOR DESENSITISATION PROTOCOL IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

INTRODUCTION

Hypoxia is a well characterized stressor that leads to a set of general responses including an elevation of blood pressure, increased systemic vascular resistance and bradycardia (Satchell, 1961; Holeton and Randall, 1967; Piiper *et al.*, 1970; Wood and Shelton, 1980; Fritsche and Nilsson, 1989, 1990; Fritsche, 1990; Bushnell and Brill, 1991; Maxime *et al.*, 1995). The physiological responses to hypoxia are initiated, in part, by peripheral chemoreceptors that are located in the gill region in teleosts (Bamford, 1974; Smith and Jones 1978; Fritsche and Nilsson, 1989; Burleson and Smatresk, 1990), in the mouth and pharynx area, and in the vasculature (De Kock, 1963; Freihofer, 1978; Vasilevskaya and Polyakova, 1979, 1981; Walker *et al.*, 1981). These receptors monitor changes in the external and internal environment (Burleson and Smatresk, 1990), and react to aquatic hypoxia by increasing ventilation to maintain a significant oxygen gradient between the water and the blood (Burleson and Smatresk, 1990; Bushnell and Brill, 1991; Kinkead *et al.*, 1991), and by altering neuronal (sympathetic and parasympathetic nerves) and humoral (circulating catecholamines) activity (Fritsche and Nilsson, 1993; Bushnell and Jones, 1992; Farrell, 1993; Olson, 1998).

Circulating and neuronal catecholamines elicit physiological changes to maintain or enhance cardiovascular function (Perry and Gilmour, 1999) and blood oxygen transport (Nikinmaa, 1992) during acute stress. Catecholamines stored in chromaffin cells are released upon stimulation by preganglionic sympathetic nerve fibres of the autonomic nervous system. The primary stimulus that elicits catecholamine secretion is thought to be *via* the nicotinic cholinergic receptors (Nilsson *et al.*, 1976).

Recently, Lapner *et al.* (2000) demonstrated that chromaffin cell nicotinic receptor desensitisation in rainbow trout, catecholamine secretion into the circulation still occurred during acute hypoxia. The protocol used to desensitise the nicotinic receptors *in vivo* involved intravenous nicotine infusion for a period of 60 min. Although this protocol was developed to target chromaffin cell nicotinic receptors, the nicotinic receptors that are present throughout the nervous system may also be susceptible to desensitisation. If this is the case (i.e. neuronal nicotinic receptors undergo desensitisation *via* nicotine infusion), this may have physiological consequences that affect the normal physiological responses to hypoxia.

Thus, the purpose of this study was to determine whether the protocol used by Lapner *et al.* (2000) to desensitise chromaffin cell nicotinic receptors has physiological consequences that interfere with the ability of fish to secrete catecholamines in response to acute hypoxia. Experiments were performed *in vivo* using an extracorporeal blood loop (EC loop).

MATERIALS AND METHODS

Experimental animals

Rainbow trout [*O. mykiss* (Walbaum)] weighing between 300 and 500 g (mean mass 562.4 ± 40.2 g; mean ± S.E.M., N = 19) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and were held indoors in large fiberglass tanks supplied with dechlorinated City of Ottawa tap water that was maintained at 13°C. Fish were allowed to acclimate to the aquarium for at least three weeks before experimentation. Fish were maintained on a 12 h: 12h L:D photoperiod and fed daily to satiation with a commercial salmonid diet until 24h prior to experimentation.

Animal preparation

Surgical procedures

Rainbow trout were anaesthetized in an aerated solution of ethyl-*P*-amino-benzoate (benzocaine; Sigma Chemical Co.; 1g benzocaine dissolved in 10 ml 95% ethanol/ 25L water; final concentration 2.4×10^{-4} mol l^{-1}) and placed on an operating table where the gills were continuously irrigated with aerated anaesthetic solution. An indwelling polyethylene cannula (Clay-Adams PE50 polyethylene tubing; internal diameter 0.580 mm, outer diameter 0.965 mm) was implanted into the dorsal aorta to permit measurement of arterial blood pressure (P_{DA}) via percutaneous puncture of the roof of the buccal cavity (Soivio *et al.*, 1975). To permit monitoring of inspired water PO_2, a heat-flared cannula (Clay-Adams PE160 polyethylene tubing; internal diameter 1.14 mm, outer diameter 1.57 mm) was inserted through the snout into the oral cavity. A lateral incision was made at the level of the caudal peduncle in order to insert a third and fourth cannula (PE 50) into the caudal artery and vein to allow blood withdrawal and return from an extracorporeal blood loop (see below). The caudal vein cannula was fastened with a three-way extension to permit nicotine or saline infusion, and periodic blood sampling. The incision was made approximately 4 mm below the lateral line and was approximately 3 cm long. The cannulae were inserted into the caudal artery and vein in the retrograde and orthograde direction, respectively. The caudal incision was sutured using a running stitch and the cannulae were secured to the body wall with silk ligatures. The opercula of these fish were fastened, using a silk ligature, with small (1 cm^2) brass plates that were connected with a cable to an impedance converter to allow for the measurement of ventilation amplitude and frequency (Peyraud and Ferret-Bouin, 1960). After surgery, trout were placed into individual opaque Perspex boxes supplied with aerated flowing water and allowed to recover for 24 h prior to experimentation.

Experimental protocol

Fish were infused (0.2 ml min^{-1}) *via* the caudal vein with nicotine ($1.3x10^{-5}$ mol $kg^{-1}h^{-1}$) or saline (control) using a syringe infusion pump (Sage Instruments) for 60 min. They were then subjected to 10 min of hypoxia (inspired PO_2=40-45 mmHg; 5.3-6.0 kPa), while the infusion continued.

Acute hypoxia was achieved by replacing the air supplying a water/gas equilibration column with N_2. The desired water PO_2 (PwO_2) (40-45 mm Hg) was pre-set and established by adjusting the rate of water and/or N_2 flow through the column. The level of PwO_2 was chosen on the basis of a previous study (Perry and Reid, 1992) that demonstrated significant catecholamine release in rainbow trout using this protocol.

The PwO_2 within the box was monitored continuously using a peristaltic pump (flow = 0.6 ml min^{-1}) that withdrew water from each individual trout box and passed it across a PO_2 electrode (Cameron Instruments) connected to an O_2 meter (Cameron Instruments). Generally, the desired PwO_2 in the experimental box was reached within 10 min and thereafter never varied more than ± 5 mm Hg (0.67 kPa). After experimentation, P_WO_2 was restored to normoxic levels and fish were allowed to recover.

Determination of plasma catecholamine levels

Blood samples collected for catecholamine analysis were taken immediately prior to beginning the infusion and after 10 and 60 min of infusion. Four blood samples were withdrawn during the hypoxic period: at 0, 3, 5, and 10 min after reaching the targeted 40 – 45 mm Hg PwO_2. Due to fluctuating P_WO_2 levels during the 10 min hypoxic period (± 5 mm Hg; 0.67 kPa), the blood sample that was withdrawn at a P_WO_2 level nearest to the target of 40 mm Hg was used to represent levels during hypoxia. The collected blood samples were placed into micro-centrifuge tubes (1.5 ml) and centrifuged (12 000 *g* for 20 s). The plasma was transferred to micro-centrifuge tubes containing 10 µl (25 units) of heparin (ammonium salt). Samples were quick-frozen in liquid N_2 and then stored at – 80° C until subsequent analysis.

Extraction of plasma catecholamine samples

All plasma catecholamine samples were subjected to alumina extraction and then analysed by high-pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). 3,4-Dihydroxybenzylamine hydrobromide (DHBA) was used as an internal standard in these analyses. Detection limits for adrenaline and noradrenaline were 0.1 nmol l^{-1}.

Extra-corporeal blood shunt

Blood was monitored continuously for arterial O_2 tension (PaO_2), arterial CO_2 tension ($PaCO_2$), and arterial pH (pHa) using an extracorporeal blood shunt (Thomas, 1994). A peristaltic pump (flow = 0.6 ml min^{-1}) was used to withdraw blood from the caudal artery and pass it through a series of O_2, CO_2 (Cameron Instruments, Inc.) and pH (Metrohm) electrodes before returning it to the fish *via* the caudal vein cannula (Thomas, 1994). The blood gas electrodes were housed in temperature controlled cuvettes and connected to a Radiometer PHM 73 blood gas analyzer.

The O_2 electrodes were calibrated with a zero solution (PO_2 = 0 mm Hg; 2% (w/v) sodium sulfite) or air-saturated water (PO_2 = 160 mm Hg) that was continuously pumped through the electrode sample compartments, using the EC loop peristaltic pump, until stable readings were obtained. The CO_2 electrode was calibrated using mixtures of 0.5% and 1.0% CO_2 in air provided by a gas-mixing flowmeter (Cameron gas flowmeter) using the same peristaltic pump. The pH electrode was calibrated using precision buffers (Sigma). All electrodes were calibrated prior to each experiment.

Immediately prior to experimentation, the extracorporeal shunt was rinsed for 20 min with a solution of ammonium heparin (540 units ml^{-1} in Cortland (Wolf, 1963) saline) to prevent blood from clotting in the tubing and electrode chambers. A period of 10 min was required to achieve stable recordings of PaO_2, $PaCO_2$, and pHa prior to commencing the experiment. The extracorporeal blood loop contained approximately 1 ml of blood representing less than 3% of the total blood volume. Analog signals from the meters were converted to digital data and collected and stored in a computer using a data acquisition system (Biopac) and accompanying software (AcqKnowledge 3.03).

Measurement of Cardiovascular and Ventilation Variables

Ventilation was assessed by monitoring opercular impedance changes using a custom-built impedance converter and amplifier. Ventilation amplitude (Vamp) was determined after conversion of the impedance data to linear opercular deflections (in cm) through appropriate calibration (performed by manually displacing the opercular covers known distances on euthanised animals).

The dorsal aorta was flushed with heparinised saline (50 units ml^{-1}) to prevent clotting and then connected to a pressure transducer (Bell and Howell) that was pre-calibrated against a static column of water. Analog blood pressure signals were measured using Harvard Biopac amplifiers (DA 100).

Analog signals for ventilation and blood pressure were converted to digital data by interfacing with a data acquisition system (Biopac Systems Inc.) using Acknowledge^{TM} data acquisition software (sampling rate of 40 sec^{-1}) and a Pentium^{TM} PC. Ventilation amplitude was determined from the difference between maximum and minimum impedance values. Ventilation frequency (Vf) was determined using an automatic rate calculation from peaks in the raw ventilation amplitude traces. Mean arterial blood pressure was calculated as (systolic pressure + diastolic pressure)/2, and cardiac frequency was determined using an automatic rate calculation from peaks in the blood pressure traces.

Statistical analyses

The catecholamine data are presented as means + 1 standard error of the mean (S.E.M). All other data are presented as means ± 1 S.E.M, and are shown as absolute changes, which were calculated by assigning a value of 0 to the 10 min value ('pre' value) and subtracting this value from all other pre and post-10 min values. Data were analysed statistically using two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, except for catecholamine data, which were analysed statistically using one-way ANOVA followed by Dunn's multiple-comparison test; if assumptions for parametric statistics were violated, an ANOVA on ranks was performed followed by Dunn's multiple-comparison test. Catecholamine data were also analysed using a Student's *t*-test, and if assumptions for parametric statistics were violated, a Mann-Whitney rank sum test was performed. All statistical analyses were performed using commercial software (SigmaStat Version 2.0; SPSS); the fiducial limits of significance were set at 5%.

RESULTS

For all data except data depicting plasma catecholamine levels (which are shown in Figure 2-1), absolute changes from a 'pre' value (10 min value) were calculated and are depicted in Figures 2-2 – 2-4. This was done in order to observe the changes within the fishes, for that particular parameter, rather than the differences between the fish (which may have been larger than the recorded differences from a 'pre' value).

To confirm the ability of fish with desensitised nicotinic receptors to release catecholamines into the circulation upon acute hypoxia, saline or nicotine (to desensitise the nicotinic receptors; Lapner *et al.* 2000) was infused continuously for 60 min prior to the onset of hypoxia and continued throughout the hypoxic period. As expected, the nicotine infusion elicited a significant increase in plasma catecholamine levels at 10 min that was not observed in saline–infused fish (Fig 2-1). Owing to receptor desensitisation, the elevated plasma catecholamine levels returned to baseline levels within 60 min, despite continuous nicotine stimulation (Fig 2-1B).

Upon acute hypoxia, a significant increase in plasma adrenaline and noradrenaline levels was observed in fish with both functional (Fig 2-1A) and non-functional/desensitised (Fig 2-1B) nicotinic receptors. However, noradrenaline levels were reduced in comparison to hypoxic control fish (Fig 2-1).

Figure 2-2 displays ventilation (A) amplitude and (B) frequency throughout a 60 min saline (control) or nicotine (to desensitize chromaffin cell nicotinic receptors) infusion. The nicotine infusion commenced at 10 min and continued throughout the experiment. Acute hypoxia was induced after 60 min infusion, however the values during 10 min of hypoxia were recorded once the water PO_2 reached 45 mm Hg. In saline-infused fish, ventilation amplitude remained constant ('pre' value = 0.38 ± 0.11 cm), but increased significantly during hypoxia to 1.10 ± 0.38 cm. The nicotine-infused fish, however, displayed an initial increase in amplitude from the 'pre' value of 0.39 ± 0.04 to 0.95 ± 0.17 cm, at 5-6 min infusion. Ventilation amplitude rapidly returned to baseline levels, however, where they remained for the rest of the infusion and hypoxia. The hypoxic values were significantly different from the control values.

Ventilation frequency (Fig. 2-2 (B)) did not differ significantly from the 'pre' value of 74 ± 4 breaths min^{-1} throughout the infusion, and hypoxic values were not altered from the pre-hypoxic value of 109 ± 4 breaths min^{-1}. The nicotine-infused fish, however, showed an increase in ventilation frequency from the 'pre' value of 74 ± 4 that remained elevated throughout the infusion, and stabilized at 110 ± 4 breaths min^{-1}. During hypoxia these values fell significantly to 67-83 ± 5 breaths min^{-1}, but only the initial 2 min of hypoxia differed significantly from control values.

