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**ANGIOTENSIN II AND THE CONTROL OF
HUMORAL CATECHOLAMINE RELEASE IN FISH**

By

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ABSTRACT

In fish, although the control of humoral catecholamine release is thought to be primarily mediated by the cholinergic fibers that innervate the chromaffin tissue, several non-cholinergic secretagogues have recently been identified. Overall, almost nothing is known about the relative contribution of these non-cholinergic secretagogues to the control of catecholamine release *in vivo*, and the physiological conditions under which they act. Therefore, the main goal of this thesis was to determine the relative importance of non-cholinergic secretagogues, primarily angiotensin II (Ang II), in the control of catecholamine release from the chromaffin tissue of fish.

In rainbow trout (*Oncorhynchus mykiss*), immunohistochemical techniques and an *in situ* posterior cardinal vein (PCV) perfusion preparation provided direct evidence that Ang II can elicit catecholamine release from the chromaffin tissue via specific Ang II binding sites. Injections of Ang II *in vivo* elicited dose-dependent increases in plasma adrenaline (Adr), and cardiovascular effects that were partially inhibited by α -adrenoceptor blockade. The establishment of relationships between plasma Adr concentrations and their cardiovascular effects provided evidence that physiological levels of Adr can significantly contribute to cardiovascular control, and that a portion of the pressor effects of exogenous Ang II can be attributed to increased levels of plasma Adr. The development and validation of a radioimmunoassay for Ang II made it possible to investigate whether endogenous Ang II contributes to the control of catecholamine release. In response to an acute hypotensive stress, endogenous Ang II, humoral catecholamines, and sympathetic nerves were recruited and played important roles in the compensatory response to hypotension. However, whereas the contribution of the renin-

angiotensin system (RAS) to blood pressure recovery was largely indirect and relied on an Ang II-mediated secretion of catecholamines, the contribution from the sympathetic nervous system (SNS) was direct and relied on both plasma catecholamines and sympathetic nerves.

In the American eel (*Anguilla rostrata*), injections of Ang II had no effect on humoral catecholamine release in either *in situ* PCV perfusion preparations or in chronically cannulated fish. Therefore, although α -adrenoceptor blockade partially inhibited Ang II-elicited increases in blood pressure, the pressor effects of exogenous Ang II could not be attributed to any change in plasma catecholamine levels. In response to an acute hypotensive stress, endogenous Ang II and humoral catecholamines were recruited, however the increase in plasma catecholamines was only a fraction of that observed in rainbow trout. Moreover, neither α -adrenoceptor nor sympathetic nerve blockade altered blood pressure recovery during a hypotensive stress. Therefore, the SNS does not appear to be an essential contributor to cardiovascular homeostasis during hypotension in the eel.

The contributions of Ang II and humoral catecholamines to cardiovascular control were also investigated in an elasmobranch, the spiny dogfish (*Squalus acanthias*). Ang II elicited dose-dependent increases in plasma catecholamines and cardiovascular effects that were abolished by α -adrenoceptor blockade. In response to an acute hypotensive stress, the RAS and humoral catecholamines were recruited and played significant roles in the compensatory response to hypotension. However, whereas the contribution of catecholamines was direct, Ang II indirectly contributed to cardiovascular control by dose-dependent stimulation of catecholamine release.

Finally, an *in situ* PCV perfusion preparation was used to assess the ability of various potential non-cholinergic secretagogues to elicit catecholamine release from the aneural chromaffin tissue of the Atlantic hagfish (*Myxine glutinosa*). Whereas injections of ACTH or serotonin elicited the release of catecholamines, Ang II or histamine did not. In addition, the secretory responses elicited by ACTH and serotonin were modified by prior treatment with an adenosine receptor agonist or antagonist. These results suggest that the control of catecholamine release from the chromaffin tissue of *M. glutinosa* can be achieved through hormonal and/or paracrine means and that ACTH, serotonin, and adenosine may all be involved.

In summary, Ang II is a potent secretagogue of humoral catecholamine release in *O. mykiss* and *S. acanthias*. In both species, Ang II plays a significant role in the control of catecholamine release during acute hypotension, and this interaction represents an important physiological response for the maintenance of cardiovascular homeostasis. On the other hand, although previously suggested, Ang II does not appear to be a secretagogue of humoral catecholamine release in either *A. rostrata* or *M. glutinosa*.

RÉSUMÉ

Chez les poissons, bien que l'on pense que le contrôle de la sécrétion des catécholamines est accompli principalement par l'intermédiaire des fibres cholinergiques qui innervent les cellules chromaffines, plusieurs sécrétagogues non-cholinergiques ont récemment été identifiés. En général, presque rien n'est connu sur la contribution relative de ces sécrétagogues non-cholinergiques au contrôle de la sécrétion des catécholamines *in vivo*, ainsi que sur les conditions physiologiques sous lesquelles ils agissent. Par conséquent, le but principal de cette thèse était de déterminer l'importance relative des sécrétagogues non-cholinergiques, principalement l'angiotensine II (Ang II), au contrôle de la sécrétion des catécholamines par les cellules chromaffines des poissons.

Chez la truite arc-en-ciel (*Oncorhynchus mykiss*), des techniques immunohistochimiques et une préparation *in situ* de perfusion de la veine cardinale postérieure (VCP) ont directement fourni des preuves que l'Ang II provoque la sécrétion des catécholamines par l'intermédiaire de récepteurs spécifiques pour l'Ang II. L'injection d'Ang II *in vivo* a provoqué une augmentation d'adrénaline (Adr) dans le plasma sanguin, et des effets cardiovasculaires qui sont en partie bloqués par une inhibition des adrénocéphes α . L'établissement de liens entre les concentrations d'Adr dans le plasma et de leurs effets cardiovasculaires ont fourni la preuve que des niveaux physiologiques d'Adr peuvent contribuer de façon significative au contrôle cardiovasculaire, et qu'une portion des effets de pression de l'Ang II peuvent être attribués à des augmentations du niveau d'Adr dans le plasma. Le développement et la validation d'une manipulation radioisotopique pour quantifier l'Ang II a permis de déterminer si l'Ang II endogène contribue également au contrôle de la sécrétion des

catécholamines. En réaction à un stress hypotensif aigu, l'Ang II endogène, les catécholamines, et les nerfs adrénergiques ont tous été recrutés et jouent un rôle important dans la réponse de compensation à l'hypotension. Cependant, alors que la contribution du système rénine-angiotensine (SRA) au redressement de la pression sanguine est principalement indirecte et dépend d'une sécrétion de catécholamines causée par l'Ang II, la contribution du système nerveux sympathique (SNS) est directe et dépend des catécholamines et des nerfs adrénergiques.

Chez l'anguille d'amérique (*Anguilla rostrata*), l'injection d'Ang II n'a pas eu d'effet sur la sécrétion des catécholamines soit à partir d'une préparation *in situ* de perfusion de la VCP, ou à partir de poissons muni de cathéter. Donc, bien qu'une inhibition adrénergique α bloque partiellement l'augmentation de pression sanguine causé par l'Ang II, les effets de pression de l'Ang II ne peuvent pas être attribués à des changements dans les niveaux de catécholamines du plasma. En réaction à un stress hypotensif aigu, l'Ang II endogène et les catécholamines ont tous deux été recrutés. Cependant l'augmentation des catécholamines fût seulement une fraction de celle obtenue chez la truite arc-en-ciel. De plus, ni le blocage des adrénoccepteurs α ou des nerfs adrénergiques a modifié le redressement de la pression sanguine à la suite d'un stress hypotensif. Donc, le SNS ne semble pas être un élément essentiel à l'homéostasie cardiovasculaire au cours de l'hypotension chez l'anguille.

La contribution de l'Ang II et des catécholamines au contrôle cardiovasculaire ont également été étudiés chez un requin, l'aiguillat commun (*Squalus acanthias*). L'injection d'Ang II a provoquée une augmentation des catécholamines du plasma et des effets cardiovasculaires qui peuvent être abolient par une inhibition adrénergique α . En

réaction à un stress hypotensif aigu, le SRA et les catécholamines ont tous deux été recrutés et joue un rôle important dans la réponse de compensation à l'hypotension. Cependant, alors que la contribution des catécholamines est directe, l'Ang II contribue indirectement au contrôle cardiovasculaire en stimulant la sécrétion des catécholamines.

Finalement, une préparation *in situ* de perfusion de la VCP a été utilisée pour évaluer la capacité de divers sécrétagogues non-cholinergiques à provoquer la sécrétion de catécholamines à partir des cellules chromaffines non-innervées de la myxine de l'atlantique (*Myxine glutinosa*). Tandis que des injections d'ACTH ou de sérotonine ont provoqués la sécrétion de catécholamines, l'Ang II et l'histamine n'ont pas eu d'effet. De plus, la sécrétion provoquée soit par l'ACTH ou par la sérotonine a été modifiée par un traitement antérieur avec un agoniste ou antagoniste des récepteurs de l'adénosine. Ces résultats semblent indiquer que le contrôle de la sécrétion des catécholamines à partir des cellules chromaffines de *M. glutinosa* peut être accompli par des moyens hormonaux et que l'ACTH, la sérotonine, et l'adénosine sont peut-être tous impliqués.

En résumé, l'Ang II est un sécrétagogue puissant de la sécrétion des catécholamines chez *O. mykiss* et *S. acanthias*. Dans ces deux espèces, l'Ang II joue un rôle considérable dans le contrôle de la sécrétion des catécholamines au cours d'hypotension aiguë, et cette interaction représente une réponse physiologique importante pour le maintien de l'homéostasie cardiovasculaire. Toutefois, bien que précédemment suggéré, l'Ang II ne semble pas être un sécrétagogue de la sécrétion des catécholamines ni chez *A. rostrata* ou *M. glutinosa*.

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LIST OF ABBREVIATIONS

- A₁ receptor, adenosine type 1 receptor
- A₂ receptor, adenosine type 2 receptor
- ACE, angiotensin converting enzyme
- ACTH, adrenocorticotropic hormone
- Adr, adrenaline
- Ang II, angiotensin II
- ANOVA, analysis of variance
- AT₁ receptor, angiotensin type 1 receptor
- AT₂ receptor, angiotensin type 2 receptor
- BSA, bovine serum albumin
- BW, body weight
- DβH, dopamine-β-hydroxylase
- dfAng II, dogfish angiotensin II
- DHBA, 3,4-dihydroxybenzylamine
- DPSPX, 1,3-dipropyl-8-*p*-sulfophenyloxanthine
- EC₅₀, half maximal effective concentration
- ED₅₀, half maximal effective dose
- EDTA, ethylenediaminetetraacetic acid
- EGTA, ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
- f*_H, heart rate
- FITC, fluorescein isothiocyanate
- HPLC, high performance liquid chromatography

IC_{50} , half maximal inhibitory concentration

IU, international units

N , number of animals or preparations

NECA, S'-(*N*-ethylcarboxamido) adenosine

Nor, noradrenaline

PaO_2 , arterial partial pressure of oxygen

PwO_2 , water partial pressure of oxygen

P_{CA} , mean caudal artery pressure

P_{DA} , mean dorsal aorta pressure

PBS, phosphate buffered saline

PCV, posterior cardinal vein

PE, polyethelyne tubing

Q , cardiac output

RAS, renin-angiotensin system

RIA, radioimmunoassay

R_G , branchial vascular resistance

R_S , systemic vascular resistance

S.E.M., standard error of the mean

SNS, sympathetic nervous system

TFA, trifluoroacetic acid

U, units of enzymatic activity

V_S , stroke volume

w/v, weight per volume

CHAPTER 1

General Introduction

RATIONALE FOR THE THESIS

Catecholamines, originating either from the sympathetic nervous system (SNS) or glandular chromaffin tissue, are involved in regulating a multitude of physiological functions throughout vertebrates (Nilsson 1983). Released from chromaffin cells in response to acute stress, humoral catecholamines, adrenaline and noradrenaline, are the key effectors of an integrated response aimed at maintaining homeostasis (Randall and Perry 1992; Thomas and Perry 1992; Wendelaar Bonga 1997). As such, the control of humoral catecholamine release from the chromaffin tissue is central to the ability of an animal to cope with life-threatening events.

Based on early investigations that identified the sympathetic pre-ganglionic cholinergic fibers innervating the adrenal gland in mammals (Young 1939; Coupland 1963), research into the control of catecholamine release from the chromaffin tissue has focused primarily on the involvement of cholinergic mechanisms (Ungar and Phillips 1983; Livett *et al.* 1983; Burgoyne 1991; Edwards and Jones 1993). Similarly, in fish, there has been considerable research aimed at establishing the cholinergic mechanisms involved in the control of catecholamine release (Nilsson 1976; Nilsson *et al.* 1976; Reid *et al.* 1994; Reid and Perry 1994; 1995; Furimsky *et al.* 1996; Al-Kharrat *et al.* 1997; Gfell *et al.* 1997; Abele *et al.* 1998; Julio *et al.* 1998). Although it has recently become clear that a large number of neuropeptides, hormones, and non-cholinergic neurotransmitters are also capable of directly stimulating humoral catecholamine secretion in vertebrates (Burgoyne 1991; Livett and Marley 1993), the importance of these non-cholinergic mechanisms in the control of humoral catecholamine release have received relatively little attention.

The multiplicity of factors involved in the control of catecholamine release is thought to reflect the precise control required to enable these hormones to play their central role in biochemical and physiological homeostasis (Livett and Marley 1993). In mammals, the mechanisms involved in the control of catecholamine release vary with the nature and severity of stress (Kuchel 1991). In short, complexity of control provides flexibility in response. However, although several potential non-cholinergic secretagogues of humoral catecholamine release have been identified in fish, their relative contribution to the control of catecholamine release *in vivo*, and the physiological conditions under which they act have yet to be characterized (Reid *et al.* 1998).

Therefore, the general aim of this thesis was to investigate the relative importance of non-cholinergic control mechanisms in the overall control of humoral catecholamine release in fish. More specifically, a series of experiments were designed to investigate the potential role of angiotensin II (Ang II), the biologically active product of the renin-angiotensin system, in the overall control of catecholamine release during both normotensive and hypotensive conditions in two teleost species (*Oncorhynchus mykiss* and *Anguilla rostrata*) and an elasmobranch (*Squalus acanthias*). Finally, in a separate study, the non-cholinergic control of catecholamine release from the aneural chromaffin tissue of hagfish (*Myxine glutinosa*) was assessed.

BACKGROUND KNOWLEDGE

1. *Humoral Catecholamines*

Humoral catecholamines, adrenaline and noradrenaline, are synthesized and stored in chromaffin cells from which they can be released into the circulation and function as hormones (Randall and Perry 1992). The synthesis of catecholamines in fish, as in other vertebrates, is achieved via a series of enzymatic reactions known as the Blaschko pathway (Jonsson and Nilsson 1983; Nilsson 1983; Randall and Perry 1992). In general, the synthesized catecholamines are stored in separate adrenaline- and noradrenaline-containing cells, but unlike the chromaffin tissue of mammals, the chromaffin cells of fish are not found in a distinct gland (Reid *et al.* 1988). In the most primitive fish, hagfish, catecholamines are stored in the systemic and portal hearts, and the posterior cardinal vein (PCV; Perry *et al.* 1993; Bernier and Perry 1996; Bernier and Perry 1998). In elasmobranchs, the chromaffin cells are found in paravertebral autonomic ganglia, the axillary bodies, which are closely associated with the venous circulation immediately behind the heart (Abrahamsson 1979; Nilsson and Holmgren 1988). In the more advanced teleosts, the chromaffin cells are located within the walls of the PCV and in the anterior region of the kidney, the head kidney (Mastrolia *et al.* 1984; Hathaway and Epple 1989; Gallo *et al.* 1993; Reid and Perry 1994).

Catecholamines are released into the circulation in response to a variety of stimuli which generally require enhanced blood oxygen transport (Perry and Wood 1989; Randall and Perry 1992; Thomas and Perry 1992; Gamperl *et al.* 1994c; Wendelaar Bonga 1997; Perry and Bernier 1998; Reid *et al.* 1998). Although a number of different conditions have been identified as being associated with elevated plasma catecholamine

levels (e.g. air exposure, environmental hypoxia and hypercapnia, metabolic acidosis, exercise, handling, physical disturbances, anemia, and anaesthesia), fish only release appreciable amounts of catecholamines in response to severe stress and not in response to mild or moderate disturbances (Randall and Perry 1992; Perry and Bernier 1998). Moreover, plasma catecholamine levels generally only increase significantly in teleosts under conditions that acutely lower blood oxygen content (Perry and Wood 1989; Randall and Perry 1992; Thomas and Perry 1992; Reid *et al.* 1998). During hypoxia, there is good evidence that catecholamine release occurs abruptly at a critical value of PaO_2 (the partial pressure of oxygen in the arterial blood) corresponding to a reduction of hemoglobin- O_2 saturation of approximately 50-60% (Perry and Reid 1992; 1994; Julio *et al.* 1998).

Once into the circulation, catecholamines are rapidly cleared via a combination of tissue uptake mechanisms and metabolic degradation (Randall and Perry 1992; Olson 1998). Although the predominant enzymes involved in the catabolism of catecholamines are found in gill, liver, and kidney tissues, only the gills have been extensively investigated for their role in catecholamine inactivation (Nekvasil and Olson 1986a, b; Colletti and Olson 1988). There is some evidence that circulating adrenaline may be an important source of neuronal catecholamine uptake (Xu and Olson 1993b). The biological half-time of injected doses of catecholamines in rainbow trout is approximately 2 min (Nekvasil and Olson 1986b; Gamperl and Boutilier 1994). Similarly, the half-life for recovery of cardiovascular parameters following an injection of catecholamines in rainbow trout is around 3-4 min (Olson *et al.* 1997a). Thus, both

physiological activation and metabolic inactivation of catecholamines in rainbow trout are rapid (Olson *et al.* 1997a).

In fish, the primary effects of humoral catecholamines are on blood oxygen transport capacity (Randall 1990; Randall and Perry 1992; Thomas and Perry 1992), cardiovascular control (Wood and Shelton 1980; Morris and Nilsson 1994; Zhang *et al.* 1998), and the regulation of metabolism (Randall and Perry 1992; Epple 1993; Fabbri *et al.* 1998). Catecholamines contribute to the maintenance of, or increase in, blood oxygen carrying capacity by increasing gill diffusing capacity (Perry *et al.* 1985), stimulating the release of erythrocytes from the spleen (Perry and Kinkead 1989), and by increasing red blood cell hemoglobin-O₂ affinity and capacity (Nikinmaa 1990). In the cardiovascular system, humoral catecholamines can modulate systemic and branchial resistances, as well as cardiac and venous functions (Wood and Shelton 1980; Bushnell *et al.* 1992; Farrell and Jones 1992; Gamperl *et al.* 1994a, b; Morris and Nilsson 1994; Zhang *et al.* 1998). Metabolically, catecholamines appear to be involved in glucose production (Moon and Foster 1995) and lipid mobilization (Sheridan 1994; Van Raaij *et al.* 1995). Although a possible role for catecholamines in the control of fish ventilation has been debated both for and against (Randall and Taylor 1991; Perry *et al.* 1992), there is no evidence that circulating catecholamines of endogenous origin act as ventilatory stimulants (Perry and Gilmour 1996).

Current evidence suggests that the control of catecholamine release in both teleosts and elasmobranchs is mediated primarily via preganglionic cholinergic fibers (Randall and Perry 1992; Reid *et al.* 1998). Electrical stimulation of the cholinergic fibers innervating the chromaffin tissue of teleosts (Nilsson *et al.* 1976; Abele *et al.* 1998)

and elasmobranchs (Abrahamsson 1979) elicits catecholamine release. Moreover, *in situ* or *in vitro*, acetylcholine or different cholinergic agonists elicit the release of catecholamines (Perry *et al.* 1991; Fritsche *et al.* 1993; Reid *et al.* 1994; Reid and Perry 1994; 1995; Al-Kharrat *et al.* 1997; Gfell *et al.* 1997; Julio *et al.* 1998; Montpetit and Perry 1998) and this response can be blocked by pre-treatment with cholinceptor antagonists (Nilsson *et al.* 1976; Fritsche *et al.* 1993; Reid and Perry 1995). While these results clearly identify the cholinergic nerves as an important pathway, there is increasing evidence that a large number of non-cholinergic pathways may also play an important role in the control of catecholamine release in fish (Reid *et al.* 1988).

In cyclostomes (hagfishes and lampreys), unlike in teleosts, the chromaffin tissue is not innervated and thus catecholamine release in these fish presumably is mediated exclusively through non-cholinergic means (Perry *et al.* 1993; Epple *et al.* 1995; Bernier and Perry 1996; Bernier and Perry 1998). In the teleost *Cyprinus carpio*, a recent microscopic investigation of the innervation pattern of the chromaffin cells in the head kidney revealed a low frequency of synaptic connections (Imagawa *et al.* 1996). Although similar data are not available for other fish species, these results suggest that at least carp chromaffin cells may be controlled predominantly by the endocrine system via the bloodstream (Imagawa *et al.* 1996).

Non-humoral agents that are known to either elicit or modulate catecholamine release in fish include blood oxygen content, blood acidosis, and blood $[K^+]$. In the Atlantic cod, *Gadus morhua*, although sectioning the spinal nerves supplying the head kidney prevents a hypoxia-induced increase in plasma noradrenaline, it has no effect on the increase in plasma adrenaline (Perry *et al.* 1991). Similarly, hypoxic blood has been

shown to directly stimulate adrenaline secretion in an *in situ* head kidney perfusion preparation of *G. morhua* (Perry *et al.* 1991), and chronic exposure to moderate hypoxia enhances the responsiveness of the chromaffin tissue to cholinergic stimulation in *O. mykiss* (Montpetit and Perry 1998). Acidosis, while not influencing basal catecholamine release in *O. mykiss*, modulates the cholinergic control of catecholamine release by enhancing the response to nicotinic receptor stimulation of chromaffin cells (Julio *et al.* 1998). In spiny dogfish (*Squalus acanthias*), physiological doses of K^+ elicits a dose-dependent increase in plasma catecholamines (Opdyke *et al.* 1981a; 1983).

One of the first humoral agents to be identified as a potential secretagogue of catecholamine release in fish was Ang II (Opdyke *et al.* 1981b; Carroll and Opdyke 1982). Intravascular injections, *in vivo*, of Ang II in either the teleost *Cyclopterus lumpus* (Carroll and Opdyke 1982) or the elasmobranch *S. acanthias* (Opdyke *et al.* 1981b) were shown to elicit an increase in plasma catecholamine levels. Indirect evidence also suggests that angiotensin II may elicit catecholamine release in the bowfin, *Amia calva* (Butler *et al.* 1995) and in the American eel, *A. rostrata* (Nishimura *et al.* 1978; Nishimura 1985b; Oudit and Butler 1995a). Since the early observations of Opdyke and co-workers on angiotensin II, a number of other humoral agents have been proposed as potential non-cholinergic secretagogues of catecholamine release. Serotonin, stored in high concentrations in the vicinity of the chromaffin tissue in the head kidney (Fritsche *et al.* 1993; Reid *et al.* 1995), has been shown to elicit catecholamine release both *in situ* and *in vivo* in *O. mykiss* (Fritsche *et al.* 1993). Although there is immunohistochemical evidence for the presence of atrial natriuretic peptides in the chromaffin cells of the common carp, *C. carpio* (Kloas *et al.* 1994), overall results from *in situ* and *in vivo*

experiments in either *O. mykiss* or *A. rostrata* do not support a role for natriuretic peptides in the control of catecholamine release in teleosts (McKendry *et al.* 1999). On the other hand, intravenous injections of C-type natriuretic peptides (CNP) in *S. acanthias* elicit an increase in plasma adrenaline (McKendry *et al.* 1999). Mixed results have also been observed with regards to the potential role of adrenocorticotrophic hormone (ACTH) in the control of humoral catecholamine release in teleosts (Kloas *et al.* 1994; Reid *et al.* 1996; Milakofsky *et al.* 1995). While there is *in situ* and *in vivo* evidence that ACTH can stimulate catecholamine release independently from cortisol in *O. mykiss* (Reid *et al.* 1996), *in vitro* ACTH has no effect on catecholamine release in either *C. carpio* (Kloas *et al.* 1994) or *A. rostrata* (Milakofsky *et al.* 1995). In addition, direct and indirect evidence suggest that humoral agents such as urotensin II (Conlon *et al.* 1996c), opioid peptides (Epple *et al.* 1993; 1994), neuropeptides (Reid *et al.* 1995), adenosine (Bernier *et al.* 1996), and catecholamines themselves (Epple and Nibio 1985; Perry *et al.* 1991) may all be involved in modulating the release of catecholamines from the chromaffin tissue of fish. Finally, there is immunohistochemical evidence that non-cholinergic neuronal agents such as vasoactive intestinal polypeptide (VIP) and pituitary adenylyl cyclase-activating polypeptide (PACAP), peptides known to stimulate and/or modulated catecholamine release in other vertebrates (Livett and Marley 1993; Yamaguchi 1993; Watanabe *et al.* 1995), may be involved in the non-cholinergic control of humoral catecholamine release in fish (Reid *et al.* 1995).

2. *The Renin-Angiotensin System*

The renin-angiotensin system (RAS) is an important element of the interrelated mechanisms that contribute to the maintenance of blood pressure and to the regulation of salt and water metabolism (Peach 1977). Investigations into the comparative physiology of this hormonal cascade are gradually unraveling the key roles of the RAS in body fluid and cardiovascular homeostasis throughout the vertebrate lineage (Nishimura 1978; 1985a; Olson 1992; Henderson and Deacon 1993; Henderson *et al.* 1993; Kobayashi and Takei 1996). Overall, the principle functions associated with the RAS include: elevation of blood pressure, stimulation of interrenal steroidogenesis, stimulation of humoral and neuronal catecholamine release, control of renal glomerular and tubular function, and stimulation of dipsogenic behaviour (Peach 1977; Wilson 1984a, b; Henderson and Deacon 1993; Kobayashi and Takei 1996).

The basic organization of the RAS system in vertebrates is as follows (Peach 1977). Renin, an enzyme produced by the juxtaglomerular cells in the glomerulus of the kidney, converts the circulating liver protein angiotensinogen into the decapeptide angiotensin I (Ang I). Angiotensin converting enzyme (ACE) hydrolyzes a dipeptide from Ang I to produce the octapeptide Ang II. Ang II is further converted into the heptapeptide angiotensin III (Ang III) and smaller peptide fragments by peptidases. Whereas Ang I is biologically inactive, Ang II and to a lesser extent Ang III have a number of different physiological actions in vertebrates (Henderson and Deacon 1993; Kobayashi and Takei 1996). In addition to this endocrine blood borne regulatory system, components of the RAS have also been described in various extrarenal tissues (Henderson and Deacon 1993; Phillips *et al.* 1993). Such extrarenal renin systems

suggest that angiotensins may also function as paracrine hormones with physiological functions of local importance (Phillips *et al.* 1993).

The renin-angiotensin system in cyclostomes

Until recently the primitive cyclostomes were thought to lack a renin-angiotensin system (Nishimura *et al.* 1970; Nishimura 1985a). Various investigations failed to reveal any sign of juxtaglomerular cells in either hagfish or lamprey species (Nishimura *et al.* 1970; see also Kobayashi and Takei 1996 for references). However, renal extracts from *Lampetra fluviatilis* incubated with canine renin substrate were shown to be pressor when assayed in rat (Henderson *et al.* 1981). Moreover, injections of mammalian Ang II in the hagfish, *Myxine glutinosa*, elicited a pressor response (Carroll and Opdyke 1982). Most notably, angiotensins from the lamprey species *L. fluviatilis* and *Petromyzon marinus* have recently been isolated (Y. Takei, personal communication). Although these findings strongly suggest the presence of an RAS in lampreys, overall the physiological significance of an RAS in cyclostomes has yet to be elucidated (Henderson *et al.* 1993).

The renin-angiotensin system in elasmobranchs

Although the presence of a complete RAS in elasmobranchs was also debated (Olson 1992; Henderson *et al.* 1993; Takei *et al.* 1993), there is now evidence for all the major components of the RAS in this vertebrate group (Lipke and Olson 1988; Lacy and Reale 1990; Uva *et al.* 1992; Takei *et al.* 1993). Overall, Ang II in elasmobranchs appears to play a role in the regulation of blood pressure (Opdyke and Holcombe 1976; Opdyke *et al.* 1982; Hazon *et al.* 1989; Hazon *et al.* 1995; Tierney *et al.* 1997), in the secretion of catecholamines (Opdyke *et al.* 1981b) and 1α -hydroxycorticosterone (Hazon and Henderson 1985), and in the control of drinking (Hazon *et al.* 1989). On the other

hand, Ang II does not seem to affect sodium excretion, glomerular filtration rate, or urine flow in elasmobranch fish (Churchill *et al.* 1985).

The renin-angiotensin system in teleosts

Many elements analogous to the mammalian RAS have been identified in teleost fish (Olson 1992). Juxtaglomerular cells have been observed in the renal arterioles of a variety of freshwater and seawater fish species (Kobayashi and Takei 1996), and renin secretion from these cells is primarily regulated by a baroreceptor mechanism that detects changes in arterial pressure (Bailey and Randall 1982). While the formation of Ang II by ACE primarily takes place in the arterioarterial pathway of the gills (Lipke and Olson 1988; Olson 1998), its inactivation and metabolism occurs in the gill's arteriovenous pathway as well as in the liver and kidney (Olson *et al.* 1986; Olson 1998). Ang II turnover is rapid with a half-time for *in vivo* recovery of 6-7 min following Ang II infusion (Olson *et al.* 1997a). In *O. mykiss*, in addition to the principal components of a systemic RAS (Olson 1992), there is physiological and molecular evidence for a local renal RAS (Brown *et al.* 1995; Brown and Balment 1997). Ang II and Ang III have an antidiuretic action in teleosts and appear to be an important component of renal function regulation (Brown and Balment 1997). Ang II is also dipsogenic and may play an important role in the regulation of drinking (Fuentes and Eddy 1997). In addition, several investigations have implicated the RAS of teleosts in cardiovascular homeostasis (Olson 1992; Platzack *et al.* 1993; Tierney *et al.* 1995b). The vasopressor response to Ang II in teleosts appears to be mediated by a direct action on the systemic microcirculation (Olson *et al.* 1994) and through an interaction with the SNS (Nishimura *et al.* 1978; Olson *et al.* 1994; Oudit and Butler 1995a). Finally, Ang II has been shown to stimulate

catecholamine release (Carroll and Opdyke 1982), and cortisol secretion (Decourt and Lahlou 1987; Perrott and Balment 1990; Arnold-Reed and Balment 1994).

3. Interactions between the Renin-Angiotensin System and Humoral Catecholamines

The studies of Opdyke and co-workers (Opdyke *et al.* 1981b; Carroll and Opdyke 1982) provided the first evidence that Ang II could elicit catecholamine release in all non-mammalian vertebrate classes. Although Carroll and Opdyke (1982) reported only a small increase in the plasma catecholamine levels of *C. lumpus* using a very high dose of Ang II ($\sim 2000 \text{ pmol kg}^{-1}$), their results have been repeatedly cited to explain the indirect SNS-mediated pressor properties of Ang II in teleosts (Wilson 1984a, b; Nishimura 1985b; Olson 1992; Platzack *et al.* 1993; Butler *et al.* 1995; Oudit and Butler 1995a). However, given the experimental design used by Carroll and Opdyke (1982), it is not possible to determine whether the origin of the increase in plasma catecholamines was from the chromaffin tissue or neuronal tissue. Therefore, there is no direct evidence that Ang II can stimulate catecholamine release from the chromaffin tissue of teleosts. On the other hand, Platzack (1995) recently provided some preliminary evidence that Ang II can facilitate catecholamine release from adrenergic nerve endings in *G. morhua*.

With the exception of birds (Wilson 1984a, b), little is known about the potential role of Ang II as a non-cholinergic secretagogue of humoral catecholamine release in non-mammalian vertebrates. Although several α -adrenoceptor blockade experiments have shown that a significant component of the pressor response attributed to Ang II is mediated by the SNS in some amphibian and reptilian species (Stephens 1981; Zehr *et al.* 1981; Sham *et al.* 1984; Harper and Stephens 1985; Silldorff and Stephens 1992a), others

have not (West *et al.* 1998). Overall, investigators have not determined the origin of the increase in plasma catecholamines originally observed by Carroll and Opdyke (1982) in these tetrapods. In the Pekin duck (*Anas platyrhynchos*), Ang II-elicited increases in blood pressure are indirect and have been shown to be mediated by the release of catecholamines from the adrenal gland and the adrenergic nerves (Wilson and Butler 1983a, b).

In mammals, Ang II has been known to directly stimulate catecholamine release from the adrenal gland since 1940 (Braun-Menendez *et al.* 1940 from Livett and Marley 1993). Since then, a large number of studies in different mammalian species have reached the same conclusion (reviewed by Peach 1977; Ungar and Phillips 1983; Reid 1992; Livett and Marley 1993). In general, the RAS has been implicated in the increased secretion of catecholamines from the adrenal medulla that occurs in response to hypotension, haemorrhage, and other stimuli (Reid 1992; Livett and Marley 1993). The chromaffin cells of the adrenal medulla are exposed to circulating Ang II generated by the systemic RAS and to Ang II generated locally by an intrinsic (local) RAS (Livett and Marley 1993). Detailed studies on the subtypes of Ang II receptors and on the intracellular mechanism of action of Ang II in chromaffin cells have also been carried out (Chiu *et al.* 1989; Livett and Marley 1993). In addition to catecholamine secretion, Ang II has a number of other actions on adrenal chromaffin cells, including secretion of opioid peptides, and increased expression and activation of the Blaschko pathway enzymes (Bommer and Herz 1989; Stachowiak *et al.* 1990; Goc and Stachowiak 1994). Ang II, in addition to stimulating catecholamine release from the mammalian adrenal gland, is known to interact with the SNS at various levels. Ang II facilitates peripheral

sympathetic neurotransmission by several mechanisms, augments sympathetic nerve outflow from the central nervous system, and attenuates baroreceptor-mediated reductions in sympathetic discharge (Reid 1992; Saxena 1992; Squire and Reid 1992).

In summary, while there is some indirect evidence that non-cholinergic secretagogues such as Ang II may be involved in the control of catecholamine release in fish, the physiological significance of these preliminary observations remains to be established. Meanwhile, previous investigations have indicated that both humoral catecholamines and the RAS may be involved in the cardiovascular control of hagfish, elasmobranchs, and teleosts. Whether under physiological conditions the cardiovascular involvement of the RAS includes a direct stimulation of humoral catecholamine release from the chromaffin tissue of fish is the central question of this thesis.

HYPOTHESES OF THE THESIS

1. General Hypothesis

Non-cholinergic secretagogues such as Ang II play a physiological role in the control of catecholamine release from the chromaffin tissue of fish.

2. Specific Hypotheses

CHAPTER 2: Angiotensins directly stimulate catecholamine release from the chromaffin tissue of *O. mykiss*.

CHAPTER 3: A portion of the cardiovascular effects of exogenous Ang II are mediated through plasma catecholamines in *O. mykiss*, and physiological concentrations of circulating catecholamines can contribute to cardiovascular regulation.

CHAPTER 4: A portion of the cardiovascular effects of endogenous Ang II are mediated through plasma catecholamines in *O. mykiss*, and humoral catecholamines contribute to cardiovascular control during hypotensive conditions.

CHAPTER 5: Ang II stimulates catecholamine release from the chromaffin tissue of *A. rostrata*, and both humoral and neuronal catecholamines contribute to blood pressure regulation during hypotension in *A. rostrata* and *O. mykiss*.

CHAPTER 6: The cardiovascular effects of endogenous Ang II are entirely mediated by plasma catecholamines in *S. acanthias*, and humoral catecholamines contribute to cardiovascular control during hypotensive conditions.

CHAPTER 7: Non-cholinergic secretagogues can elicit catecholamine release from the chromaffin tissue of *M. glutinosa*.

CHAPTER 2.**Angiotensins Stimulate Catecholamine Release from the
Chromaffin Tissue of the Rainbow Trout**

ABSTRACT

Immunohistochemical and pharmacological techniques were utilized to investigate the relationships between angiotensins and catecholamine release from the chromaffin tissue of rainbow trout (*Oncorhynchus mykiss*). Double labelling with [Asp¹,Ile⁵]-angiotensin II fluorescein (Ang II-FITC) and anti dopamine- β -hydroxylase (D β H) revealed specific Ang II binding sites on chromaffin cells. Injection (1 nmol kg⁻¹) of either Ang II-FITC, [Asn¹,Val⁵,Asn⁹]-Ang I, [Asp¹,Ile⁵,His⁹]-Ang I, [Asn¹,Val⁵]-Ang II, [Asp¹,Val⁵]-Ang II, or [Asp¹,Ile⁵]-Ang II, elicited catecholamine release from *in situ* perfusion preparations of the head kidney. Catecholamine release elicited by [Asn¹,Val⁵]-Ang II (10⁻¹³ to 10⁻⁷ mol kg⁻¹) was dose-dependent, and the secretion of adrenaline (Adr) was greater than that of noradrenaline (Nor). Relative to the results obtained with the [Asn¹,Val⁵]-Ang II treatment (1 nmol kg⁻¹), Adr release was 72 and 82% lower in response to injections (1 nmol kg⁻¹) of [Asn¹,Val⁵]-Ang I (AA's 1-7) and [Asn¹,Val⁵]-Ang I (AA's 1-6), respectively. Pre-treatment with either Losartan (10⁻⁵ M), PD123319 (10⁻⁵ M), or hexamethonium (10⁻³ M), had no effect on [Asn¹,Val⁵]-Ang II-elicited catecholamine release. Pre-treatment with captopril (10⁻⁴ M) significantly reduced [Asn¹,Val⁵,Asn⁹]-Ang I-elicited Adr and Nor release, and decreased basal catecholamine release. These results provide direct evidence that angiotensins can elicit catecholamine release from the chromaffin tissue via specific Ang II binding sites, and indicate that the synthesis of Ang II may be either local or systemic.

INTRODUCTION

Angiotensins, the regulatory peptides of the renin-angiotensin system (RAS), are important mediators of blood pressure and fluid volume homeostasis in teleosts (for review see Olson 1992). Similarly, the catecholamines stored in the chromaffin tissue of the head kidney in teleost fish, and released during acute stress (Randall and Perry 1992), play a key role in the regulation of blood flow and distribution (Bushnell *et al.* 1992). The ability of intravascular injections of mammalian angiotensin II (Ang II) to increase circulating levels of catecholamines in the lumpfish (*Cyclopterus lumpus*; Carroll and Opdyke 1982), suggests that at least part of the vascular effects of the RAS in fish may be mediated by circulating catecholamines.

In mammals, numerous studies indicate that Ang II stimulates the adrenal medulla to release catecholamines both *in vivo* and *in vitro* (for reviews see Peach 1977; Reid 1992). These effects appear to be mediated by a direct action of Ang II on the adrenal medulla, since they are not prevented by surgical denervation or by ganglionic blockade (Peach 1977). Ang II binding sites have been identified in isolated bovine chromaffin cells (Marley *et al.* 1989). In response to Ang II stimulation (Bunn and Marley 1989), catecholamine secretion is blocked by the specific AT₁-receptor antagonist, Losartan (Timmermans *et al.* 1993). The physiological relevance of the interaction between the RAS and catecholamine release in mammals is shown by several studies where Ang II elicits the release of catecholamines from the adrenal medulla in response to hemorrhage or hypotension (Badder *et al.* 1985; MacLean and Ungar 1986).

In teleosts, there is indirect evidence that Ang II may elicit catecholamine release (Butler *et al.* 1995; Carroll and Opdyke 1982; Nishimura *et al.* 1978; Oudit and Butler 1995a). However, unlike the situation in mammals, it is not known whether angiotensins elicit catecholamine secretion by a direct action on the chromaffin cells. Although it is clear that the Ang II-induced

pressor response in fish is partially mediated by secondary stimulation of the sympathetic nervous system (Butler *et al.* 1995; Olson 1992; Oudit and Butler 1995a), there is ambiguity as to whether the catecholamines originate from peripheral sympathetic nerves or from the chromaffin tissue. In teleosts, unlike mammals, adrenaline dominates over noradrenaline in both the adrenergic neurons and the chromaffin tissue (Nilsson 1983).

The goal of this study, therefore, was to provide direct evidence that angiotensins stimulate catecholamine secretion from chromaffin cells in the teleost, rainbow trout. This was achieved by using a combination of immunohistochemical techniques on postcardinal vein cross sections, and pharmacological techniques on *in situ* perfusion preparations of the head kidney.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex weighing between 165 and 430 g (mean mass = 246.9 ± 3.0 g; experimental $N = 191$) were obtained from Linwood Acres Trout Farms (Campbellcroft, Ontario) and transported to the fish holding facilities of the University of Ottawa. The fish were held indoors in large fiberglass tanks supplied with flowing, aerated and dechloraminated city of Ottawa tap water. The fish were maintained at a temperature of 10-12°C, on a 12 h light:12 h dark photoperiod cycle, and fed daily with a commercial salmonid diet.

Immunohistochemistry

A) Preparation of the PCV Tissues Sections

Rainbow trout were killed by a blow to the head. The fish were then placed dorsally on ice and after a ventral incision the kidney was exposed by removing the overlying organs. The posterior cardinal vein (PCV) was removed from the head kidney region, with a narrow strip of kidney tissue surrounding the vein. Five mm sections of the PCV were fixed by immersion in 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS) adjusted to pH 7.2 for 12 h. The tissues were then washed with PBS (3 X 5 min), and immersed in a series of PBS solutions containing 5, 10, 15, and 20% sucrose (w/v) for one hour each. Once cryoprotected, the sections of the tissue were embedded in Tissue-Tek (Miles Inc., Elkhart, IN) and quick frozen by immersion in liquid nitrogen. Cross sections (10 μm) were cut on a cryostat and thaw-mounted on poly-L-lysine (1 mg ml⁻¹) coated slides. In order to facilitate the histochemical techniques, a barrier of rubber cement was applied to surround the sections on the slides. The prepared slides were kept in the dark, at -20°C, until needed.

B) Histochemical Techniques

Tissue sections were first rehydrated in PBS (3 X 5 min), and reduced in sodium metabisulfite (1 mg ml⁻¹ in PBS; 3 X 5 min) to decrease autofluorescence. The sections were then rinsed with PBS (3 X 5 min), incubated for one hour with 5% bovine serum albumin (BSA; ICN Biomedicals, Aurora, OH) in PBS (to decrease non-specific binding), and rinsed again with PBS (3 X 5 min). Following these steps, the slides were submitted to indirect immunofluorescence. The sections were incubated for two hours with a primary antiserum to the catecholamine synthesis enzyme dopamine- β -hydroxylase (D β H; code: TE 103; dilution 1:100; host: rabbit; source: Eugene Tech International, Allendale, NJ), rinsed in PBS (3 X 5 min), and further incubated for one hour with the conjugated secondary purified antibody Dar-Cy3 (dilution: 1:400; host: donkey; source: Jackson ImmunoResearch Laboratories,

West Grove, PA). After rinsing in PBS (3 X 5 min), the tissues were also labelled with [Asp¹,Ile⁵]-angiotensin II fluorescein (Ang II - FITC; Cat. # A-150, RBI, Natick, MA) by incubating the sections for two hours with the conjugated probe at a concentration of 1.4×10^{-6} M. The sections were then washed in PBS (3 X 5 min), and mounted with coverslips using 30 μ l of a buffered glycerol solution containing 0.1% paraphenylenediamine (to prevent fading of the fluorochromes). To preserve the slides, the coverslips were sealed with nailpolish and stored at -20°C.

To distinguish specific from non-specific binding of Ang II - FITC, Ang II receptors were saturated with an excess unlabelled [Asp¹,Ile⁵]-Ang II (Cat. # A-9525, Sigma Chemical Co., St. Louis, MO) prior to the Ang II - FITC labelling procedure. This was achieved by incubating sections with 100 μ l of an [Asp¹,Ile⁵]-Ang II solution (9.5×10^{-4} M), and 10 minutes later with an additional 100 μ l of the Ang II - FITC (1.4×10^{-6} M). The specificity of secondary antisera labelling was also determined by incubating some sections with conjugated Cy3 alone. Finally, to determine the amount of background fluorescence, the primary, secondary, and fluorescent probe were replaced with PBS during incubation.

All incubations were performed in a sealed moist chamber at room temperature, consistently using 100 μ l aliquots of the various solutions. The PBS, sodium metabisulfite, and BSA solutions were freshly prepared before use. [Asp¹,Ile⁵]-Ang II, Ang II - FITC, and the DBH antiserum were diluted in 0.9% PBS containing 0.05 mg ml⁻¹ BSA, 0.05 mg ml⁻¹ thyroglobulin, 2.5 mg ml⁻¹ sodium azide, and 20 mg ml⁻¹ EDTA (pH 7.6).

C) Fluorescence Microscopy

For combined phase contrast and epifluorescence microscopy, tissue sections were visualised with a Zeiss Axiophot Photomicroscope using either 10X Ph1, 20X Ph2, or 40X Ph2 Plan Neofluar objectives. Photographs were taken using Ilford XP2-400 film.

In situ Postcardinal Vein Perfusion

To assess the ability of the chromaffin tissue to release catecholamines in response to a variety of angiotensins, the *in situ* saline-perfused postcardinal vein preparation of Fritsche *et al.* (1993) was employed with the following modifications. Trout killed by a blow to the head were dissected ventral side up on ice. An incision was made from the vent to the pectoral girdle to expose the head kidney and the bulbus arteriosus. An outflow cannula (PE 160) was inserted through the bulbus and into the ventricle. This cannula was secured in place with a ligature around the walls of the bulbus. An inflow cannula (PE 160) was inserted into the PCV approximately two-thirds along the length of the kidney in the anterograde direction. The body cavity was filled with lint-free wipes and a ligature was placed around the entire fish to secure the inflow cannula and prevent leakage. The cannulated trout was placed on ice and perfused with modified Cortland saline (Fritsche *et al.* 1993) gassed with air (final pH = 7.8). Perfusion was achieved by siphon resulting from the positive pressure difference between the saline and the outflow cannula (10 mmHg), and the perfusion flow rate was approximately 1 ml min⁻¹. Each preparation was perfused for 20 min prior to sample collection. After this stabilization period, a control pre-sample was taken to assess basal catecholamine concentrations. The perfusion saline was then altered in accordance to the treatments described below over a period of one min, and seven post-samples were collected 1, 2, 3, 4, 5, 7.5, and 10 min after the intervention. All

samples were frozen immediately in liquid nitrogen and kept frozen at -80°C until analysis of catecholamine content.

Series 1: Bioactivity of Ang II - FITC on catecholamine release

After collection of the pre-sample, a bolus injection (300 μl) of Ang II - FITC (1 nmol kg^{-1}) was administered to the preparation via a valve in the inflow cannula and the post-samples collected.

Series 2: Dose-response relationship between [Asn¹,Val⁵]-Ang II and catecholamine release

Using the same procedures as in series 1, head kidney preparations were given bolus injections of the teleost octapeptide [Asn¹,Val⁵]-Ang II (Cat. # A-6402, Sigma), and their effects on catecholamine release were monitored. The doses of [Asn¹,Val⁵]-Ang II ranged from 10^{-13} to 10^{-7} mol kg^{-1} , allowing dose-response relationships to be established. A single dose of [Asn¹,Val⁵]-Ang II was assessed per preparation. Curve fits for each of the dose response relationships were generated using the software program Sigma Plot for Windows (Jandel, San Rafael, CA) nonlinear curve fitting options.

Series 3: Effects of teleost, tetrapod, and mammalian angiotensins on catecholamine release

Using the same procedures as in series 1, and doses of 1 nmol kg^{-1} , the following angiotensins were assessed for their effects on catecholamine release: the teleost decapeptide [Asn¹,Val⁵,Asn⁹]-Ang I (Cat. # A-2928, Sigma), the teleost heptapeptide [Asn¹,Val⁵]-Ang I (AA's 1-7) (Cat. # 7042, Peninsula Laboratories, Inc. Belmont, CA), the teleost hexapeptide [Asn¹,Val⁵]-Ang I (AA's 1-6) (Cat. # 7041, Peninsula Laboratories), the tetrapod octapeptide [Asp¹,Val⁵]-Ang II (Cat. # 05-23-0106, Calbiochem, LaJolla, CA), the mammalian decapeptide [Asp¹,Ile⁵,His⁹]-Ang I (Cat. # A-9650, Sigma), and the mammalian octapeptide [Asp¹,Ile⁵]-Ang II (Cat. # A-9525, Sigma).

Series 4: Effects of captopril on catecholamine release

A) Using the same procedures as in series 1, the stimulatory effects of the teleost decapeptide [Asn¹,Val⁵,Asn⁹]-Ang I (1 nmol kg⁻¹) on catecholamine release were assessed with the angiotensin converting enzyme inhibitor, captopril (Cat. # C-157, RBI), added to the perfusion saline (final concentration = 10⁻⁴ M).

B) The effects of captopril on basal catecholamine release were assessed in head kidney preparations perfused with either saline alone or saline containing captopril (10⁻⁴ M). After the period of stabilization the pre- and post-samples were collected at the set times given above without further intervention.

C) The effects of saline injection in head kidney preparations were also assessed in this experimental series. After collection of the pre-sample, a bolus injection (300 µl) of saline was administered to the preparation via a valve in the inflow cannula and the post-samples collected.

Series 5: Characterization of the Ang II receptor involved in catecholamine release

Using the same procedures as in series 1, the stimulatory effects of the teleost octapeptide [Asn¹,Val⁵]-Ang II (1 nmol kg⁻¹) on catecholamine release were assessed with each one of the following receptor antagonists added to the perfusion saline: the non-peptide human Ang II AT₁ receptor antagonist, Losartan (10⁻⁵ M) (= DuP753, 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[2'-(¹H-tetrazol-5-yl) biphenyl-4-yl]-methyl-imidazole, DuPont Pharmaceuticals, Wilmington, DE), the non-peptide human Ang II AT₂ receptor antagonist, PD123319 ditrifluoroacetate (10⁻⁵ M) (S(+)-1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-¹H-imidazo[4,5-*c*]pyridine-6-carboxylic acid ditrifluoroacetate, Cat. # P-186, RBI), and the cholinergic receptor antagonist, hexamethonium (10⁻³ M; hexamethonium dichloride, Cat. # H-

132, RBI). The stimulatory effects of the teleost octapeptide [Asn¹,Val⁵]-Ang II (1 nmol kg⁻¹) on catecholamine release were also assessed with a calcium-free saline containing 1 mM EGTA.

Analytical procedures

Perfusate adrenaline (Adr) and noradrenaline (Nor) levels were determined on alumina-extracted saline samples (200 µl) using high performance liquid chromatography (HPLC) with electrochemical detection. The HPLC incorporates a Varian Star 9012 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments Corporation, Princeton, NJ). The extracted samples were passed through an SP-C18-5 Varian MicroPak column, and the separated amines were integrated with the Star Chromatography software program (version 4.0, Varian). Concentrations were calculated relative to appropriate standards, and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations.

Statistical analyses

All data are presented as means \pm one standard error. To assess the effects of a given treatment on the release of catecholamines from the *in situ* postcardinal vein preparations, a paired t-test was used to compare the mean of the pre-values (control release) with the mean of the maximum values observed within the 10 min period post-intervention (maximum release). This approach was chosen in order to account for temporal differences in the release process between preparations. The statistical significance of observed effects of a given treatment within a group were tested by one-way repeated measures ANOVA. Dunnett's test was used to compare the time 0 mean with the means at subsequent times. The statistical significance of observed differences between the means of several treatments were tested by one-way ANOVA. The significance level for all statistical test was $P < 0.05$.

RESULTS

Immunohistochemistry

Phase contrast light microscopy revealed a distinct and continuous layer of cells in the immediate vicinity of the walls of the PCV (Fig. 2.1A). Immunohistochemistry confirmed the presence of chromaffin cells in the same location (Fig. 2.1B). Chromaffin cells were identified by examining the pattern of labelling obtained with antisera raised against D β H, an enzyme of the catecholamine-synthesizing Blaschko pathway (Randall and Perry 1992). The pattern of labelling suggested that most chromaffin cells were in close contact with each other, with only a few individual cells lying outside of these aggregates (Fig. 2.1B). Double labelling with the Ang II - FITC probe (Fig. 2.1C) and the antisera against D β H (Fig. 2.1B) revealed that a large number of D β H-positive chromaffin cells exhibited Ang II binding sites. The pattern of Ang II - FITC (Fig. 2.1C) suggested that Ang II binding sites were restricted to enzyme-positive chromaffin cells (Fig. 2.1B).

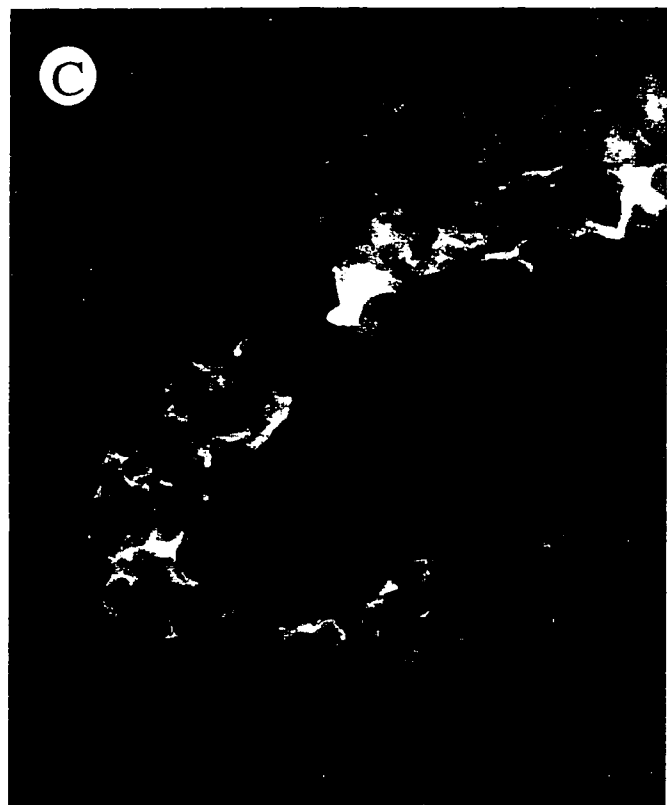
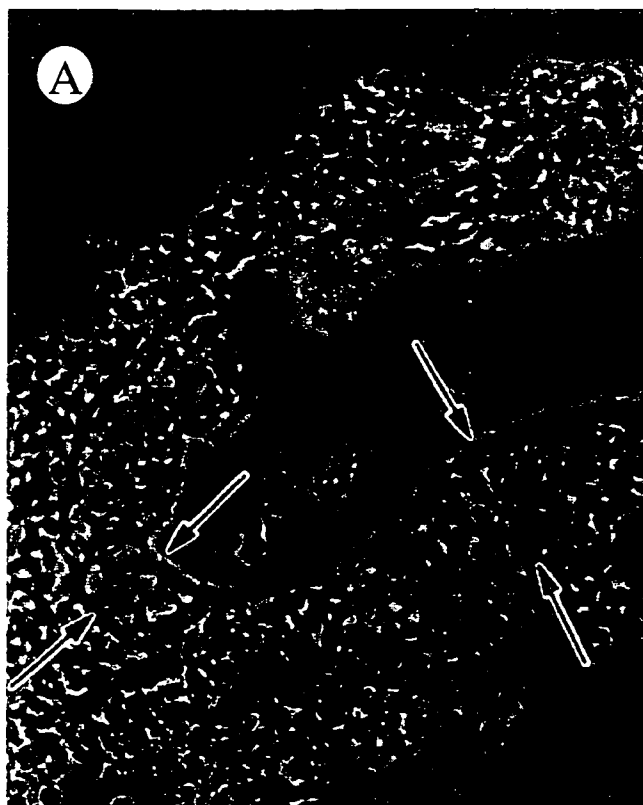
Control tissue sections treated with the standard histochemical techniques, except for the omission of the primary and secondary antibodies, and the labelled Ang II probe, exhibited dull non-specific autofluorescence in comparison to the bright specific fluorescence displayed in Fig. 2.1. Incubation of other sections without the primary antisera TE-103, but with the secondary antisera Darcy3, did not produce any specific labelling. Finally, excess unlabelled [Asp¹,Ile⁵]-Ang II eliminated the specific fluorescence of the conjugated Ang II - FITC probe in D β H-positive chromaffin cells.

In situ Postcardinal Vein Perfusion

Series 1: Bioactivity of Ang II - FITC

Bolus injections of the Ang II - FITC probe into the *in situ* postcardinal vein preparations

Figure 2.1 A-C: Fluorescent labelling of angiotensin II (Ang II) receptors in the chromaffin tissue of rainbow trout posterior cardinal vein (PCV). PCV cross sections were incubated with antisera to dopamine β -hydroxylase (D β H), processed for indirect immunofluorescence with Cy3, and double labelled with a fluorescein conjugated [Asp¹,Ile⁵]-angiotensin II probe (Ang II-FITC). (A) is a phase contrast image showing the PCV lumen surrounded by a layer of chromaffin cells (layer delineated by arrows). (B) is a fluorescent image of D β H-positive cells from the section shown in (A). The white bar represents 50 μ m. (C) is a fluorescent image of Ang II labelling in the PCV section shown in (A). D: Perfusate [catecholamine] from *in situ* head-kidney preparations of rainbow trout treated with 1 nmol kg⁻¹ Ang II-FITC (Nor, noradrenaline; Adr, adrenaline). The open and solid bars represent the control and maximum [catecholamine] before and after treatment, respectively. Values are means \pm 1 S.E.M. ($N = 6$). * Significantly different from control value ($P < 0.05$).



D

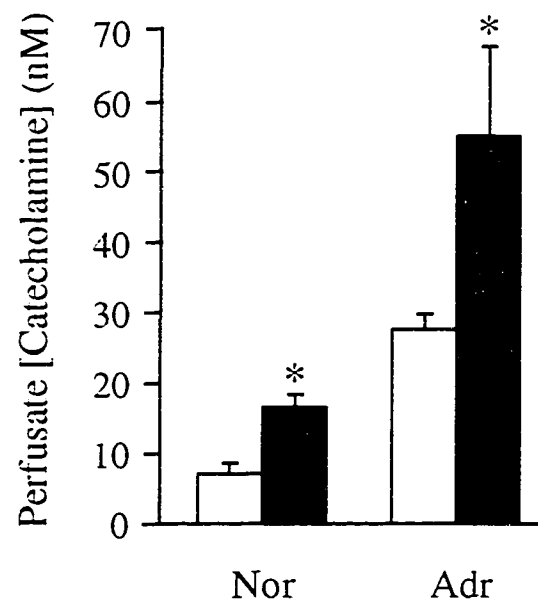


Figure 2.1

elicited a significant release of both NA and AD (Fig. 2.1D). Adr was the predominant catecholamine released either before or after the injection of the probe.

Series 2: Dose-response relationship between [Asn¹,Val⁵]-Ang II and catecholamine release

Bolus injections of [Asn¹,Val⁵]-Ang II ranging from 10⁻¹³ to 10⁻⁷ mol kg⁻¹ elicited a dose-dependent release of both catecholamines (Fig. 2.2). Adr secretion was stimulated at all doses of [Asn¹,Val⁵]-Ang II (Fig. 2.2A), whereas NA secretion was stimulated at doses equal to or exceeding 10⁻¹² mol kg⁻¹ (Fig. 2.2B). The curve fits depicted in figure 2.2 reveal maximum Adr and Nor concentrations of 413 and 27 nM, respectively. The Hill plots corresponding to the dose-response relationships are shown in the insets. They demonstrate half-maximal effective [Asn¹,Val⁵]-Ang II doses (ED₅₀) of 0.34 and 0.92 nmol kg⁻¹ for Adr and Nor, respectively. Overall, this analysis indicates that [Asn¹,Val⁵]-Ang II preferentially stimulates the release of Adr over Nor.

Series 3: Effects of teleost, tetrapod, and mammalian angiotensins on catecholamine release

Bolus injections of a 1 nmol kg⁻¹ dose of either [Asn¹,Val⁵,Asn⁹]-Ang I, [Asn¹,Val⁵]-Ang I (AA's 1-7), [Asn¹,Val⁵]-Ang I (AA's 1-6), [Asp¹,Val⁵]-Ang II, [Asp¹,Ile⁵,His⁹]-Ang I, or [Asp¹,Ile⁵]-Ang II all elicited a significant release of Adr and Nor (Table 2.1). The catecholamine values obtained in series 2 with [Asn¹,Val⁵]-Ang II are shown in Table 2.1 for comparative purposes. Overall there was no difference between all the treatments listed above in the control Adr or Nor values. There was also no difference between the maximum Adr or Nor values elicited by the 3 different Ang II agonists, nor by the two Ang I agonists. The maximum Adr release elicited by [Asn¹,Val⁵]-Ang I (AA's 1-6) was significantly less than the release obtained with [Asn¹,Val⁵,Asn⁹]-Ang I or [Asn¹,Val⁵]-Ang II, and the release elicited by [Asn¹,Val⁵]-Ang I (AA's 1-7) was intermediate between the two (Table 2.1). Meanwhile, there was no difference

Figure 2.2 Dose response curves of (A) perfusate [adrenaline] ([Adr]) and (B) perfusate [noradrenaline] ([Nor]) as a function of log [Asn¹-Val⁵]-Ang II dose in *in situ* head-kidney preparations of rainbow trout. The open circles and solid squares represent the control and maximum [catecholamine] before and after treatment, respectively. Values are means \pm 1 S.E.M. ($N = 6-9$). * Significantly different from control value for a given dose ($P < 0.05$). Curve fits depicted in each panel reveal maximum catecholamine values ($Adr_{max} = 413$ nM; $Nor_{max} = 27$ nM). Insets are Hill plots demonstrating half-maximal effective dose (ED_{50} ; 0.34 nmol kg^{-1} for Adr; 0.92 nmol kg^{-1} for Nor).

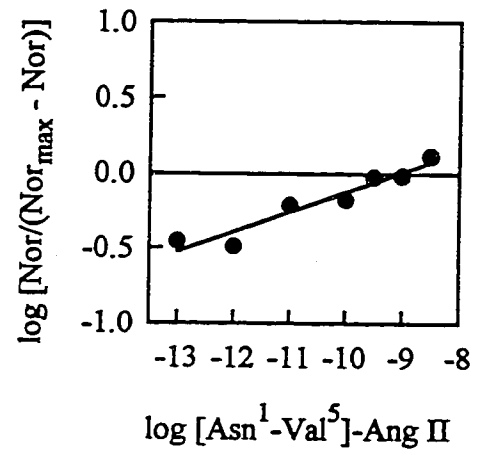
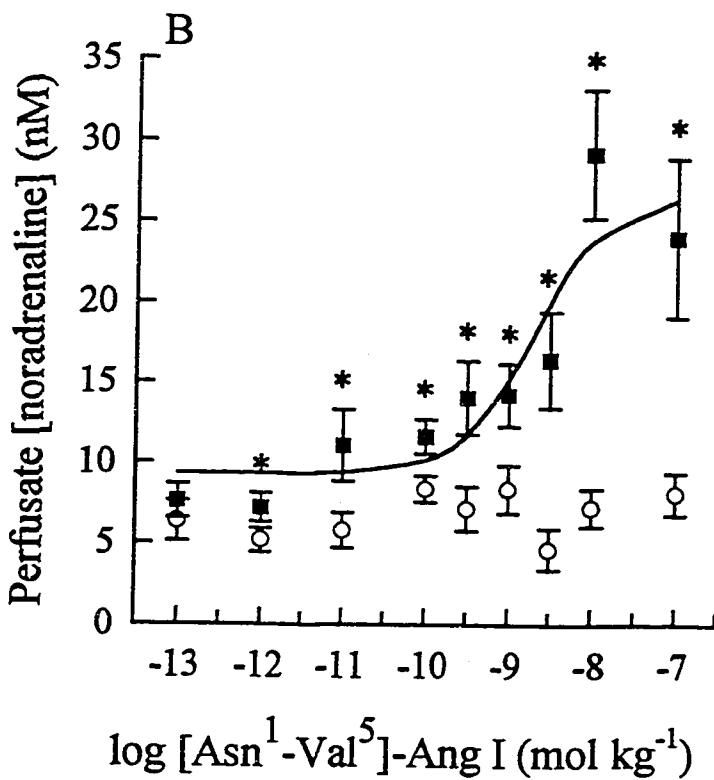
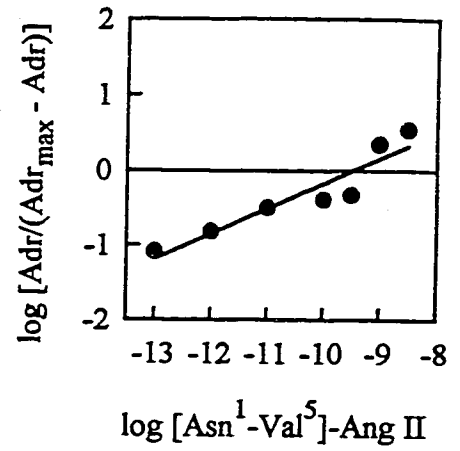
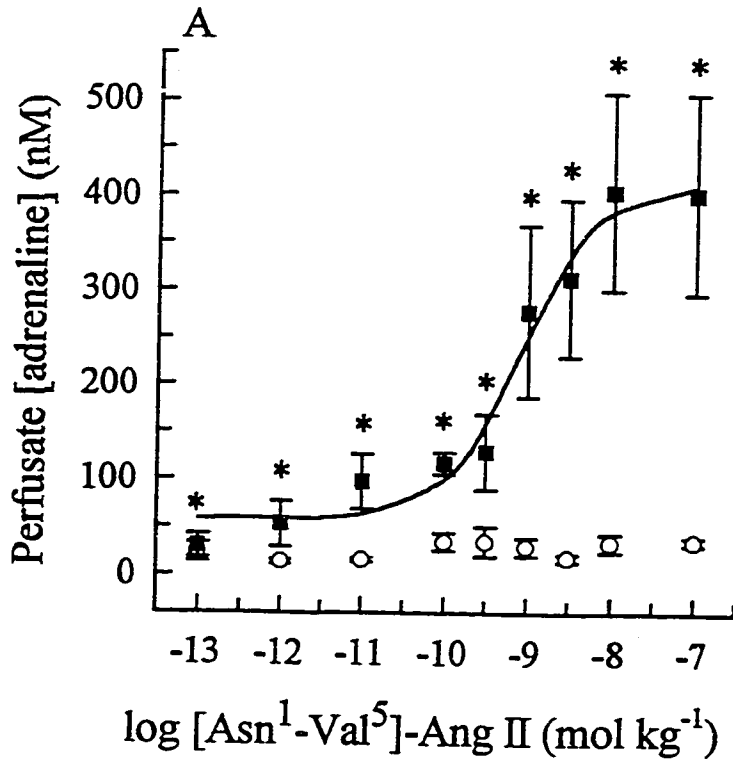


Figure 2.2

Table 2.1 Effects of teleost, tetrapod, and mammalian angiotensins (1 nmol kg^{-1}) on perfusate [adrenaline] and [noradrenaline] from *in situ* head-kidney preparations of rainbow trout.

| Treatment | N | Perfusate Adrenaline (nM) | | Perfusate Noradrenaline (nM) | |
|---|---|---------------------------|--------------|------------------------------|-----------|
| | | Control | Maximum | Control | Maximum |
| Teleost | | | | | |
| [Asn ¹ , Val ⁵ , Asn ⁹]-Ang I | 8 | 21.6±3.0 | 222.4±56.4*a | 7.6±1.6 | 15.5±2.8* |
| [Asn ¹ , Val ⁵]-Ang II | 8 | 29.8±9.4 | 277.6±90.4*a | 8.2±1.5 | 14.3±2.0* |
| [Asn ¹ , Val ⁵]-Ang I (1-7) | 8 | 32.9±4.7 | 78.5±12.3*ab | 5.8±0.9 | 12.2±1.8* |
| [Asn ¹ , Val ⁵]-Ang I (1-6) | 8 | 30.9±4.3 | 50.6± 7.9*b | 4.3±0.6 | 8.0±2.0* |
| Captopril / [Asn ¹ , Val ⁵ , Asn ⁹]-Ang I | 8 | 6.9±1.8† | 11.7± 2.6*† | 3.4±0.7† | 4.2±0.8† |
| Tetrapod | | | | | |
| [Asp ¹ , Val ⁵]-Ang II | 9 | 46.4±16.9 | 443.0±78.8* | 7.1±1.2 | 15.2±3.5* |
| Mammalian | | | | | |
| [Asp ¹ , Ile ⁵ , His ⁹]-Ang I | 8 | 32.1±7.0 | 226.7±67.1* | 5.1±0.5 | 10.1±1.7* |
| [Asp ¹ , Ile ⁵]-Ang II | 8 | 32.9±7.4 | 191.3±46.1* | 6.6±1.4 | 18.1±3.9* |

Values are means ± S.E.M.; N, no. of fish. Control values are the basal [catecholamine] after 20 min of perfusion with saline. Maximum values are the peak [catecholamine] during a 10 min period after injection of the angiotensin agonists. In the captopril treatment, fish were perfused with the ACE inhibitor throughout the trial. * Significantly different from control value for a given treatment. † Significantly different from the [Asn¹, Val⁵, Asn⁹]-Ang I treatment for a given column. Treatments which do not share a common letter in a given column are significantly different from each other ($P < 0.05$).

between the maximum Nor values obtained with the four teleost angiotensins. Adr was the predominant catecholamine released with all the angiotensins tested.