Figure 2-3 depicts (A) the water PO_2 (PwO_2), (B) arterial PO_2 (PaO_2), (C) arterial PCO_2 ($PaCO_2$), and (D) pH throughout a nicotine or saline infusion, followed by acute hypoxia while the infusion continued. As described, PwO_2 remained at 'pre' levels (148.9 ± 5.2 mm Hg) throughout the infusion period in both experimental and control fish. During acute hypoxia, PwO_2 levels fell to 40-45 mm Hg in both groups of fish and did not vary more than ± 3 torr during this period.

Arterial PO_2 mirrored the PwO_2 and remained constant (122.1 ± 6.3 mm Hg) throughout the saline infusion prior to hypoxia, and fell to 17 – 29 ± 4 mm Hg during hypoxia. In nicotine-infused fish, however, there was a reduction in PaO_2 levels from the 'pre' value (118.5 ± 5.5 mm Hg), that became apparent at 24-25 min following the commencement of the nicotine infusion, and remained at these levels (95 ± 6 mm Hg) until hypoxia, at which time they fell significantly (11-16 ± 2 mm Hg). These values did not differ significantly from saline-infused PaO_2 levels during hypoxia.

Figure 2-3 (C) depicts the $PaCO_2$ in control fish that, throughout the 60 min pre-hypoxic saline infusion, did not differ significantly from the 'pre' value (2.4 ± 0.4 mm Hg). During acute hypoxia, $PaCO_2$ levels fell significantly to 1.7 ± 0.1 mm Hg. In nicotine-infused fish, $PaCO_2$ levels fell and re-stabilized at a higher level than the 'pre' value (2.7 ± 0.2 mm Hg), although there was no statistically significant elevation from the 'pre' value. During hypoxia in nicotine-infused fish, only the initial 2 min of hypoxia showed a significant reduction in $PaCO_2$ from the pre-hypoxic value, and the last 8 min of hypoxia (2.6 ± 0.2 mm Hg) did not differ significantly from the infusion levels but were significantly different from the control hypoxic values.

The pH values shown in Figure 2-3 (D) remained constant throughout the saline infusion ('pre' value = 7.79 ± 0.03) and did not change significantly during acute hypoxia. In nicotine-infused fish, an initial drop in pH was apparent between 10 and 23 min after the commencement of the infusion ('pre' value = 7.94 ± 0.09), which differed from the control value at 20-21 min., but returned to baseline levels prior to hypoxia. During the hypoxic period, however, pH levels in nicotine-infused fish were significantly different from the control fish. The last 5 min. of hypoxia were significantly reduced from the pre-hypoxic value of 7.88 ± 0.12 to levels of 7.68-7.74 ± 0.14.

Figure 2-4 depicts (A) arterial blood pressure and (B) heart frequency throughout the nicotine or saline infusion and hypoxia. Blood pressure was not altered from the saline infusion in control fish ('pre' value = 33.4 ± 3.0 mm Hg) or during the hypoxic period. Nicotine-infused fish, however, displayed a significant but transient elevation in blood pressure following the commencement of the nicotine infusion. During hypoxia however, blood pressure was reduced significantly in nicotine-infused fish to values that differed from the control values (30 ± 5 mm Hg). The heart frequency decreased during the initial six min of nicotine infusion but returned to pre-infusion levels ('pre' value = 32 ± 3 beats min^{-1}) for the remainder of the infusion. During hypoxia, the heart frequency of the nicotine-infused fish did not change significantly compared to pre-hypoxic levels. In control fish, however, heart frequency remained at pre-infusion levels (32 ± 3 beats min^{-1}) throughout the saline infusion but decreased significantly during hypoxia. Heart frequency did not differ significantly between control and experimental groups.

Figure 2-1. The effects of acute hypoxia (40 mm Hg; 5.3 kPa; at the vertical dashed line) on plasma levels of adrenaline (filled columns) or noradrenaline (open columns) in rainbow trout (*O. mykiss*) previously infused (0.2 ml min^{-1}) with (A) saline (N=9); or (B) nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}; N=10) for 60 min under conditions of normoxia. Plasma catecholamine levels were taken at 'Pre', 10 min, and 60 min of infusion, and during acute hypoxia. Values are shown as means + SEM. * denotes a statistically significant increase ($P<0.05$) from the corresponding 'Pre' value or '60 min' pre-hypoxia value.

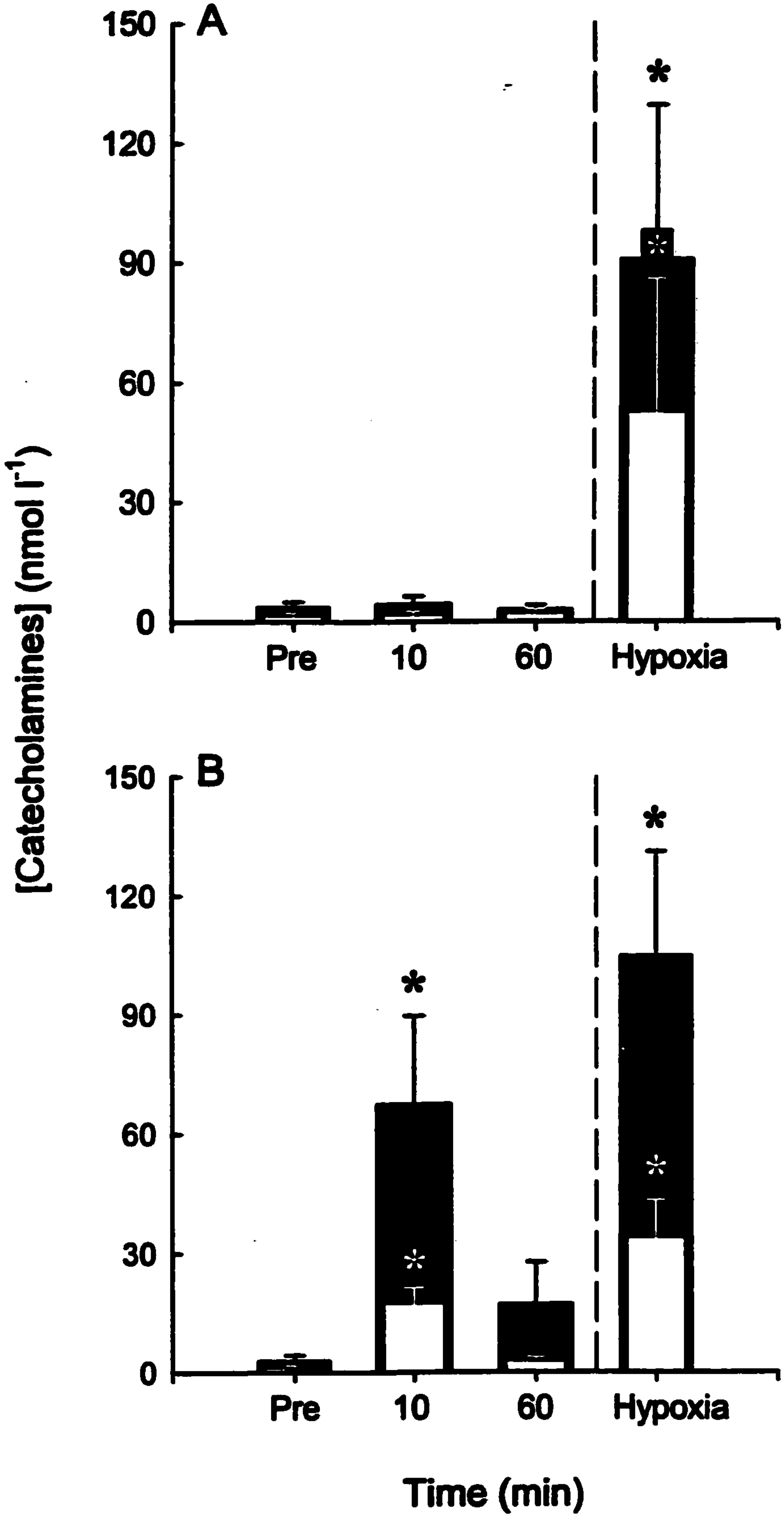

A
B
150
120
90
60
30
0
[Catecholamines] (nmol l^{-1})
Pre
10
60
Hypoxia
*
Time (min)

Figure 2-2. Changes in ventilatory parameters including (A) amplitude (Vamp; cm) and (B) frequency (Vf; breaths min^{-1}) over time (minutes) during a continuous infusion (0.2 ml min^{-1}) of nicotine (1.3 x 10^{-5} mol kg^{-1} h^{-1}; N=10, black circles); or saline (N=9, open circles) for 60 min followed by acute hypoxia (40 torr) as the infusion continued. The first vertical dashed line indicates the commencement of the infusion and was assigned a value of zero to calculate overall changes before and after the commencement of infusion. The second dashed line represents a drop in water PO_2 to 40 torr. Values are shown as means ± SEM. * denotes a statistically significant difference ($P<0.05$) from the corresponding pre-infusion or pre-hypoxia value. † indicates a statistically significant difference ($P<0.05$) from the control value (saline infusion).

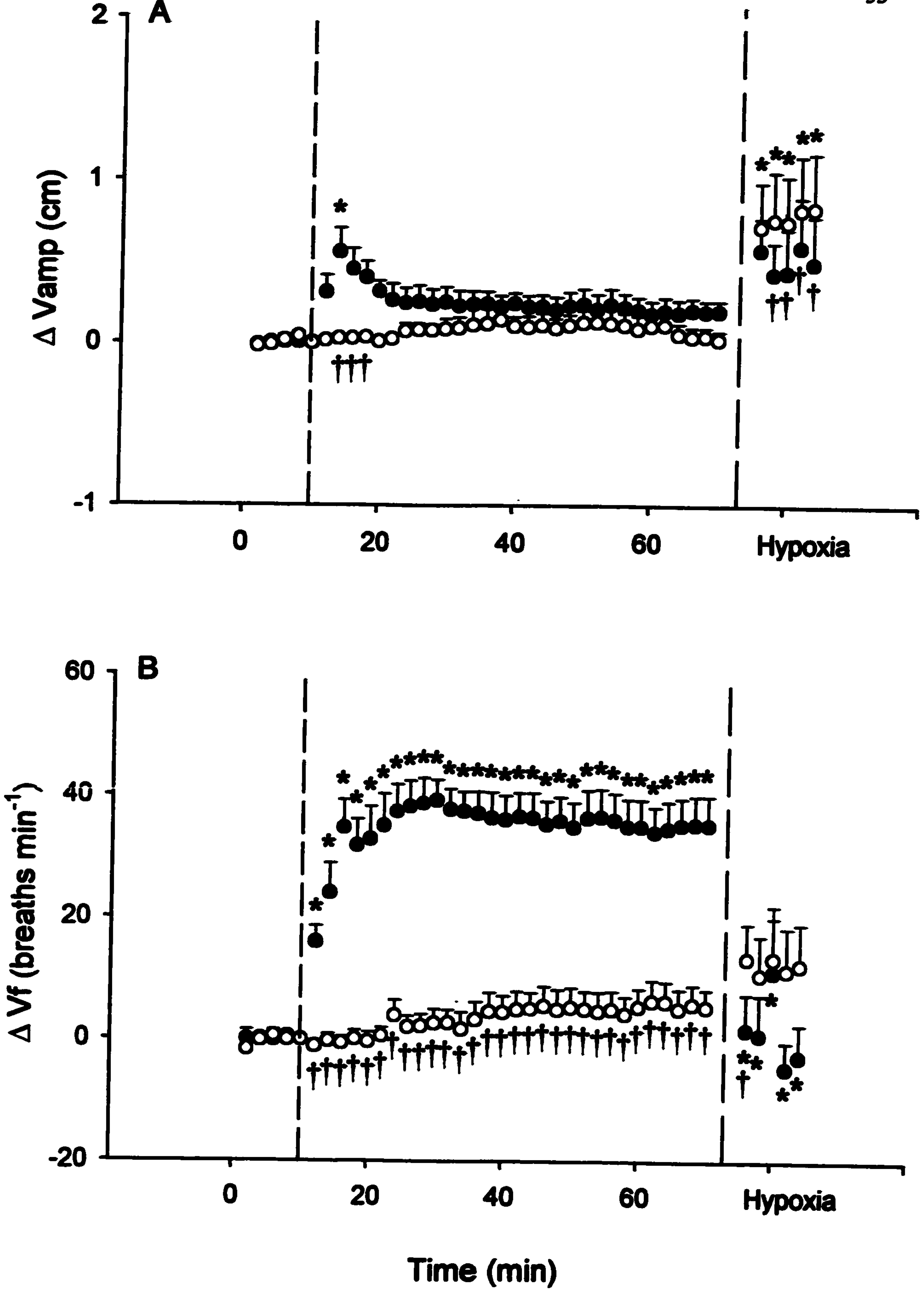

A
2
1
0
-1
Δ Vamp (cm)
0
20
40
60
Hypoxia
B
60
40
20
0
-20
Δ Vf (breaths min^{-1})
0
20
40
60
Hypoxia
Time (min)

Figure 2-3. Changes in water PO_2 (Torr) and the accompanying changes in arterial blood gases over time (minutes) including arterial (B) PO_2 (Torr), (C) PCO_2 (Torr), and (D) pH, during a continuous infusion (0.2 ml min^{-1}) of nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}; N=10, black circles); or saline (N=9, open circles) for 60 min followed by acute hypoxia (40 torr) as the infusion continued. The first vertical dashed line indicates the commencement of the infusion and was assigned a value of zero to calculate overall changes before and after the commencement of infusion. The second dashed line represents a drop in water PO_2 to 40 torr. Values are shown as means ± SEM. * denotes a statistically significant difference ($P<0.05$) from the corresponding pre-infusion or pre-hypoxia value. † indicates a statistically significant difference ($P<0.05$) from the control value (saline infusion).