Series 4: Effects of captopril on catecholamine release

Captopril treatment abolished the stimulatory effects of [Asn¹,Val⁵,Asn⁹]-Ang I on Nor release, and significantly reduced Adr release (Table 2.1). In comparison to the [Asn¹,Val⁵,Asn⁹]-Ang I treatment of series 3, the presence of captopril in the perfusion saline significantly decreased both the control and the maximum values elicited by [Asn¹,Val⁵,Asn⁹]-Ang I (Table 2.1). A decrease in control catecholamine values with captopril treatment was also observed in preparations where the basal catecholamine release was recorded over time (Fig. 2.3). The reduction in catecholamine release through a 10 min perfusion period was more pronounced in the fish perfused with saline only than in the captopril-treated fish (Fig. 2.3). In comparison to the saline-perfused group, captopril treatment did not affect flow through the perfusion preparations. Injections of saline alone had no effect on the reduction in catecholamine release recorded over time (Fig. 2.3).

Series 5: Characterization of the Ang II receptor involved in catecholamine release

The presence of either Losartan, PD123319, or hexamethonium in the perfusion saline had no effect on either the control catecholamine values or the maximum values elicited by [Asn¹,Val⁵]-Ang II (Table 2.2). Bolus injections of [Asn¹,Val⁵]-Ang II to preparations perfused with Ca²⁺-free saline caused a small but significant increase in Adr and no increase in Nor. Perfusing with Ca²⁺-free saline significantly decreased both the control and the maximum values elicited by [Asn¹,Val⁵]-Ang II (Table 2.2).

Figure 2.3 Time course of (A) perfusate [adrenaline] and (B) perfusate [noradrenaline] in *in situ* head-kidney preparations of rainbow trout pre-perfused for 20 min either with (squares) or without (circles and triangles) the angiotensin converting enzyme inhibitor, captopril (10^{-4} M). The preparations were either injected with saline at time 0 (circles) or left undisturbed (triangles and squares). Values are means \pm 1 S.E.M. ($N = 8-14$). * Significantly different from the 0 min value for a given treatment. Where letters are used to denote statistical differences, treatments which do not share a common letter at a given time are significantly different from each other ($P < 0.05$).

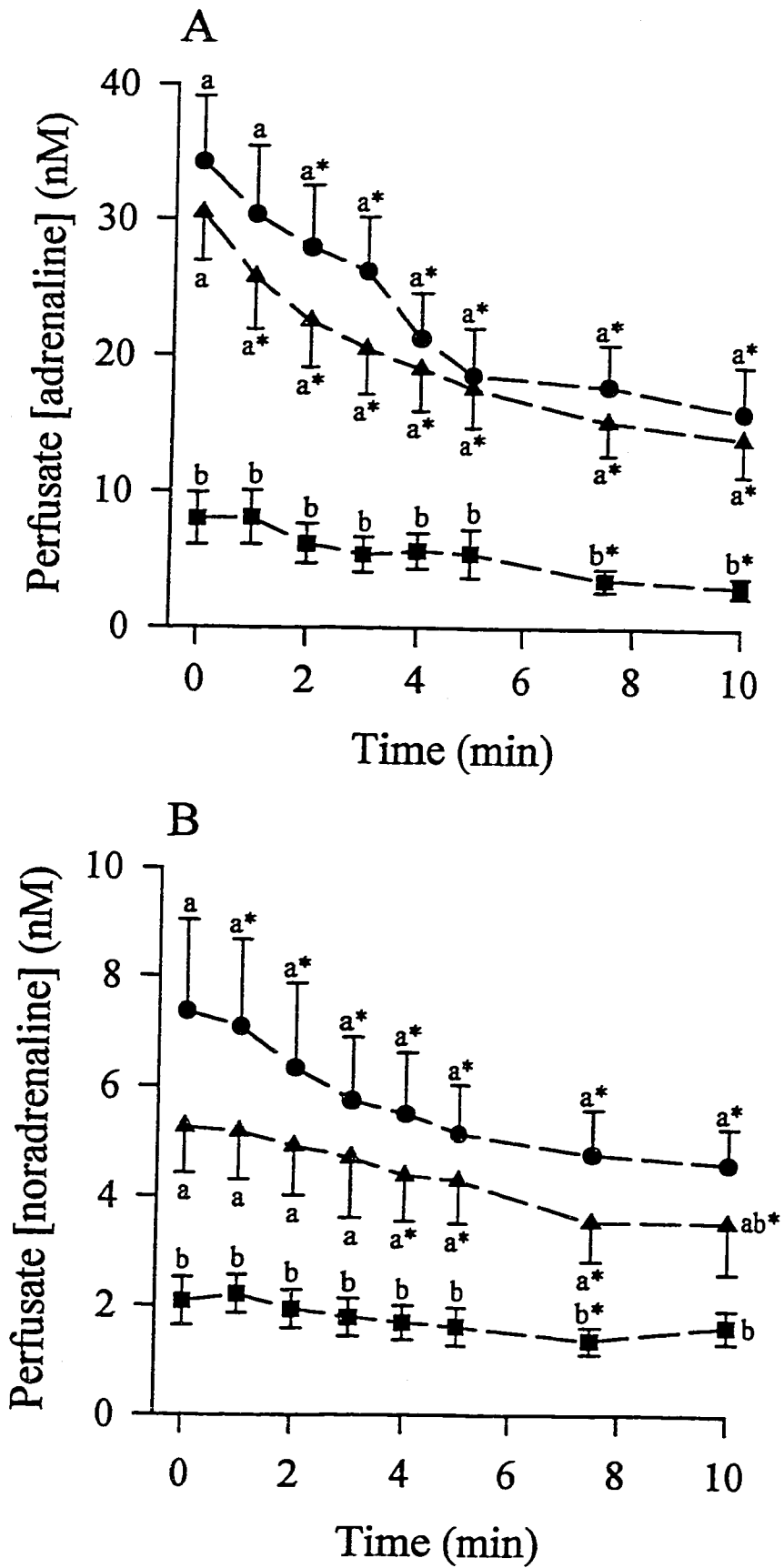


Figure 2.3

Table 2.2 Effects of Losartan, PD123319, hexamethonium, and a calcium free saline on perfusate [adrenaline] and [noradrenaline] elicited by 1 nmol kg⁻¹ of [Asn¹,Val⁵]-Ang II from *in situ* head-kidney preparations of rainbow trout.

| Treatment | N | Perfusate Adrenaline (nM) | | Perfusate Noradrenaline (nM) | |
|---|---|---------------------------|----------------|------------------------------|-------------|
| | | Control | Maximum | Control | Maximum |
| [Asn ¹ ,Val ⁵]-Ang II | 8 | 29.8 ± 9.4 | 277.6 ± 90.4* | 8.2 ± 1.5 | 14.3 ± 2.0* |
| Losartan / [Asn ¹ ,Val ⁵]-Ang II | 8 | 35.1 ± 6.5 | 231.9 ± 50.1* | 8.4 ± 1.6 | 21.6 ± 4.1* |
| PD123319 / [Asn ¹ ,Val ⁵]-Ang II | 8 | 31.9 ± 6.1 | 316.6 ± 48.0* | 7.1 ± 2.2 | 13.0 ± 2.9* |
| Hexamethonium/ [Asn ¹ ,Val ⁵]-Ang II | 9 | 31.0 ± 11.6 | 497.1 ± 176.9* | 5.6 ± 1.5 | 20.5 ± 5.3* |
| Calcium free / [Asn ¹ ,Val ⁵]-Ang II | 8 | 2.8 ± 0.6† | 6.0 ± 1.3*† | 2.6 ± 0.4† | 3.8 ± 0.7† |

Values are means ± S.E.M.; N, no. of fish. Control values are the basal [catecholamine] after 20 min of perfusion with only saline. Maximum values are the peak [catecholamine] during a 10 min period after injection of [Asn¹,Val⁵]-Ang II. In the Losartan, PD123319, and hexamethonium treatments, fish were perfused with the receptor antagonists throughout the trials. * Significantly different from control value for a given treatment. † Significantly different from the [Asn¹,Val⁵]-Ang II treatment for a given column ($P < 0.05$).

DISCUSSION

The presence of specific labelling for Ang II binding sites in piscine chromaffin tissue is reported for the first time. The distribution of chromaffin cells in a discrete layer either adjacent to the PCV or embedded within the connective tissue of the PCV wall, supports the results of several histological studies (Mastrolia *et al.* 1984; Reid *et al.* 1995). Using autoradiography, Ang II binding sites have also been localized in a broad array of rainbow trout tissues (Cobb and Brown 1992). Within the kidney, Ang II has been shown to bind to the afferent and efferent arteries of glomeruli (Cobb and Brown 1994), to isolated glomeruli (Cobb and Brown 1994), and to adrenocortical tissue of the head kidney (Cobb and Brown 1992). These receptors are thought to mediate the ability of Ang II to reduce glomerular filtration rate (Gray and Brown 1985), and to modulate cortisol secretion (Perrott and Balment 1990). In the present study, we have demonstrated that the chromaffin tissue Ang II binding sites can mediate the stimulatory effects of Ang II on catecholamine secretion. This was achieved by establishing the biological activity of the Ang II-FITC probe in the *in situ* preparations of the head kidney. The stimulatory effects of the fluorescein conjugated Ang II on catecholamine release, corroborates earlier reports of the biological activity of this immunohistochemical probe (Landas *et al.* 1980).

Bolus injections of the native rainbow trout Ang II, [Asn¹,Val⁵]-Ang II (Conlon *et al.* 1996b), caused the dose-dependent release of Adr and Nor from the chromaffin tissue of the PCV. The Ang II ED₅₀ values and the maximum catecholamine secretion values obtained from the dose-response curves, showed that Ang II preferentially stimulates the secretion of Adr in trout. In bovine chromaffin cells, although the secretion elicited by Ang II is also dose-dependent (Bunn and Marley 1989), and Ang II binding sites are present in higher density over Adr- than Nor- storing chromaffin cells (Marley *et al.* 1989), Ang II stimulates Adr and Nor with equal

potency (Bunn and Marley 1989). In contrast, several *in vivo* experiments have shown that the effects of Ang II on catecholamine release from the adrenal medulla of mammals are either exclusive to Adr, or more pronounced on Adr than on Nor secretion (Badder *et al.* 1985; Butler *et al.* 1994).

In trout, as in bovine chromaffin cells (Bunn and Marley 1989), Ang II-elicited catecholamine release occurs over a wide range of doses (10^{-13} to 10^{-7} mol kg⁻¹). In this study, the doses of [Asn¹,Val⁵]-Ang II used in the *in situ* preparations correspond to estimated maximum concentrations of 8.2×10^{-5} to 8.2×10^{-11} M (injection volume = 300 μ l; average weight = 246.9 g). Although few studies have reported the plasma concentration of Ang II in teleosts, it appears to vary from 10^{-12} M at rest to 10^{-10} M following either a hypotensive (Tierney *et al.* 1995a) or a hyperosmotic (Takei *et al.* 1988) shock. These observations suggest that the chromaffin cells of the PCV in trout may be exposed to Ang II concentrations that will elicit catecholamine release *in vivo*.

Native and non-native Ang I and Ang II agonists stimulate the release of catecholamines with equal potency in rainbow trout. This conservation of agonist potency across various angiotensins has been observed previously with regards to the vasopressor properties of angiotensins in eels (*Anguilla rostrata*; Nishimura *et al.* 1978), and bowfin (*Amia calva*; Butler *et al.* 1995). Because the amino acid sequence of the angiotensins utilized in these studies show structural differences only at positions one, five, and nine, these amino acids are usually considered to have little influence on the vasopressor activity of Ang I. A similar result was obtained in this study with regards to the catecholamine-releasing activity of angiotensins in rainbow trout.

In comparison to the biological activity of [Asn¹,Val⁵]-AngII on Adr release, removal of the Phe⁸ residue caused a 72% reduction in the activity of [Asn¹,Val⁵]-Ang I (AA's 1-7), and further removal of the Pro⁷ residue caused an 82% reduction in the activity of [Asn¹,Val⁵]-Ang I (AA's 1-6). Meanwhile, relative to the [Asn¹,Val⁵]-AngII treatment, although the amino acid deletions in [Asn¹,Val⁵]-Ang I (AA's 1-7) and [Asn¹,Val⁵]-Ang I (AA's 1-6) resulted in a 15 and 44% decrease in Nor release respectively, these changes were not significant. In each treatment the chromaffin tissue was exposed to a similar dose of 1 nmol kg⁻¹. These differences, although difficult to explain, may be the result of different agonist / receptor binding interactions between angiotensins and Adr-containing chromaffin cells versus angiotensins and Nor-containing chromaffin cells. In mammals, the presence the Phe⁸ residue apparently is a requirement for stimulation of catecholamine release (Peach 1977). The Phe⁸ residue of mammalian Ang II also appears to be important in determining the affinity of angiotensin to receptors and the activation of secondary messenger mechanisms (Bumpus 1977).

In the American eel, *Anguilla rostrata*, intravenous injections of either 0.03 or 0.17 nmol kg⁻¹ of [Asn¹,Val⁵]-Ang I (AA's 1-7) had no measurable effects on cardiovascular function, while injections of similar doses of either eel-Ang I ([Asn¹,Val⁵,Gly⁹]-Ang I) or eel Ang III (AA's 2-8) had pronounced effects (Butler and Oudit 1995). Differences between these results and ours (i.e. absence of activity of [Asn¹,Val⁵]-Ang I (AA's 1-7) in the eel, versus reduced biological activity in trout) are most likely explained by the differences in dosages between the two studies. The dose of [Asn¹,Val⁵]-Ang I (AA's 1-7) used in this study, 1 nmol kg⁻¹, corresponds to an estimated maximum concentration of 8.2×10^{-7} M. *In vivo*, at physiological concentrations (10^{-10} to 10^{-12} M) of angiotensins, [Asn¹,Val⁵]-Ang I (AA's 1-7) probably has no biological activity on the chromaffin tissue of the trout.

Although various angiotensin agonists, including human and tetrapod Ang II, stimulate catecholamine secretion with equal potency in the trout, the highly selective mammalian antagonists Losartan and PD123319 (Timmermans *et al.* 1993) do not block the stimulatory effects of rainbow trout Ang II. Losartan and PD123177 (a structural analog to PD123319; Timmermans *et al.* 1993) have previously been used to determine the specific angiotensin receptor subtypes of the mammalian adrenal medulla. The IC_{50} of these ligands for displacement of Ang II binding in rat and bovine adrenal preparations range from 10^{-8} to 10^{-7} M (Balla *et al.* 1991). Similarly, the IC_{50} for Losartan inhibition of Ang II binding in isolated rainbow trout glomeruli ($\sim 10^{-8}$ M) also is in the same concentration range (Cobb and Brown 1993). Hence, if the Ang II binding sites of the rainbow trout chromaffin cells share the same characteristics as the mammalian Ang II receptor subtypes, the antagonist concentrations used in this study, 10^{-5} M, should have been sufficient to inhibit [Asn¹,Val⁵]-Ang II binding. Our results suggests that the Ang II receptors on the chromaffin cells of rainbow trout are not of the AT₁ or AT₂ subtype. In previous studies, mammalian peptide-substituted angiotensin receptor antagonists have been shown to be ineffective in fish (Cobb and Brown 1993; Conklin and Olson 1994a; see also review by Olson 1992). Meanwhile, Losartan, the non-peptide angiotensin receptor antagonist, is ineffective in some tissues of rainbow trout (Conklin and Olson 1994a; Olson *et al.* 1994), but effective in others (Cobb and Brown 1993; Le Mevel *et al.* 1994). Although the above studies suggests that rainbow trout have more than one Ang II receptor subtype, the functional characteristics of these Ang II binding sites, especially those relating to the inhibitory sites of the receptors (Olson *et al.* 1994), are different than the ones described for the mammalian Ang II receptors (Timmermans *et al.* 1993).

The results obtained with the cholinergic receptor antagonist, hexamethonium, indicate that Ang II-elicited catecholamine release is independent of the cholinergic receptors found on the surface of rainbow trout chromaffin cells (Furimsky *et al.* 1996). These findings corroborate work done on cultured bovine chromaffin cells, where the secretion elicited by Ang II also is independent of cholinergic receptor blockade (Bunn and Marley 1989). The extracellular Ca^{2+} requirement of the Ang II-elicited catecholamine release in trout also is similar to results obtained with cultured bovine chromaffin cells (Bunn and Marley 1989), and confirms the crucial role of Ca^{2+} entry at the plasma membrane for the activation of exocytosis (Furimsky *et al.* 1996).

The stimulatory effects of $[Asn^1, Val^5, Asn^9]$ -Ang I on Nor release were abolished by captopril treatment, whereas, although significant, these effects were incomplete on Adr release. It is possible that the remaining stimulatory effect of $[Asn^1, Val^5, Asn^9]$ -Ang I on Adr release in the presence of captopril resulted from incomplete inhibition of the ACE activity found in the kidney of trout (Lipke and Olson 1988). However, it is worth noting that Ang I, in the presence of ACE inhibitors, can still stimulate catecholamine secretion in isolated, perfused mammalian adrenal glands (Peach 1977). Therefore, although our results suggests that most of the Ang I-elicited catecholamine release is indirect and requires conversion to Ang II, it is possible that a small component of the stimulatory effects of angiotensins on catecholamine release in trout is mediated by Ang I.

Captopril treatment also resulted in lower basal release of both catecholamines from the *in situ* head-kidney preparations. This observation suggests that the RAS may be chronically stimulating catecholamine release in the *in situ* head-kidney preparations. Since there is no renal circulation to the kidney in these preparations, and therefore no substrate (angiotensinogen) for

the RAS, an intra-renal RAS (including locally produced angiotensinogen) may be operative in the trout under the specific conditions of this experiment.

In mammals, the juxtaglomerular cells, respond to a drop in blood pressure or volume in the afferent renal arteriole by secreting renin (Hackenthal *et al.* 1990). Similarly, in teleosts, renin secretion from the juxtaglomerular cells appears to be primarily regulated by a baroreceptor mechanism that detects changes in arterial pressure (Bailey and Randall 1981; Nishimura *et al.* 1979). In both rainbow trout and toadfish (*Opsanus tau*) a reduction in renal perfusion pressure, following either hemorrhage or smooth muscle relaxation, results in an increase in plasma renin activity (Bailey and Randall 1981; Nishimura *et al.* 1979). In the *in situ* head-kidney preparation used in this study, the ventral aorta is ligated during the cannulation procedures and thus renal perfusion pressure in the afferent renal arteriole is probably zero. Therefore, it is possible that the kidney is chronically synthesizing renin under the experimental conditions of the *in situ* head-kidney preparations.

Overall, results from this study and from other recent investigations (Brown *et al.* 1995; Lipke and Olson 1988) suggest the presence of an intra-renal RAS in rainbow trout. In response to a drop in blood pressure in the afferent renal arterioles, renin may be secreted by the juxtaglomerular apparatus (Baily and Randall 1981) and act on angiotensinogen produced locally (Brown *et al.* 1995) to form Ang I. Ang I may then be further converted to Ang II by the angiotensin converting enzyme of the kidney (Lipke and Olson 1988), and stimulate catecholamine secretion. The Ang II and catecholamine thus formed may both cause vasoconstriction in the microcirculation of the kidney (Bushnell *et al.* 1992; Olson *et al.* 1994). This closed-loop monitoring system of blood pressure, with negative feedback control of Ang II

on renin secretion (Bailey and Randall 1981), may be an important component of the anti-drop roles in blood pressure regulation attributed to the RAS in teleosts (Olson *et al.* 1994).

In summary, the present study demonstrates that angiotensins can elicit the release of catecholamines from the chromaffin tissue of rainbow trout via specific Ang II binding sites. The sources of Ang II in the head kidney may be either local or systemic, and the pathways of Ang II synthesis sensitive to perfusion pressure. Therefore, *in vivo*, as part of its role as a tonic anti-drop regulator of arterial blood pressure, the RAS may also interact with the chromaffin tissue to elicit catecholamine release under acute conditions of hypotension. In trout, the supportive role of circulating catecholamines in the neuronal regulation of vascular resistance (Xu and Olson 1993b) and the stimulatory effects of catecholamines on the heart (Randall and Perry 1992), may complement the pressor effects of Ang II on the microcirculation (Olson *et al.* 1994) and its effects as a neuromodulator of cardiovascular control (Le Mevel *et al.* 1994). Although the physiological significance of an intra-renal RAS has yet to be established in trout, the dual control mechanisms offered through paracrine and endocrine pathways may allow more flexibility in cardiovascular homeostasis.

PERSPECTIVES

The afferent limb of the acute adrenergic stress response in fish (i.e. the processes initiating catecholamine release) likely involves multiple mechanisms incorporating a variety of neural and humoral pathways. The multiplicity and apparent redundancy of catecholamine secretagogues probably reflects the importance of adrenergic control mechanisms in fish and the diverse array of stress situations that elicit catecholamine secretion. Thus, catecholamine secretion may be controlled by a number of independent pathways depending upon the nature of

the acute stressor. The renin-angiotensin system has a key role in blood pressure regulation in fish and thus it seems likely that angiotensin II elicits catecholamine release during conditions of acute hypotension. Thus, in addition to a direct effect on the cardiovascular system, angiotensin II will promote an elevation of blood pressure indirectly owing to catecholamine secretion and the resultant adrenergic effects on the systemic vasculature and heart. Future research should address the relative contributions of angiotensin II and catecholamines in restoring blood pressure during hypotension.

CHAPTER 3.**Cardiovascular effects of angiotensin II-mediated adrenaline release in rainbow trout**

Abstract

To determine the contribution of plasma catecholamines to the cardiovascular effects of elevated levels of angiotensin II (Ang II) in trout, this study investigated 1) the stimulatory effects of [Asn¹-Val⁵]-Ang II on plasma catecholamine levels, 2) the cardiovascular effects of Ang II with and without α -adrenoceptor blockade, and 3) the relationship between plasma adrenaline (Adr) concentrations and their cardiovascular effects. Bolus intravascular injections of Ang II (25-1200 pmol kg⁻¹) elicited dose-dependent (75-1200 pmol kg⁻¹) increases in plasma Adr levels without affecting plasma noradrenaline levels. Ang II-elicited increases in plasma Adr ranged from 3.3±0.3 to 125.1±40.0 nM for the 75 to 1200 pmol kg⁻¹ doses, respectively. Ang II injections (25-1200 pmol kg⁻¹) also elicited dose-dependent increases in dorsal aortic pressure (P_{DA}), systemic resistance (R_S), cardiac output (Q), and stroke volume (V_S). In fish first treated with the α -adrenoceptor blocker phenoxybenzamine, Ang II injections elicited a decrease in Q and V_S , and the increases in P_{DA} and R_S following the 600 and 1200 pmol kg⁻¹ Ang II doses were reduced significantly. Bolus injections of Adr (1.8×10^{-10} to 1.4×10^{-8} mol kg⁻¹) elicited dose-dependent increases in P_{DA} and R_S starting at plasma Adr concentrations of 16.5 and 50.5 nM, respectively. Adr injections also elicited increases in Q and V_S starting at plasma Adr concentrations of 50.5 nM, however, higher plasma Adr concentrations were not associated with further increases in either Q or V_S . These results demonstrate that *in vivo*, Ang II can act as a potent non-cholinergic secretagogue of humoral adrenaline in trout, and that a portion of the cardiovascular effects of exogenous Ang II can be attributed to increase levels of plasma Adr. The present data also indicate that the cardiovascular effects of Ang II-mediated humoral catecholamines are recruited in a dose-dependent manner, and as such may require an acute stimulation of the renin-angiotensin system in order to significantly contribute to the pressor activity of endogenous angiotensins.

Introduction

Regulation of the cardiovascular system by the sympathetic nervous system (SNS) in mammals is modulated at various levels by the renin-angiotensin system (RAS; for reviews see Peach 1977; Saxena 1992; Reid 1992; Head 1996). Angiotensin II (Ang II), the active product of the RAS, is involved centrally in the autonomic control of the cardiovascular system, and peripherally in enhancing sympathetic neurotransmission and eliciting secretion from the adrenal medulla. Interactions between these two mediators of cardiovascular homeostasis have been reported in all vertebrate classes (Carroll and Opdyke 1982; Wilson 1984a; Nishimura 1985b) and attest to the evolutionary significance of the relationship between the RAS and the SNS.

In teleosts, peripheral injection of Ang II elicits a pressor response that is partially inhibited by α -adrenoceptor blockade (*Amia calva*, Butler *et al.* 1995; *Anguilla rostrata*, Nishimura *et al.* 1978; Nishimura 1985a; Oudit and Butler 1995a; *Cyclopterus lumpus*, Carroll and Opdyke 1982; *Gadus morhua*, Platzack *et al.* 1993; *Oncorhynchus mykiss*, Lipke *et al.* 1990; Olson *et al.* 1994). While these studies implicated the SNS in the cardiovascular effects of Ang II in fish, they could not reveal whether the interaction between the RAS and the SNS occurs at the level of the adrenergic nerve terminals, the chromaffin tissue, or both (Nishimura *et al.* 1978).

In previous experiments, we have provided immunohistochemical evidence for the presence of Ang II binding sites on the catecholamine-containing chromaffin cells of *O. mykiss* (Bernier and Perry, 1997). Furthermore, bolus injections of Ang II in an *in situ* perfused posterior cardinal vein preparation of *O. mykiss*, elicits a dose-dependent release of catecholamines (Bernier and Perry, 1997). The only *in vivo* evidence that Ang II can evoke catecholamine secretion in fish stems from a single study in which intravascular injections of high doses of heterologous Ang II

(~1940 pmol kg⁻¹) increased plasma catecholamines in *Cyclopterus lumpus* (Carroll and Opdyke 1982). However, the maximum plasma catecholamine concentrations recorded in *C. lumpus* following Ang II injection (adrenaline ~3.2 nM; noradrenaline ~0.7 nM; Carroll and Opdyke 1982) are similar to the basal resting catecholamine levels reported in most teleost species (Randall and Perry, 1992). Therefore, while it is generally accepted that a portion of the cardiovascular effects of Ang II in teleosts are mediated through an interaction with the SNS, it remains to be ascertained whether humoral catecholamines represent a significant portion of this interaction.

Although it is known that angiotensins can elicit catecholamine release from the chromaffin tissue of teleosts, it is unclear whether catecholamines of humoral origin can contribute to cardiovascular regulation. There is convincing evidence that in resting rainbow trout (*O. mykiss*) the adrenergic control of vascular resistance has a neuronal origin (Wood and Shelton 1975; Smith 1978; Xu and Olson 1993b). However, there is considerable debate as to whether physiological concentrations of circulating catecholamines can influence systemic resistance (R_s). Although results obtained from perfused tissues and isolated vessels (Wood and Shelton 1975; Xu and Olson 1993b) indicate that plasma catecholamines are of minor importance in the regulation of R_s , intravascular injections of adrenaline doses (≤ 3 nmol kg⁻¹), which presumably yield physiological plasma concentrations, have resulted in significant increases in R_s (Wood and Shelton 1980; Gamperl *et al.* 1994a, 1994b). Previous studies have identified the problems associated with estimating plasma catecholamine levels after such injected doses (Gamperl *et al.* 1994c). Thus, in studies employing catecholamine injections, there is a clear need to correlate the realized (i.e. measured) plasma concentrations with the resultant physiological

effects to determine the true contribution of humoral catecholamines. This approach, however, has not been used in any previous investigation.

Therefore, while there is some evidence to suggest that a portion of the cardiovascular effects of Ang II in teleosts may be mediated through plasma catecholamines, this hypothesis has yet to be tested rigorously. Towards this goal, the present study 1) assessed whether homologous rainbow trout angiotensin II can elicit a dose-dependent increase in plasma catecholamines, 2) determined the cardiovascular effects of Ang II with and without α -adrenoceptor blockade, and 3) established relationships between intravascular doses of adrenaline, realised plasma levels, and cardiovascular responses. These experiments were performed to assess whether Ang II-elicited increases in humoral catecholamines can contribute to cardiovascular control.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex were obtained from Linwood Acres Trout Farms (Campbellcroft, Ontario) and transported to the fish-holding facilities of the University of Ottawa. The fish were held indoors in large fiberglass tanks supplied with flowing, aerated, and dechloraminated city of Ottawa tap water. The fish were maintained at a temperature of 14°C, on a 12:12-h light-dark photoperiod cycle, and fed daily with a commercial salmonid diet. The trout of series 1 (see below) had a mean mass of 325.4±9.6 g (experimental $N=56$), and the trout of series 2 and 3 together had a mean mass of 743.0±39.7 g (experimental $N=26$).

Surgical procedures

Series 1: the effects of Ang II on plasma catecholamines

Rainbow trout were anaesthetised in an oxygenated and buffered (NaHCO_3 ; 0.16 g l^{-1}) solution of ethyl-*m*-aminobenzoate (0.08 g l^{-1} ; MS-222; Syndel, Vancouver, B.C.) until cessation of breathing movements. The fish were then transferred to an operating table where the gills were force ventilated with the same anaesthetic solution. To permit injections and repeated blood sampling, the dorsal aorta was cannulated with polyethylene tubing (PE 50; Clay Adams) using the technique of Soivio *et al.* (1975). After surgery, fish were placed into individual flow-through opaque perspex boxes and left to recover for 48 h prior to experimentation.

Series 2 and 3: cardiovascular responses to Ang II and to adrenaline

Rainbow trout were anaesthetized as in series 1 prior to surgery. In order to measure dorsal aortic blood pressure (P_{DA}), or to carry out repeated blood sampling, fish were equipped with a dorsal aortic cannula (PE 50; Soivio *et al.* 1975). To permit drug injections, a lateral incision was made in the caudal peduncle to expose the caudal vein, dissect it free from overlying tissue, and to cannulate (PE 50) this vessel in the direction towards the heart. In addition, the pericardial cavity was exposed with a midline ventral incision and the pericardium was dissected to expose the bulbus arteriosus. To allow measurement of cardiac output (Q), a 3S or 4S ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY) was placed non-occlusively around the bulbus. Lubricating jelly was used with the perivascular flowprobe as an acoustic couplant. Silk sutures were used to close the ventral and caudal peduncle incisions, and to anchor the cardiac output probe lead and the caudal vein cannula to the skin. After surgery, fish were placed into individual flow-through perspex boxes and left to recover for 24 h prior to experimentation.

All cannulae were filled and flushed with heparinized (50 IU ml⁻¹ sodium heparin; Sigma, St Louis, MI) teleost Cortland saline (Wolf, 1963).

Experimental Protocol

Series 1: the effects of Ang II on plasma catecholamines

Seven different experimental groups of eight fish each ($N = 56$) were used to investigate the effects of homologous [Asn¹-Val⁵]-Ang II (0, 25, 75, 150, 300, 600, 1200 pmol kg⁻¹; Sigma) on the circulating plasma catecholamine concentrations of rainbow trout. Within a given trial, after removal of a blood sample (0.3 ml) to assess basal plasma catecholamines, fish were given a bolus injection (0.3 ml) of [Asn¹-Val⁵]-Ang II over a period of 30 sec, and the injection was followed by 0.2 ml of saline to clear the cannula. Five more blood samples (0.3 ml) were then taken 1, 2, 3, 5, and 10 min after the beginning of the injection. Each blood sample was replaced by an equivalent volume of saline, collected in a 1.5 ml microcentrifuge tube, spun down immediately at 10,000 g for 15 sec, and the plasma was frozen in liquid nitrogen and stored at -80°C for later analysis of catecholamines.

Series 2: the cardiovascular effects of Ang II with and without α -adrenoceptor blockade

After monitoring stable P_{DA} and Q traces for one hour, control parameters were recorded for 5 min and the fish were given a bolus injection (0.3 ml) of [Asn¹-Val⁵]-Ang II through the caudal vein cannula over a period of 30 sec. Cardiovascular responses to the injection were monitored continuously, and following recovery of cardiovascular variables to control levels for a 1 hour period, a second dose of [Asn¹-Val⁵]-Ang II was injected. Repeating this protocol, seven doses of [Asn¹-Val⁵]-Ang II (0, 25, 75, 150, 300, 600, 1200 pmol kg⁻¹) were randomly and sequentially tested on each fish ($N = 10$). Each injection was followed by 0.2 ml of saline to clear the cannula.

In a second group ($N = 8$), the same experimental protocol and doses of [Asn¹-Val⁵]-Ang II as above were tested on fish first treated with the α -adrenoceptor blocker, phenoxybenzamine hydrochloride (RBI, Natick, MA). Alpha-adrenergic blockade was achieved by slowly (over a 15 min period) giving two doses of 3 mg kg⁻¹ phenoxybenzamine 12 and 6 h prior to experimentation (Xu and Olson 1993a). Phenoxybenzamine was dissolved in 100 μ l ethanol and diluted in saline prior to injection (3 mg ml⁻¹). Before and after the seven Ang II doses, the effectiveness of the α -adrenergic blockade was tested by injection of a catecholamine cocktail (0.375 ml kg⁻¹) prepared in a 0.9% NaCl solution and consisting of 3.5×10^{-6} mol l⁻¹ noradrenaline bitartrate (Arterenol, Sigma) and 1.1×10^{-5} mol l⁻¹ adrenaline bitartrate (Sigma).

Series 3: cardiovascular responses to adrenaline

Once stable baseline cardiovascular parameters were established, fish were given a bolus injection (0.3 ml) of either saline or adrenaline bitartrate via the caudal vein cannula and the responses monitored. The doses of adrenaline ranged from 1.8×10^{-10} to 1.4×10^{-8} mol kg⁻¹. Spaced by inter-injection periods which allowed cardiovascular parameters to recover to control levels for 60 min, four injections of adrenaline were tested on each fish ($N = 8$). The range of adrenaline doses tested in this series was selected to achieve a continuum of arterial plasma adrenaline concentrations between 10 and 1000 nM. This was achieved by varying the lowest dose tested in each fish (1.8×10^{-10} to 1.1×10^{-9} mol kg⁻¹) and multiplying this dose by 2, 5, and 12.5 to prepare the 3 other doses. All injections were randomly tested, given through the caudal vein cannula over a period of 30 sec, and followed by 0.2 ml of saline to clear the cannula.

After 24 h of recovery, in order to determine the maximum arterial plasma adrenaline concentration achieved with each injection, each fish once again received an injection of saline and the same four doses of adrenaline, in the same sequence and with the same time interval between

each injection. Within a given trial, a control blood sample (0.3 ml) was taken to assess basal plasma catecholamines, the fish were then given a bolus injection of adrenaline or saline over a period of 30 sec, and four more blood samples (0.3 ml) were taken 1, 2, 3, and 4 min after the beginning of the injection. While the adrenaline injections were made via the caudal vein cannula, all the blood samples were collected via the dorsal aorta cannula. Each blood sample was replaced by an equivalent volume of saline, collected in a 1.5 ml microcentrifuge tube, spun down immediately at 10,000 g for 15 sec, and the plasma was frozen in liquid nitrogen and stored at -80°C for later analysis of catecholamines.

Analytical procedures

Plasma catecholamines (adrenaline and noradrenaline) were determined on alumina-extracted plasma samples (0.2 ml) using high-pressure liquid chromatography (HPLC) with electrochemical detection (Bernier and Perry 1997). P_{DA} was measured with a UFI model 1050BP (UFI, Morro Bay, CA) pressure transducer which was calibrated against a static water column. Mean blood pressure was calculated as: (systolic pressure + diastolic pressure) / 2. The perivascular flow probes used to measure Q were connected to a Transonic T106 small animal blood flow meter. These probes were pre-calibrated in the factory and verified in the laboratory by pump perfusion of the heart with saline at known flow rates in an immersed euthanized fish. Both P_{DA} and Q signals were recorded with a data acquisition system (Biopac System Inc., Goleta, CA) and collected at 0.04 sec intervals using Acknowledge IIITM (Biopac System Inc.) data acquisition software. Systemic resistance (R_{S}) was calculated as mean P_{DA} divided by Q (i.e. $R_{\text{S}} = P_{\text{DA}} / Q$), heart rate (f_{H}) was derived from the dorsal aortic pressure pulse trace, and stroke volume (V_{S}) was calculated as Q divided by f_{H} (i.e. $V_{\text{S}} = Q / f_{\text{H}}$).

Statistical analyses

Data are presented as mean values \pm one standard error of the mean (S.E.M). The statistical significance of the observed effects of a given Ang II injection within a group were tested using one-way repeated-measures analysis of variance (ANOVA). Dunnett's *post-hoc* multiple-comparison test was used to compare the pre-injection control data point with values at subsequent and previous times. Control and maximum plasma catecholamine levels following a given Ang II injection were analysed by paired *t*-test. Differences between the changes in a cardiovascular variable after the different Ang II injections within a given treatment were determined using one-way repeated-measures ANOVA followed by Student-Newmen-Keuls test for multiple-comparison. The statistical significance of observed differences between the means of two treatments was determined by *t*-test. In order to assess the effects of the adrenaline injections on the cardiovascular, post-injection increases for a given cardiovascular variable were separated into four groups based on the maximum arterial plasma adrenaline concentration achieved by each adrenaline injection (0-30 nM, 31-100 nM, 101-250 nM, 251-1000 nM adrenaline). Differences between the mean increases in a cardiovascular variable after the adrenaline injections were determined using one-way ANOVA followed by Student-Newmen-Keuls test for multiple-comparison. The significance level for all statistical tests was $P < 0.05$.

Results

Series 1: the effects of Ang II on plasma catecholamines

Bolus injections of Ang II between 75 and 1200 pmol kg⁻¹ elicited a dose-dependent increase in plasma adrenaline (Figs. 3.1A-F, 3.2A). The time taken to attain the peak plasma

Figure 3.1 Plasma adrenaline (A-F) and noradrenaline (G-L) concentrations of rainbow trout given a bolus injection of homologous [Asn¹-Val⁵]-Ang II with doses ranging between 25 and 1200 pmol kg⁻¹ (*N*=8 for each dose). The open bars indicate the catecholamine concentrations prior to the Ang II injection, and the closed bars indicate the catecholamine concentrations in response to the injection. An asterisk denotes a significant difference from the pre-treatment value for a given dose (*P*<0.05). Values are means + 1 S.E.M.

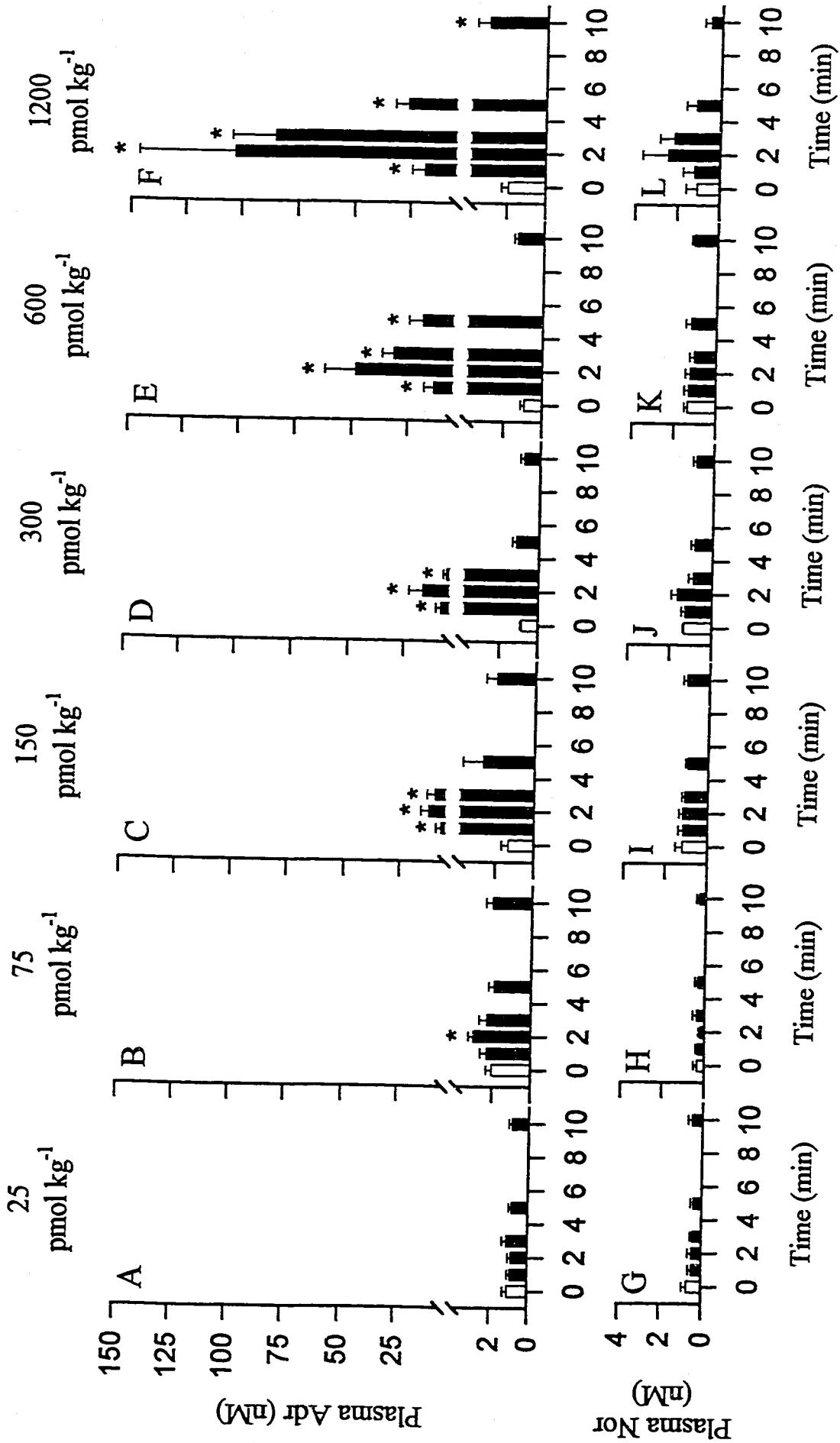


Figure 3.1

Figure 3.2 Effects of bolus injections of homologous [Asn¹-Val⁵]-Ang II on plasma adrenaline (A) and noradrenaline (B) concentrations in rainbow trout ($N=8$ for each Ang II dose). The open bars indicate the catecholamine concentrations prior to the Ang II injection, and the closed bars indicate the maximum catecholamine concentrations in response to the injection. An asterisk denotes a significant difference between the maximum and the control concentration for a given dose ($P<0.05$). Values are means + 1 S.E.M.

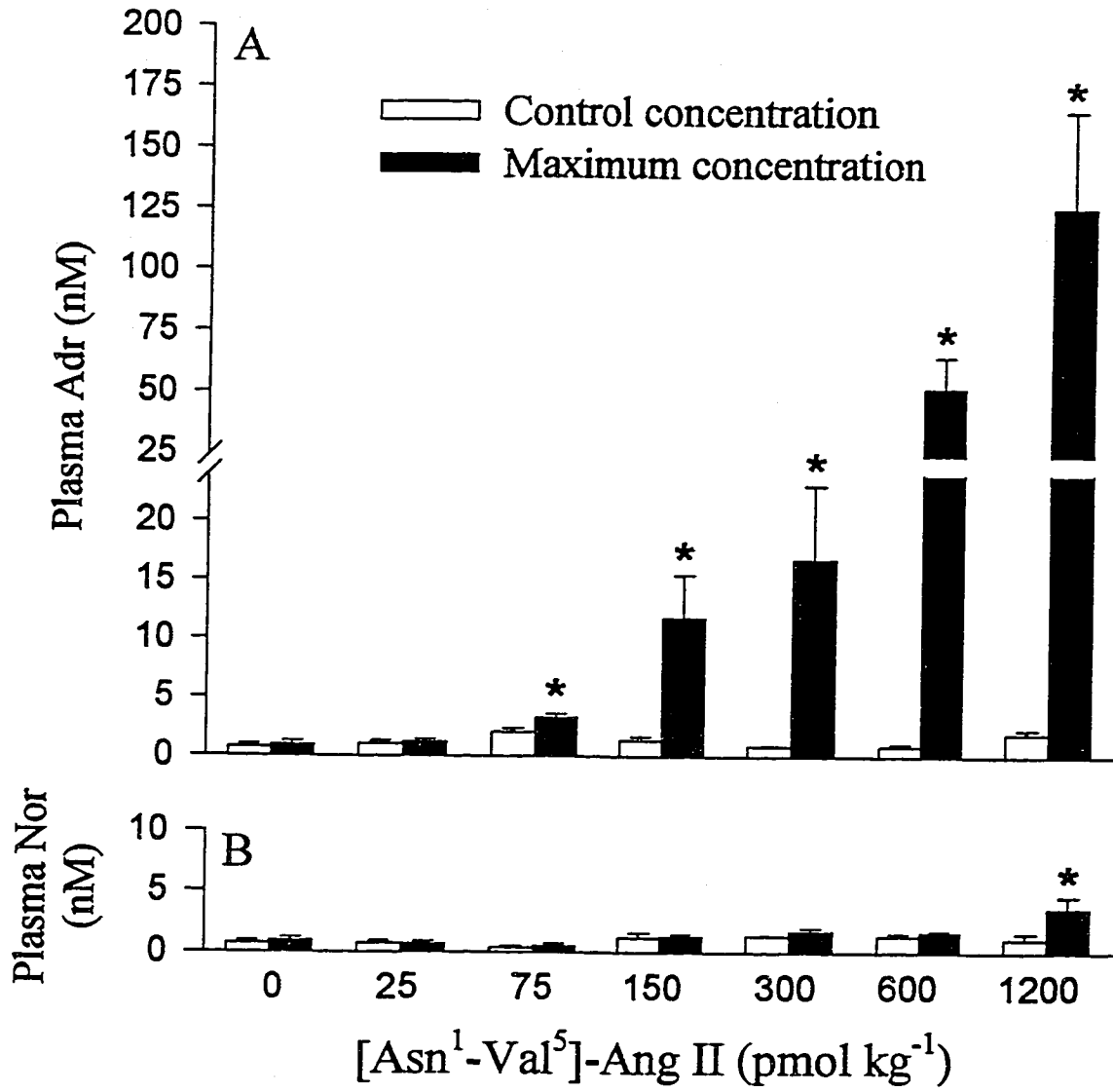


Figure 3.2

adrenaline concentration was similar for all Ang II doses and occurred 2 min post-injection (Fig. 3.1A-F). In contrast, none of the Ang II doses tested in this study had a significant effect on the mean plasma noradrenaline concentration (Fig. 3.1G-L). Only the 1200 pmol kg⁻¹ Ang II dose elicited a small increase in the mean of the maximum plasma noradrenaline concentration achieved by each fish (Fig. 3.2B). Bolus injection of saline had no effect on basal plasma adrenaline and noradrenaline (Fig. 3.2A, B).

Series 2: the cardiovascular effects of Ang II with and without α -adrenoceptor blockade

In comparison to the resting cardiovascular variables of the control group, fish treated with the α -adrenergic antagonist phenoxybenzamine had significantly lower resting mean P_{DA} and R_S , higher resting Q (Table 3.1) and V_S (Table 3.2), and similar f_H (Table 3.3). Relative to the control group, phenoxybenzamine treatment reduced the pressure response to a bolus injection of catecholamines by 67%, abolished the increase in R_S , and reduced the increase in Q by 42% (Table 3.1).

Bolus injections of homologous rainbow trout Ang II resulted in a dose-dependent increase in P_{DA} (25-1200 pmol kg⁻¹; Fig. 3.3A-F and Fig. 3.5A) and R_S (75-1200 pmol kg⁻¹; Fig. 3.4A-F and Fig. 3.5B) in both the control and phenoxybenzamine-treated fish. For any given Ang II dose, the duration of the pressor response in the control treatment was longer than the duration of the increase in R_S (Figs. 3.3, 3.4). In contrast, the duration of the Ang II-mediated pressor response in the phenoxybenzamine treated fish was of equal duration to the increase in R_S (Figs. 3.3, 3.4). In comparison to the control group, the increase in mean P_{DA} and R_S were significantly reduced by 33% and 43%, respectively, following the 600 pmol kg⁻¹ Ang II dose, and by 33% and 53%, respectively, following the 1200 pmol kg⁻¹ Ang II dose in the phenoxybenzamine-treated

Table 3.1 The effects of a bolus injection of catecholamines¹ on the cardiovascular variables² of intact control and phenoxybenzamine-treated rainbow trout

| Treatment | <i>N</i> | <i>P</i> _{DA} (cm H ₂ O) | <i>R</i> _S (cm H ₂ O ml ⁻¹ min ⁻¹ kg ⁻¹) | <i>Q</i> (ml min ⁻¹ kg ⁻¹) |
|------------------|----------|---|---|--|
| Control | 10 | | | |
| Resting values | | 30.4±2.0 | 1.3±0.2 | 25.3±2.5 |
| Post-injection | | 41.1±4.4* | 1.7±0.3* | 29.5±2.1* |
| Phenoxybenzamine | 8 | | | |
| Resting values | | 22.5±2.6† | 0.6±0.1† | 44.6±3.8† |
| Post-injection | | 26.0±3.3* | 0.6±0.1 | 47.1±3.8* |

1 – The catecholamine injection consisted of a 0.375 ml kg⁻¹ dose of 3.5×10⁻⁶ mol l⁻¹ noradrenaline bitartrate and 1.1×10⁻⁵ mol l⁻¹ adrenaline bitartrate.

Values are mean ± 1 S.E.M.

* Significantly different from resting value for a given treatment. † Significantly different from resting value of the control treatment (*P*<0.05).

2 – *P*_{DA}, mean dorsal aortic pressure; *R*_S, systemic resistance; *Q*, cardiac output.

Table 3.2 The effects of a bolus injection of homologous [Asn¹-Val⁵]-Ang II on the stroke volume of intact control and phenoxybenzamine-treated rainbow trout

| Ang II dose (pmol kg ⁻¹) | Treatment | Stroke Volume (ml kg ⁻¹ beat ⁻¹) | | | |
|---|-----------|---|------------------------|------------------------|------------------------|
| | | Pre-injection | | Post-injection | |
| | | 0 min | 2.5 min | 5 min | 10 min |
| 0 | Control | 0.36±0.05 | 0.37±0.04 | 0.37±0.04 | 0.36±0.04 |
| | Phen. | 0.60±0.06 [†] | 0.59±0.06 [†] | 0.61±0.07 [†] | 0.61±0.07 [†] |
| 25 | Control | 0.39±0.04 | 0.42±0.05* | 0.41±0.05* | 0.40±0.04 |
| | Phen. | 0.53±0.04 [†] | 0.56±0.04 [†] | 0.56±0.04 [†] | 0.54±0.04 [†] |
| 75 | Control | 0.37±0.04 | 0.42±0.05* | 0.42±0.05* | 0.39±0.05 |
| | Phen. | 0.54±0.05 [†] | 0.51±0.05 | 0.53±0.05 | 0.54±0.05 [†] |
| 150 | Control | 0.38±0.05 | 0.43±0.06* | 0.45±0.06* | 0.41±0.05* |
| | Phen. | 0.56±0.05 [†] | 0.52±0.06 | 0.54±0.05 | 0.55±0.05 |
| 300 | Control | 0.35±0.04 | 0.37±0.04 | 0.43±0.05* | 0.39±0.05* |
| | Phen. | 0.60±0.06 [†] | 0.54±0.07 [†] | 0.54±0.06 | 0.59±0.06 [†] |
| 600 | Control | 0.37±0.05 | 0.38±0.04 | 0.43±0.05* | 0.41±0.05* |
| | Phen. | 0.59±0.05 [†] | 0.53±0.06 | 0.54±0.06 | 0.56±0.06 |
| 1200 | Control | 0.37±0.05 | 0.36±0.05 | 0.42±0.05* | 0.42±0.05* |
| | Phen. | 0.55±0.05 [†] | 0.49±0.06 | 0.48±0.05 | 0.52±0.05 |

Values are mean ± 1 S.E.M. Sample size for control and phenoxybenzamine (Phen.) treatments are 10 and 8, respectively. † Significantly different from control treatment at given time. * Significantly different from pre-injection value for a given treatment ($P < 0.05$).

Table 3.3 The effects of a bolus injection of homologous [Asn¹-Val⁵]-Ang II on the heart rate of intact control and phenoxybenzamine-treated rainbow trout

| Ang II dose (pmol kg ⁻¹) | Treatment | Heart Rate (beats min ⁻¹) | | | |
|---|-----------|---------------------------------------|----------|----------------|----------|
| | | Pre-injection | | Post-injection | |
| | | 0 min | 2.5 min | 5 min | 10 min |
| 0 | Control | 72.0±2.2 | 73.4±2.4 | 71.4±2.5 | 73.7±2.6 |
| | Phen. | 72.2±3.9 | 74.1±3.1 | 72.5±3.7 | 73.4±3.9 |
| 25 | Control | 73.1±3.0 | 72.3±2.6 | 72.6±2.6 | 71.8±2.4 |
| | Phen. | 77.9±2.9 | 76.2±3.3 | 74.2±2.6 | 77.7±3.8 |
| 75 | Control | 74.5±2.7 | 72.8±2.5 | 73.9±2.5 | 74.6±3.0 |
| | Phen. | 75.3±2.3 | 77.6±3.3 | 76.0±3.2 | 75.0±2.6 |
| 150 | Control | 73.7±2.8 | 71.6±2.5 | 71.0±2.1 | 71.9±2.2 |
| | Phen. | 76.1±3.4 | 75.9±3.9 | 74.9±2.8 | 76.3±2.7 |
| 300 | Control | 75.1±3.1 | 77.6±1.8 | 74.0±2.9 | 75.8±4.3 |
| | Phen. | 71.2±2.3 | 73.8±3.2 | 74.9±2.4 | 72.3±3.0 |
| 600 | Control | 72.3±2.3 | 72.8±1.8 | 72.3±2.0 | 72.6±2.9 |
| | Phen. | 73.9±3.0 | 74.2±3.1 | 74.3±3.4 | 76.1±3.1 |
| 1200 | Control | 73.7±2.7 | 72.7±3.2 | 71.6±3.4 | 72.7±3.8 |
| | Phen. | 73.8±2.8 | 74.5±3.7 | 74.6±1.7 | 76.2±2.9 |

Values are mean ± 1 S.E.M. Sample size for control and phenoxybenzamine (Phen.) treatments are 10 and 8, respectively. † Significantly different from control treatment at given time. * Significantly different from pre-injection value for a given treatment ($P < 0.05$).

Figure 3.3 Mean dorsal aortic pressure (P_{DA}) in intact control ($N=10$; solid circles) and phenoxybenzamine-treated ($N=8$; open squares) rainbow trout given a bolus injection of homologous [Asn¹-Val⁵]-Ang II with doses ranging between 25 and 1200 pmol kg⁻¹. The dashed line in each graph indicates the time of Ang II injection. The solid lines above and below the control and phenoxybenzamine-treated groups, respectively, indicate the time interval during which the mean P_{DA} values were significantly different from the resting value immediately preceding the dashed line ($P<0.05$). Values are means \pm 1 S.E.M.

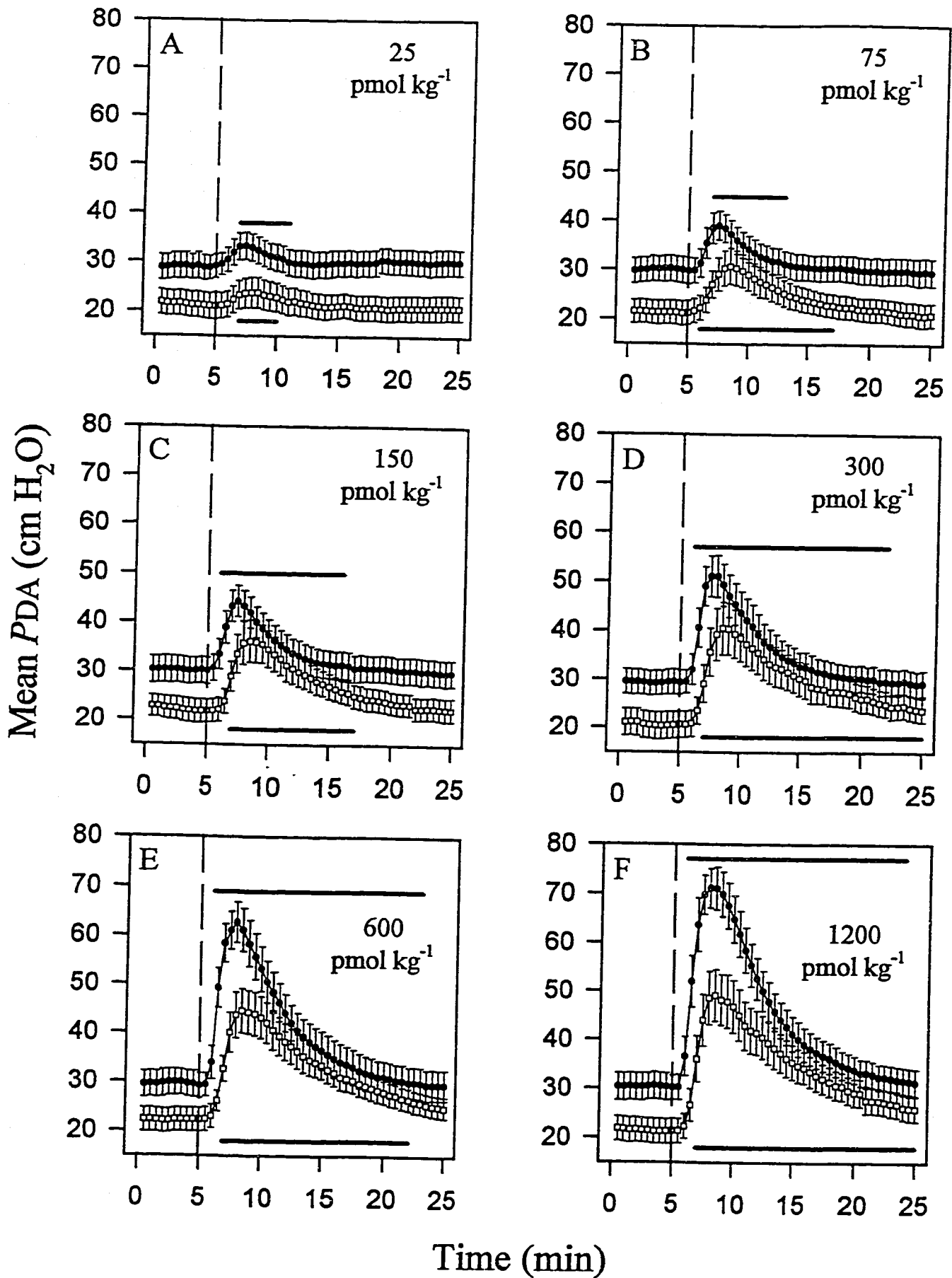


Figure 3.3

Figure 3.4 Systemic resistance (R_s) in intact control ($N=10$; solid circles) and phenoxybenzamine-treated ($N=8$; open squares) rainbow trout given a bolus injection of homologous [Asn¹-Val⁵]-Ang II with doses ranging between 25 and 1200 pmol kg⁻¹. The dashed line in each graph indicates the time of Ang II injection. The solid lines above and below the control and phenoxybenzamine-treated groups, respectively, indicate the time interval during which the R_s values are significantly different from the resting value immediately preceding the dashed line ($P<0.05$). Values are means \pm 1 S.E.M.

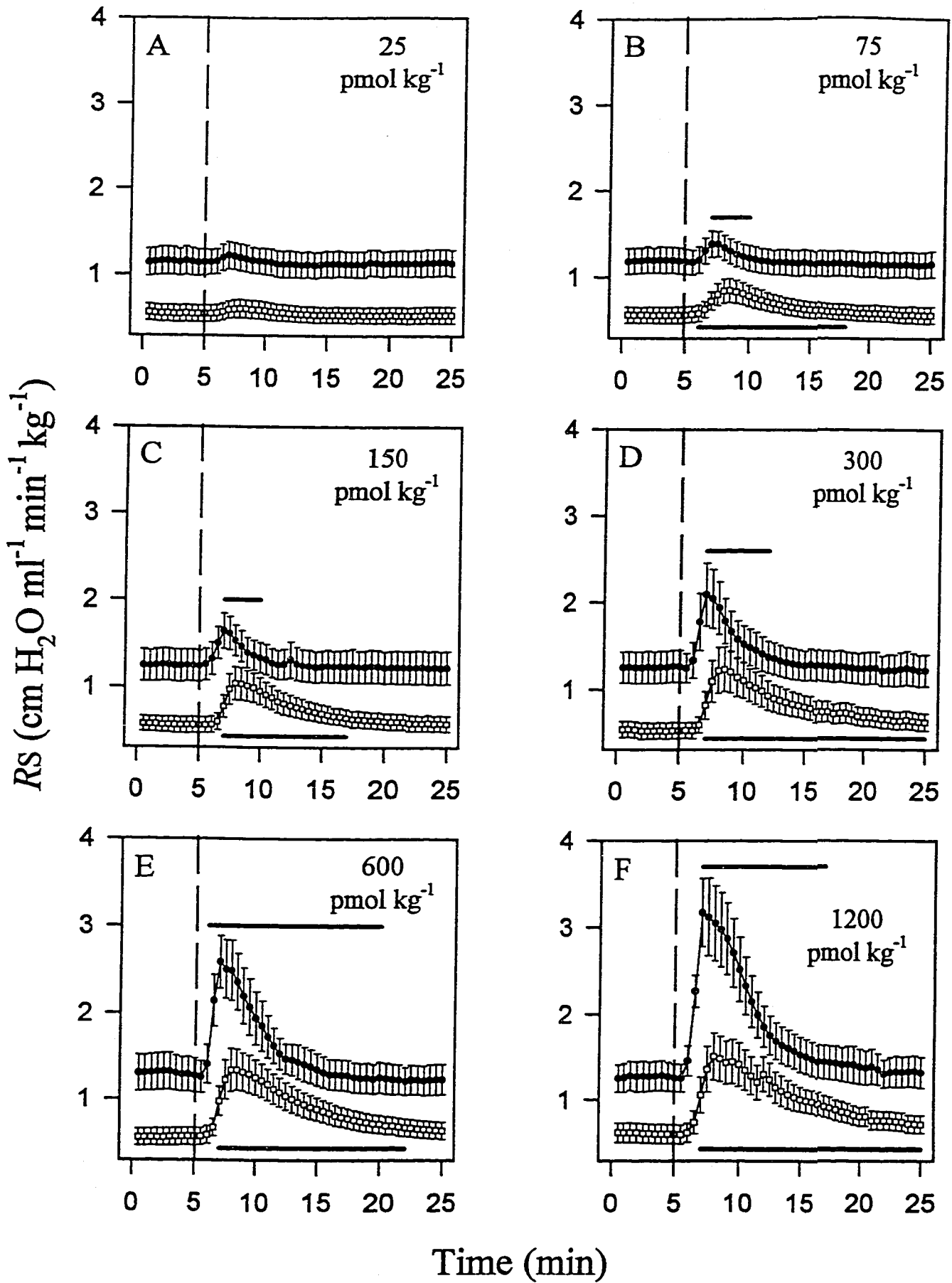


Figure 3.4

Figure 3.5 Absolute change in mean dorsal aortic pressure (P_{DA} ; A) and systemic resistance (R_S ; B) in intact control ($N=10$; solid circle) and phenoxybenzamine-treated ($N=8$; open squares) rainbow trout after intravenous injections of graded homologous [Asn^1 - Val^5]-Ang II doses. An asterisk denotes a significant difference between the control and phenoxybenzamine-treated fish at a given dose ($P<0.05$). Dissimilar letters indicate significant differences between doses within a group ($P<0.05$). Values are means \pm 1 S.E.M.

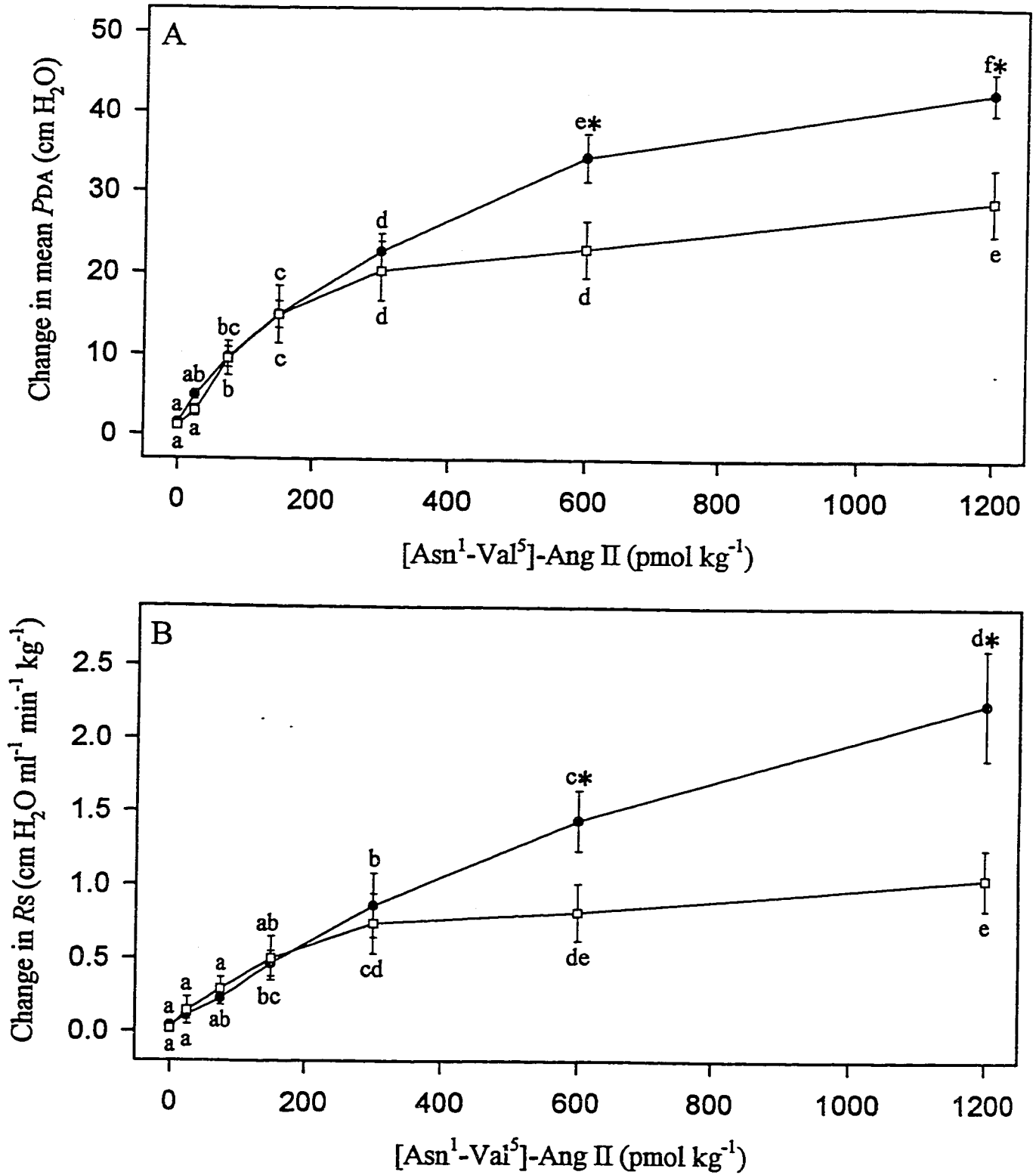


Figure 3.5

fish (Fig. 3.5A-B). Saline injection alone had no significant effect on mean P_{DA} and R_S in the control and phenoxybenzamine-treated fish.