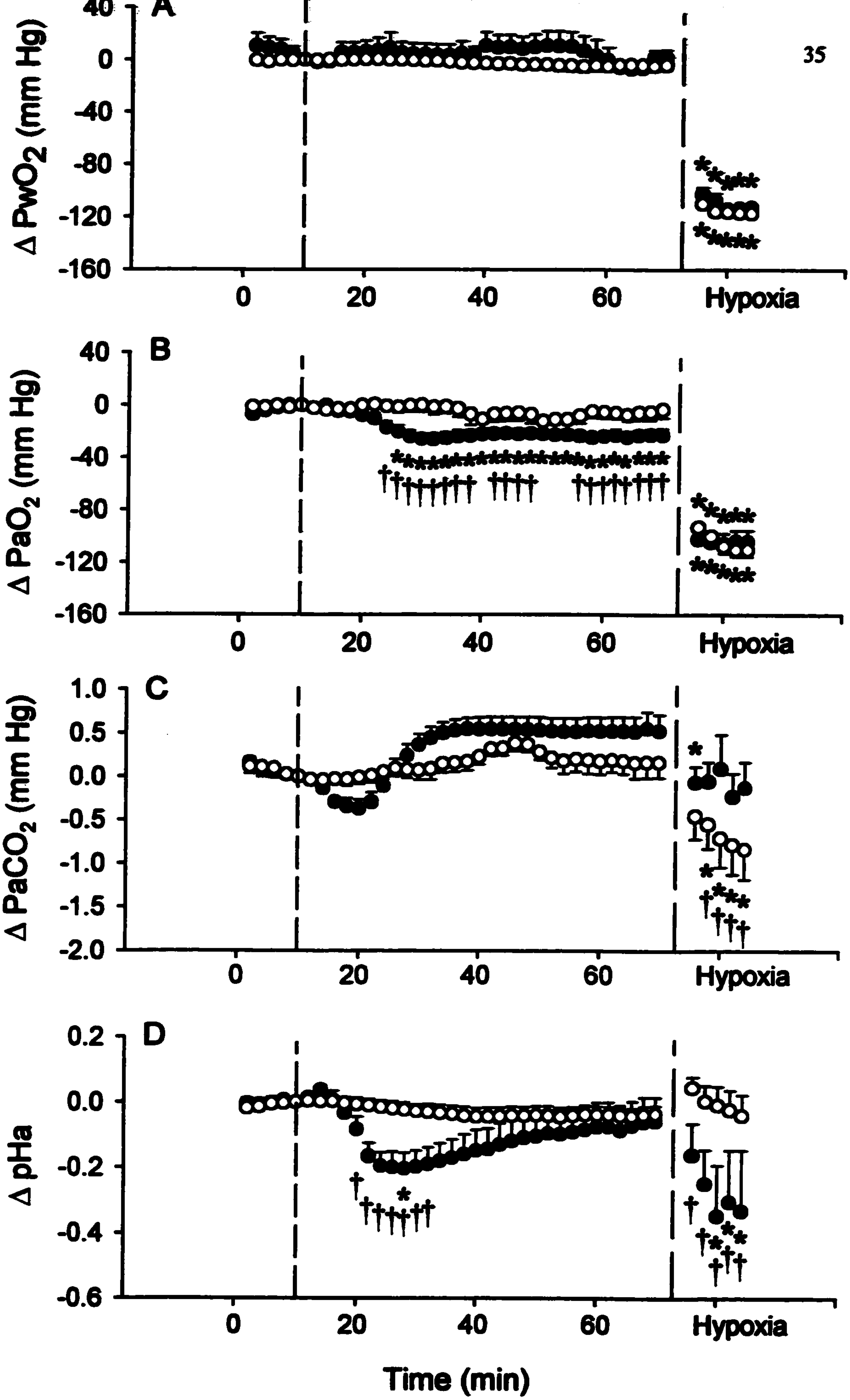
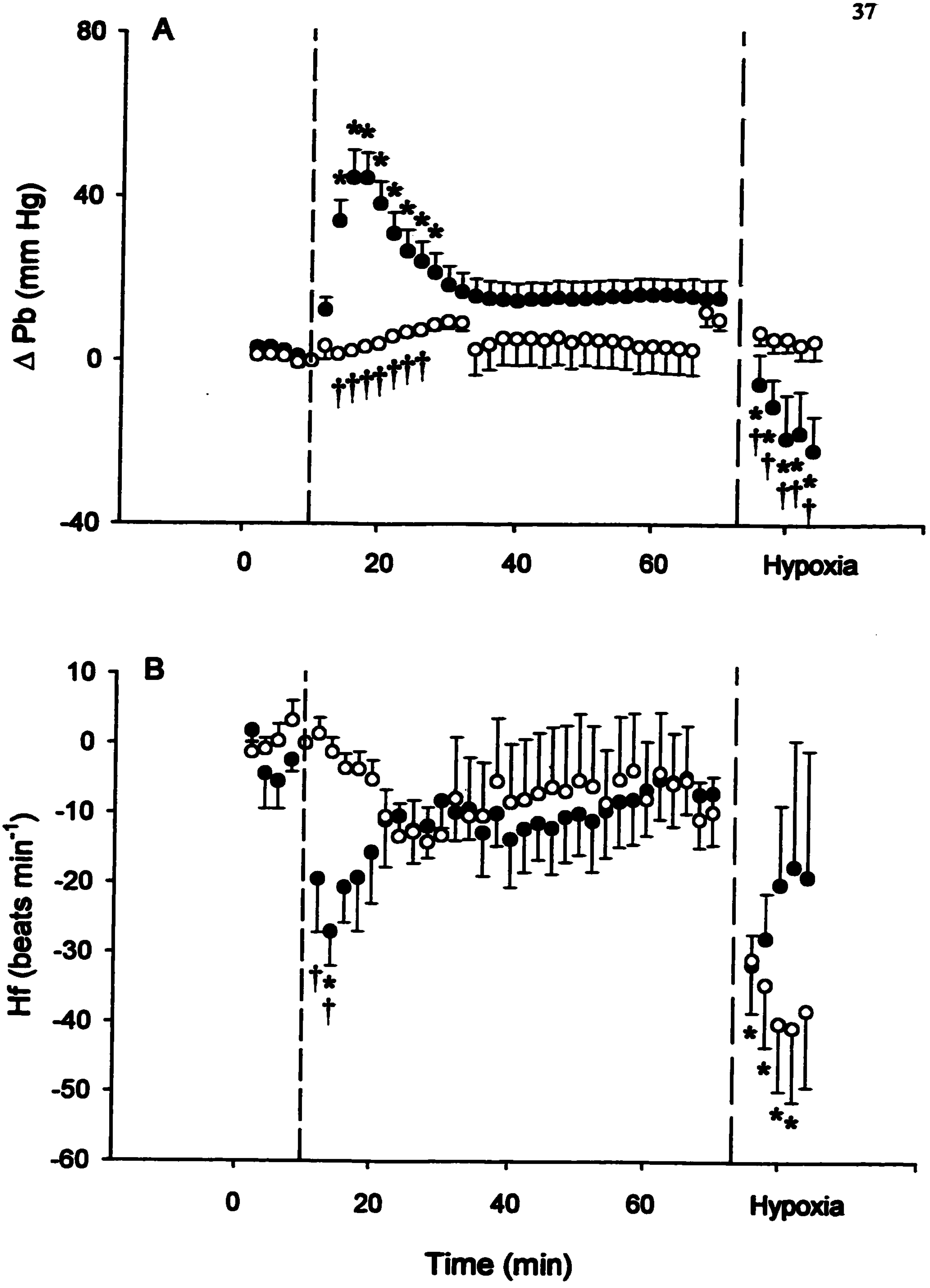

A
Δ PwO$_2$ (mm Hg)
40
0
-40
-80
-120
-160
0
20
40
60
Hypoxia
B
Δ PaO$_2$ (mm Hg)
40
0
-40
-80
-120
-160
0
20
40
60
Hypoxia
C
Δ PaCO$_2$ (mm Hg)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0
20
40
60
Hypoxia
D
Δ pHa
0.2
0.0
-0.2
-0.4
-0.6
0
20
40
60
Hypoxia
Time (min)

Figure 2-4. Changes in (A) arterial blood pressure P_{DA} (cm H_2O) and (B) heart frequency (beats min^{-1}) over time (minutes) during a continuous infusion (0.2 ml min^{-1}) of nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}; N=10, black circles); or saline (N=9, open circles) for 60 min followed by acute hypoxia (40 torr) as the infusion continued. The first vertical dashed line indicates the commencement of the infusion and was assigned a value of zero to calculate overall changes before and after the commencement of infusion. The second dashed line represents a drop in water PO_2 to 40 torr. Values are shown as means ± SEM. * denotes a statistically significant difference ($P<0.05$) from the corresponding pre-infusion or pre-hypoxia value. † indicates a statistically significant difference ($P<0.05$) from the control value (saline infusion).


A
80
40
0
-40
Δ Pb (mm Hg)
0
20
40
60
Hypoxia
B
10
0
-10
-20
-30
-40
-50
-60
Hf (beats min-1)
0
20
40
60
Hypoxia
Time (min)

DISCUSSION

A protocol employing intravenous nicotine infusion was recently developed by Lapner *et al.* (2000) to desensitize rainbow trout chromaffin cell nicotinic receptors. Although the infusion protocol was successful at desensitising the nicotinic receptors and is intended to be used in future studies (e.g. Chapter 3), little is known about the effects of a prolonged nicotine infusion on fish cardiovascular and respiratory physiology, and how this might affect their ability to mount an appropriate response to acute hypoxia. Recently, the effects of acetylcholine and nicotine injections on cardiorespiratory variables in rainbow trout (*O. mykiss*) were investigated *in vivo* by Burleson and Milsom (1995). They found that acetylcholine or nicotine injections caused an increase in opercular pressure, gill ventilation rate, blood pressure, and heart rate. Results from the present study are in agreement with their findings for all commonly measured variables except heart rate, which in the present study, was reduced significantly within the first six min. of infusion.

Upon stimulation from nicotine, the nicotinic receptors of trout chromaffin cells elicit catecholamine secretion. Plasma catecholamine levels were elevated at 10 min infusion but then declined to baseline levels during the remaining 50 min period of infusion, indicative of chromaffin cell receptor desensitisation (Lapner *et al.*, 2000).

Nicotine from the infusion may have also affected peripheral chemoreceptor output to the respiratory centers of the medulla (for review see Fritsche and Nilsson, 1993), as ventilation amplitude and frequency were significantly elevated during the infusion. Mechanistic studies of O_2 chemoreceptors performed in mammals have shown that Type I (glomus) cells release neurotransmitters onto the nerve endings that are in synaptic contact with the glomus cells, to modify or control the response to low oxygen levels (Jones and Milsom, 1982). Dempsey *et al.* (1986) showed that acetylcholine stimulates respiration through a nicotine-like action on peripheral chemoreceptors, and other experiments that have tested the effects of neurotransmitters on chemoreceptor activity on isolated perfused gill arches of rainbow trout have shown that acetylcholine is the most potent factor in stimulating receptor discharge (Burleson, 1991). Thus, in the present study, nicotinic-receptor stimulation to the afferent nerves of the peripheral chemoreceptors that convey chemosensory output may have elicited the increases in ventilation. The resultant increase in ventilation from nicotine infusion may have desensitised the peripheral chemoreceptor sensitivity to neurotransmitters prior to hypoxia, which may have in turn blunted the ventilatory response during hypoxia that was observed in control fish.

A reduction in ventilation amplitude and frequency in nicotinic receptor-desensitised fish during hypoxia would impact blood gas transfer at the gills. Elevated $PaCO_2$ levels in nicotine-infused fish compared to control, and reduced pH levels compared to control during hypoxia confirm that CO_2 diffusion out across the gill lamellar epithelium was impaired and a respiratory acidosis likely resulted from the reductions in ventilation. PaO_2 during hypoxia, however, did not differ from control values, and although lower plasma pH can enhance nicotine-evoked catecholamine secretion from chromaffin tissue *in situ* (Julio *et al.*, 1998), the main stimulator of catecholamine secretion during hypoxia is a reduction in blood oxygen content at or below the P_{50} (Perry and Reid, 1992). Thus, differences in $PaCO_2$ and pH levels caused by reduced ventilation during hypoxia would not likely affect the ability to recruit other catecholamine secretagogues during hypoxia.

In addition to the indirect effects that the nicotine infusion had on blood gas $PaCO_2$ and pH during hypoxia, circulating catecholamines released from chromaffin tissue also affect the cardio-respiratory physiology of the fish. During the infusion, catecholamines are able to bind ß-adrenergic Na^+/H^+ exchangers (ßNHE) that are present on red blood cells (RBCs; Nikinmaa, 1990). The ßNHE extrudes protons in exchange for sodium ions on the RBC plasma membrane, resulting in an increased intracellular RBC pH (Nikinmaa 1990; Motais, 1990), and an increase in the affinity of Hb for oxygen (Bohr effect) and capacity to carry oxygen (Root effect; see General Introduction). Thus, plasma pH declines due to the extruded RBC protons, but in the present study, plasma pH returned to baseline prior to hypoxia, likely because circulating catecholamine levels declined as the nicotinic receptors desensitised. It is not understood, at present, why PaO_2 levels stabilized 20 mm Hg below 'pre' levels throughout the infusion (Figure 2-3B). However, during hypoxia, PaO2 levels in desensitised fish are not different from control values. This is important as another effect of the ßNHE is a right-shift in the oxygen-dissociation curve, which increases the P_{50} (partial pressure of oxygen at which 50% of Hb is bound with oxygen). Because rainbow trout release catecholamines at or below the P_{50} (Perry and Reid, 1992), an increase in the P_{50} might initiate catecholamine release at a higher PaO_2 than control fish during hypoxia. This possibility can be discounted, however, as circulating catecholamines returned to baseline levels during the 60 min infusion and hence adrenergic stimulation to RBCs likely ceased prior to hypoxia.