The administration of graded Ang II doses elicited an increase in Q in the control fish at all doses (25-1200 pmol kg⁻¹; Fig. 3.6A-F), and the maximum increase in Q was attained with the 600 pmol kg⁻¹ dose (Fig. 3.7A). In the phenoxybenzamine-treated fish, Ang II injections elicited a significant decrease in Q at the two highest doses (600 and 1200 pmol kg⁻¹; Fig. 3.6A-F), and the reduction in Q was dose-dependent between the 25 and 1200 pmol kg⁻¹ doses (Fig. 3.7A). While the Ang II injections had no significant effect on the f_H of the control and phenoxybenzamine-treated fish, they elicited an increase in V_S in the control fish and a decrease in V_S in the phenoxybenzamine-treated fish (Table 3.2 and Fig. 3.7B). Injection of saline alone elicited a significant increase in Q of 1.00 and 1.14 ml min⁻¹ kg⁻¹ in the control and phenoxybenzamine-treated fish, respectively, and had no statistical effect on f_H or V_S in either group (Tables 3.2, 3.3).

The temporal features of the Ang II-elicited changes in P_{DA} , R_S and Q are shown in figure 3.8. In the control treatment, the time taken to achieve peak pressor response (2.5-3.0 min) and peak R_S (1.9-2.4 min) remained constant for the different Ang II doses, and the time taken to achieve peak Q (3.4-7.1 min) increased dose-dependently (Fig. 3.8A). In the phenoxybenzamine treatment, the time taken to achieve peak pressor response (3.0-3.8 min), peak R_S (2.4-4.2 min), and peak Q (2.9-5.4 min) remained constant for the different Ang II doses (Fig. 3.8B). While the time taken to achieve peak P_{DA} and peak R_S were shorter in the control group than in the phenoxybenzamine group (75-1200 pmol kg⁻¹), the time taken to achieve peak Q at the three highest Ang II doses (300-1200 pmol kg⁻¹) was longer in the control group than in the phenoxybenzamine group (Fig. 3.8). Although the increase in Q in the control group significantly lagged behind the increase in P_{DA} and R_S at all doses, and in general peak R_S took place prior to

Figure 3.6 Cardiac output (Q) in intact control ($N=10$; solid circles) and phenoxybenzamine-treated ($N=8$; open squares) rainbow trout given a bolus injection of homologous [Asn¹-Val²]-Ang II with doses ranging between 25 and 1200 pmol kg⁻¹. The dashed line in each graph indicates the time of Ang II injection. The solid lines below the control group indicate the time interval during which the Q values are significantly different from the resting value immediately preceding the dashed line ($P<0.05$). P values resulting from the one-way repeated-measures ANOVA analysis of the phenoxybenzamine-treated fish (E, F) are shown when results from the multiple comparison test failed to determine area of significance. Values are means \pm 1 S.E.M.

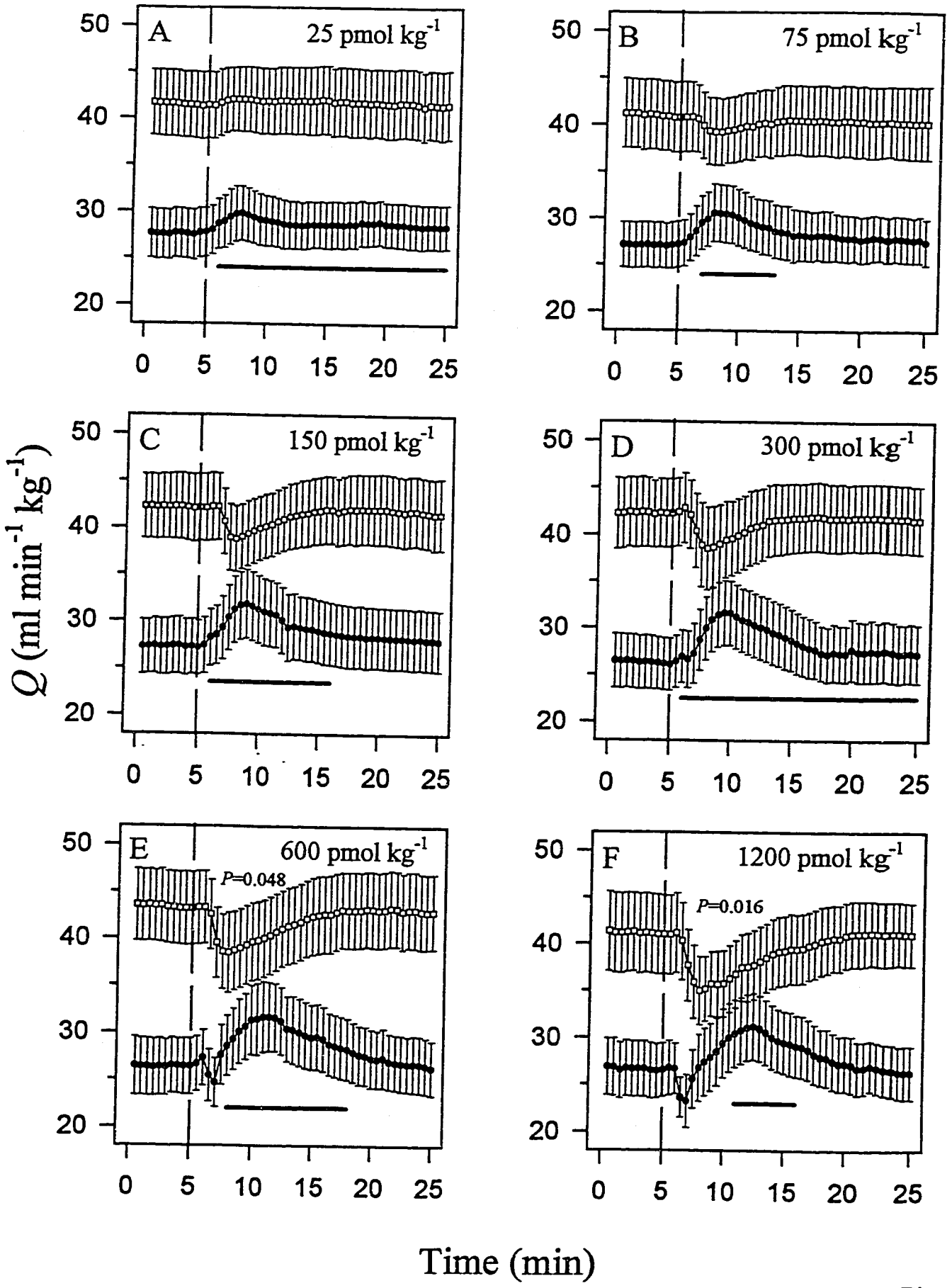


Figure 3.6

Figure 3.7 Absolute change in cardiac output (Q ; A) and stroke volume (V_s ; B) in intact control ($N=10$; solid circle) and phenoxybenzamine-treated ($N=8$; open squares) rainbow trout after intravenous injections of graded homologous [Asn¹-Val⁵]-Ang II doses. An asterisk denotes a significant difference between the control and phenoxybenzamine-treated fish at a given dose ($P<0.05$). Dissimilar letters indicate significant differences between doses within a group ($P<0.05$). Values are means \pm 1 S.E.M.

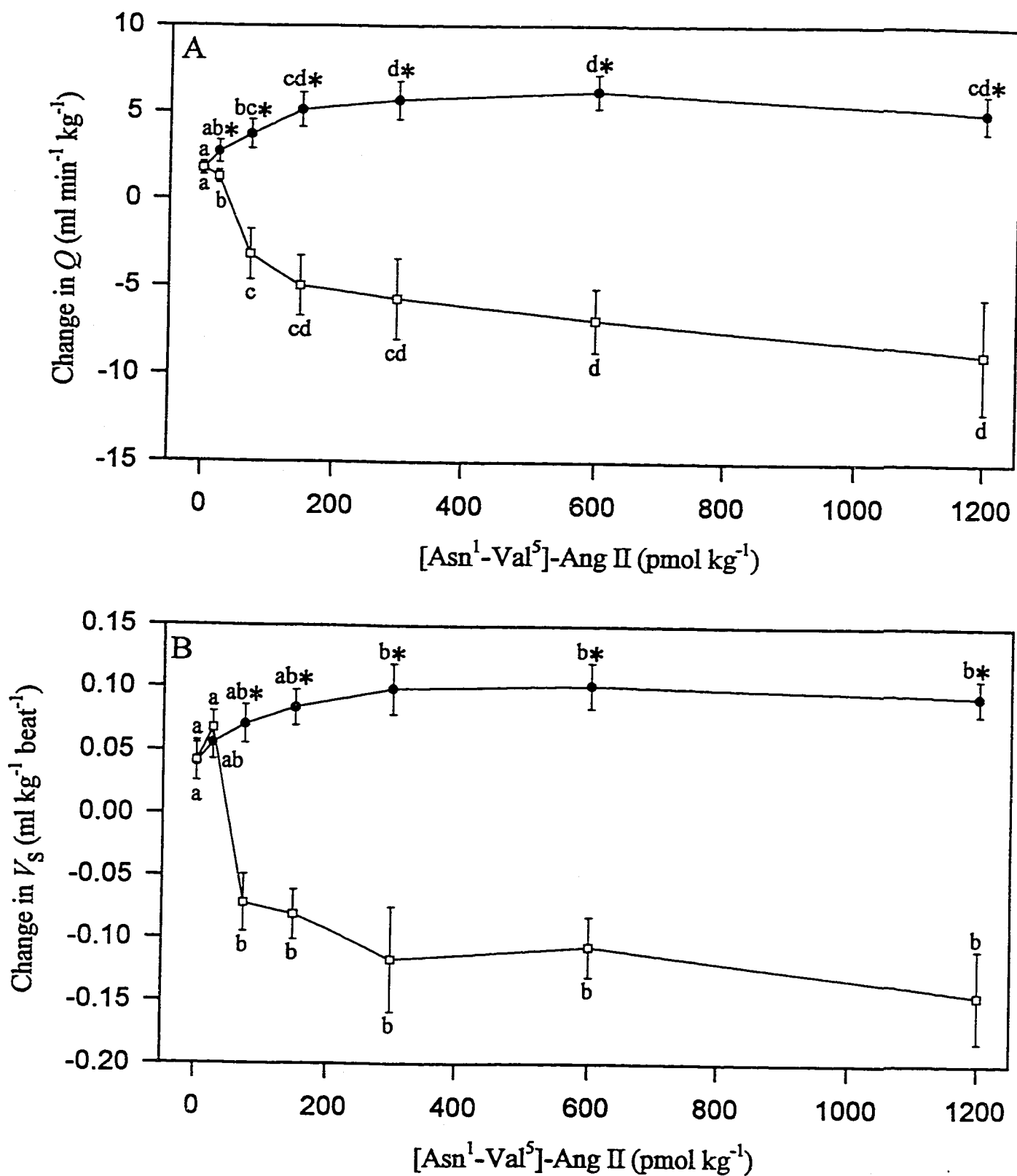


Figure 3.7

Figure 3.8 Effects of intravenous injections of graded homologous [Asn¹-Val⁵]-Ang II doses on time taken for peak pressure (open bar), peak R_s (hatched bar), and peak Q (solid bar) in intact control (A; $N=10$) and phenoxybenzamine-treated (B; $N=8$) rainbow trout. Dissimilar letters indicate significant differences between doses for a given cardiovascular parameter and treatment. Dissimilar numbers indicate significant differences between cardiovascular parameters for a given dose and treatment. † denotes a significant difference between the control and phenoxybenzamine treatments at a given dose ($P<0.05$). Values are means \pm 1 S.E.M.

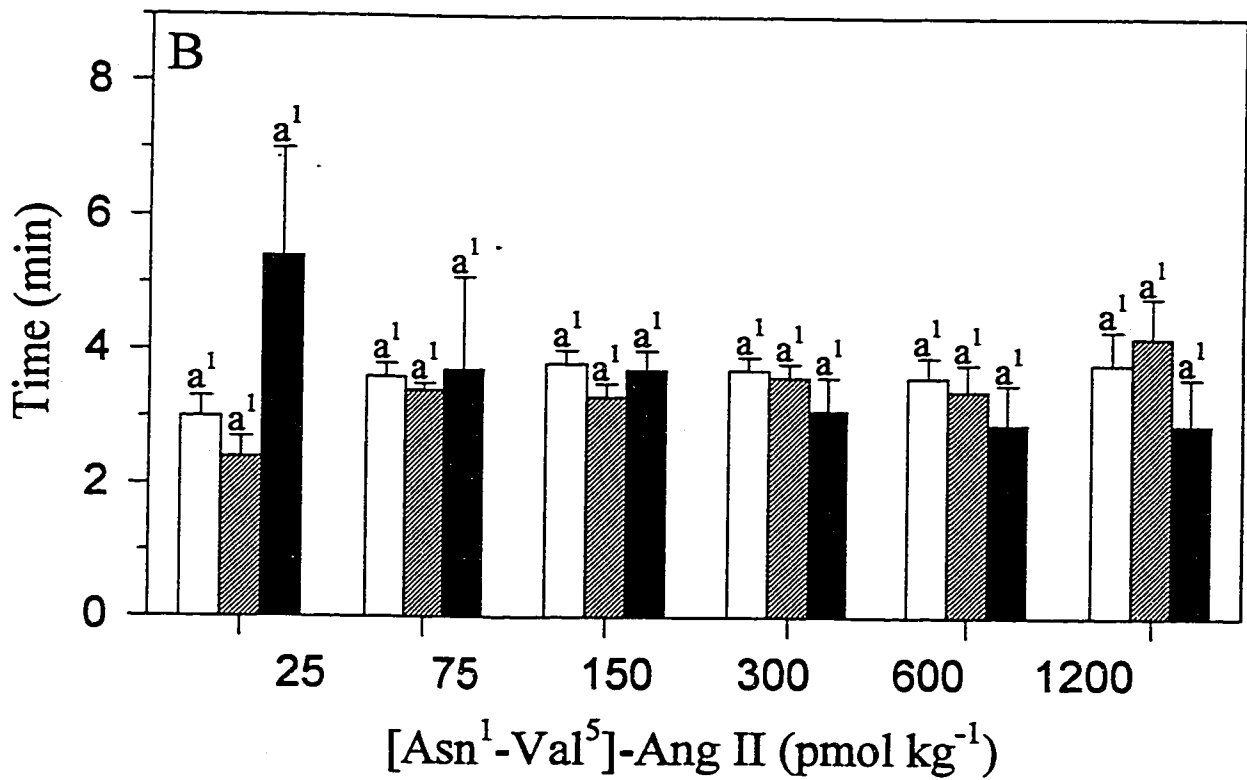
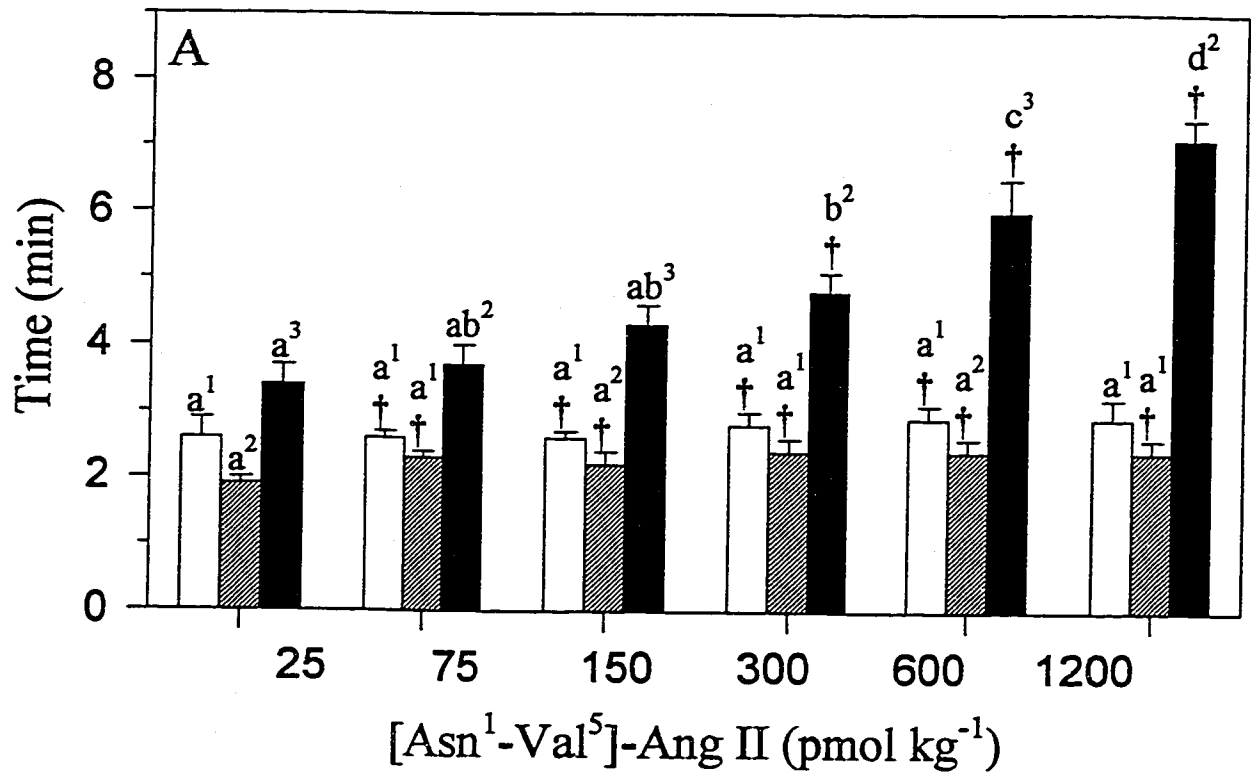


Figure 3.8

peak P_{DA} , in the phenoxybenzamine treatment, all three cardiovascular variables peaked at similar times (Fig. 3.8).

Series 3: cardiovascular responses to adrenaline

Caudal vein injection of adrenaline doses ranging between 1.8×10^{-10} and 1.4×10^{-8} mol kg⁻¹ resulted in arterial plasma adrenaline concentrations between 9.2 nM and 785.6 nM ($r^2=0.941$, Fig. 3.9). The dose-response relationship between injected adrenaline dose and realised plasma concentration is described by the following linear equation: $Y = 56.62X - 4.27$, where Y is in nM and X is in nmol kg⁻¹. The adrenaline injections had no effect on basal plasma noradrenaline concentrations ($r^2=0.028$; Fig. 3.9). Adrenaline injections elicited significant and dose-dependent increases in mean P_{DA} between the plasma adrenaline concentrations of 16.5 ± 1.8 and 487.6 ± 98.2 nM (Fig. 3.10A). The increases in R_S following administration of adrenaline were significant and dose-dependent between the plasma adrenaline concentrations of 50.5 ± 5.2 and 487.6 ± 98.2 nM (Fig. 3.10B). A significant increase in Q and V_S was achieved with adrenaline injections which yielded plasma adrenaline concentrations of 50.5 ± 5.2 nM (Fig. 3.10C-D). Higher plasma adrenaline concentrations did not result in further increases in either Q or V_S (Fig. 3.10C-D). Although in some fish, adrenaline injections were followed by a drop in f_H , overall the changes in f_H were not significant. While injections of saline alone had no significant effect on mean P_{DA} , R_S , f_H or V_S , they elicited a small increase in Q (Fig. 3.10A-D).

Figure 3.9 Effects of intravenous injections of adrenaline on maximal arterial plasma adrenaline (solid circle) and noradrenaline (open triangle) concentrations in resting rainbow trout ($N=8$). Linear regression for plasma adrenaline, $Y = 56.62X - 4.27$ ($r^2 = 0.941$); for plasma noradrenaline, $Y = -0.03X + 0.10$ ($r^2 = 0.028$). Confidence interval of 95% is indicated by dotted line.

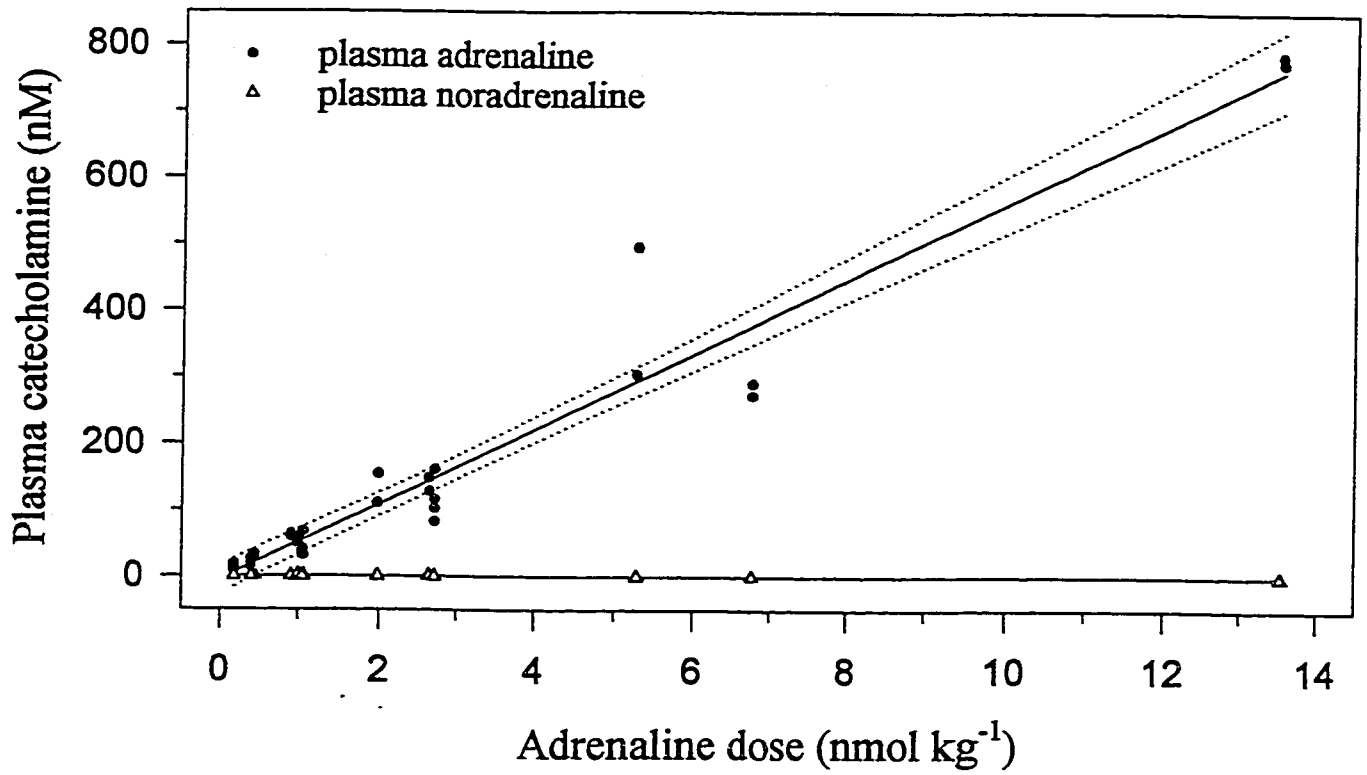


Figure 3.9

Figure 3.10 Increase in mean dorsal aortic pressure (P_{DA} ; A), systemic resistance (R_s ; B), cardiac output (Q ; C), and stroke volume (V_s ; D) as a function of arterial plasma adrenaline concentration in rainbow trout ($N=8$). Open circles represent individual adrenaline injections ranging between 1.8×10^{-10} and 1.4×10^{-8} mol kg⁻¹. Open triangles represent mean values (± 1 S.E.M) for saline injections. Solid triangles represent mean values (± 1 S.E.M) for adrenaline injections. Dissimilar letters indicate significant differences between means for a given cardiovascular variable ($P < 0.05$).

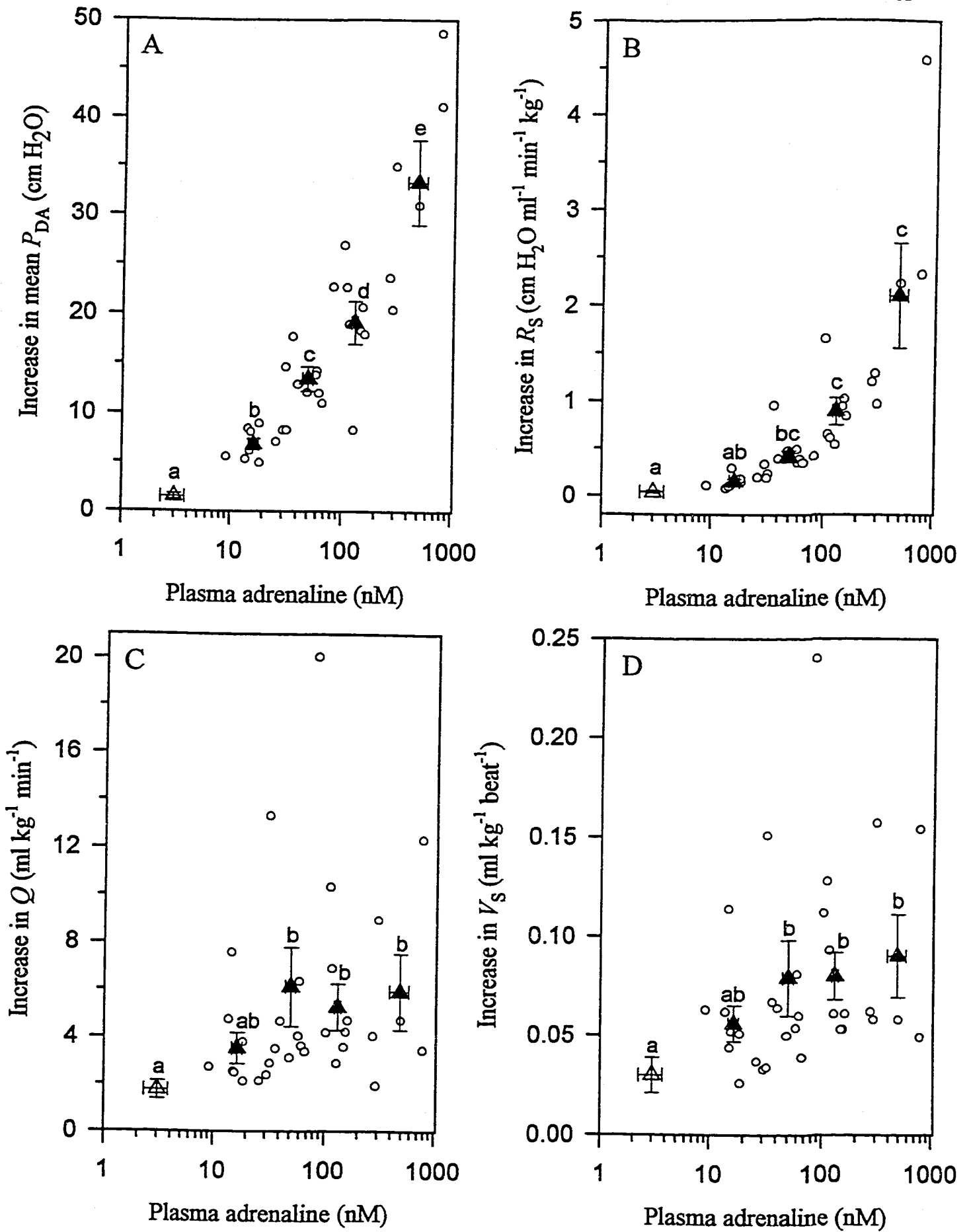


Figure 3.10

Discussion

The effects of Ang II on plasma catecholamines

The stimulatory effects of Ang II on plasma adrenaline reported in this study, supports the original observation of Carroll and Opdyke (1982) that Ang II can elicit the release of catecholamines in teleost fish *in vivo*, and establishes for the first time the dose-dependent nature of the relationship. The preferential increase in plasma adrenaline over noradrenaline with Ang II injections also supports the findings of Carroll and Opdyke (1982) in the lumpfish (*C. lumpus*), and our previous observations in *in situ* preparations of rainbow trout (Bernier and Perry 1997). Since a 1200 pmol kg⁻¹ dose of Ang II elicited an increase in plasma noradrenaline which was only marginally greater than basal resting levels, our results suggest that under physiological conditions, the cardiovascular effects of Ang II-mediated catecholamine release can be attributed primarily to plasma adrenaline in rainbow trout. While an Ang II dose of 1940 pmol kg⁻¹ was required to elicit a plasma adrenaline concentration of 3.3 nM in *C. lumpus*, the same adrenaline concentration was achieved with an Ang II dose of 75 pmol kg⁻¹ in the trout. Hence, although results are only available from two species, they do suggest considerable variability in the responsiveness of the chromaffin tissue to Ang II among teleosts. Relative to other secretagogues of the chromaffin tissue (Fritsche *et al.* 1993; Reid and Perry 1994; Reid *et al.* 1996, 1998), the maximum adrenaline concentration achieved with Ang II in this study, 125 nM, suggest that Ang II can be a potent secretagogue of humoral adrenaline in rainbow trout.

Although few studies have measured circulating levels of Ang II in teleosts, available data suggest that an Ang II-elicited increase in plasma adrenaline can be achieved with physiological concentrations of Ang II. While basal plasma Ang II concentrations vary from approximately 10 to 220 pM in eels (Henderson *et al.* 1985; Kobayashi *et al.* 1980; Okawara *et al.* 1987; Takei *et*

al. 1988; Tierney *et al.* 1995a, 1995b), Lipke *et al.* (1990) reported a mean resting value of 824 pM in rainbow trout. From these resting values, experiments carried out with eels have shown that plasma Ang II concentrations can increase more than 15 fold (~ 380 to 3490 pM) following various acute hypotensive stress (Henderson *et al.* 1985; Kobayashi *et al.* 1980; Tierney *et al.* 1995a, 1995b). In this study, Ang II doses ranging from 75 to 1200 pmol kg⁻¹ elicited a significant increase in plasma adrenaline. Given that the half-life of Ang II in trout is 3-7 minutes (Olson 1992) and assuming that Ang II, after injection, was distributed rapidly throughout the extracellular fluid volume (300 ml kg⁻¹) prior to reaching the chromaffin tissue, the 75-1200 pmol kg⁻¹ Ang II doses likely elicited physiological levels of Ang II in the plasma. Whether activation of the proposed intrarenal RAS of rainbow trout (Brown *et al.* 1995; Bernier and Perry 1997) may result in higher concentrations of Ang II in the vicinity of the chromaffin tissue has yet to be investigated.

The cardiovascular effects of Ang II with and without α -adrenoceptor blockade

Once Q₁₀ effects are taken into consideration, the resting cardiovascular variables (mean P_{DA} , R_s , Q , f_H , V_s) of the cannulated and instrumented fish in the control group are comparable to those previously reported for rainbow trout (Kiceniuk and Jones 1977; Wood and Shelton 1980; Gamperl *et al.* 1994a, 1994b). Phenoxybenzamine treatment, in addition to blocking humoral α -receptor mediated increases in R_s and P_{DA} (Wood 1976; Randall and Stevens 1967; Wood and Shelton 1980; Xu and Olson 1993a), caused a drop in resting mean P_{DA} and R_s , and a compensatory increase in resting Q and V_s . While phenoxybenzamine-associated vasodepression has been observed by some authors (Wood and Shelton 1980), others have concluded that phenoxybenzamine has no significant effect on blood pressure (Randall and Stevens 1967; Olson and Duff 1992; Xu and Olson 1993a). Based on the latter observations, Xu and Olson (1993a)

suggested that the α -antagonistic effects of phenoxybenzamine are limited to the luminal receptors of the vasculature that are exposed only to humoral catecholamines. In contrast, our results suggest that phenoxybenzamine treatment, as other α -adrenergic antagonists (Wood and Shelton 1980; Xu and Olson 1993a), blocks both neuronal and humoral α -adrenoceptors.

The pressor effect of intravascular Ang II injections has been documented in a variety of teleosts species (for reviews see Nishimura 1985b; Olson 1992) including rainbow trout (Le Mevel *et al.* 1993; 1994; Olson *et al.* 1994; Fuentes and Eddy 1998). A significant component of this Ang II-elicited pressor response is mediated indirectly through an activation of the SNS (Nishimura *et al.* 1978; Carroll and Opdyke 1982; Nishimura 1985b; Lipke *et al.* 1990; Platzack *et al.* 1993; Olson *et al.* 1994; Butler *et al.* 1995; Oudit and Butler 1995a). In the dorsal aorta of perfused trunk preparations of rainbow trout, approximately 40% of the overall vasoconstrictory effect of a pharmacological dose of Ang II (10^{-7} mol l^{-1}) is abolished by either the α -adrenoceptor antagonist phentolamine or the adrenergic nerve toxin bretylium (Olson *et al.* 1994). Since catecholamines from the chromaffin tissue do not circulate in the isolated trunk preparation, and bretylium only blocks the release of catecholamines from adrenergic neurons, these results suggest that a significant portion of the Ang II pressor response can be mediated via sympathetic nerves. However, since the Ang II pressor response in the celiacomesenteric artery of the same preparation was unaffected by pharmacological blockade, the interaction between Ang II and sympathetic nerves may be vascular bed specific (Olson *et al.* 1994). *In vivo*, α -adrenoceptor blockade with phenoxybenzamine also inhibits a significant portion (33%, this study; 60%, Lipke *et al.* 1990) of the Ang II-mediated pressor response in rainbow trout. While this may result in part from blocking a possible interaction between Ang II and the sympathetic nerves, our results suggest that the reduced Ang II-elicited pressor response with α -adrenoceptor blockade can also

be ascribed to an inhibition of the Ang II-mediated increase in humoral catecholamines. Indeed, the maximum plasma adrenaline concentrations recorded following the 600 and 1200 pmol kg⁻¹ doses of Ang II, are concentrations that can elicit significant increases in P_{DA} , R_S , and Q .

In the trout, increases in both R_S and Q contribute to the Ang II-elicited pressure response. However, since the increase in R_S slightly precedes peak P_{DA} and the increase in Q lags behind it, R_S is the principle cause of the pressor response in trout. Comparisons of Ang II pressor responses in perfused tissues and large isolated vessels of rainbow trout have shown that the direct vasoconstrictory response to Ang II occurs in the systemic microcirculation (Conklin and Olson 1994a; Olson *et al.* 1994). In contrast, experiments using ventricular rings *in vitro*, and *in situ* perfused heart preparations of rainbow trout, have concluded that Ang II does not directly affect cardiac performance in this species (Olson *et al.* 1994). In the American eel (*Anguilla rostrata*), increases in R_S and Q also contribute to the Ang II-elicited pressure response, and the temporal changes in R_S , P_{DA} , and Q are similar to those in the trout (Oudit and Bulter 1995a). However, in the eel, Ang II has both direct and indirect stimulatory effects on the heart (Oudit and Bulter 1995a). Ang II injections in the Antarctic fish *Pagothenia borchgrevinki* also produced increases in R_S and P_{DA} , but unlike either the eel or the trout, they elicited no change in Q as bradycardia cancelled out a rise in V_S (Axelsson *et al.* 1994).

Although there is no evidence for a direct effect of Ang II on the cardiac performance of the trout (Olson *et al.* 1994), several indirect mechanisms may contribute to the stimulatory effects of Ang II on Q . For example, Ang II-elicited catecholamines may have chronotropic and inotropic effects (Farrell and Jones 1992; Gamperl *et al.* 1994a). Ang II can also act centrally to suppress vagal tone (Le Mevel *et al.* 1994) or to facilitate sympathetic neurotransmission (Reid 1992). In addition, Ang II may increase Q by affecting venous return (Oudit and Butler, 1995a).

However, while an Ang II dose of 25 pmol kg^{-1} elicited a significant increase in Q , it had no effect on plasma adrenaline levels. Also, although near maximal stimulation of Q was attained with a 300 pmol kg^{-1} dose of Ang II, this dose elicited a plasma adrenaline concentration of only 16.8 nM. So while humoral catecholamines may contribute to the stimulatory effects of Ang II on Q , the importance of circulating catecholamines may be relatively minor and secondary to other mechanisms. Even though in trout (Le Mevel *et al.* 1994) and mammals (Reid 1992) Ang II can act centrally to suppress baroreceptor-mediated reflexive bradycardia, it is unlikely that peripheral injections of Ang II inhibit vagal tone or reduced the baroreceptor reflex. The Ang II-elicited increase in Q was mediated entirely by an increase in V_s in this study, and peripheral injections of Ang II in trout have also been associated with moderate bradycardia (Le Mevel *et al.* 1993). Although inotropic and chronotropic effects have been attributed to interactions between Ang II and sympathetic ganglia in mammals (Saxena 1992), overall the role of adrenergic nerves in teleost cardiac control is not clear (Nilsson 1994). Even though interactions between Ang II and sympathetic nerves have been observed in parts of the vasculature of rainbow trout *in situ* (Olson *et al.* 1994), the specific effects of Ang II on the adrenergic nervous control of the heart remain to be investigated. In *A. rostrata*, Ang II may also increase Q by affecting venous return (Oudit and Butler, 1995a). In rainbow trout, this is supported by the importance of cardiac filling pressures in determining V_s (Graham and Farrell 1989). However, the venous system of trout appears to be refractory to Ang II *in vitro* (Conklin and Olson 1994a; 1994b), and angiotensin-converting enzyme inhibition does not affect mean circulatory filling pressure *in vivo* (Zhang *et al.* 1995). Nevertheless, the stimulatory effects of Ang II on sympathetic nerves and humoral catecholamines may increase the sensitivity of the rainbow trout heart to filling pressures (Graham

and Farrell 1989), and increase venous return through a mobilization of blood from the unstressed volume (Conklin and Olson 1994b; Zhang *et al.* 1998).

The dose-dependent delay in the peak Q response to Ang II, behind peak P_{DA} , probably results from a passive inhibition of systolic emptying caused by the elevated outflow pressures (Wood and Shelton 1980), especially at higher Ang II concentrations (600 and 1200 pmol kg⁻¹, see Fig. 3-6). Although end-systolic volume of the ventricle is usually independent of mean aortic output pressure (homeometric regulation), at very high pressures the intrinsic ability of the heart to maintain V_S breaks down and end-systolic volume increases (Farrell 1992). The break down of homeometric regulation and the resulting decrease in V_S is especially noticeable in α -adrenoceptor blocked fish. Without the potential Q stimulation resulting from the interaction between Ang II and the SNS, the Q and V_S of phenoxybenzamine-treated fish decreased in proportion and simultaneously to the increase in P_{DA} and R_S . The response is also probably exacerbated by the higher resting V_S that α -adrenoceptor blocked fish must maintain in order to compensate for the lost of neuronal tone.

Cardiovascular responses to adrenaline

In support of other *in vivo* studies that have investigated the cardiovascular responses of physiological doses of adrenaline (Wood and Shelton 1980; Gamperl *et al.* 1994a, 1994b), our data provides direct evidence that physiological levels of plasma adrenaline can contribute to cardiovascular control in rainbow trout. The discrepancy between these *in vivo* results and the observation that physiological concentrations of catecholamines do not contribute to systemic vascular resistance *in vitro* (Wood and Shelton 1975), may be resolved through the observation that neuronal sympathetic tone is enhanced by circulating catecholamines (Xu and Olson 1993b). As previously observed (Randall and Stevens 1967; Wood and Shelton 1980; Gamperl *et al.*

1994b), the effects of adrenaline on P_{DA} are dose-dependent within and beyond the physiological range of plasma adrenaline. Our results also confirm the previously described variable nature of the trout Q response to adrenaline (Wood and Shelton 1980; Gamperl *et al* 1994a), and support the observations that near maximal increases in Q and V_s are achieved at relatively low physiological concentrations of adrenaline (Farrell *et al.* 1986; Gamperl *et al.* 1994a).

The plasma adrenaline levels which elicit significant increases in P_{DA} , R_s , and Q , are characteristic of the circulating catecholamine levels observed following a variety of disturbances (for review see Randall and Perry 1992; Gamperl *et al.* 1994c). The common link between these physical and environmental disturbances is their severity. As observed by Randall and Perry (1992), plasma catecholamine levels do not rise substantially unless the degree of stress is severe. In support of this, we observed that only Ang II doses ≥ 600 pmol kg⁻¹ elicit plasma adrenaline levels that contribute to the cardiovascular effects of Ang II. Based on experiments carried out on eels (Henderson *et al.* 1985; Kobayashi *et al.* 1980; Tierney *et al.* 1995a, 1995b), the plasma Ang II concentrations that correspond to these Ang II doses may only be approached following acute hemorrhage, hypotension, or transfer to seawater. Hence, based solely on circulating Ang II levels, our results suggest that the cardiovascular effects of Ang II will only be supplemented by humoral catecholamines under conditions that acutely stimulate the renin angiotensin system. Whether circulating catecholamines are elevated during such disturbances remains to be investigated.

Finally, our dose-response relationship between caudal vein injected adrenaline dose and realised arterial plasma concentration can only be used for predictive purposes if the same drug injection and blood sampling sites are used. In comparison to the study of Gamperl *et al.* (1994c) where both adrenaline injection and blood sampling were done via a dorsal aorta cannula, the

realised plasma concentration for a given adrenaline dose was approximately 10 times lower than in the current study. Also, in support of other studies in the trout (Perry and Vermette 1987; Gamperl *et al.* 1994c), but in contrast to the observation of Epple and Nibbio (1985) in the American eel (*Anguilla rostrata*), adrenaline injections had no catecholaminotropic effects on plasma noradrenaline.

In summary, this study demonstrates that Ang II can be a potent non-cholinergic secretagogue of adrenaline secretion from the chromaffin tissue of the trout *in vivo*. However, given the exogenous Ang II doses required to elicit plasma adrenaline levels that contribute to cardiovascular regulation, the interaction between Ang II and adrenaline secretion may only have physiological significance following stressful disturbances that acutely stimulate the renin-angiotensin system. Since mammalian studies suggest that the Ang II-SNS interactions that result from exogenous Ang II are of little significance in comparison to those from endogenous Ang II (Reid 1992), it is possible that the present results underestimate the impact of Ang II-elicited catecholamine release to fish cardiovascular control. In addition to the role of endogenous plasma Ang II, future experiments investigating Ang II-elicited catecholamine release should take into account the role of the intrarenal RAS and the potential neuromodulatory actions of central Ang II in the neural regulation of catecholamine release.

CHAPTER 4.

**Mediation of humoral catecholamine secretion by the renin-angiotensin system
in hypotensive rainbow trout (*Oncorhynchus mykiss*)**

Abstract

The individual contributions of, and potential interactions between, the renin-angiotensin system (RAS) and the humoral adrenergic stress response to blood pressure regulation were examined in rainbow trout. Intravenous injection of the smooth muscle relaxant papaverine (10 mg kg^{-1}) elicited a transient decrease in dorsal aortic blood pressure (P_{DA}) and systemic vascular resistance (R_{S}), and significant increases in plasma angiotensin II (Ang II) and catecholamine levels. Blockade of α -adrenoceptors prior to papaverine treatment prevented P_{DA} and R_{S} recovery, had no effect on the increase in plasma catecholamines, and resulted in higher plasma Ang II levels. Administration of the angiotensin converting enzyme inhibitor lisinopril ($10^{-4} \text{ mol kg}^{-1}$) prior to papaverine treatment attenuated the increases in the plasma concentrations of Ang II, adrenaline, and noradrenaline by 90, 79, and 40%, respectively and also prevented P_{DA} and R_{S} recovery. By itself, lisinopril treatment caused a gradual and sustained decrease in P_{DA} and R_{S} , and reductions in basal plasma Ang II and adrenaline concentrations. Bolus injection of a catecholamine cocktail (4 nmol kg^{-1} noradrenaline plus 40 nmol kg^{-1} adrenaline) in the lisinopril + papaverine treated trout, to supplement their circulating catecholamine levels and mimic those observed in fish treated only with papaverine, resulted in a temporary recovery in P_{DA} and R_{S} . These results indicate that the RAS and the acute humoral adrenergic response are both recruited during an acute hypotensive stress, and play important roles in the compensatory response to hypotension in rainbow trout. However, whereas the contribution of the RAS to P_{DA} recovery is largely indirect and relies on an Ang II-mediated secretion of catecholamines, the contribution from the adrenergic system is direct and relies at least in part on plasma catecholamines.

Introduction

Studies in comparative endocrinology point to a central role for the renin-angiotensin system (RAS) in the maintenance of blood pressure and fluid volume homeostasis among all classes of vertebrates (Wilson 1984b; Henderson and Deacon 1993; Kobayashi and Takei 1996). Indeed, the vasopressor and dipsogenic actions of angiotensin II (Ang II), the biologically active product of the RAS, have been described from elasmobranchs to mammals. In teleost fish, a reduction in renal perfusion pressure, or in blood volume following hemorrhage, leads to an increase in plasma renin activity, the catalyst of the RAS cascade (Nishimura *et al.* 1979; Bailey and Randall 1980), and plasma Ang II (Kobayashi *et al.* 1980). Similarly, administration of the hypotensive agent papaverine elicits an increase in plasma renin activity, plasma Ang II levels, and drinking rate (Nishimura *et al.* 1979; Balment and Carrick 1985; Tierney *et al.* 1995b). Preventing the formation of Ang II with an angiotensin-converting enzyme (ACE) inhibitor abolishes the papaverine-induced dipsogenic response and hinders recovery from the hypotensive stress (Balment and Carrick 1985; Perrott and Balment 1990; Tierney *et al.* 1995b). Blockade of the RAS with an ACE inhibitor also has been shown to lower resting blood pressure in a variety of teleost species (Olson 1992; Platzack *et al.* 1993; Tierney *et al.* 1995b). Taken together, this evidence suggests that the RAS of teleosts has a tonic role in blood pressure regulation and mediates some of the physiological responses that counteract hypotensive stressors.

In addition to the RAS, an important contributor to cardiovascular control in teleosts is the sympathetic nervous system (SNS). While resting blood pressure is under the control of an adrenergic tonus of neuronal origin, plasma catecholamines originating from the chromaffin tissue in the head kidney can provide an additional adrenergic influence on the cardiovascular system during periods of stress (Nilsson 1994; Bernier and Perry 1999). Furthermore, experiments

employing α -adrenoceptor blockade have shown that a significant component of the cardiovascular effects of exogenous Ang II injections are mediated indirectly via interactions with the SNS (Olson 1992; Platzack *et al.* 1993; Olson *et al.* 1994; Butler *et al.* 1995; Oudit and Butler 1995a; Bernier and Perry 1999). However, the potential contributions of the SNS to cardiovascular control during a hypotensive stress have yet to be investigated in fish.

In rainbow trout (*Oncorhynchus mykiss*), there is mounting evidence that Ang II may be a potent activator of the humoral component of the SNS. In a preceding study, we have provided immunohistochemical evidence for the presence of Ang II binding sites on the catecholamine-containing chromaffin cells of *O. mykiss* (Bernier and Perry 1997). Moreover, bolus injections of homologous Ang II in trout elicit a dose-dependent release of catecholamines in an *in situ* perfusion preparation of the head kidney, and a dose-dependent increase in plasma adrenaline concentrations *in vivo* (Bernier and Perry 1997; 1999). While it is known that Ang II can exert a direct vasoconstrictor action in the systemic microcirculation of rainbow trout (Olson *et al.* 1994), a portion of the cardiovascular effects of exogenous Ang II can also be attributed to increased levels of plasma adrenaline (Bernier and Perry 1999). However, given that the cardiovascular effects of Ang II-mediated humoral catecholamines are recruited in a dose-dependent manner (Bernier and Perry 1999), it remains to be ascertained whether endogenous recruitment of Ang II can elicit an increase in plasma catecholamines capable of contributing to cardiovascular homeostasis.

Therefore, the present study was undertaken to determine whether the acute humoral adrenergic stress response contributes to cardiovascular control following a hypotensive stress, and to assess the relative importance of the interactions between the RAS and the SNS for blood pressure regulation in rainbow trout.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) of either sex were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported to the fish-holding facilities of the University of Ottawa. The fish were held indoors in large fiberglass tanks supplied with flowing, aerated, and dechloraminated city water. The fish were maintained at a temperature of 13°C, on a 12:12 h light-dark photoperiod cycle, and fed daily with a commercial salmonid diet. The trout of series 1 and 2 (see below) had a mean mass of 767.9 ± 21.8 g (experimental $N=44$) and 430.0 ± 8.4 g (experimental $N=35$), respectively.

Surgical procedures

Rainbow trout were anaesthetised in an oxygenated and buffered (NaHCO_3 ; 0.16 g l^{-1}) solution of ethyl-*m*-aminobenzoate (0.08 g l^{-1} ; MS-222; Syndel, Vancouver, B.C.) until cessation of breathing movements. The fish were then transferred to an operating table where the gills were force ventilated with the same anaesthetic solution. In series 1, in order to measure dorsal aortic blood pressure (P_{DA}), fish were equipped with a dorsal aortic cannula (PE 50, Clay Adams; Soivio *et al.* 1975). To permit drug injections and repeated blood sampling, a lateral incision was made in the caudal peduncle to expose the caudal vein, dissect it free from overlying tissue, and to cannulate (PE 50) this vessel in the anterograde direction. In addition, the pericardial cavity was exposed with a midline ventral incision and the pericardium was dissected to expose the bulbus arteriosus. To allow measurement of cardiac output (Q), a 3S or 4S ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY) was placed non-occlusively around the bulbus. Lubricating jelly was used with the perivascular flow probe as an acoustic couplant. Silk sutures were used to close the ventral and caudal peduncle incisions, and to anchor the cardiac output probe lead and

the caudal vein cannula to the skin. In series 2, trout were anaesthetized as above and equipped with a caudal vein cannula to permit drug injections and serial blood sampling. After surgery, all fish were placed into individual opaque boxes supplied with flowing water and left to recover for 24 h prior to experimentation. All cannulae were filled and flushed with teleost Cortland saline (Wolf, 1963).

Experimental protocol

Series 1: The effects of hypotension on cardiovascular control and plasma catecholamines

Six separate experimental groups of rainbow trout were used to investigate the relative contributions of catecholamines and the renin-angiotensin system to cardiovascular control during a hypotensive stress. Trout were monitored during an initial period of 30 to 60 min to assess the stability of P_{DA} and Q traces. Upon stabilisation, control baseline cardiovascular parameters were recorded for 10 min, after which trout were administered one of the following intravenous injection: (a) 0.9% NaCl (0.375 ml kg^{-1}) over a 10 min period (control treatment; $N=7$), (b) papaverine (0.375 ml kg^{-1} ; RBI, Natick, MA) at 10 mg kg^{-1} over a 10 min period (papaverine treatment; $N=8$), (c) papaverine (0.375 ml kg^{-1}) at 10 mg kg^{-1} over a 10 min period after phenoxybenzamine (RBI) pre-treatment (phenoxybenzamine + papaverine treatment; $N=6$), (d) lisinopril (1 ml kg^{-1} ; Sigma Chemical, St. Louis, MO) at $10^{-4} \text{ mol kg}^{-1}$ over a 2 min period (lisinopril treatment; $N=8$), (e) lisinopril (1 ml kg^{-1}) at $10^{-4} \text{ mol kg}^{-1}$ over a 2 min period followed 10 min later by an injection of papaverine (0.375 ml kg^{-1}) at 10 mg kg^{-1} over a 10 min period (lisinopril + papaverine treatment; $N=8$), and (f) lisinopril (1 ml kg^{-1}) at $10^{-4} \text{ mol kg}^{-1}$ over a 2 min period followed 10 min later by an injection of papaverine (0.375 ml kg^{-1}) at 10 mg kg^{-1} over a 10 min period and 7.5 min later by a bolus injection of catecholamines (4 nmol kg^{-1} noradrenaline bitartrate and 40 nmol kg^{-1} adrenaline bitartrate; Sigma; lisinopril + papaverine + catecholamine

treatment; $N=7$). Each injection was followed by 0.2 ml of saline to clear the caudal vein cannula, and the cardiovascular effects of these treatments were monitored continuously over the following 90 min. In each treatment, a blood sample (0.3 ml) was taken at the end of the initial 10 min control baseline period, as well as 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 90 min into the treatment for subsequent analysis of plasma catecholamines. Each blood sample was replaced by an equivalent volume of saline containing 3% bovine serum albumin (BSA), collected in a microcentrifuge tube containing 5 μ l of 10% $\text{Na}_2\text{-EDTA}$, and centrifuged immediately at 10,000 g for 15 sec. The separated plasma was quick frozen in liquid nitrogen and stored at -80°C for later analysis of catecholamines.

In the phenoxybenzamine + papaverine treatment, α -adrenoceptor blockade was achieved by slowly (over a 15 min period) giving two injections of 3 mg kg^{-1} phenoxybenzamine 12 and 6 h prior to experimentation. Phenoxybenzamine was dissolved in 100 μ l ethanol and diluted in saline prior to injection (3 mg ml^{-1}). The effectiveness of this protocol to achieve α -adrenoceptor blockade in rainbow trout was confirmed previously (Bernier and Perry 1999).

Series 2: The effects of hypotension on plasma Ang II

Five of the experimental groups outlined in *Series 1* above were repeated in order to assess circulating concentrations of plasma $[\text{Asn}^1\text{-Val}^5]\text{-Ang II}$ in hypotensive rainbow trout: (a) control treatment ($N=7$), (b) papaverine treatment ($N=7$), (c) phenoxybenzamine + papaverine treatment ($N=7$), (d) lisinopril treatment ($N=7$), and (e) lisinopril + papaverine treatment ($N=7$). In each treatment, a blood sample (0.15 ml) was taken at the end of the initial 10 min control baseline period, as well as 10, 15, 20, 25, 30, 40, 50, 70, and 90 min into the treatment for subsequent analysis of plasma $[\text{Asn}^1\text{-Val}^5]\text{-Ang II}$. Each blood sample was replaced by an equivalent volume of saline containing 3% BSA, collected in a microcentrifuge tube containing 5

μl of 10% $\text{Na}_2\text{-EDTA}$, and centrifuged immediately at 10,000 g for 15 sec. The separated plasma was quick frozen in liquid nitrogen and stored at -80°C for later analysis of $[\text{Asn}^1\text{-Val}^5]\text{-Ang II}$.

Analytical procedures

Dorsal artery pressure (P_{DA}) was measured with a UFI model 1050BP (UFI, Morro Bay, CA) pressure transducer that was calibrated against a static water column. Mean blood pressure was calculated as: (systolic pressure + diastolic pressure) / 2. The perivascular flow probes used to measure Q were connected to a Transonic T106 small animal blood flow meter (Transonic Systems, Inc., Ithaca, NY). These probes were pre-calibrated in the factory and verified in the laboratory by pump perfusion of the heart of an immersed euthanized fish with saline at known flow rates. Both P_{DA} and Q signals were recorded with a data acquisition system (Biopac System Inc., Goleta, CA) and collected at 0.04 sec intervals using Acknowledge IIITM (Biopac System Inc.) data acquisition software. Systemic vascular resistance (R_{S}) was calculated as mean P_{DA} divided by Q (i.e. $R_{\text{S}} = P_{\text{DA}} / Q$).

Plasma catecholamines (adrenaline and noradrenaline) were determined on alumina-extracted plasma samples (0.2 ml) using high-pressure liquid chromatography (HPLC) with electrochemical detection (Bernier and Perry 1997). The extracted samples were passed through an Ultratechsphere ODS-C18 5 μm column (HPLC Technology Ltd), using a catecholamine and metanephrine mobile phase (Chromosystems, Munich, Germany). The separated amines were integrated using the Star Chromatography software program (version 4.0, Varian, Walnut Creek, CA). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations.

Development and validation of a radioimmunoassay for trout Ang II

Source of antisera and specificity.

The antisera used for the current radioimmunoassay (RIA) was initially raised against mammalian Ang II ([Asp¹, Ile⁵]-Ang II; Yamaguchi, 1981) and has previously been shown to exhibit a high degree of cross-reactivity with teleost Ang II ([Asn¹, Val⁵]-Ang II; Takei et al. 1988). In this study, the specificity of the antiserum was checked by comparing the standard curve obtained with [Asp¹, Ile⁵]-Ang II (Sigma) against those obtained with the following peptides: [Asp¹, Val⁵]-Ang II (Sigma), [Asn¹, Val⁵]-Ang II (Sigma), [Asn¹, Pro³, Ile⁵]-Ang II (Peptide Institute Inc., Osaka, Japan), [Asp¹, Ile⁵, His⁹]-Ang I (Sigma), [Asn¹, Val⁵, Asn⁹]-Ang I (Sigma), [Asn¹, Val⁵, Gly⁹]-Ang I (Peninsula Laboratories, Belmont, CA), and [Asn¹, Pro³, Ile⁵, Gln⁹]-Ang I (Peptide Institute). The cross-reaction of these peptides with the Ang II antiserum was calculated as the ratio of the amount of [Asp¹, Ile⁵]-Ang II which produced 50% of ¹²⁵I-Ang II bound to the amount of each peptide which produced 50% of ¹²⁵I-Ang II bound.

The specificity of the antisera for [Asn¹, Val⁵]-Ang II was also assessed by a combination of reverse-phase HPLC and RIA. In order to determine the elution position of standard ligands, 1 ml injection volumes of distilled water containing either 100 pmol [Asn¹, Val⁵]-Ang II and 300 pmol [Asn¹, Val⁵, Asn⁹]-Ang I or 100 pmol [Asn¹, Val⁵, Asn⁹]-Ang I, were subjected to reverse-phase HPLC. The standard ligands were applied to an ODS-120T column (4.6 × 250 mm; Tosoh, Japan) and eluted with a linear gradient from 10 to 60% CH₃CH in 0.1% trifluoroacetic acid for 40 min at 1 ml min⁻¹. A 0.8 ml trout plasma sample was extracted (see below for procedure), reconstituted in 1 ml of distilled water, and subjected to the same chromatographic separation as the standard ligands. Fractions of the eluted plasma sample were collected at a 1

min interval throughout the 40 min gradient. Each fraction was lyophilized, reconstituted in 1 ml of assay buffer, and analysed for immunoreactive Ang II with the RIA.

Procedures for radioimmunoassay.

The RIA was performed by a double antibody method (Takei *et al.* 1992). The incubation mixture for the standard curve consisted of 0.1 ml of standard [Asn¹, Val⁵]-Ang II ligand, 0.1 ml Ang II antiserum (1/400 000 dilution), and 0.1 ml of normal rabbit serum (1/250; Sigma). After allowing the mixture to stand for 20 h at 4°C, 0.05 ml of ¹²⁵I-labelled [Asp¹, Ile⁵]-Ang II (approximately 5000 c.p.m.; specific activity, 2000 Ci mmol⁻¹; Amersham) was added to the mixture and incubated for another 24 h at 4°C. Precipitation was then achieved with a combination of goat anti-rabbit IgG serum (0.1 ml; 1/40 dilution; code 111-001-003; Jackson ImmunoResearch Laboratories, West Grove, PA) and 16% w/v polyethylene glycol (PEG) 8000 (0.1 ml; Sigma). The resulting mixture was centrifuged at 1500 g for 60 min at 4°C to separate the bound from the free ligands. The supernatant was discarded via aspiration, and the radioactivity in the precipitate was counted in a γ -counter (1271 RIAGamma, LKB) for 1 min. In the incubation mixture of unknown samples, the 0.1 ml of standard ligand was replaced with extracted trout plasma (10 to 40 μ l) diluted with assay buffer to a final volume of 0.1 ml. The buffer used throughout the RIA consisted of: 10 mmol l⁻¹ phosphate-buffer, pH 7.4, containing 140 mmol l⁻¹ NaCl, 0.1 % w/v NaN₃, 40 mmol l⁻¹ K₂EDTA, 10 mmol l⁻¹ ϵ -amino-n-caproic acid, 0.05% Triton X-100, and 1% BSA. The amount of Ang II in the unknown samples was calculated using a 3-parameter sigmoid curve regression equation (Sigma Plot; Jandel, San Rafael, CA) obtained from the standard curve of [Asn¹, Val⁵]-Ang II. All measurements were done on triplicates.

Extraction of plasma samples.

Plasma samples were extracted according to the method of Phillips *et al.* (1991) with modifications. Briefly, 0.1 ml of just thawed plasma and acidic acetone (acetone/H₂O/1 mol l⁻¹ HCl, 40/5/1) were mixed and vortexed vigorously. The mixture was centrifuged for 10 min at 10 000 g and 4°C. The supernatant was collected in a new tube, the pellet re-solubilized and re-extracted with 0.1 ml of acidic acetone. Once combined, the supernatants were lyophilized. For the RIA, the extracted pellet was re-suspended in 0.1 ml of assay buffer. The recovery rate of Ang II through this extraction procedure, as measured with ¹²⁵I-labelled [Asn¹, Val⁵]-Ang II, was 89.8% (N=6).

Statistical analyses

Data are presented as mean values \pm one standard error of the mean (S.E.M). The statistical significance of the observed effects of an injection within a treatment was tested using a one-way repeated-measures analysis of variance (ANOVA). Dunnett's *post-hoc* multiple-comparison test was used to compare the pre-injection control data point with values at subsequent times. For a given parameter and time, differences between the control and papaverine treatments, or between the control and lisinopril treatments were assessed with *t*-tests. Differences among the four experimental groups treated with papaverine (papaverine, lisinopril + papaverine, lisinopril + papaverine + catecholamine, and phenoxybenzamine + papaverine) were assessed with a one-way ANOVA followed by Dunnett's multiple-comparisons test. The significance level for all statistical tests was $P < 0.05$.

Results

Development and validation of an RIA for trout Ang II

The standard curves obtained with tetrapod-, teleost-, and dogfish-Ang II were virtually superimposable with the curve obtained with mammalian-Ang II, indicating a high % of cross reaction of the antiserum for a wide spectrum of Ang II peptides (Table 4.1). On the other hand, the cross reactivity of the antiserum with mammalian-, salmon-, eel-, and dogfish-Ang I was almost negligible. The reverse-phase HPLC of the extracted trout plasma revealed that the major peak of immunoreactive Ang II (Fig. 4.1B) was observed at the position corresponding to the standard [Asn¹, Val⁵]-Ang II ligand (Fig. 4.1A). Only a minor peak (Fig. 4.1B), representing 4.75% of the total assayed immunoreactivity, corresponded to the standard [Asn¹, Val⁵, Asn⁹]-Ang I ligand (Fig. 4.1A). The least detectable amount of Ang II in the RIA was 0.1 fmol tube⁻¹ ($N = 11$; $P = 0.03$). The intra-assay and interassay coefficients of variation were 4.81% ($N = 6$) and 5.09% ($N = 6$), respectively. The dilution curve of immunoreactive Ang II in extracted *O. mykiss* plasma was parallel to the standard curve of [Asn¹, Val⁵]-Ang II (Fig. 4.1C)

Series 1 & 2: The effects of hypotension on cardiovascular control, plasma catecholamines, and plasma Ang II

In the control treatment, intravenous injection of 0.9% NaCl and repeated blood sampling resulted in a small decrease in P_{DA} (Fig. 4.2A) and R_S (Fig. 4.3A) over the course of the experiment, and had no effect on Q (Fig. 4.4A). The injection of saline alone and the sampling protocol had no effect on basal plasma catecholamine (Fig. 4.5A) and Ang II concentrations (Fig. 4.6A).

The smooth muscle relaxant papaverine elicited a rapid decrease in P_{DA} (from 33.3 ± 1.8 to 16.2 ± 1.7 cm H₂O after 10 min) followed by a full recovery (33.5 ± 4.9 cm H₂O; Fig. 4.2B)

only 25 min after the treatment. Papaverine treatment also elicited a rapid decrease in R_S (from 1.4 ± 0.1 to 0.9 ± 0.1 cm H₂O ml⁻¹ min⁻¹ kg⁻¹ after 15 min) with a full recovery after 30 min (1.4 ± 0.2 cm H₂O ml⁻¹ min⁻¹ kg⁻¹; Fig. 4.3B), and a slower increase in Q (from 25.1 ± 2.5 to 32.6 ± 1.6 ml min⁻¹ kg⁻¹ after 50 min; Fig. 4.4B). In addition, administering papaverine resulted in marked and sustained increases in plasma adrenaline (from 2.2 ± 0.6 to 588.7 ± 135.1 nM after 20 min; Fig. 4.5B), plasma noradrenaline (from 1.2 ± 0.6 to 139.9 ± 32.9 nM after 15 min; Fig. 4.5B) and plasma Ang II concentrations (from 125 ± 12 to 4098 ± 1214 pM after 20 min; Fig. 4.6B).

Fish treated with the α -adrenergic blocker, phenoxybenzamine, had significantly lower R_S (0.8 ± 0.1 cm H₂O ml⁻¹ min⁻¹ kg⁻¹; Fig. 4.3C) and higher Q (38.0 ± 2.8 ml min⁻¹ kg⁻¹; Fig. 4.4C) than those in the papaverine treatment. Intravenous injection of papaverine in phenoxybenzamine-treated rainbow trout elicited rapid and sustained decreases in P_{DA} (from 28.5 ± 2.7 to 13.9 ± 0.7 cm H₂O after 10 min; Fig. 4.2C) and R_S (from 0.8 ± 0.1 to 0.4 ± 0.1 cm H₂O ml⁻¹ min⁻¹ kg⁻¹ after 15 min; Fig. 4.3C), and sustained increases in Q (from 38.0 ± 2.8 to 52.2 ± 4.2 ml min⁻¹ kg⁻¹ after 50 min; Fig. 4.4C), plasma adrenaline (from 3.2 ± 0.4 to 516.8 ± 82.0 nM after 15 min; Fig. 4.5C), plasma noradrenaline (from 1.9 ± 0.5 to 185.0 ± 37.0 nM after 15 min; Fig. 4.5C), and plasma Ang II concentrations (from 188 ± 55 to 9866 ± 1914 pM after 40 min; Fig. 4.6C). Overall, during recovery from the hypotensive stress in the phenoxybenzamine + papaverine treatment, P_{DA} and R_S values were significantly lower, and Q and plasma Ang II values significantly higher than those obtained in the papaverine treatment.