Finally, nicotine from the infusion may have stimulated autonomic sympathetic nerve synapses that innervate the systemic vasculature and regulate vasoconstriction via constrictory α-, and dilatory ß-adrenergic receptors (Nilsson, 1983). Upon stimulation of the neuronal nicotinic receptors present at the pre-to-post ganglionic synapses, noradrenaline is released from nerve endings onto the receptors in the systemic vasculature. In teleosts, the α-receptor constrictory response predominates and generally results in an increase in systemic resistance and elevated blood pressure (P_{DA}; Wood and Shelton, 1980). An increase in P_{DA} was indeed observed in the present study during the nicotine infusion, and this idea may also explain the significant drop in P_{DA} that occurs in nicotine-infused fish during acute hypoxia. The nicotinic receptors present at the pre-to-post ganglionic synapses may become desensitised and cease to stimulate the constrictory α-adrenoceptors (Nilsson, 1983). Thus, during hypoxia, adrenaline, the predominant circulating catecholamine, may have bound the dilatory ß-adrenoceptors and resulted in significantly reduced blood pressure (Nilsson, 1983). The possibility that a reduction in P_{DA} might interfere with the secretion of catecholamines during hypoxia or the recruitment of catecholamine secretagogues may be discounted, however, as both nicotinic receptor-desensitised and control fish are able to recruit the same system to elicit catecholamine secretion during hypoxia (see Chapter 3).

During the nicotine infusion, the heart frequency reflected the changes in blood pressure by displaying a bradycardia coincident with peak blood pressures (P_{DA}). However during hypoxia, no changes in Hf were evident in nicotine-infused fish regardless of a significant drop in P_{DA}, whereas control fish displayed a significant bradycardia. This result may be attributed to a barostatic reflex in desensitised fish.

Although baroreceptors have not yet been localized in fish, there is evidence suggesting the existence of a barostatic reflex (Jones and Milsom, 1982). Nilsson (1983) and Farrell and Jones (1992) found that increased heart frequency (tachycardia) associated with hypotension does not exceed the Hf that results from zero vagal tone and concluded that cholinoceptors were exclusively involved in this mechanism. Thus, in the present study, a barostatic reflex may have overridden a hypoxic bradycardia, as the nicotine infusion may have acted neuronally to reduce heart frequency prior to hypoxia, but desensitised the nicotinic receptors and thus disabled them from responding with the typical bradycardia during acute hypoxia. Although nicotine-infused fish may thus have been unable to respond to hypoxia with a bradycardia, this would not likely affect the secretion of catecholamines or the recruitment of potential secretagogues.

Altogether, the infusion protocol to desensitise chromaffin cell nicotinic receptors *in vivo* does not appear to impair the ability of fish to release catecholamines as part of appropriate stress response to hypoxia. Although catecholamine, respiratory and cardiovascular measurements taken throughout the desensitisation protocol indicate that non-chromaffin, neuronal nicotinic receptors also appear to be stimulated and to desensitise, this *in vivo* method appears to be a reliable technique for desensitising chromaffin cell nicotinic receptors that will permit future studies to be carried out in the field of catecholamine secretion.

Chapter 3

THE ROLE OF ANGIOTENSIN II IN REGULATING CATECHOLAMINE SECRETION DURING HYPOXIA IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

INTRODUCTION

In teleost fish, the catecholamine hormones (noradrenaline and adrenaline) are secreted from the chromaffin tissue into the bloodstream in response to acute stress (for reviews see Randall and Perry, 1992; Reid *et al.* 1998). Catecholamines are stored in chromaffin cells that line the walls of the posterior cardinal vein (PCV) in the region of the head kidney (Nandi, 1961; Nakano and Tomlinson, 1967). Once released into the circulation, these hormones serve to maintain or enhance a variety of physiological processes including cardiovascular function (Perry and Gilmour, 1999) and blood oxygen transport (Nikinmaa, 1992). Stressors capable of causing catecholamine secretion include hypoxia (Ristori and Laurent, 1989), hypercapnia (Perry *et al.* 1987), intensive exercise (Primmett *et al.* 1986), air exposure (Walhqvist and Nilsson, 1980) and hypotension (Bernier *et al.* 1999b).

The control of catecholamine secretion in teleosts is achieved through several cholinergic and non-cholinergic mechanisms (Reid *et al.* 1998). However, it has generally been accepted that the primary mechanism initiating catecholamine release in trout involves increased neuronal stimulation by pre-ganglionic sympathetic nerve fibres that innervate chromaffin cell cholinergic receptors (Nilsson *et al.* 1976; Montpetit and Perry, 1999; Reid *et al.* 1998). The subsequent release of the neurotransmitter acetylcholine predominantly stimulates nicotinic receptors to elicit a series of Ca^{2+}-dependent events leading to catecholamine secretion (Nilsson *et al.* 1976; Montpetit and Perry, 1999; Furimsky *et al.* 1996). Recently, however, it was demonstrated that catecholamine secretion into the circulation still occurred in hypoxic rainbow trout that possessed non-functional (desensitised) nicotinic receptors (Lapner *et al.* 2000). Thus, clearly other mechanisms are contributing to catecholamine secretion at times when nicotinic receptors are non-functional. With this background, the goal of the present study was to identify the mechanism(s) promoting catecholamine release in rainbow trout subjected to acute hypoxia under conditions of nicotinic receptor desensitisation. Three mechanisms were evaluated on the basis of previous studies demonstrating their potential involvement in catecholamine secretion in trout. First, the contribution of muscarinic receptors was assessed because it was recently shown that muscarinic cholinergic stimulation enhances nicotinic-evoked catecholamine secretion and may, under intense stimulation, cause direct secretion (Julio *et al.* 1998; Montpetit and Perry, 1999). Second, the involvement of serotonin, a secretagogue of catecholamine release in trout (Fritsche *et al.* 1993), was assessed because of the known localisation of serotonergic cells in the vicinity of chromaffin tissue (Fritsche *et al.* 1993; Reid *et al.* 1995) and the sensitivity of similar gill serotonergic cells to oxygen (Dunel-Erb *et al.* 1982). Finally, the role of angiotensin II (Ang II), the biologically active product of the RAS, was investigated. Ang II is known to cause catecholamine release in rainbow trout (Bernier and Perry, 1997). Further, it was recently demonstrated that hypoxia is a powerful stimulant of renin secretion and renin gene expression in rats (Ritthaler *et al.* 1997). If a similar mechanism was operative in rainbow trout, Ang II could play an important role in eliciting catecholamine secretion during acute hypoxia.

MATERIALS AND METHODS

Experimental animals

Rainbow trout [*O. mykiss* (Walbaum)] weighing between 200 and 500 g (mean mass = 366.5 ± 13.6 g; N = 107) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and were held indoors in large fibreglass tanks supplied with dechlorinated City of Ottawa tap water that was maintained at 13° C. Fish were allowed to acclimate to the aquarium for at least three weeks before experimentation. Fish were maintained on a 12 h: 12h L:D photoperiod and fed daily to satiation with a commercial salmonid diet until 24 h before experimentation.

Surgical procedures

Rainbow trout were anaesthetised in a solution of ethyl-*P*-amino-benzoate (benzocaine; Sigma; final concentration 2.4 x 10^{-4} mol l^{-1}) and placed onto an operating table where the gills were continuously irrigated with aerated anaesthetic solution. An indwelling polyethylene cannula (Clay-Adams PE 50 polyethylene tubing; internal diameter 0.580 mm, outer diameter 0.965 mm) was implanted into the dorsal aorta (Soivio *et al.* 1975) to permit injections and periodic blood sampling. A second cannula (Clay-Adams PE 160 polyethylene tubing; internal diameter 1.14 mm, outer diameter 1.57 mm) was inserted through the snout into the oral cavity to permit monitoring of inspired water PO_2. To allow nicotine or saline infusion, a third cannula (PE 50) was inserted into the caudal vein at the level of the caudal peduncle in the orthograde direction using standard surgical procedures (Axelsson and Fritsche, 1994). The caudal incision was sutured using a running stitch and the cannula was secured to the body wall with silk ligatures.

After surgery, trout were placed into individual opaque Perspex boxes supplied with aerated flowing water and allowed to recover for 24 h prior to experimentation.

Experimental protocol

To desensitise nicotinic receptors fish were infused (0.2 ml min^{-1}) *via* the caudal vein with nicotine (1.3 x 10^{-5} mol kg^{-1} h^{-1}) using a syringe infusion pump (Sage Instruments) for 60 min (Lapner *et al.* 2000); control fish were infused with Cortland saline (Wolf, 1963). During continuous infusion, they were subjected to 10 min of hypoxia (beginning when the inspired PO_2 had fallen to 40 - 45 mm Hg; 5.3 - 6.0 kPa).

Acute hypoxia was achieved by replacing the air supplying a water/gas equilibration column with N_2. The desired water PO_2 (P_WO_2) (40 - 45 mm Hg) was pre-set and established by adjusting the rate of water and/or N_2 flow through the column. This level of hypoxia was chosen on the basis of a previous study (Perry and Reid, 1992) that demonstrated significant catecholamine release in rainbow trout using this protocol. The P_WO_2 within the box was monitored continuously using a peristaltic pump (flow = 0.6 ml min^{-1}) that withdrew water from each individual trout box and passed it across a PO_2 electrode (Cameron Instruments) connected to an O_2 meter (Cameron Instruments). Generally, the desired P_WO_2 in the experimental box was reached within 10 min and thereafter never varied more than ± 5 mm Hg (0.67 kPa). After experimentation, PwO_2 was restored to normoxic levels and the fish were allowed to recover.

The involvement of muscarinic receptors, serotonin or the RAS in promoting catecholamine release

After 40 min of infusion (nicotine or saline) under normoxic conditions, fish were injected via the dorsal aorta with the muscarinic receptor antagonist atropine (1 μmol kg^{-1}). The infusion continued for a further 20 min prior to the commencement of hypoxia (see above). Using the same protocol, a separate group of fish was injected with the serotonin receptor antagonist methysergide (1 x 10^{-8} mol kg^{-1}) prior to hypoxia. Finally, a third group was treated (as above) with the Ang II converting enzyme (ACE) inhibitor lisinopril (1 x 10^{-4} mol kg^{-1}) to prevent the formation of Ang II (Bernier *et al.* 1999b). Control fish were injected with saline after 40 min of infusion.

The effectiveness of atropine in blocking muscarinic receptors was demonstrated by monitoring P_{DA} on the extracorporeal blood loop to determine changes in Hf prior to and following an atropine injection. The effectiveness of lisinopril in blocking the conversion of Ang I to its biologically active form Ang II by the angiotensin converting enzyme (ACE) was also demonstrated by monitoring P_{DA} on the extracorporeal blood loop. The effects of an Ang I injection were monitored both prior to and following a lisinopril injection.

Determination of plasma catecholamine and Ang II levels

Blood samples collected for catecholamine analysis were taken immediately prior to beginning the infusion and after 10 and 60 min of infusion. Prior to hypoxia, samples withdrawn for Ang II analysis were only taken at 60 min of infusion. For measurement of plasma catecholamine and Ang II levels, four blood samples were withdrawn during the hypoxic period, at 0, 3, 5, and 10 min after reaching the targeted 40 – 45 mm Hg P_WO_2. Due to fluctuating P_WO_2 levels during the 10 min hypoxic period (± 5 mm Hg; 0.67 kPa), the blood sample that was withdrawn at a P_WO_2 level nearest to the target of 40 mm Hg was used to represent levels during hypoxia. The collected blood samples were placed into micro-centrifuge tubes (1.5 ml) and centrifuged (12 000 *g* for 20 s). The plasma was transferred to micro-centrifuge tubes containing 10 µl (25 units) of heparin (ammonium salt). Samples were quick-frozen in liquid N_2 and then stored at – 80° C until subsequent analysis.

Extraction of plasma catecholamine samples

All plasma catecholamine samples were subjected to alumina extraction and then analysed by high-pressure liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). 3,4-Dihydroxybenzylamine hydrobromide (DHBA) was used as an internal standard in these analyses. Detection limits for adrenaline and noradrenaline were 0.1 nmol l^{-1}.

Determination of Ang II levels by RIA

Plasma samples were extracted according to the method of Phillips *et al.* (1991), with incorporated modifications by Bernier *et al.* (1999a) and by us (as follows). 0.1 ml of just-thawed plasma was mixed with acidic acetone (volume ratio of acetone:H_2O:1 mol l^{-1} HCl = 40:5:1) and vortexed vigorously for 10 sec. The mixture was centrifuged for 10 min at 10 000 *g* and 4° C. The supernatant was collected in a new tube and the pellet re-solubilised and re-extracted with 0.1 ml acidic acetone. Once combined, the supernatants were centrifuged for 10 min as above. The supernatant was collected into a new tube and lyophilized. For the RIA, the extracted pellet was re-suspended in 0.025 ml of RIA buffer (diluent; see below). The recovery rate of Ang II through this extraction procedure, as measured with ^{125}I-labelled [Asn^1, Val^5]-Ang II, was 89.8%.

A single antibody method was used that employed an Ang II antibody that was designed for the detection of mammalian Ang II ([Asp^1, Ile^5]-Ang II). Recently, however, it was shown that a similar mammalian antibody cross-reacts (~70%) with rainbow trout Ang II ([Asn^1, Val^5]-Ang II) and does not cross react with piscine or mammalian Ang I (Bernier *et al.* 1999a).

Ninety-six well microtitre plates (Wallac; Rigid Plates) were prepared by coating each well with 100 µl Protein A/G (1 µg ml^{-1} prepared in 0.1 mol l^{-1} $NaHCO_3^-$; pH 9.0). Plates were wrapped in ^{TM}Parafilm and stored at 4° C overnight. The following day, the plates were washed twice with wash buffer [0.1% Tween-20 in diluent (0.5% BSA in 0.08 mol l^{-1} barbitol buffer)] for 2 min each at room temperature. Plates were then washed once with diluent for 20 min at room temperature and were blot dried on paper towel for 1 min. Antibody (1:200,000 dilution of rabbit anti-Ang II serum; Amersham Pharmacia Biotech) or normal rabbit serum (to determine non-specific binding) was added to the appropriate wells. The plates were wrapped in ^{TM}Parafilm and incubated at 4° C overnight. The following day, the plates were washed with wash buffer three times for 2 min each at room temperature. Plates were blot dried on paper towel for 1 min before adding 50 µl of RIA buffer (diluent) to each well. Radio-labelled hormone [25 µl of ^{125}I-Ang II ([Asp^1, Ile^5]-Ang II; Amersham Pharmacia Biotech; reconstituted to 100 µCi ml^{-1} with water, and diluted to a final concentration of 400 DPM µl^{-1} with diluent)] and 25 µl of standard (unlabeled [Asn^1, Val^5]-Ang II) or sample were added to the appropriate wells. Where required, diluent was added to wells such that each well contained a total volume of 100 µl. Plates were wrapped in ^{TM}Parafilm and incubated at 4° C for 48 h with intermittent gentle shaking after 24 h. After the 48 h incubation period, the plates were washed three times with wash buffer for 2 min each at room temperature. Plates were blot dried on paper towel before adding 100 µl of scintillation cocktail to each well. 25 µl of labelled hormone was added to the appropriate wells for determination of 'total' radioactivity. Plates were incubated at room temperature for 2 h before counting in a MicroBeta Liquid Scintillation and Luminescence Counter, using a MicroBeta Windows Workstation; data were analysed in MS-DOS using a WIA Level 5.M MultiCalc Advanced program.

Statistical analyses

The data are presented as means ± 1 standard error of the mean (SEM). Where appropriate, data were analysed statistically using one-way analysis of variance (ANOVA) followed by Dunn's multiple-comparison test. If assumptions for parametric statistics were violated, an ANOVA on ranks was performed followed by Dunn's multiple-comparison test. In other instances, data were analysed using Student's *t*-tests, and if assumptions for parametric statistics were violated, a Mann-Whitney rank sum test was performed. All statistical analyses were performed using commercial software (SigmaStat Version 2.0; SPSS); the fiducial limits of significance were set at 5%.

RESULTS

Continued chromaffin cell responsiveness during nicotinic receptor desensitisation

To confirm the ability of fish with desensitised nicotinic receptors to release catecholamines into the circulation upon acute hypoxia, saline or nicotine (to desensitise the nicotinic receptors; Lapner *et al.* 2000) was infused continuously for 60 min prior to the onset of hypoxia and continued throughout the hypoxic period. As expected, the nicotine infusion elicited a significant increase in plasma catecholamine levels at 10 min that was not observed in saline–infused fish (Fig 3-1). Owing to receptor desensitisation, the elevated plasma catecholamine levels returned to baseline levels within 60 min, despite continuous nicotine stimulation (Fig 3-1B).

Upon acute hypoxia, a significant increase in plasma adrenaline and noradrenaline levels was observed in fish with both functional (Fig 3-1A) and non-functional/desensitised (Fig 3-1B) nicotinic receptors. However, noradrenaline levels were reduced in comparison to hypoxic control fish (Fig 3-1). Subsequent experiments were designed to elucidate the mechanisms causing the elevation in plasma catecholamine levels during hypoxia in fish with desensitised nicotinic receptors.

Mechanisms eliciting catecholamine release during nicotinic receptor desensitisation

Regardless of treatment, all fish displayed the characteristic transient increase in plasma catecholamine levels at 10 min of nicotine infusion that is indicative of nicotinic receptor desensitisation (Fig 3-2). Blockade of muscarinic (Fig 3-2A) or serotonergic (Fig 3-2B) receptors did not diminish plasma catecholamine levels during acute hypoxia (compare with Fig 3-1A). However, blockade of the RAS abolished the ability of desensitised fish to mobilize circulating catecholamines during hypoxia (fig 3-2C). Figure 3-3 effectively illustrates the importance of the RAS in promoting catecholamine secretion during hypoxia in desensitised fish.

To ensure that catecholamine release during acute hypoxia in desensitised fish was indeed a specific consequence of activation of the RAS (rather than indirect effects of lisinopril), plasma Ang II levels were measured before and after hypoxia (Fig 3-4). Fish were infused with nicotine or saline for a 60 min period prior to hypoxia, however data from these two experiments were compiled owing to a lack of any statistical differences between the two groups. A saline (control) or lisinopril injection was made after 40 min of infusion. The results demonstrated a significant increase in plasma Ang II levels (from 456 ± 166 to 1338 ± 391 pmol l^{-1}) during hypoxia in control fish possessing a functional RAS (Fig 3-4). In contrast, however, fish experiencing RAS blockade displayed significantly lower Ang II levels during normoxia and did not exhibit an increase in plasma Ang II levels during acute hypoxia.

The involvement of the RAS in eliciting catecholamine release from non-desensitised chromaffin cells

To further test the involvement of the RAS in eliciting catecholamine release from chromaffin tissue during hypoxia, ACE activity was inhibited in fish possessing functional nicotinic receptors. Although plasma catecholamine levels were increased significantly upon acute hypoxia in both groups (Fig 3-5), the ACE-blocked fish displayed lower plasma noradrenaline levels during hypoxia than control fish. Further, total plasma catecholamine levels (adrenaline plus noradrenaline) were significantly reduced during hypoxia in the ACE-blocked fish (93.6 ± 33.7 *versus* 205.5 ± 35.5 nmol l^{-1}; $P < 0.05$).

Efficacy of atropine and lisinopril

A representative trace demonstrating the efficacy of atropine to block muscarinic receptors is shown in Figure 3-6. Hf increased from (A) 1.0 beats sec^{-1} prior to an atropine injection to (B) 1.4 beats sec^{-1} following an atropine injection. Figure 3-7 shows a representative trace depicting an increase in P_{DA} elicited from the conversion of an Ang I injection (represented by grey arrows) to Ang II by ACE that was eliminated following the lisinopril injection (represented by a black arrow).

Figure 3-1. The effects of acute hypoxia (40 mm Hg; 5.3 kPa; at the vertical dashed line) on plasma levels of adrenaline (filled columns) or noradrenaline (unfilled columns) in rainbow trout (*O. mykiss*) previously infused (0.2 ml min^{-1}) with (A) saline (N = 9); or (B) nicotine (1.3 x 10^{-5} mol kg^{-1} h^{-1}; N = 17) for 60 min under conditions of normoxia. Plasma catecholamine levels were measured at 'Pre', 10 min, and 60 min of infusion, and during acute hypoxia. Values are shown as means + SEM. * denotes a statistically significant increase ($P < 0.05$) from the corresponding 'Pre' value or '60 min' pre-hypoxia value.

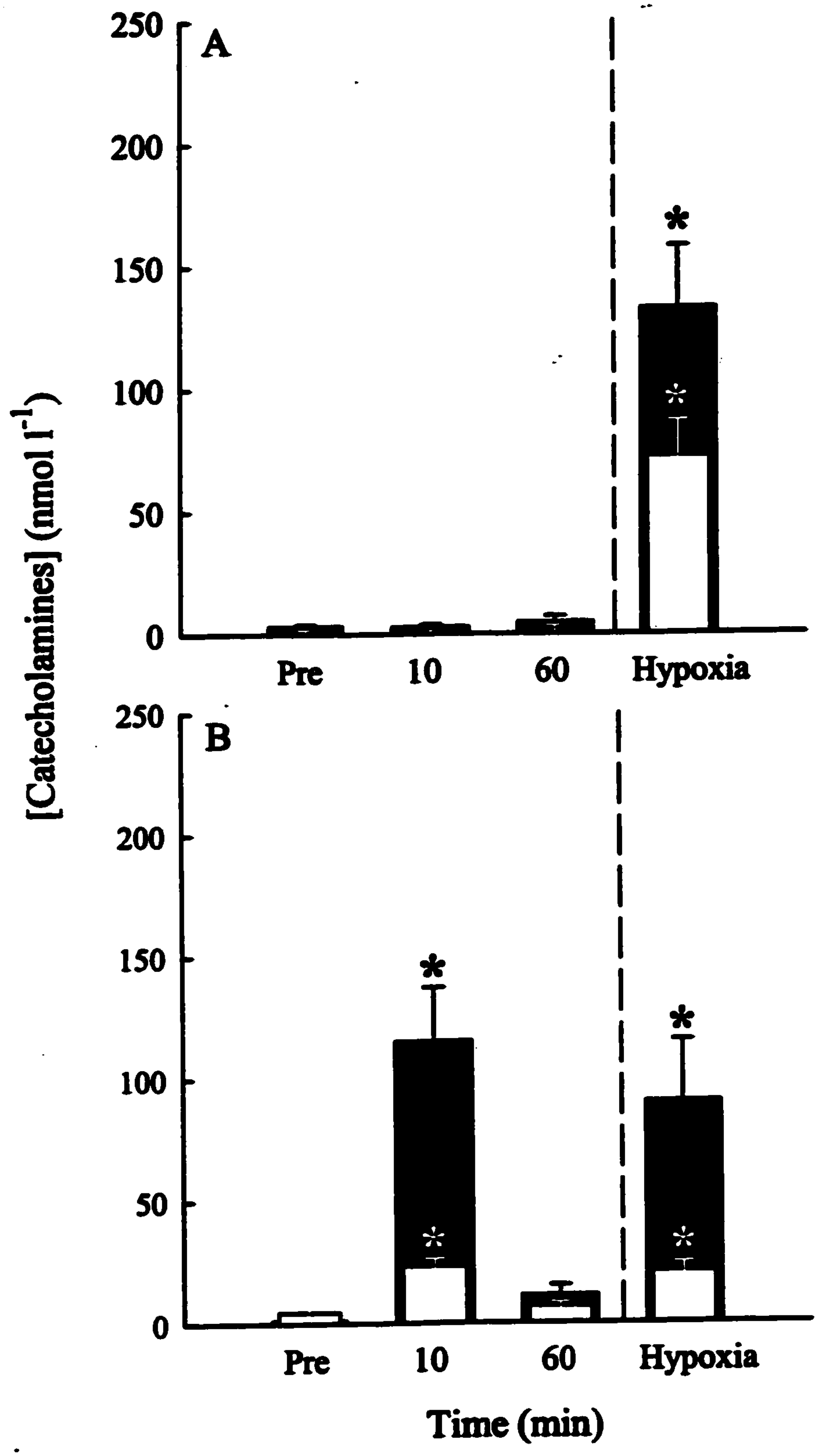

A
B
[Catecholamines] (nmol l^{-1})
250
200
150
100
50
0
Pre
10
60
Hypoxia
*
Time (min)

Figure 3-2. The effects of acute hypoxia (40 mm Hg; 5.3 kPa; at the vertical dashed line) on plasma levels of adrenaline (filled columns) or noradrenaline (unfilled columns) in rainbow trout (*O. mykiss*) previously infused (0.2 ml min^{-1}) with nicotine (1.3 x 10^{-5} mol kg^{-1} h^{-1}) for 60 min under conditions of normoxia. An injection of (A) atropine (1 µmol kg^{-1}; N = 9), (B) methysergide (1 x 10^{-8} mol kg^{-1}; N = 9), or (C) lisinopril (1 x 10^{-4} mol kg^{-1}; N = 20) was administered after 40 min of nicotine infusion. Plasma catecholamine levels were measured at 'Pre', 10 min, and 60 min of infusion, and during acute hypoxia. Values are shown as means + SEM. * denotes a statistically significant increase ($P < 0.05$) from the corresponding 'Pre' value or '60 min' pre-hypoxia value. † indicates a statistically significant difference ($P < 0.05$) from the control value [saline injection; Fig. 1 (B)].

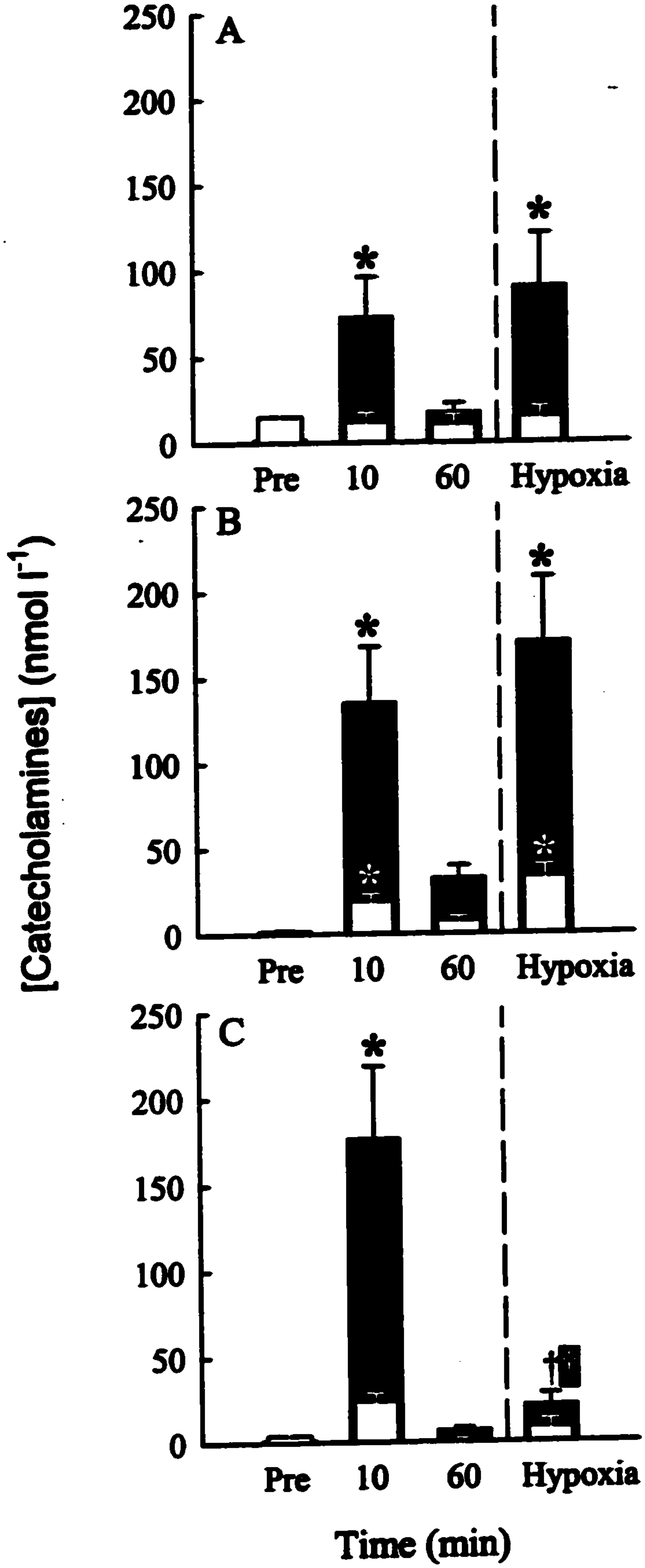

A
B
C
250
200
150
100
50
0
*
†
Pre
10
60
Hypoxia
[Catecholamines] (nmol l-1)
Time (min)

Figure 3-3. Plasma levels of adrenaline (filled columns) or noradrenaline (unfilled columns) during acute hypoxia (40 mm Hg; 5.3 kPa) in rainbow trout (*O. mykiss*) that were previously infused (0.2 ml min^{-1}) with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) for 60 min under conditions of normoxia. To block specific receptors or enzymes, injections were administered at 40 min (20 min prior to hypoxia) using saline (control; N = 17); atropine (muscarinic receptor antagonist; 1 μmol kg^{-1}; N = 9), methysergide (serotonin receptor antagonist; 1×10^{-8} mol kg^{-1}; N = 9), or lisinopril (ACE inhibitior; 1×10^{-4} mol kg^{-1}; N = 20). Values are shown as means + SEM. † indicates a statistically significant difference ($P < 0.05$) from the control value.