Administration of the angiotensin-converting-enzyme inhibitor lisinopril produced gradual and sustained decreases in P_{DA} (from 33.4 ± 1.3 to 15.7 ± 1.0 cm H₂O after 100 min; Fig. 4.2D) and R_S (from 1.5 ± 0.2 to 0.6 ± 0.1 cm H₂O ml⁻¹ min⁻¹ kg⁻¹ after 100 min; Fig. 4.3D), and no significant change in Q (Fig. 4.4D). While lisinopril treatment had no effect on plasma

Table 4.1 Cross-reaction of Ang II antiserum¹ with a variety of angiotensin peptides

| Peptide | Cross-reaction (%) |
|--|--------------------|
| [Asp ¹ , Ile ⁵]-Ang II (mammalian) | 100 |
| [Asp ¹ , Val ⁵]-Ang II (tetrapod) | 86.5 |
| [Asn ¹ , Val ⁵]-Ang II (teleost) | 62.7 |
| [Asn ¹ , Pro ³ , Ile ⁵]-Ang II (<i>Triakis scyllia</i>) ⁴ | 73.4 |
| [Asp ¹ , Ile ⁵ , His ⁹]-Ang I (mammalian) | 0.32 |
| [Asn ¹ , Val ⁵ , Asn ⁹]-Ang I (<i>Oncorhynchus keta</i>) ² | 0.10 |
| [Asn ¹ , Val ⁵ , Gly ⁹]-Ang I (<i>Anguilla japonica</i>) ³ | 0.16 |
| [Asn ¹ , Pro ³ , Ile ⁵ , Gln ⁹]-Ang I (<i>Triakis scyllia</i>) ⁴ | 0.08 |

1- Yamaguchi (1981); 2- Takemoto *et al.* (1983); 3- Hasegawa *et al.* (1983); 4- Takei *et al.* (1993)

Figure 4.1 (a) Reverse-phase HPLC chromatogram of standard ligands. The dotted line shows linear gradient elution from 10 to 60% CH₃CN in 0.1% TFA. Flow rate was 1 ml min⁻¹. Arrows indicate elution positions of [Asn¹, Val⁵]-Ang II and [Asn¹, Val⁵, Asn⁹]-Ang I. (b) Elution pattern of immunoreactive [Asn¹, Val⁵]-Ang II from extracted *Oncorhynchus mykiss* plasma submitted to reverse-phase HPLC. Elution conditions as in (a). Fraction size was 1 ml tube⁻¹. (c) Standard curve for radioimmunoassay of [Asn¹, Val⁵]-Ang II and serial dilution curve of extracted *O. mykiss* plasma.

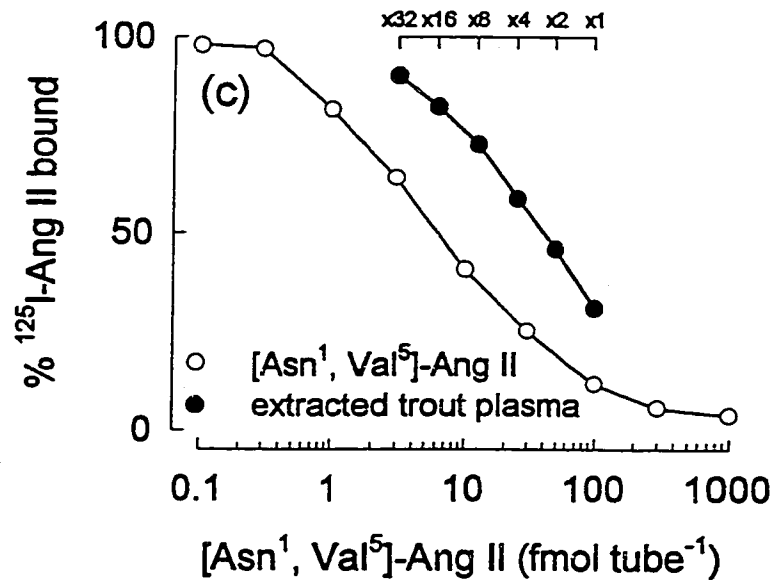
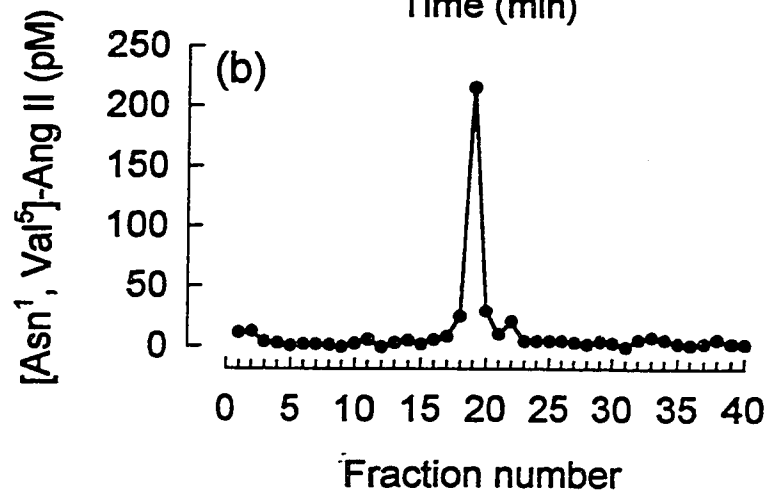
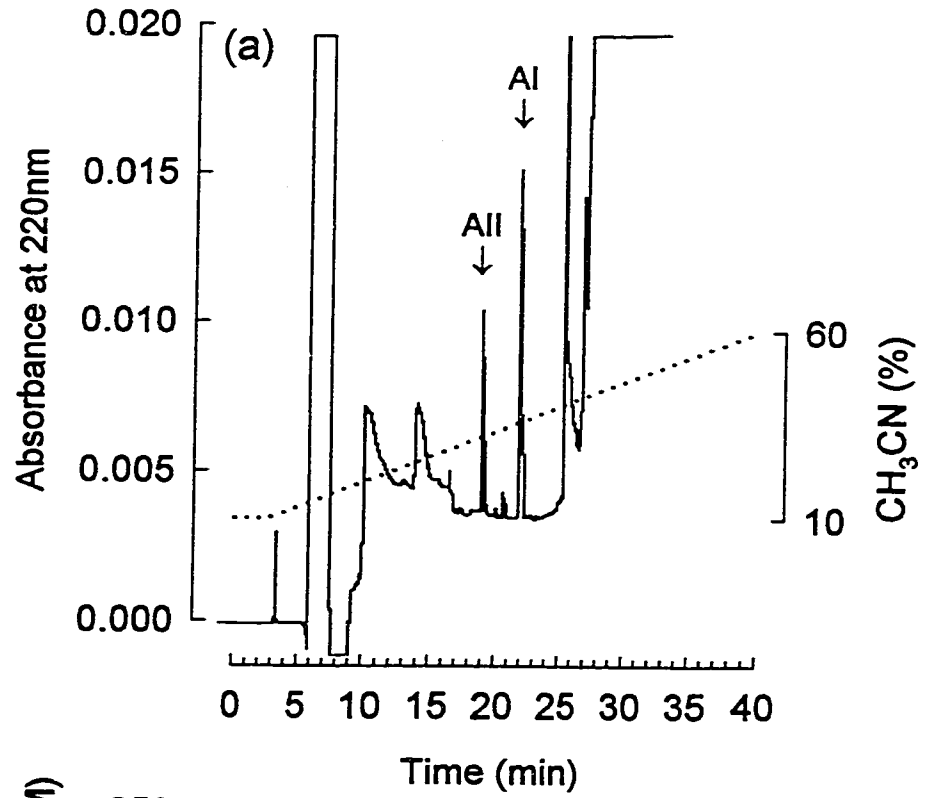


Figure 4.1

Figure 4.2 Time-course of changes in mean dorsal aortic pressure (P_{DA} , cm H₂O) in rainbow trout administered with either (a) 0.9% NaCl (control; $N=7$), (b) the smooth muscle relaxant papaverine (10 mg kg⁻¹; $N=8$), (c) papaverine (10 mg kg⁻¹) after pre-treatment with the α -adrenoceptor antagonist, phenoxybenzamine ($N=6$), (d) the angiotensin-converting enzyme inhibitor, lisinopril (10⁻⁴ mol kg⁻¹; $N=8$), (e) lisinopril (10⁻⁴ mol kg⁻¹) followed by papaverine (10 mg kg⁻¹; $N=8$), or (f) lisinopril (10⁻⁴ mol kg⁻¹) followed by papaverine (10 mg kg⁻¹) and by a bolus of catecholamines (4 nmol kg⁻¹ noradrenaline bitartrate and 40 nmol kg⁻¹ adrenaline bitartrate; $N=7$). The time during which papaverine was injected is shown by a gray box in graphs (b), (c), (e), and (f). The time during which lisinopril was injected is shown by a hatched bar in graphs (d), (e), and (f). The solid line in (f) indicates injection of catecholamines. For a given treatment, an asterisk denotes a significant difference from the 0 min value in graphs (a), (b), and (c), and from the -10 min value in graphs (d), (e), and (f). † denotes a significant difference from the corresponding value in the papaverine-treated (b) animals ($P<0.05$). Values are means \pm 1 S.E.M.

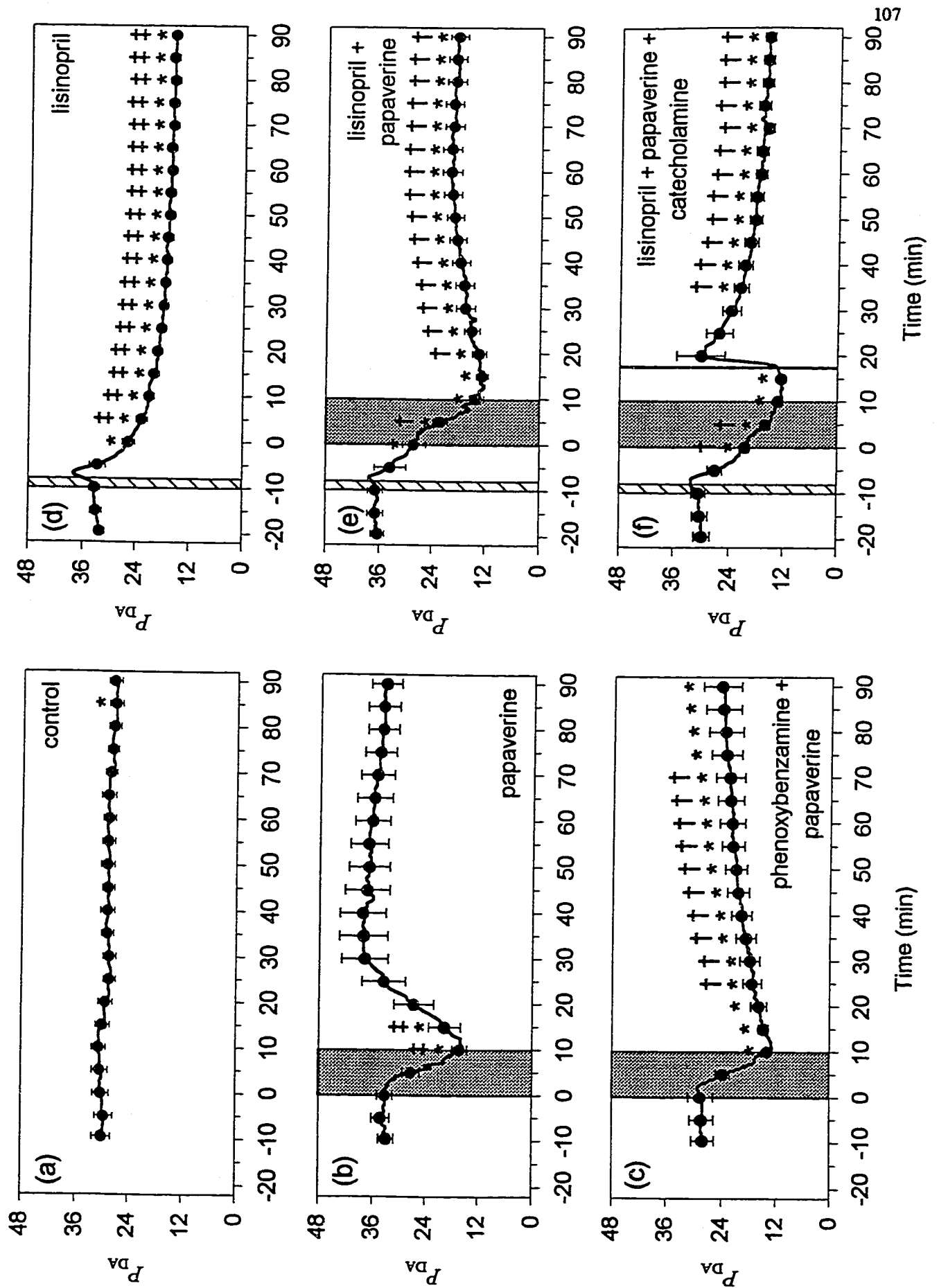


Figure 4.2

Figure 4.3 Time-course of changes in systemic vascular resistance (R_s , cm H₂O ml⁻¹ min⁻¹ kg⁻¹) in rainbow trout administered with either (a) 0.9% NaCl (control; $N=7$), (b) the smooth muscle relaxant papaverine (10 mg kg⁻¹; $N=8$), (c) papaverine (10 mg kg⁻¹) after pre-treatment with the α -adrenoceptor antagonist, phenoxybenzamine ($N=6$), (d) the angiotensin-converting enzyme inhibitor, lisinopril (10⁻⁴ mol kg⁻¹; $N=8$), (e) lisinopril (10⁻⁴ mol kg⁻¹) followed by papaverine (10 mg kg⁻¹; $N=8$), or (f) lisinopril (10⁻⁴ mol kg⁻¹) followed by papaverine (10 mg kg⁻¹) and by a bolus of catecholamines (4 nmol kg⁻¹ noradrenaline bitartrate and 40 nmol kg⁻¹ adrenaline bitartrate; $N=7$). The time during which papaverine was injected is shown by a gray box in graphs (b), (c), (e), and (f). The time during which lisinopril was injected is shown by a hatched bar in graphs (d), (e), and (f). The solid line in (f) indicates injection of catecholamines. For a given treatment, an asterisk denotes a significant difference from the 0 min value in graphs (a), (b), and (c), and from the -10 min value in graphs (d), (e), and (f). † denotes a significant difference from the corresponding value in the papaverine-treated (b) animals ($P<0.05$). Values are means \pm 1 S.E.M.

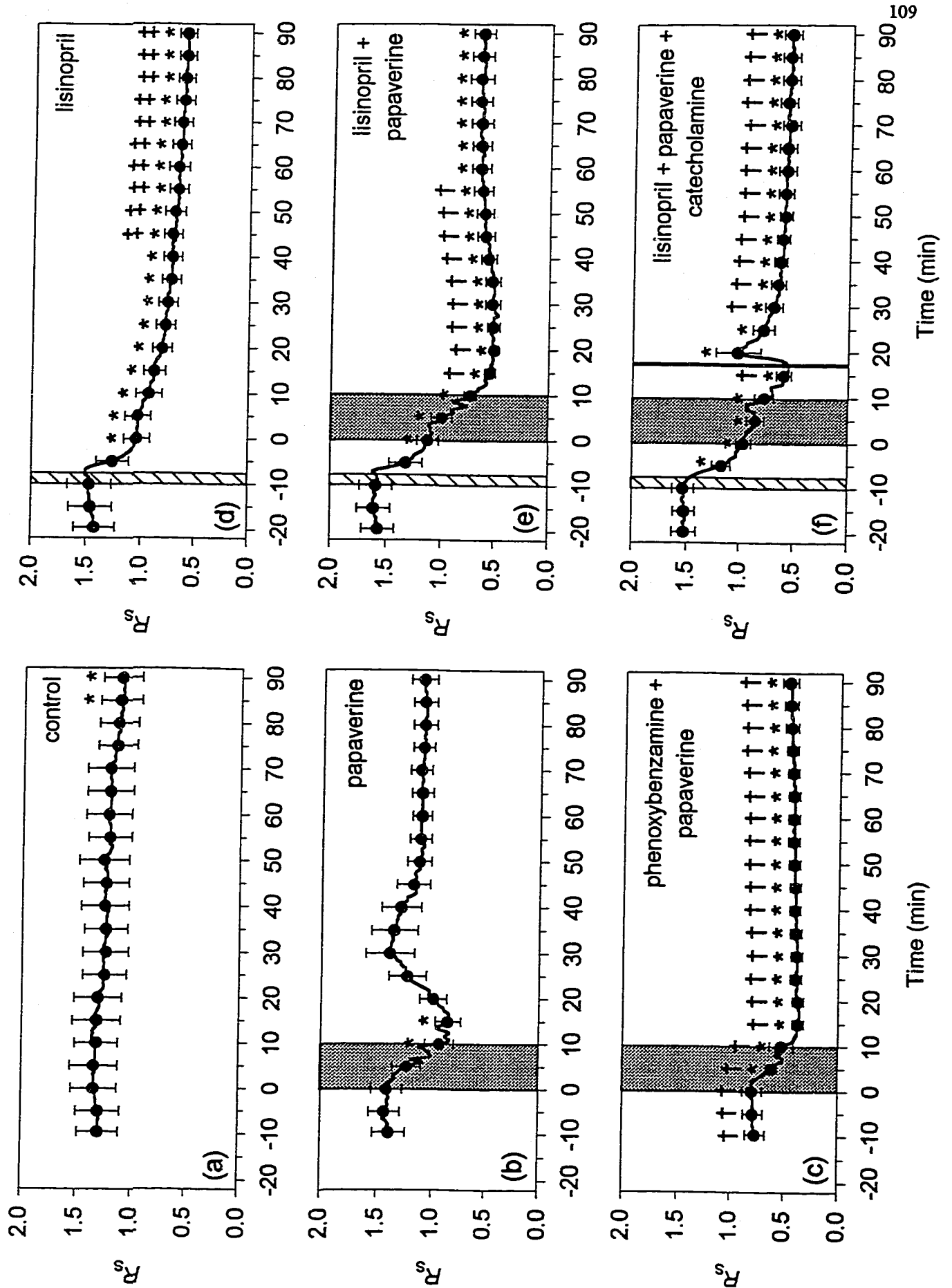


Figure 4.3

Figure 4.4 Time-course of changes in cardiac output (Q , ml min⁻¹ kg⁻¹) in rainbow trout administered with either (a) 0.9% NaCl (control; $N=7$), (b) the smooth muscle relaxant papaverine (10 mg kg⁻¹; $N=8$), (c) papaverine (10 mg kg⁻¹) after pre-treatment with the α -adrenoceptor antagonist, phenoxybenzamine ($N=6$), (d) the angiotensin-converting enzyme inhibitor, lisinopril (10⁻⁴ mol kg⁻¹; $N=8$), (e) lisinopril (10⁻⁴ mol kg⁻¹) followed by papaverine (10 mg kg⁻¹; $N=8$), or (f) lisinopril (10⁻⁴ mol kg⁻¹) followed by papaverine (10 mg kg⁻¹) and by a bolus of catecholamines (4 nmol kg⁻¹ noradrenaline bitartrate and 40 nmol kg⁻¹ adrenaline bitartrate; $N=7$). The time during which papaverine was injected is shown by a gray box in graphs (b), (c), (e), and (f). The time during which lisinopril was injected is shown by a hatched bar in graphs (d), (e), and (f). The solid line in (f) indicates injection of catecholamines. For a given treatment, an asterisk denotes a significant difference from the 0 min value in graphs (a), (b), and (c), and from the -10 min value in graphs (d), (e), and (f). † denotes a significant difference from the corresponding value in the papaverine-treated (b) animals ($P<0.05$). Values are means \pm 1 S.E.M.

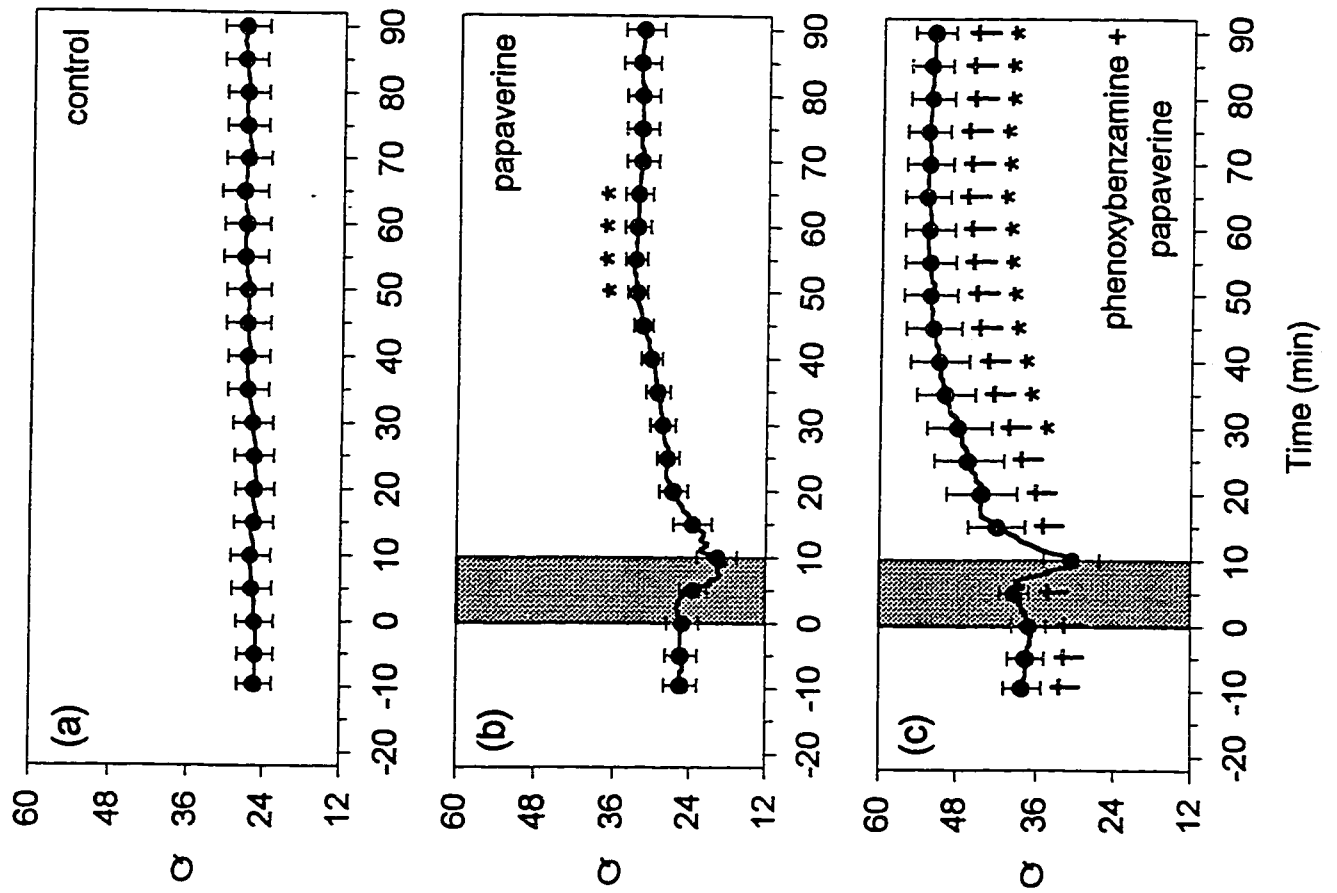


Figure 4.4

Figure 4.5 Time-course of changes in plasma catecholamines, adrenaline (unfilled bars) and noradrenaline (filled bars; nM), in rainbow trout administered with either (a) 0.9% NaCl (control; $N=7$), (b) the smooth muscle relaxant papaverine (10 mg kg^{-1} ; $N=8$), (c) papaverine (10 mg kg^{-1}) after pre-treatment with the α -adrenoceptor antagonist, phenoxybenzamine ($N=6$), (d) the angiotensin-converting enzyme inhibitor, lisinopril ($10^{-4} \text{ mol kg}^{-1}$; $N=8$), (e) lisinopril ($10^{-4} \text{ mol kg}^{-1}$) followed by papaverine (10 mg kg^{-1} ; $N=8$), or (f) lisinopril ($10^{-4} \text{ mol kg}^{-1}$) followed by papaverine (10 mg kg^{-1}) and by a bolus of catecholamines (4 nmol kg^{-1} noradrenaline bitartrate and 40 nmol kg^{-1} adrenaline bitartrate; $N=7$). Saline, papaverine and lisinopril injections were carried out prior to the 10 min sampling time. In (f) the injection of catecholamines took place at 17.5 min. For a given treatment, an asterisk denotes a significant difference from the 0 min value in graphs (a), (b), and (c), and from the -10 min value in graphs (d), (e), and (f). † denotes a significant difference from the corresponding value in the papaverine-treated (b) animals ($P<0.05$). Values are means + 1 S.E.M.

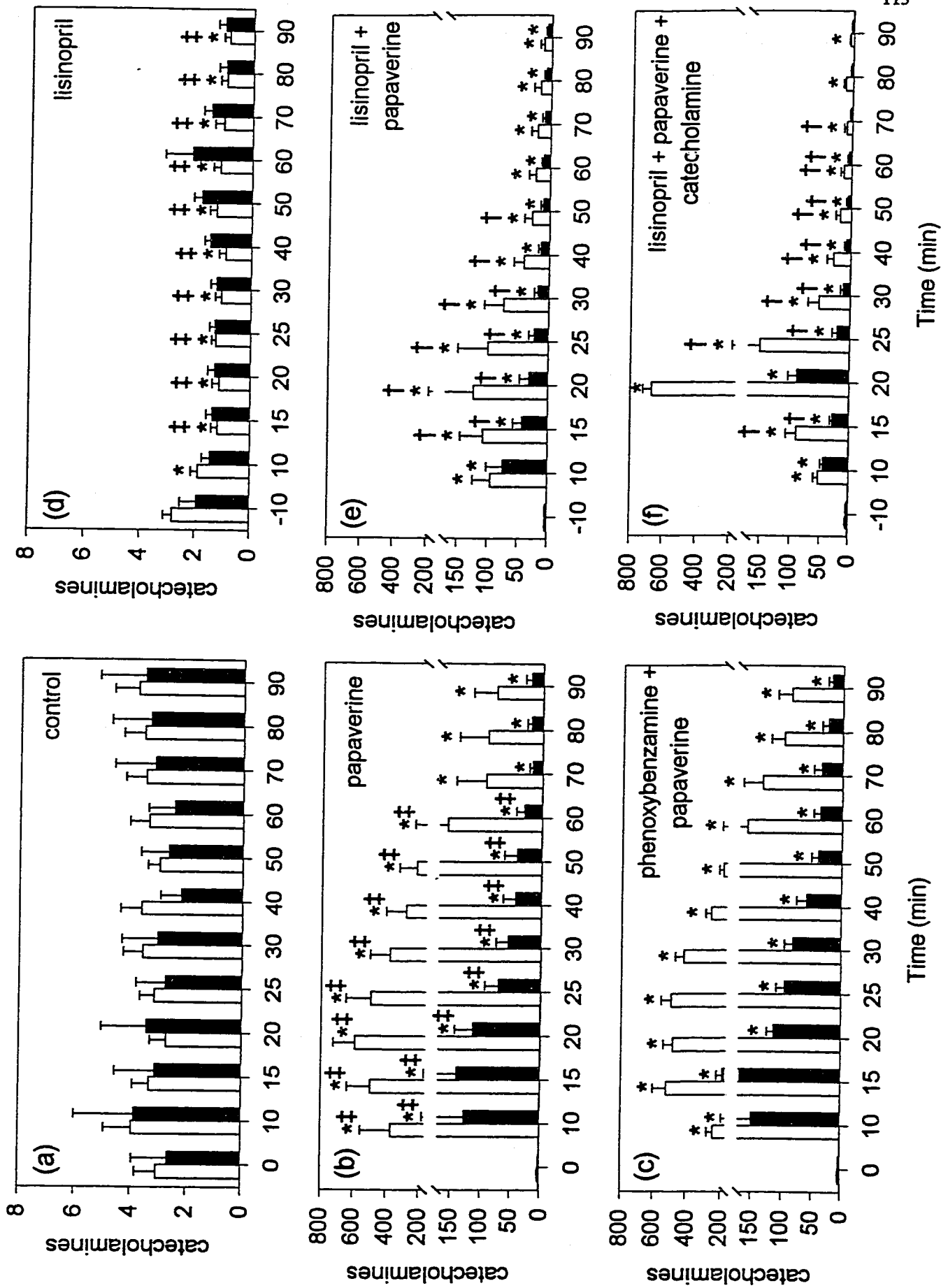


Figure 4.5

Figure 4.6 Time-course of changes in plasma [Asn¹-Val⁵]-Ang II (pM) in rainbow trout administered with either (a) 0.9% NaCl (control; *N*=7), (b) the smooth muscle relaxant papaverine (10 mg kg⁻¹; *N*=7), (c) papaverine (10 mg kg⁻¹) after pre-treatment with the α -adrenoceptor antagonist, phenoxybenzamine (*N*=7), (d) the angiotensin-converting enzyme inhibitor, lisinopril (10⁻⁴ mol kg⁻¹; *N*=7), or (e) lisinopril (10⁻⁴ mol kg⁻¹) followed by papaverine (10 mg kg⁻¹; *N*=7). Saline, papaverine and lisinopril injections were carried out prior to the 10 min sampling time. For a given treatment, an asterisk denotes a significant difference from the 0 min value in graphs (a), (b), and (c), and from the -10 min value in graphs (d), and (e). † denotes a significant difference from the corresponding value in the papaverine-treated (b) animals (*P*<0.05). Values are means + 1 S.E.M.

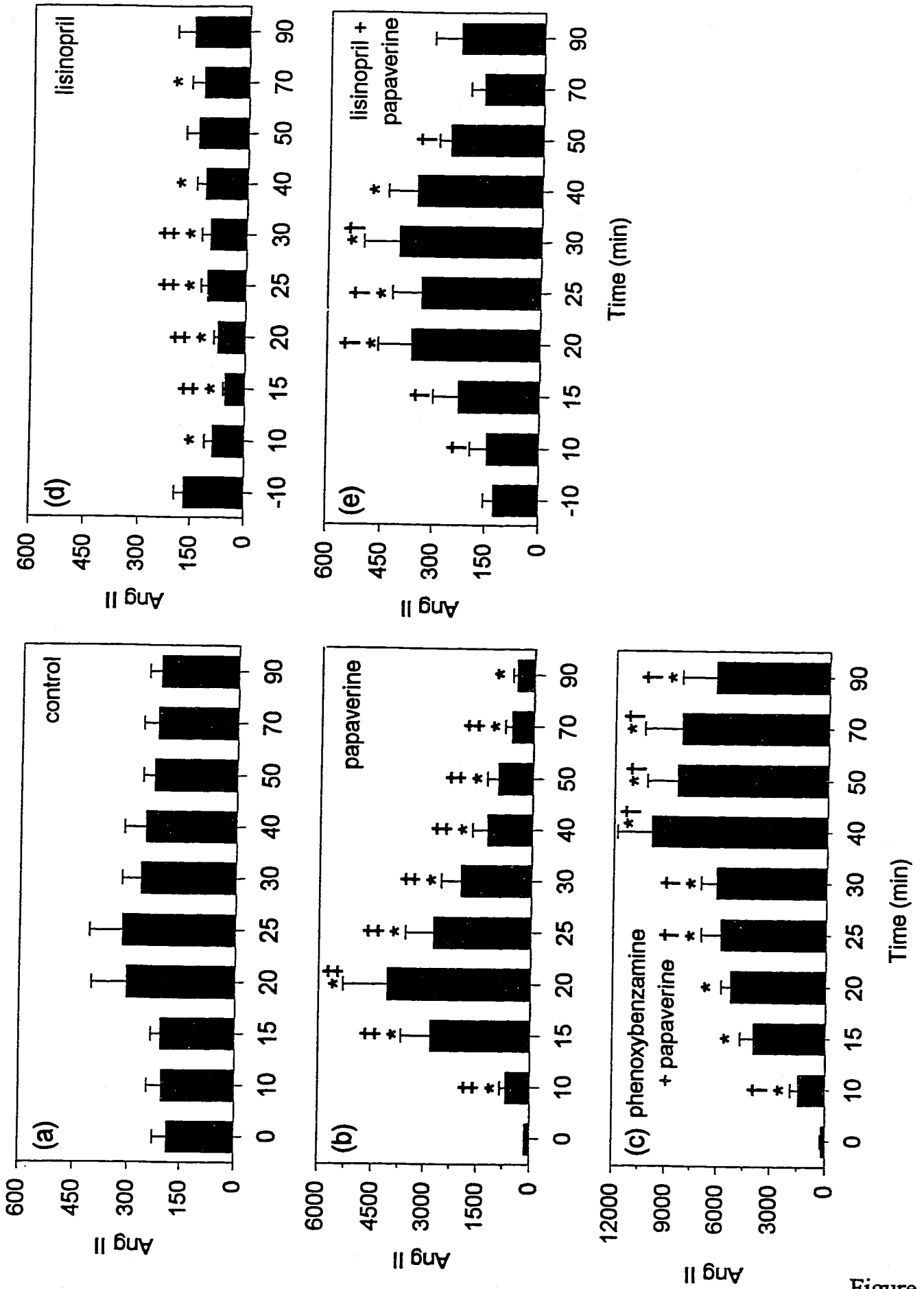


Figure 4.6

noradrenaline concentrations, ACE blockade was characterized by chronic decreases in basal plasma adrenaline (from 2.8 ± 0.3 to 1.1 ± 0.2 nM after 25 min; Fig. 4.5D) and plasma Ang II concentrations (from 166 ± 28 to 55 ± 6 pM after 25 min; Fig. 4.6D).

Papaverine injection in lisinopril-treated fish elicited rapid and sustained decreases in P_{DA} (from 36.8 ± 1.7 to 12.6 ± 1.4 cm H₂O after 25 min; Fig. 4.2E) and R_S (from 1.6 ± 0.2 to 0.5 ± 0.0 cm H₂O ml⁻¹ min⁻¹ kg⁻¹ after 30 min; Fig. 4.3E), and a slower increase in Q (from 24.5 ± 2.3 to 34.1 ± 4.0 ml min⁻¹ kg⁻¹ after 60 min; Fig. 4.4B). The combined lisinopril + papaverine treatment also produced sustained increases in plasma adrenaline (from 2.3 ± 0.9 to 123.5 ± 66.2 nM after 30 min; Fig. 4.5E) and noradrenaline concentrations (from 2.2 ± 0.6 to 73.8 ± 27.4 nM after 20 min; Fig. 4.5E), and a temporary increase in plasma Ang II concentration (from 124 ± 29 to 400 ± 100 pM after 40 min; Fig. 4.6E). Overall, while the papaverine-elicited decreases in P_{DA} and R_S in the lisinopril-treated trout lasted longer and were more pronounced than in the papaverine treatment, the increases in plasma catecholamines and Ang II concentrations in the lisinopril + papaverine treatment were significantly less than those observed in the papaverine treatment.