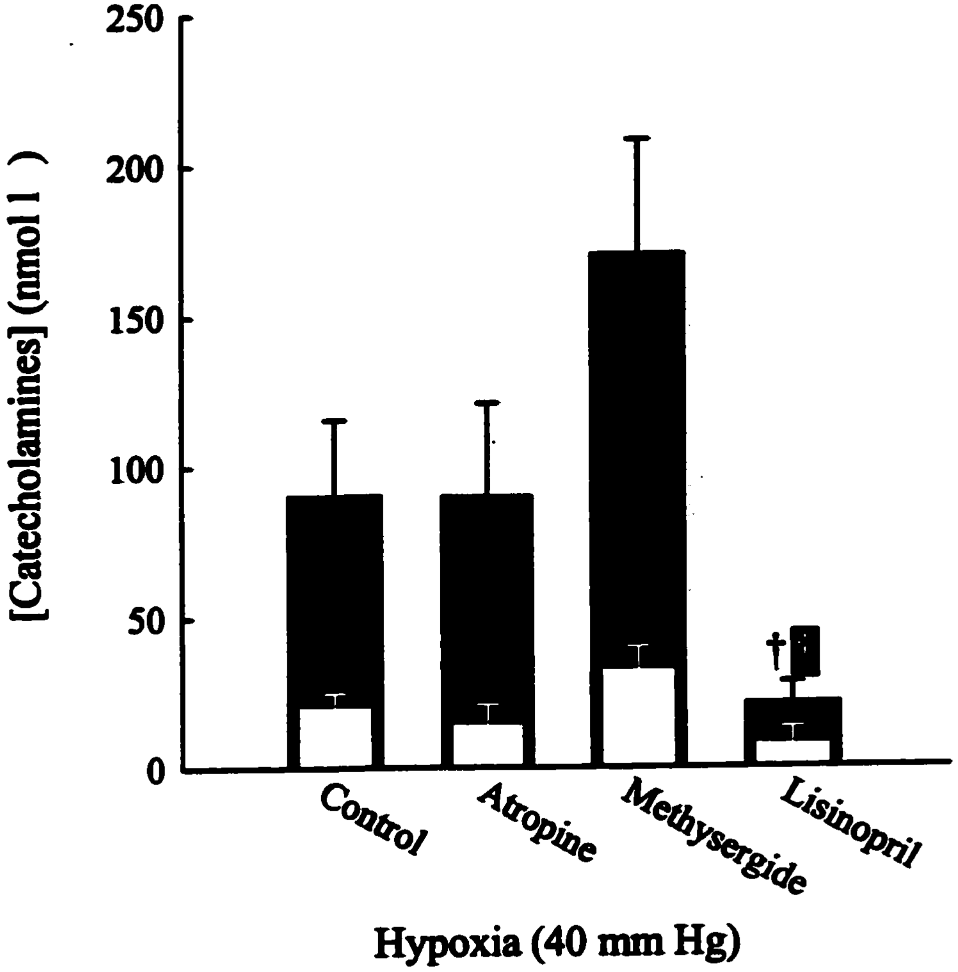

250
200
150
100
50
0
[Catecholamines] (nmol l^{-1})
Control
Atropine
Methysergide
Lisinopril
†
Hypoxia (40 mm Hg)

Figure 3-4. The effect of acute hypoxia (40 mm Hg; 5.3 kPa) on plasma angiotensin II levels in rainbow trout (*O. mykiss*) previously infused (0.2 ml min^{-1}) with nicotine (1.3×10^{-5} mol kg^{-1} h^{-1}) or saline (data were combined as there were no significant differences in [angiotensin II] between the two groups) for 60 min under conditions of normoxia. An injection of saline (filled columns; N = 15) or lisinopril (1×10^{-4} mol kg^{-1}; unfilled columns; N = 15) was made prior to hypoxia at 40 min infusion. 'Pre' samples were taken immediately prior to the onset of hypoxia. Values are shown as means + SEM. * denotes a statistically significant increase ($P < 0.05$) from the corresponding 'Pre' value. † indicates a statistically significant difference ($P < 0.05$) from the control value.

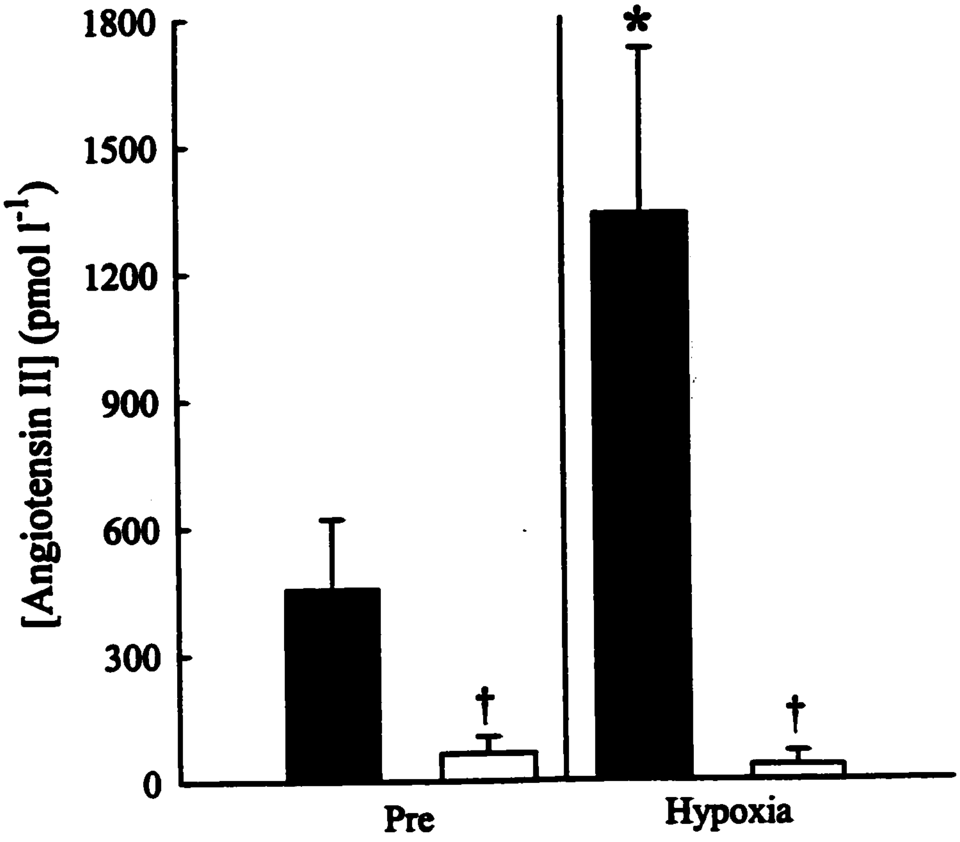

1800
1500
1200
900
600
300
0
[Angiotensin II] (pmol l^{-1})
*
†
†
Pre
Hypoxia

Figure 3-5. The effects of acute hypoxia (40 mm Hg; 5.3 kPa; at the vertical dashed line) on plasma levels of adrenaline (filled columns) or noradrenaline (unfilled columns) in rainbow trout (*O. mykiss*) previously infused (0.2 ml min^{-1}) with saline for 60 min under conditions of normoxia. An injection of (A) saline (control; N = 9) or (B) lisinopril (1×10^{-4} mol kg^{-1}; N = 10) was made prior to hypoxia at 40 min saline infusion. Plasma catecholamine levels were measured at 'Pre', 10 min, and 60 min of infusion, and during acute hypoxia. Values are shown as means + SEM. * denotes a statistically significant increase ($P < 0.05$) from the corresponding '60 min' pre-hypoxia value. † indicates a statistically significant difference ($P < 0.05$) from the control value.

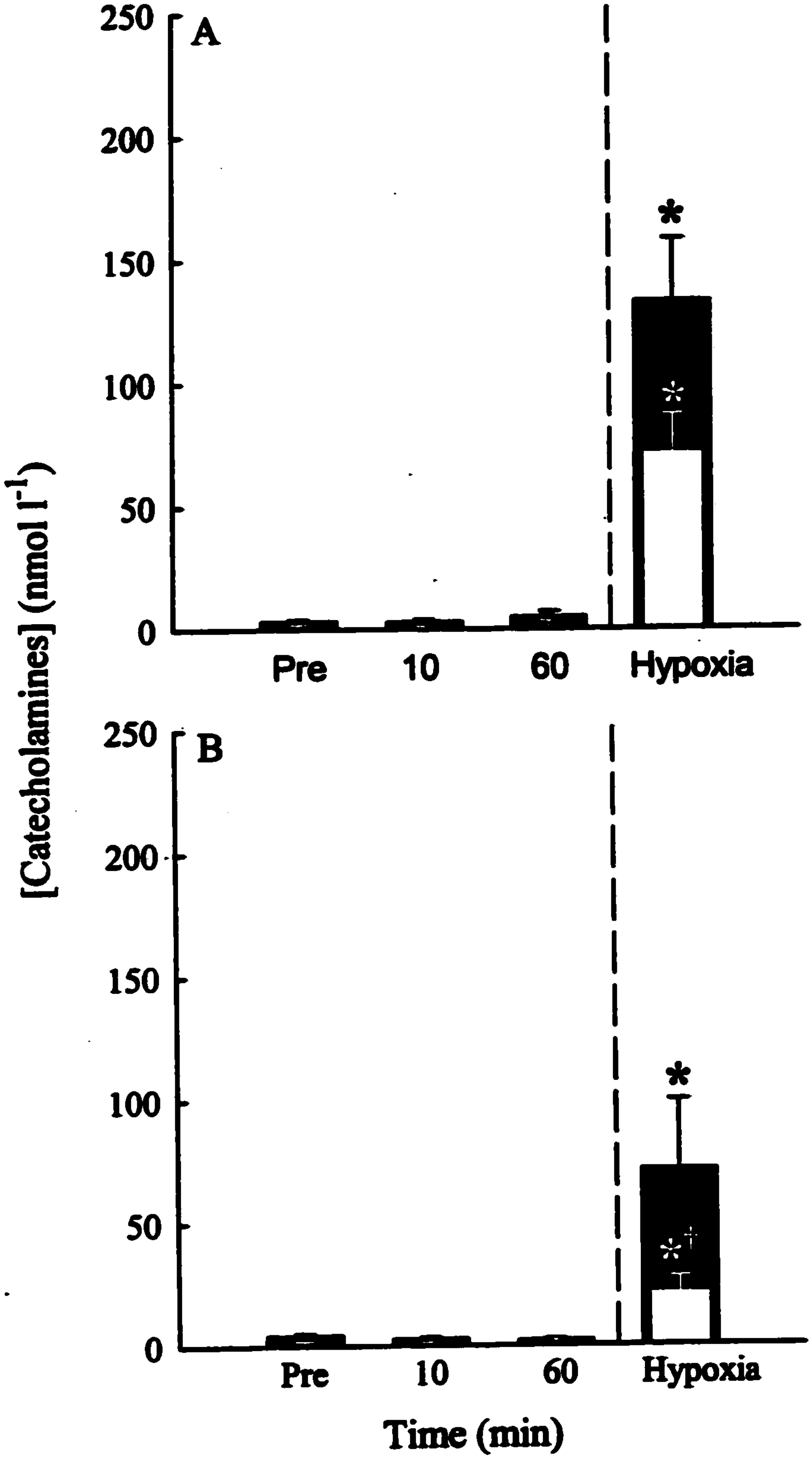

A
B
[Catecholamines] (nmol l^{-1})
250
200
150
100
50
0
Pre
10
60
Hypoxia
*
Time (min)
*†

Figure 3-6. Representative data acquisition traces illustrating the effectiveness of the muscarinic receptor antagonist atropine (1 μm kg^{-1}) in blocking muscarinic receptors affecting heart frequency detected by changes in blood pressure (P_{DA}) in (A) control and (B) atropinised rainbow trout (*O. mykiss*).

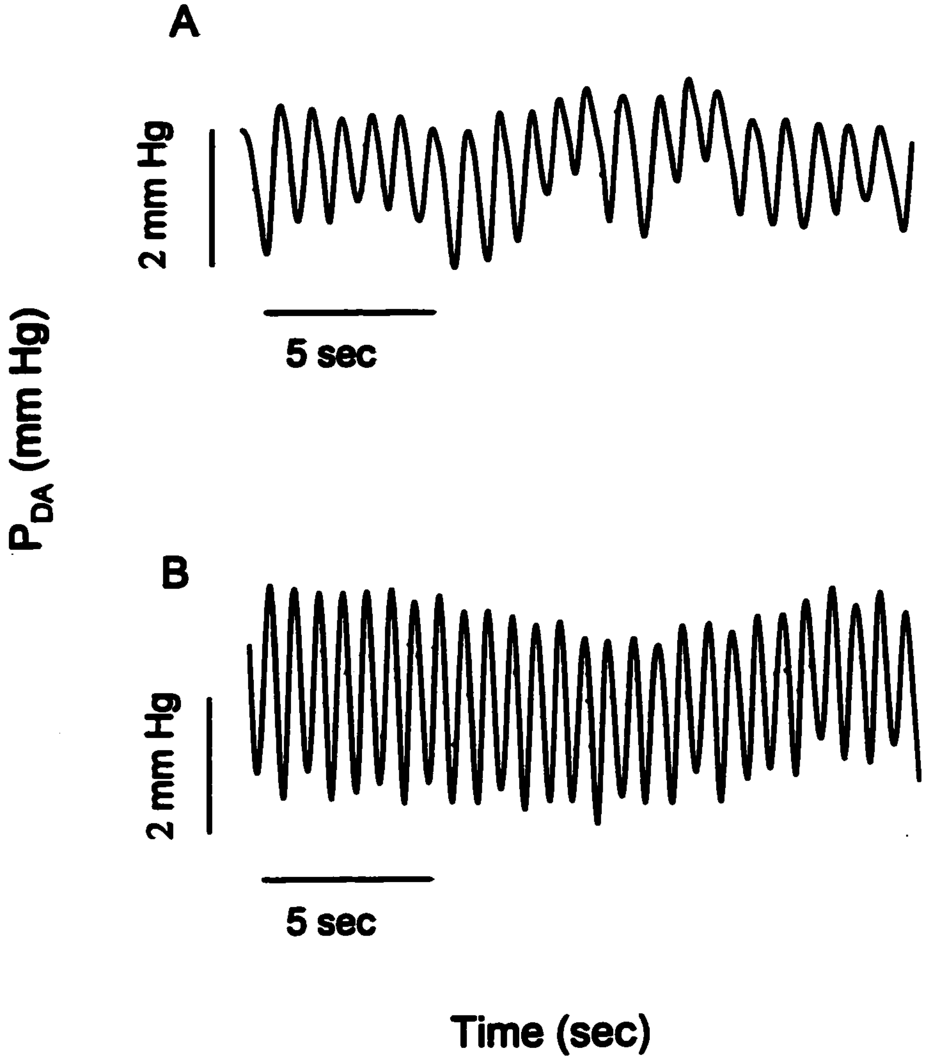
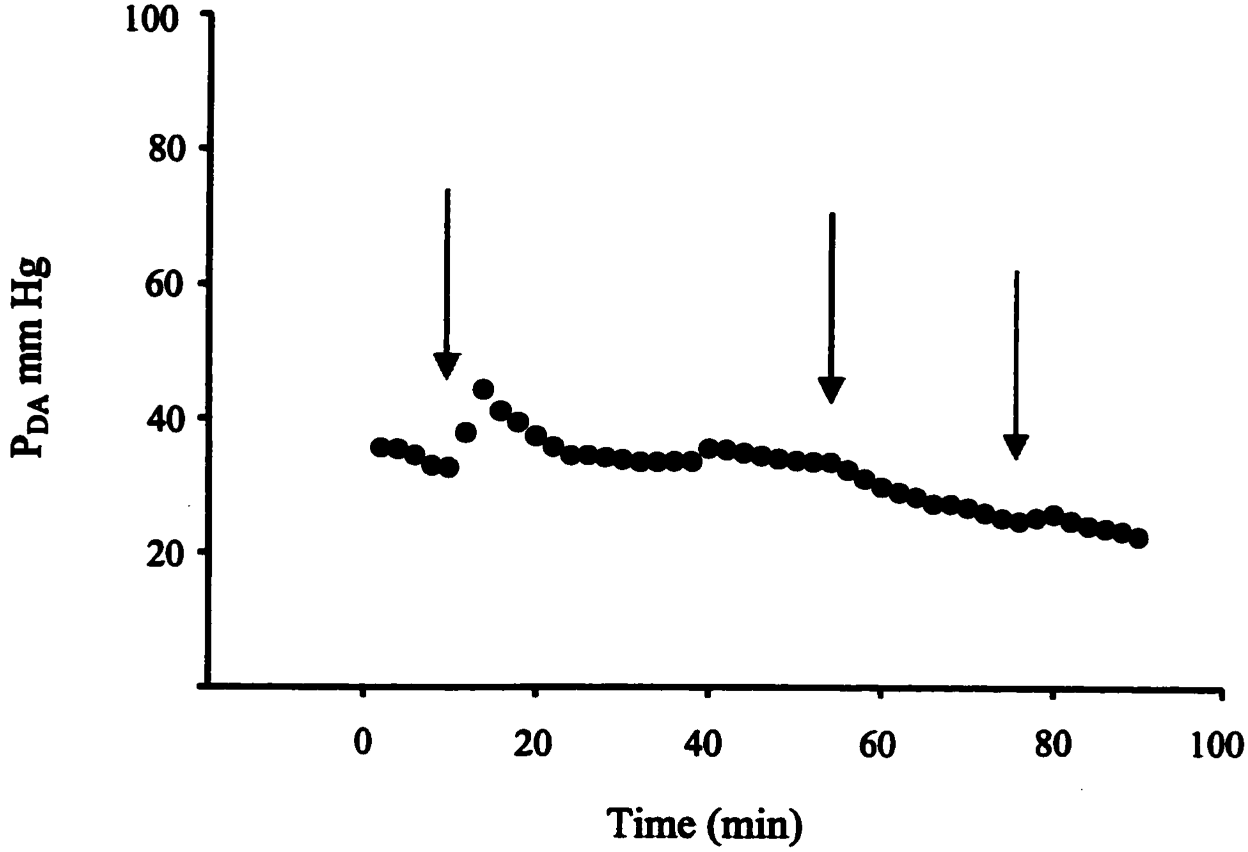

A
2 mm Hg
5 sec
P_{DA} (mm Hg)
B
2 mm Hg
5 sec
Time (sec)

Figure 3-7. Representative data acquisition trace of blood pressure (P_{DA}) illustrating the effectiveness of the angiotensin converting enzyme (ACE) inhibitor lisinopril (1×10^{-4} mol kg^{-1}) in preventing Ang II formation from an injection of Ang I (1×10^{-9} mol kg^{-1}; indicated by grey arrows) prior to and following a lisinopril injection (indicated by black arrow) in rainbow trout (*O. mykiss*).


100
80
60
40
20
P_DA mm Hg
0
20
40
60
80
100
Time (min)

DISCUSSION

This study demonstrates that the RAS in rainbow trout is activated during hypoxia and leads to a significant elevation of plasma Ang II levels. Although Ang II is a potent activator of catecholamine release in trout (Bernier and Perry, 1997), its elevation is not required to elicit catecholamine release during acute hypoxia in intact fish. However, using a protocol that we previously developed to desensitise chromaffin cell nicotinic receptors (Lapner *et al.* 2000), the results of this study demonstrate that Ang II plays an essential role in eliciting catecholamine release in hypoxic fish experiencing nicotinic receptor desensitisation. Activation of muscarinic or serotonergic receptors, on the other hand, does not appear to contribute to catecholamine release in desensitised fish.

Catecholamine release in desensitised fish

Upon stimulation, the nicotinic receptor of trout chromaffin cells, as in mammals (Boksa and Livett, 1984), undergoes rapid desensitisation (Lapner *et al.* 2000). The re-sensitisation process is gradual (e.g. as long as 40 min in rainbow trout; Lapner *et al.* 2000). Thus, although it is believed to be a mechanism preventing excessive catecholamine secretion, nicotinic receptor desensitisation, if prolonged, could potentially impair catecholamine release in animals experiencing repetitive bouts of acute stress. The single previous study to address this issue, however, demonstrated that the ability of trout to release catecholamines during acute hypoxia was not impaired during periods of nicotinic receptor desensitisation (Lapner *et al.* 2000). This finding indicated that other pathways that are not reliant on nicotinic receptors were activated to elicit catecholamine secretion. The goal of this study was to attempt to identify one or more of these pathways. It was necessary, however, to first confirm that catecholamine release during hypoxia was indeed unimpaired in trout experiencing nicotinic receptor desensitisation. Thus, using a protocol of intravascular nicotine infusion to desensitise nicotinic receptors (Lapner *et al.* 2000), initial experiments revealed similar elevations of plasma catecholamine levels during hypoxia regardless of the state of the nicotinic receptor. Because local hypoxia in the vicinity of chromaffin cells does not evoke catecholamine secretion in rainbow trout (Perry *et al.* 2000), subsequent experiments were designed to establish alternative mechanisms of catecholamine release in desensitised fish.

Potential involvement of serotonin

Although serotonergic receptors are present on rainbow trout chromaffin cells and the ability of serotonin to elicit catecholamine secretion was previously demonstrated *in vivo* and *in situ* (Fritsche *et al.* 1993), serotonin does not appear to be a ‘secondary’ catecholamine secretagogue during hypoxia when nicotinic receptors are desensitised. Previous work by Fritsche *et al.* (1993) showed that injection of the serotonergic receptor antagonist methysergide blocked serotonin-induced adrenaline release *in situ* but not *in vivo*. It was suggested that this discrepancy could be attributed to serotonin acting on methysergide-insensitive receptors within higher control centres *in vivo*. Furthermore, Perry *et al.* (2000) showed that localised hypoxia inhibited chromaffin cell responsiveness to nicotine *in situ* but had an opposite, enhancing effect *in vivo*, further supporting the idea that higher control centres are involved in catecholamine release *in vivo*. Therefore, although our results cannot rule out the possibility that serotonin is indirectly acting on higher control centres to elicit catecholamine secretion during nicotinic receptor desensitisation, we can exclude any direct effect of serotonin on chromaffin cells.

The possible involvement of muscarinic receptors

Previous *in situ* studies have revealed that activation of chromaffin cell muscarinic receptors can enhance nicotine-evoked catecholamine secretion and it has been suggested that under intense stimulation, muscarinic receptors might directly cause catecholamine secretion (Montpetit and Perry, 1999). The results of this study, however, demonstrate that muscarinic receptors do not contribute to catecholamine release during hypoxia in nicotinic receptor-desensitised fish. In contrast, stimulation of chromaffin cell muscarinic receptors in mammals can evoke significant catecholamine secretion during periods of nicotinic receptor desensitisation (Malhotra *et al.* 1989). In trout, it seems that chromaffin cell muscarinic receptors serve only to enhance the nicotinic-evoked catecholamine secretion (Montpetit and Perry, 1999) but are incapable of independently eliciting catecholamine release during stress (at least acute hypoxia).

Potential involvement of the renin-angiotensin-system

Although chromaffin cell serotonergic and muscarinic receptors do not appear to be involved in eliciting catecholamine secretion during hypoxia in trout experiencing nicotinic receptor desensitisation, the results of the present study indicate an essential role of the RAS. Under normal conditions, the RAS is an important regulator of cardiovascular function in fish (Olson, 1992). Recently, a link between the RAS and circulating catecholamines has been established in cardiovascular control whereby Ang

II, mobilised during hypotension, dose-dependently stimulates catecholamine release (Bernier and Perry, 1997; Bernier *et al.* 1999b). Thus typically, Ang II and catecholamines function together as vasopressive agents to regulate blood pressure during periods of hypotension (Nishimura *et al.* 1978; Oudit and Butler, 1995; Fuentes and Eddy, 1998; Bernier and Perry, 1999). The present paper provides the first evidence of RAS recruitment during acute hypoxia, a condition that is not associated with hypotension. Further, the results demonstrate that activation of the RAS is a prerequisite for catecholamine secretion in hypoxic trout possessing desensitised nicotinic receptors.

Circulating Ang II is derived via activation of systemic and/or regional renin-angiotensin systems (Bernier and Perry, 1997). In mammals, key components indicative of a local RAS have been demonstrated in multiple tissues of rodents (Campbell and Habener, 1986; Dzau *et al.* 1987, Leung *et al.* 1999). Interestingly, secretory granules in adrenaline-containing chromaffin cells of rat adrenal medulla contain both renin and pro-renin (Berka *et al.* 1996). Furthermore, chronic or acute hypoxia were recently shown to result in the enhanced expression of local RAS component genes in rat pancreas (Chan *et al.* 2000) and primary cultures of renal juxtaglomerular cells (Ritthaler *et al.* 1997), to increase numbers of lung Ang II receptors (Zhao *et al.* 1996), and to increase cardiac ACE activity (Morrell *et al.* 1997). In rat renal juxtaglomerular cells, renin secretion and renin gene expression were indirectly stimulated by acute hypoxia, as their expression was enhanced *in vivo* but not *in situ*. The origin of the increased circulating Ang II levels was not investigated in the present study. Further experiments are required to determine whether, as in mammals, local or systemic RAS components exhibit increased expression during hypoxia.

Angiotensin II levels were elevated equally during hypoxia in control fish and in fish experiencing nicotinic receptor desensitisation. However, unlike in the desensitised fish, blockade of the RAS did not prevent catecholamine release in control fish exposed to hypoxia. This result reinforces the notion that stimulation of nicotinic receptors via activation of sympathetic nerve fibres is the dominant pathway controlling catecholamine secretion in trout under normal conditions. Angiotensin II, however, may be contributing to the overall response in control fish based on the significant reduction in total plasma catecholamine levels after RAS blockade. Previous studies have demonstrated that Ang II preferentially stimulates adrenaline secretion from fish chromaffin cells (e.g. Bernier and Perry, 1997). Interestingly, however, RAS blockade prevented the release of both catecholamines in desensitised fish and had a greater effect on noradrenaline secretion in control fish.

Most teleosts that have been studied, including trout, exhibit pronounced cardiovascular adjustments when exposed to hypoxia including bradycardia and hypertension (see review by Fritsche and Nilsson, 1993). Traditionally, the hypertension has been attributed to increased systemic vascular resistance owing to increased activity of sympathetic nerve fibres as well as elevated circulating catecholamine levels. In light of the results of the present study showing elevated Ang II levels in hypoxic trout, additional mechanisms may be contributing to the hypoxic hypertension including direct vasoconstrictory effects of Ang II and indirect effects of Ang II on evoking catecholamine secretion.

As in mammals, stimulation of the pre-ganglionic nerve fibres that innervate the chromaffin cells leads to the release of both cholinergic (i.e. acetylcholine) and non-cholinergic neurotransmitters (Montpetit and Perry, 2000). In particular, vasoactive intestinal polypeptide (VIP) and pituitary adenylyl cyclase activating peptide (PACAP) are potent catecholamine secretagogues in rainbow trout (Montpetit and Perry, 2000). Because blockade of the RAS abolished catecholamine secretion during hypoxia in fish with desensitised nicotinic receptors, it would appear that these non-cholinergic neurotransmitters were not secreted in sufficient quantities to evoke catecholamine release. The relative rates of secretion of cholinergic *versus* non-cholinergic neurotransmitters are partially dependent on the frequency of neuronal action potentials. Specifically, in trout, the secretion of non-cholinergic neurotransmitters is favoured under conditions of low frequency nerve activity (Montpetit and Perry, 2000), a situation that might not exist during acute hypoxic stress. Thus, while catecholamine release in trout is thought to be controlled by multiple redundant pathways (see Reid *et al.* 1998), the specific involvement of each of these pathways may depend on the precise nature of the stressor. For example, while it is clear that serotonin, VIP, PACAP and muscarinic receptor stimulation are capable of independently eliciting catecholamine secretion (Reid *et al.* 1998), they appear to play no role during hypoxia. Thus during hypoxic stress, they cannot complement or replace the nicotinic receptor-mediated pathway of catecholamine secretion when nicotinic receptors are desensitised. Their possible role during other types of stress, however, cannot be ruled out. On the other hand, Ang II, usually considered to be a catecholamine secretagogue during periods of hypotension (Bernier *et al.* 1999a; 1999b), also contributes to catecholamine release during hypoxia and indeed is essential to allow catecholamine release in fish experiencing nicotinic receptor desensitisation.

Chapter 4

GENERAL DISCUSSION

Although previous experiments have shown that nicotinic receptor desensitisation occurs in rainbow trout chromaffin cells and that the stimulation of nicotinic receptors by preganglionic sympathetic cholinergic nerve fibres is not the sole mechanism for eliciting catecholamine secretion during hypoxia, this study is the first to assess the involvement of other known catecholamine secretagogues that may elicit secretion in nicotinic receptor-desensitised rainbow trout (*O. mykiss*) under conditions of acute hypoxia. The infusion protocol to desensitise chromaffin cell nicotinic receptors *in vivo* was also assessed by monitoring the effects on fish cardiovascular and respiratory physiology to determine whether the physiological ability of fish to respond to acute hypoxia was compromised. The results indicate that although nicotine infusion likely stimulates and desensitises nicotinic receptors throughout the nervous system, the physiological effects do not appear to impair the acute stress response to hypoxia in rainbow trout. In fact, this study also demonstrates that the RAS is activated during hypoxia and that elevated levels of plasma Ang II play an essential role in eliciting catecholamine release in hypoxic fish experiencing nicotinic receptor desensitisation. The role of the RAS is secondary to the activation of nicotinic receptors. Finally, this study also shows that serotonergic and muscarinic receptors do not play a role in eliciting an increase in circulating catecholamines during hypoxia in nicotinic receptor-desensitised fish.

Nicotinic Receptor Desensitisation Protocol

The first goal of this thesis was to determine whether *in vivo* nicotine infusion to desensitise chromaffin cell nicotinic receptors impairs the ability of fish to show the appropriate physiological responses to acute hypoxia. The results indicate that although chromaffin cell nicotinic receptors become desensitised by nicotine infusion (Lapner *et al.*, 2000), the infusion also elicits changes in the measured ventilatory, arterial, and cardiac variables that likely result from stimulation of nicotinic receptors present in the nervous system. The neuronal nicotinic receptors also appear to desensitise prior to hypoxia, however this does not appear to impair the ability of fish to secrete catecholamines. In a separate study, Burleson and Milsom (1995) measured the short-term effects (10 min) of a nicotine injection on cardiorespiratory variables in rainbow trout (*O. mykiss*). Their results were in agreement with the results of the present study, showing that nicotine causes an increase in gill ventilation rate and blood pressure. The effects of nicotine on heart rate were different between the two studies, however, as it decreased during the initial 6 min nicotine infusion in the present study. The discrepancy between the two studies may reflect differences in protocol (continuous infusion versus a single injection). The larger fluid volume associated with an infusion protocol may have reduced the Hf in the present study to accommodate the increased volume prior to the initiation of volume control mechanisms (i.e. ANP; see below).

The two peptides responsible for maintaining fluid balance are atrial natriuretic peptide (ANP) and angiotensin II (Ang II). Any deviation from the homeostatic balance causes the release of ANP to reduce fluid volume, or Ang II to increase fluid volume. ANP controls volume increases by acting as a diuretic and lowers blood pressure by dilating blood vessels, whereas Ang II has the opposite effects (reviewed by Galli and Phillips, 1996). The possibility that an increase in fluid volume may affect the response to acute hypoxia is not of concern, as the effects of acute hypoxia on rainbow trout pre-infused with saline in the present study displayed the same continuously measured arterial blood and ventilatory variables as a study by Perry and Gilmour (1996) that measured the responses of rainbow trout (*O. mykiss*) to the same level of acute hypoxia (6.0 kPa). The similar results between the two studies indicates that, although fish in the present experiment were pre-infused (0.2 ml min^{-1}) with saline (1.3 x 10^{-5} mol kg^{-1} h^{-1}) for 60 min, this did not affect the general response to acute hypoxia. Furthermore, arterial blood pressure (P_{DA}), which is a reflection of blood volume, remained constant throughout the saline infusion, indicating that the fish must have readily excreted the excess fluid volume from the infusion. Although the role of ANP was not measured in the present study, it was likely activated throughout the experiment to reduce the increased fluid volume caused by the infusion. Interestingly, in mammals, the actions of ANP have been shown to override the effects of Ang II (reviewed by Johnston *et al.*, 1989). In *Cyprinus carpio*, ANP may have an autocrine and/or paracrine role in the control of catecholamine secretion (Kloas *et al.*, 1994), whereas in rainbow trout (*O. mykiss*), rat ANP is unable to significantly affect basal or carbachol-elicited catecholamine release (McKendry *et al.*, 1999). The reduced catecholamine levels following ACE blockade (Chapter 3) indicate that ANP does not prevent significant RAS-induced catecholamine secretion during hypoxia in nicotinic receptor-desensitised fish.

The nicotine infusion appears to stimulate and desensitise non-chromaffin cell nicotinic receptors present in the nervous system, as indicated by continually monitored cardiovascular and respiratory variables. Although the neuronal nicotinic receptors appear to desensitise prior to hypoxia and alter some of the physiological responses to acute hypoxia, the acute stress response of circulating catecholamines was unaffected.

Non-Nicotinic Catecholamine Secretagogues

The present study demonstrates that Ang II plays an essential role in eliciting catecholamine release in hypoxic fish experiencing nicotinic receptor desensitization. However, despite the ability of serotonergic receptors to elicit catecholamine release and of muscarinic receptors to enhance nicotinic receptor-induced secretion *in situ*, these receptors do not appear to contribute to the release of catecholamines during nicotinic receptor desensitisation *in vivo*. It has previously been discussed (see Chapter 3) that higher control centers may be involved in catecholamine release *in vivo*. Thus, although we can exclude any direct effect of serotonin or acetylcholine on chromaffin cell serotonergic and muscarinic receptors, indirect effects mediated through higher control centers remain a possibility. However, the general systemic nicotinic receptor desensitisation caused by the infusion protocol may have prevented any observable effects from occurring.

Although serotonergic and muscarinic receptors are not involved in eliciting catecholamine release during nicotinic receptor desensitisation, stimulation of these receptors, or the actions of Ang II on chromaffin cell binding sites, may modulate the nicotinic responses of chromaffin cells to inhibit or alleviate desensitisation. Although the stimulatory effects of these secretagogues were investigated following the desensitisation of the chromaffin cell nicotinic receptors, they may nevertheless act to alleviate nicotinic receptor desensitisation *via* a similar modulatory role. Studies in bovine adrenal chromaffin cells have shown that serotonin inhibits nicotinic responses by binding to a noncompetitive site on the nicotinic receptor, and may protect from agonist-induced desensitisation in a manner that is similar to substance P (Vijayaraghavan *et al.*, 1993). Angiotensin II has also been shown to have a modulatory effect on nicotinic receptor-induced responses by suppressing Na^+ currents in bovine adrenal chromaffin cells (Cui and Pun, 1994). Ang II reversibly and dose-dependently reduces peak Na^+ conductance without affecting the steady-state activation or steady-state inactivation characteristics of the Na^+ conductance (Cui and Pun, 1994). Stimulation of functional chromaffin cell nicotinic receptors causes an increase in the conductance of Na^+ through the receptor-linked ion channel. The resultant influx of Na^+ elicits membrane depolarization which, in turn, causes the opening of voltage-dependent Na^+ and Ca^{2+} channels (Brandt *et al.*, 1976; Wada *et al.*, 1985; Liu and Kao, 1990). When desensitised, the nicotinic receptor-linked ion channel is inactivated and membrane depolarization is prevented. Any step in the post-receptor intracellular reactions leading to catecholamine release may also be modified (Marley, 1988; Ochoa *et al.*, 1989). Thus, serotonin or the recruitment of the RAS may serve a/n (additional) role of inhibiting downstream (nicotinic receptor-evoked) intracellular events to allow resensitisation of the nicotinic receptors; further studies might clarify this issue.

The Renin Angiotensin System and Catecholamine Secretion

Although the involvement of the RAS in catecholamine release in rainbow trout has only recently been discovered, it is known that Ang II and catecholamines typically function together as vasopressive agents to regulate blood pressure during hypotension (Olson, 1992; Bernier and Perry, 1997; Bernier *et al.*, 1999a,b). Studies on the role of the RAS in mammalian systems, however, have progressed rapidly due to molecular techniques and the availability of adrenal chromaffin cell cultures, and recent findings have implicated a role of the RAS in responding to hypoxia (see Chapter 3 Discussion). In mammals, the intracellular events that occur following the stimulation of the Ang II receptor ultimately lead to increases in intracellular free calcium ($[Ca^{2+}]_i$) by two separate mechanisms (reviewed by Houchi *et al.*, 1995). The first is by increasing phosphatidylinositol turnover that results in an accumulation of inositol phosphates and increased $[Ca^{2+}]_i$; the second method is by eliciting the influx of extracellular Ca^{2+} into the cells, and both mechanisms ultimately lead to catecholamine secretion. After stimulation of cultured bovine adrenal chromaffin cells by Ang II, $[Ca^{2+}]_i$ levels are then returned to physiological levels to maintain the ability to respond to further stimuli by stimulating Ca^{2+} efflux in a manner dependent on extracellular Na^+, likely *via* a Na^+/Ca^{2+} exchange (Houchi *et al.*, 1995). Thus, if a similar process occurs in rainbow trout chromaffin cells, then Ang II may circumvent the pathway elicited by nicotinic receptors to ultimately cause the same rise in $[Ca^{2+}]_i$ that leads to exocytosis of chromaffin granules.

Future Directions

In mammalian systems, desensitisation of the nicotinic receptor causes an up-regulation of nicotinic receptor numbers (Schwartz and Kellar, 1983). It is conceivable that in trout, desensitisation of the nicotinic receptor also leads to an increase in the number of chromaffin cell nicotinic receptors and possibly also angiotensin II receptors. It has recently been shown that in rat lungs, transient increases in angiotensin II receptors (AT2 and AT1 receptor types) are attributed to hypoxia-dependent transcriptional and post-transcriptional regulatory mechanisms (Chassagne *et al.*, 2000). Future experiments that focus on isolating chromaffin cell responses to various secretagogues (i.e. using an *in situ* PCV preparation) on fish that have been previously desensitised *in vivo* might expose chromaffin cell receptor up-regulation.

Recently, Montpetit and Perry (2000) used an *in situ* nerve-stimulating technique that they previously validated in rainbow trout to demonstrate that VIP and/or PACAP may directly stimulate adrenaline secretion from trout chromaffin cells at low levels of neuronal activity. The same study demonstrated that cholinergic stimulation predominates during high frequency electrical stimulation. Thus, neuronal control of catecholamine secretion in teleosts may not be restricted to cholinergic-evoked events (Montpetit and Perry, 2000). Using the same *in situ* nerve-stimulating technique, it may be possible to characterize nicotinic receptor-desensitisation *in situ* by exposing fish to prolonged high (cholinergic) or low frequency stimulations and measuring catecholamine secretion. This technique may also be advantageous in studying nicotinic receptor desensitisation, as it stimulates the release of acetylcholine, the natural cholinergic agonist from nerves that innervate the chromaffin cells and removes any uncertainty regarding actual amounts of nicotine that diffuses to the chromaffin cells during infusions/perfusions and its rate of clearance. Finally, this technique may be employed to characterize nicotinic receptor desensitisation administering a second stimulation to determine the level of desensitisation, or time until resensitisation.

Conclusions

Several secretagogues are capable of eliciting catecholamine secretion from chromaffin tissue *in situ*. In addition to nicotinic receptor stimulation, muscarinic receptors in rainbow trout (Julio *et al.*, 1998) and other teleosts (Gfell *et al.*, 1997; Abele *et al.*, 1998) may enhance nicotinic receptor-evoked secretion. Angiotensin II (Bernier and Perry, 1997), serotonin (Fritsche *et al.*, 1993), adrenocorticotropic hormone (ACTH; Reid *et al.*, 1996), and non-cholinergic neurotransmitters such as vasoactive intestinal polypeptide (VIP) and pituitary adenylate-cyclase-activating polypeptide (PACAP) have also been shown to cause *in situ* catecholamine release in rainbow trout (Reid *et al.*, 1995; Montpetit and Perry, 2000), and a variety of autocrine/paracrine agents (Epple *et al.*, 1993, 1994; Reid *et al.*, 1996). Although these secretagogues function to release catecholamines *in situ*, and may reflect a large dependence of rainbow trout on the release of catecholamines for survival during acute stressful situations, little is known about the actual *in vivo* situations that elicit the recruitment of particular catecholamine secretagogues. It is possible that some secretagogues capable of eliciting *in situ* catecholamine release *in situ* are merely evolutionary remnants that retain *in situ* secretory ability but lose physiological relevance *in vivo*. Although the present study is the first to implicate a role of the RAS in contributing to catecholamine release during hypoxia that is secondary to nicotinic receptor-evoked release, and indicate that muscarinic receptors only serve to enhance nicotinic-evoked secretion, the natural situations that predominantly involve serotonergic or muscarinic receptors remain possible. Future studies that implicate the role of higher control centers in the control of catecholamine release *in vivo* (i.e. *in vivo* experiments using other types of acute stress), perhaps in combination with *in situ* nerve-stimulating techniques might help characterize nicotinic receptor desensitisation and reveal physiologically significant secretagogues in response to specific stressors.

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