In the lisinopril + papaverine + catecholamine treatment, changes in P_{DA} (Fig. 4.2F), R_S (Fig. 4.3F), Q (Fig. 4.4F), and plasma adrenaline and noradrenaline concentrations (Fig. 4.5F) prior to the bolus injection of catecholamines were similar to those observed in the lisinopril + papaverine treatment. The ensuing catecholamine injection temporarily increased plasma adrenaline (from 89.6 ± 18.0 to 663.7 ± 56.6 nM) and noradrenaline (from 28.6 ± 4.4 to 89.1 ± 15.5 nM) concentrations to levels comparable to those obtained in the papaverine treatment. This increase in circulating catecholamines was associated with a recovery in P_{DA} , a transient increase in R_S , and a faster increase in Q than the one observed in the lisinopril + papaverine treatment. However, as the catecholamine concentrations decreased once again to levels significantly lower

than those recorded in the papaverine treatment, there were parallel and sustained decreases in P_{DA} and R_S .

Discussion

Results from the present study show that the RAS and the humoral component of the SNS are both recruited during an acute hypotensive stress in rainbow trout and play important roles in the compensatory response to hypotension. However, whereas blockade of the RAS prolonged a papaverine-induced hypotension and significantly reduced the recruitment of plasma catecholamines, inhibition of α -adrenoceptors impeded the recovery of blood pressure despite higher concentrations of plasma Ang II. Additional support for the importance of plasma catecholamines to blood pressure recovery following a hypotensive stress is provided by the observation that exogenous supplementation of plasma catecholamine concentrations prevents the chronic hypotensive effects of RAS blockade in papaverine-treated trout. This study provides therefore the first evidence that the acute humoral adrenergic stress response contributes to cardiovascular control during a hypotensive stress in fish, and suggests that a significant component of the RAS compensatory response to hypotension is mediated via interactions with the SNS.

As indicated by a marked increase in plasma Ang II concentrations, the RAS of rainbow trout is activated following treatment with the smooth muscle relaxant papaverine. This result corroborates previous observations that papaverine treatment in fish can increase plasma renin activity (Nishimura *et al.* 1979) and plasma Ang II (Tierney *et al.* 1995b), and supports the general contention that the RAS of teleosts can be activated by a reduction in blood pressure (Olson 1992). Relative to fish treated only with papaverine, the prolonged recovery of P_{DA} in

ACE-blocked and papaverine-treated trout (this study), eels (*Anguilla anguilla*; Tierney *et al.* 1995b), and flounder (*Platichthys flesus*; Perrott and Balment 1990), highlights the importance of an increase in plasma Ang II to blood pressure restoration following a hypotensive stress. The dipsogenic and antidiuretic effects of Ang II in fish (Tierney *et al.* 1995b; Kobayashi and Takei 1996; Brown and Balment 1997), and the contribution of Ang II to R_s in trout (Olson *et al.* 1994; Bernier and Perry 1999), all suggest that the RAS may contribute directly to blood pressure regulation during hypotensive conditions. On the other hand, given that Ang II does not have any inotropic or chronotropic effect on the *in situ* perfused heart of trout (Olson *et al.* 1994), the RAS is unlikely to contribute directly to the increase in Q after papaverine treatment.

The efficacy of lisinopril as an ACE inhibitor in the present study is similar to that previously observed for ACE inhibitors both in fish (Tierney *et al.* 1995b) and mammals (Campbell 1996). The incomplete inhibition of Ang II formation despite ACE blockade in the combined lisinopril + papaverine treatment has previously been observed using a similar experimental protocol in *Anguilla anguilla* (Tierney *et al.* 1995b). Mammalian studies have shown that tissue-specific differences in the efficacy of ACE inhibitors, alternative pathways of Ang II formation, and marked elevation of circulating renin and Ang I levels may all contribute to the incomplete inhibition of ACE following clinical doses of ACE inhibitors (Campbell 1996). In fish, as in mammals (Campbell 1996), renin secretion by the kidney is subject to negative feedback regulation by Ang II (Bailey and Randall 1981). Therefore, ACE inhibitors result in increased renin secretion and increased angiotensin peptide formation.

The hypotensive effects of papaverine, in addition to stimulating the RAS, resulted in a marked increase in the circulating concentrations of plasma catecholamines. In fact, relative to other studies that monitored plasma catecholamines following various types of stress (Randall and

Perry 1992; Thomas and Perry 1992), the magnitude of the increase in plasma catecholamines following papaverine treatment suggests that hypotension may be one of the most potent activators of the humoral adrenergic stress response in fish. Because overflow from peripheral nerve terminals does not appear to significantly contribute to the elevation of plasma catecholamine levels in fish (Perry *et al.* 1991), the increase in plasma catecholamines following an acute hypotensive stress most likely originates from the chromaffin tissue in the head kidney. Whereas catecholamine secretion from the chromaffin tissue in fish is generally associated with an acute reduction in blood oxygen content (Randall and Perry 1992; Reid *et al.* 1998), this study provides evidence that acute hypotension can result in an activation of the humoral adrenergic stress response independent of blood oxygen status. Previous experiments have demonstrated that increases in circulating plasma adrenaline concentrations of equivalent or smaller magnitude than those observed in the papaverine-treated fish can significantly contribute to cardiovascular control in *O. mykiss* by elevating P_{DA} , R_S , and Q (Gamperl *et al.* 1994a; Bernier and Perry 1999). In support of these results, the present study showed that exogenous catecholamine supplementation in the lisinopril + papaverine treated fish leads to a temporary recovery in P_{DA} and R_S that is similar to the recovery experienced by fish treated with papaverine only. Overall, despite a pronounced increase in Q and plasma Ang II levels, the blunted P_{DA} recovery and chronic depression in R_S following papaverine treatment in α -adrenoceptor blocked fish suggest that the SNS plays a key role in the compensatory response of rainbow trout to a hypotensive stress.

In support of previous investigations which established Ang II as a secretagogue of catecholamine release in rainbow trout (Bernier and Perry 1997, 1999), the current results suggest that an endogenous increase in plasma Ang II can stimulate the secretion of

catecholamines from the chromaffin tissue. While papaverine treatment elicited parallel increases in plasma Ang II and catecholamine concentrations, ACE blockade prior to papaverine treatment attenuated the increase in the plasma concentrations of Ang II, adrenaline, and noradrenaline by 90, 79, and 40%, respectively. The preferential inhibition of plasma adrenaline with ACE blockade in papaverine-treated fish suggests that Ang II may be a more potent secretagogue of adrenaline secretion than noradrenaline secretion. This is consistent with the much greater increase in plasma adrenaline than noradrenaline following papaverine treatment, and with previous observations showing that bolus injections of Ang II, either *in situ* or *in vivo*, elicit a preferential secretion of adrenaline in fish (Carroll and Opdyke 1982; Bernier and Perry 1997, 1999). Although the increase in plasma Ang II was accompanied by increases in plasma catecholamines in the papaverine-treated fish, significantly higher and sustained concentrations of Ang II in the phenoxybenzamine + papaverine treatment did not result in higher plasma catecholamine levels. These results contrast with the previously observed Ang II-mediated dose-dependent release of catecholamines from the chromaffin tissue of trout (Bernier and Perry 1997, 1999), and suggest either a potential desensitization of the catecholamine secretion process following chronic stimulation by Ang II or an inhibitory influence of α -adrenoceptor blockade on catecholamine release from the chromaffin tissue. While desensitization of catecholamine release from the chromaffin tissue of trout has been observed previously following repeated stress (Reid *et al.* 1994; Perry *et al.* 1996), the inhibitory effects of α -adrenoceptor blockade on catecholamine release in *Anguilla rostrata* (Abele *et al.* 1998) have yet to be investigated in trout.

Although Ang II can directly elicit catecholamine secretion from the chromaffin tissue in both fish (Bernier and Perry 1997) and mammals (Reid 1992), Ang II can also stimulate the adrenal medullary response via the central nervous system in the latter (Corwin *et al.* 1985; Reid

1992). Because Ang II has known central actions in trout via specific Ang II binding sites (Pamantung *et al.* 1997), it is possible that at least part of the increase in plasma catecholamines following hypotension is mediated via central effects of Ang II. In addition, because ACE blockade was associated only with a partial reduction of the hypotension-elicited increase in plasma catecholamines, secretagogues other than Ang II are likely involved in stimulating catecholamine release during a hypotensive stress in trout. While stimulation of catecholamine release via pre-ganglionic cholinergic fibers is one such possibility, a variety of non-cholinergic secretagogues may also play a role in the overall control of catecholamine release following an acute stress in teleosts (Reid *et al.* 1988).

The RAS, in addition to stimulating catecholamine release from the chromaffin tissue, is known to interact with the SNS of vertebrates at the level of the sympathetic nerves (Wilson 1984b; Reid 1992). In rainbow trout, approximately 40% of the vasoconstrictory effect of Ang II in a perfused trunk preparation is abolished by the adrenergic nerve toxin bretylium (Olson *et al.* 1994). Thus, it is likely that a portion of the Ang II pressor response is mediated via sympathetic nerves. Similarly, perfusion experiments in the Atlantic cod (*Gadus morhua*) also suggest that Ang II may modulate sympathetic nerves by facilitating the release of catecholamines (Platzack 1995). In the present study, although we did not specifically assess the possible interactions between the RAS and sympathetic nerves in trout, the reduction in basal adrenaline levels observed with ACE inhibition suggest that the RAS may tonically stimulate the release of adrenaline from sympathetic nerves and / or from the chromaffin tissue. While the specific effects of Ang II on sympathetic nerves have yet to be assessed in rainbow trout, we have previously observed that ACE inhibition lowers the basal release of both adrenaline and noradrenaline secretion from *in situ* head kidney preparations of *O. mykiss* (Bernier and Perry 1997). Because

basal plasma adrenaline levels may be an important source of neuronal catecholamine uptake and may be required to sustain neuronal sympathetic tone (Xu and Olson 1993b), our results suggest that the RAS may modulate sympathetic nerve activity indirectly via a tonic stimulation of catecholamine release from the chromaffin tissue. Hence, in addition to the direct role of Ang II in maintaining systemic arteriolar resistance (Olson *et al.* 1994; Zhang *et al.* 1995), a number of different interactions between the RAS and the SNS may explain, at least in part, the hypotensive effects of ACE blockade in resting trout.

The chronic hypotensive effects of ACE blockade observed in this study concur with similar observations in rainbow trout (Olson *et al.* 1997) and other teleosts (Olson 1992; Platzack *et al.* 1993; Tierney *et al.* 1995b). Unlike other hypotensive treatments (e.g. papaverine, phenoxybenzamine), ACE blockade in trout is not associated with a reflexive increase in Q (this study; Olson *et al.* 1997). The absence of a compensatory increase in Q to stabilize blood pressure following ACE blockade is a characteristic feature of ACE inhibitor therapy in mammals (Squire and Reid 1992). Although the mechanisms involved in the cardiac response to ACE blockade remain unclear, the available data in mammals suggest that ACE inhibition reduces sympathetically mediated baroreflex responses while augmenting those that are parasympathetically mediated (Squire and Reid 1992). In trout, given that intracerebroventricular injections of Ang II appear to stimulate heart rate via inhibitory effects on cardiac vagal motor neuron activity (Le Mével *et al.* 1994; Pamantung *et al.* 1997), the cardiac effects of ACE blockade may be mediated by similar mechanisms as in mammals.

ACE blockade, in addition to preventing the conversion of Ang I to Ang II, also inhibits the inactivation of the biologically active component of the kallikrein kinin system (KKS), bradykinin (Olson 1992). Therefore, lisinopril treatment in trout may be expected to increase

plasma bradykinin concentrations. However, unlike the potent depressor action of bradykinin in mammals, kinins are pressor in fish (Olson 1992; Conlon *et al.* 1996a). Hence, although the physiological functions of the KKS in fish are unclear (Olson 1992), an ACE inhibitor-elicited increase in circulating bradykinins should in theory counteract the hypotensive effects of blocking the RAS.

While previous studies have shown that exogenous Ang II can elicit an increase in plasma catecholamines in fish (Carroll and Opdyke 1982; Bernier and Perry 1999), we have provided evidence that a hypotension-induced endogenous increase in plasma Ang II can quickly stimulate the release of large quantities of catecholamines. Furthermore, our data suggests that resting levels of plasma Ang II may assist in the regulation of basal catecholamine secretion from the chromaffin tissue of trout. While the physiological significance of maintaining circulating catecholamine levels for blood pressure regulation has previously been addressed (Xu and Olson 1993b), the marked increase in plasma catecholamines associated with the acute hypotensive stress of this study may only be relevant during conditions of acute hemorrhage which are life-threatening. Whether recruitment of the RAS under less severe physiological conditions e.g. transfer to a hyperosmotic environment (Kobayashi *et al.* 1980) or exercise-induced hypotension (Platzack *et al.* 1993) also involves interactions with the SNS should be addressed in future investigations. It should also be noted that whereas Ang II stimulates catecholamine release in teleosts, there is no evidence for the opposite effect. Unlike the situation in mammals where the adrenergic nervous system plays a role in the control of renin release (Kobayashi and Takei 1996), catecholamines do not contribute to renin release from the juxtaglomerular cells in teleosts (Bailey and Randall 1981; Nakamura *et al.* 1992).

Overall, the role of the RAS in mediating the physiological response to a hypotensive stress in trout is akin to the critical role of the mammalian RAS in the defense against hemorrhagic hypotension (Robertson 1992). In mammals, this defense is not only achieved via the direct pressor effect of Ang II and its interactions with the neuronal and humoral components of the SNS, but also through an Ang II stimulation of aldosterone and possibly vasopressin (Robertson 1992). While aldosterone is typically absent in fish (Kobayashi and Takei 1996), the circulating concentrations of arginine vasotocin (the homologue of mammalian vasopressin) increase following hemorrhage or Ang II injections in fresh water eels (Henderson *et al.* 1985), and papaverine treatment in flounder (Brown and Balment 1997). Because arginine vasotocin is a potent vasopressor and antidiuretic hormone in fish (Le Mével *et al.* 1993; Brown and Balment 1997; Conklin *et al.* 1997), it may also make a significant contribution to the restoration of blood pressure following a hypotensive stress in trout. In addition, a number of other vasoactive factors secreted from the endothelial cells of the vasculature, e.g. endothelins (Olson *et al.* 1991), may also be recruited in response to stress and contribute to cardiovascular homeostasis. Therefore, despite their key role in blood pressure regulation, the physiological effects of Ang II and plasma catecholamines in cardiovascular homeostasis should not be viewed in isolation.

In conclusion, we have presented evidence for an Ang II-mediated secretion of plasma catecholamines following an acute hypotensive stress in rainbow trout. Physiological significance of this interaction is suggested by the finding that ACE blockade prior to a hypotensive treatment substantially reduces the contribution of the chromaffin tissue and prevents restoration of blood pressure. Reliance of the RAS on the SNS for full expression of its pressor attributes is also suggested by the observations that α -adrenoceptor blockade prevents recovery from hypotension

despite elevated Ang II levels, and that exogenous catecholamine supplementation supports blood pressure recovery in ACE-blocked trout.

CHAPTER 5

**Blood pressure regulation during hypotension in two teleost species:
differential involvement of the renin-angiotensin and sympathetic nervous systems**

Summary

The stimulatory effects of angiotensin II (Ang II) on catecholamine release, and the contributions of the renin angiotensin system (RAS), humoral catecholamines, and sympathetic nerves to blood pressure regulation, were investigated in rainbow trout (*Oncorhynchus mykiss*) and American eel (*Anguilla rostrata*). In trout, bolus injections of homologous [Asn¹, Val⁵]-Ang II (100 or 500 pmol kg⁻¹) increased catecholamine secretion rates and plasma catecholamine concentrations from *in situ* posterior cardinal vein preparations and chronically cannulated fish, respectively. In contrast, *in situ* or *in vivo* injections of similar doses of Ang II in eel did not affect catecholamine release. α -Adrenoceptor blockade (prazosin; 1 mg kg⁻¹) reduced the pressor effect of exogenous Ang II (500 pmol kg⁻¹) in both species. In eel, intravenous injection of the smooth muscle relaxant papaverine (10 mg kg⁻¹) elicited a rapid decrease in dorsal aortic pressure (P_{DA} ; 58%) followed by a gradual recovery back to baseline 85 min after the treatment. In trout, papaverine elicited a similar decrease in blood pressure (62%), however P_{DA} recovered fully 20 min after. Blockade of either α -adrenoceptors with prazosin or sympathetic nerves with bretylium (10 mg kg⁻¹) prior to papaverine treatment did not alter P_{DA} recovery in eel. In trout, α -adrenoceptor and sympathetic nerve blockades prior to the papaverine treatment prevented and attenuated P_{DA} recovery, respectively. In both species, papaverine treatments elicited significant increases in plasma catecholamine and Ang II concentrations. However, the increases in plasma catecholamine concentrations were markedly higher in trout than in eel. Similarly, the papaverine-elicited increase in plasma Ang II levels occurred earlier and were greater in trout than in eel. Thus, while Ang II stimulates humoral catecholamine release in trout, there is no evidence for a similar interaction in eel. Moreover, during hypotensive stress, although the RAS is recruited in both species, an essential involvement of sympathetic nerves and humoral

catecholamines to blood pressure restoration is only apparent in trout.

Introduction

The control of catecholamine release from the chromaffin tissue of fish, as in mammals (Livett and Marley 1993), may be achieved through a variety of cholinergic and non-cholinergic pathways (for review see Reid *et al.* 1998). In addition to the cholinergic control provided by the preganglionic sympathetic fibers which innervate the chromaffin tissue in the head kidney (Nilsson 1976; Nilsson *et al.* 1976), various hormones are known to stimulate catecholamine release in fish (Reid *et al.* 1998). One such humoral factor that has been implicated in the control of catecholamine release in various species is the biologically active product of the renin-angiotensin system (RAS), angiotensin II (Ang II; Opdyke *et al.* 1981b; Carroll and Opdyke, 1982; Bernier and Perry 1997; Bernier *et al.* 1999a).

In rainbow trout (*Oncorhynchus mykiss*), Ang II directly elicits catecholamine release from the chromaffin tissue via specific Ang II binding sites (Bernier and Perry 1997). In other fish species, however, although Ang II may also stimulate catecholamine release, the evidence is indirect. While exogenous Ang II injections increase plasma catecholamine concentrations in some fish (Opdyke *et al.* 1981b; Carroll and Opdyke 1982; Bernier *et al.* 1999a), in others the implication of Ang II in the control of catecholamine release is based on the observation that Ang II-elicited pressor responses are partially inhibited by α -adrenoceptor blockade (Nishimura *et al.* 1978; Nishimura 1985b; Platzack *et al.* 1993; Butler *et al.* 1995; Oudit and Butler 1995a). In the American eel (*Anguilla rostrata*), for example, although a portion of the cardiovascular effects of Ang II are indirectly mediated by catecholamines (Nishimura *et al.* 1978; Nishimura 1985b; Oudit and Butler 1995a), it is not known whether the adrenergic effects are caused by

humoral catecholamines arising from chromaffin tissue, sympathetic nerves, or both. Furthermore, marked differences in the cholinergic control of humoral catecholamine release between *O. mykiss* and *A. rostrata* (Reid and Perry 1994, 1995; Al-Kharrat *et al.* 1997; Abele *et al.* 1998; Julio *et al.* 1998), suggest that their may also be similar inter-species differences in the Ang II-mediated control of catecholamine release from the chromaffin tissue.

The physiological significance of an interaction between Ang II and catecholamine release stems from the importance of the RAS and the sympathetic nervous system (SNS) in the homeostatic regulation of blood pressure in teleosts (Olson 1992; Nilsson 1994; Bernier and Perry 1999). In *O. mykiss* and *A. rostrata*, both Ang II and catecholamines are potent vasopressors (Nishimura *et al.* 1978; Gamperl *et al.* 1994a; Oudit and Butler 1995a, 1995b; Fuentes and Eddy 1998; Bernier and Perry 1999) and thereby key effectors of cardiovascular control. During a hypotensive stress in trout, the RAS and humoral catecholamines are both recruited and play significant roles in the compensatory response to hypotension (Bernier *et al.* 1999b). However, the contribution of the RAS to blood pressure recovery in trout is largely indirect and relies on an Ang II-mediated secretion of catecholamines (Bernier *et al.* 1999b). In the European eel (*Anguilla anguilla*), the RAS plays a significant role in the compensatory response to hypotension (Tierney *et al.* 1995b). However, the contribution of the SNS to blood pressure regulation during hypotension or the significance of a potential interaction between the RAS and the SNS during such conditions have not been investigated in eel. Lastly, the relative importance of neurally-derived catecholamines to blood pressure regulation during a hypotensive stress has not been characterized in either *O. mykiss* or *A. rostrata*.

Therefore, in this study, using *O. mykiss* and *A. rostrata* in parallel treatments, we characterized the potential interactions between Ang II and catecholamines and their respective

involvement in blood pressure regulation. Specifically, we investigated 1) the direct effects of homologous [Asn¹, Val⁵]-Ang II on catecholamine release using *in situ* perfused posterior cardinal vein preparations, 2) whether exogenous Ang II injections can elicit an increase in plasma catecholamines, 3) the pressor effects of Ang II with and without α -adrenoceptor blockade, and 4) the relative contributions of endogenous Ang II, humoral catecholamines, and neuronal catecholamines to the regulation of blood pressure during a hypotensive stress.

Materials and Methods

Experimental Animals

Rainbow trout, *Oncorhynchus mykiss*, of either sex weighing between 394 and 730 g (mean mass 569.4 ± 10.2 , experimental $N = 48$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and transported in oxygenated water to the fish-holding facilities of the University of Ottawa. American eels, *Anguilla rostrata*, of either sex weighing between 149 and 487 g (mean mass 289.9 ± 11.5 , experimental $N = 48$) were obtained from a commercial supplier (Lancaster Ontario) and were transported on ice to the same destination. Both species were held indoors in large 1300 l fiberglass tanks supplied with flowing, aerated, and dechloraminated tap water. The fish were maintained at a temperature of 14°C, on a 12:12-h light-dark photoperiod cycle. While trout were fed a commercial diet of Martin trout feed grower pellets, eels were not fed.

In situ experiments

To investigate the tissue-specific effects of Ang II on catecholamine release at the level of the chromaffin cells, an *in situ* posterior cardinal vein (PCV) perfusion preparation (Reid and Perry 1994) was employed with the following modifications. Rainbow trout and American eels

were euthanized by anaesthetic overdose using 2-phenoxy-ethanol (15 ml l^{-1} ; Sigma Chemical Co., St. Louis, MI) and dissected ventral side up on ice. An incision was made from the vent to the pectoral girdle and the internal organs were pushed aside in order to expose the head kidney and to cannulate the PCV and the bulbus arteriosus. An inflow cannula (PE 160; Clay Adams) was inserted into the PCV approximately two-thirds along the length of the kidney in the anterograde direction. The body cavity was filled with lint-free wipes, and a ligature was placed around the entire fish to secure the inflow cannula. An outflow cannula (PE 160) was inserted into the bulbus arteriosus and secured in place with a ligature around the walls of the bulbus. The head kidney of the cannulated fish was then perfused with aerated modified Cortland saline (Reid and Perry 1995) with a final pH of 7.8 and 8.0 for the trout and eel preparations, respectively. Perfusion flow rates of $2\text{--}4 \text{ ml min}^{-1}$ were achieved by siphon resulting from the positive pressure difference between the saline source, positioned approximately 30 cm above the preparation, and the outflow cannula. Each preparation was perfused for 20 min before commencing an experiment. After this stabilization period, experiments were initiated by collecting a sample of outflow perfusate to assess basal catecholamine concentrations. A bolus injection ($500 \mu\text{l}$) of $[\text{Asn}^1, \text{Val}^5]\text{-Ang II}$ (Sigma) was then administered to the preparation by way of a three-way valve in the inflow cannula over the course of 1 min and five perfusate samples were collected 1, 2, 3, 4, and 5 min after the intervention. With a single dose per preparation, two doses of $[\text{Asn}^1, \text{Val}^5]\text{-Ang II}$, 100 and 500 pmol kg^{-1} , were assessed for their effects on catecholamine release in rainbow trout and American eels ($N=6$ for each dose). In each preparation, after another stabilization period of 10 min, a second control perfusate sample was collected (15 min) and the preparation was given a bolus injection ($500 \mu\text{l}$) of the cholinergic agonist carbachol ($10^{-5} \text{ mol kg}^{-1}$; Research Biochemicals International, Natick, MA)

over the course of 1 min. Immediately following the carbachol injection, perfusate samples were collected each minute for another 5 min (17, 18, 19, 20, 21 min). Carbachol has previously been shown to elicit catecholamine release from *in situ* PCV perfusion preparations of both species (Reid and Perry 1994) and was therefore used to confirm the suitability of each preparation to investigate the effects of Ang II on catecholamine release. Each perfusate sample was collected in pre-weighed vials while recording filling times, immediately placed in liquid nitrogen, and later stored at -80°C until they could be analyzed for catecholamine and Ang II concentrations. Pre- and post-sampling weights were subsequently divided by filling time to determine perfusate flow rates and catecholamine secretion rates.

In vivo experiments

Surgical procedures - Series 1

Rainbow trout were anaesthetized in an oxygenated solution of ethyl p-amino-benzoate (40 mg l^{-1} ; benzocaine; Sigma) until cessation of breathing movements. The fish were then transferred to an operating table where the gills were force ventilated with the same anaesthetic solution. To permit Ang II injections and repeated blood sampling, a lateral incision was made in the caudal peduncle below the lateral line to expose, separate from the surrounding tissue, and cannulate (PE 50) the caudal vein in the anterograde direction. The incision was closed with a running stitch and the protruding cannula was secured to the side of the trout with silk ligatures.

American eels were immersed in an anesthetic solution of ethyl p-amino-benzoate (1.6 g l^{-1}) for approximately 5 min and placed on a dissection tray without gill irrigation. An incision was made parallel to the lateral line in the caudal region to expose, clear away from the surrounding tissue, and cannulate (PE 50) the caudal vein in the anterograde direction. The incision was closed with a running stitch, and the free end of the cannula was threaded through a

small hole punctured through the skin posterior to the incision and secured with silk ligatures. After surgery, both fish species were placed into individual flow-through opaque Perspex boxes and left to recover for 24 h prior to experimentation.

Surgical procedures - Series 2 & 3

Rainbow trout and American eels were anaesthetized as above prior to surgery. In order to measure dorsal aortic blood pressure (P_{DA}) both species were equipped with a dorsal aortic polyethylene cannula. In the trout the dorsal aorta was cannulated (PE 50) according to the technique of Sovio *et al.* (1975). In the eel, a lateral incision was made immediately below and parallel to the lateral line approximately 3-4 cm behind the pectoral fin in order to expose the pneumogastric artery and dissect it free from overlying connective tissue. A cannula (PE 10) was then occlusively inserted into the pneumogastric artery and advanced anteriorly into the dorsal aorta. In addition, in order to permit drug injection and / or repeated blood sampling, the caudal vein of both species was cannulated (PE 50) as outline above in *Series 1*. After surgery, fish were placed into individual flow-through opaque Perspex boxes and left to recover for 24 h prior to experimentation. All cannulae were filled and flushed with heparinized (50 IU ml⁻¹ sodium heparin; Sigma) teleost Cortland saline.

Experimental protocol

Series 1: the effects of Ang II on plasma catecholamines

Experimental groups of six fish each were used to investigate the effects of bolus injections of saline or homologous [Asn¹, Val⁵]-Ang II on the circulating plasma catecholamine concentrations of rainbow trout and American eels. Within a given trial, after removal of a blood sample (0.3 ml) to assess basal plasma catecholamines, fish were first given a bolus injection (0.3 ml) of saline over a period of 30 sec, and the injection was followed by 0.25 ml of saline to

clear the cannula. Four more blood samples (0.3 ml) were then taken 1, 2, 3, and 5 min after the beginning of the injection. Following a 4 hour recovery interval, the same injection and sampling regime was used to assess the effects of a bolus injection (0.3 ml) of Ang II (100 pmol kg^{-1}) on each fish. Finally, after a second 4 hour recovery period, the effects of a 500 pmol kg^{-1} dose of Ang II were assessed. Throughout this experiment each blood sample was replaced by an equivalent volume of saline containing 3% bovine serum albumin (BSA), collected in a microcentrifuge tube containing 5 μl of 10% $\text{Na}_2\text{-EDTA}$, and centrifuged immediately at 10,000 g for 15 sec. The separated plasma was quick frozen in liquid nitrogen and stored at -80°C for later analysis of catecholamines.

Series 2: the vasopressor effects of Ang II with and without α -adrenoceptor blockade

Both eels ($N=6$) and trout ($N=6$) were monitored during an initial period of 30 to 60 min to assess the stability of blood pressure. Upon stabilisation, control P_{DA} was recorded for 5 min and the fish were given a bolus injection (0.3 ml) of 100 pmol kg^{-1} Ang II through the caudal vein over a period of 30 sec. The vasopressor response to the injection was monitored continuously, and following recovery of P_{DA} to control baseline level for a 1 hour period, a 500 pmol kg^{-1} dose of Ang II was injected. Once the response to this second injection recorded and a further period of recovery elapsed, each fish was slowly treated (over a 15 min period) with the α -adrenoceptor antagonist, prazosin hydrochloride (1 mg kg^{-1} ; RBI). Then, 60 min after prazosin treatment, using the same experimental protocol as above, the 100 and 500 pmol kg^{-1} Ang II injections were repeated.

The effectiveness of the prazosin treatment to achieve α -adrenoceptor blockade was tested by comparing the pressor effects of a catecholamine cocktail given before and after the prazosin injection. The catecholamine cocktail (0.1 ml kg^{-1}) was prepared in a 0.9% NaCl

solution and consisted of 2.5×10^{-5} mol l⁻¹ adrenaline and noradrenaline bitartrate (Sigma).

Series 3: the effects of hypotension on blood pressure, plasma catecholamines and Ang II

Four separate experimental groups of rainbow trout ($N=6$) and American eels ($N=6$) were used to investigate the relative contributions of humorally-derived and neurally-derived catecholamines to blood pressure regulation during a hypotensive stress. Once the stability of blood pressure established with an initial period of monitoring, control baseline P_{DA} was recorded for 10 min and fish were administered one of the following intravenous injection: (a) 0.9 % NaCl (0.4 ml kg^{-1}) over a 10 min period (control treatment), (b) the smooth muscle relaxant papaverine hydrochloride (0.4 ml kg^{-1} ; RBI) at 10 mg kg^{-1} over a 10 min period (papaverine treatment), (c) papaverine hydrochloride (0.4 ml kg^{-1}) at 10 mg kg^{-1} over a 10 min period after pre-treatment with the α -adrenoceptor antagonist prazosin hydrochloride (prazosin + papaverine treatment), and (d) papaverine hydrochloride (0.4 ml kg^{-1}) at 10 mg kg^{-1} over a 10 min period after pre-treatment with the adrenergic neuron blocking agent bretylium tosylate (Burroughs Wellcome Inc., Kirkland, P.Q.; bretylium + papaverine treatment). Each injection was followed by 0.3 ml of saline to clear the caudal vein cannula, and the effects of these treatments on P_{DA} were monitored continuously over the following 90 min. In each treatment, a blood sample (0.5 ml) was taken at the end of the initial 10 min control baseline period, as well as 10, 20, 40, 60, and 90 min into the treatment for subsequent analysis of plasma catecholamine and Ang II concentrations. Each blood sample was replaced by an equivalent volume of saline containing 3% BSA, collected and treated as in *Series 1*, and stored at -80°C for later analysis. In the prazosin + papaverine treatment, α -adrenoceptor blockade was achieved by slowly injecting (over a 15 min period) a 1 mg kg^{-1} dose of prazosin hydrochloride 60 min prior to the papaverine injection. In the bretylium + papaverine treatment, 24 hours prior to the papaverine

injection, a 10 mg kg^{-1} dose of bretylium tosylate (50 mg ml^{-1}) was diluted with saline to a final volume of 5 ml and slowly infused ($50 \text{ } \mu\text{l min}^{-1}$) with a syringe pump over a 100 min period. The effectiveness of bretylium to prevent adrenergic transmission has previously been demonstrated in trout (Campbell and Ganon 1976) and in the eel (Hipkins *et al.* 1986).

Analytical techniques

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

Perfusate and plasma Ang II concentrations were determined on extracted plasma samples and measured by radioimmunoassay (Bernier *et al.* 1999b). The antibody used in this assay exhibited 62.7% cross-reactivity with $[\text{Asn}^1, \text{Val}^5]$ -Ang II and negligible (0.1-0.2%) cross-reactivity with trout $[\text{Asn}^1, \text{Val}^5, \text{Asn}^9]$ -Ang I and eel $[\text{Asn}^1, \text{Val}^5, \text{Gly}^9]$ -Ang I (Bernier *et al.* 1999b). The dilution curve of immunoreactive Ang II in extracted *A. rostrata* and *O. mykiss* plasma were parallel to the standard curve of $[\text{Asn}^1, \text{Val}^5]$ -Ang II. The intra- and inter-assay coefficients of variation were 5.2% and 10.9%, respectively.

Dorsal aortic blood pressure (P_{DA}) was measured with a UFI model 1050BP (UFI, Morro

Bay, CA) pressure transducer that was calibrated against a static water column. Mean blood pressure was calculated as: (systolic pressure + diastolic pressure)/2.

Statistical Analysis

Data are presented as mean values \pm one standard error of the mean (S.E.M.). The statistical significance of the observed effects of an injection within a group was tested using a one-way repeated-measures analysis of variance (ANOVA). Dunnett's *post-hoc* multiple comparison test was used to compare the pre-injection control data point with values at subsequent times. Following a given injection, differences between the control and maximum response were analysed using a Student's paired *t*-test. Among various treatments, differences at a given time were determined using a one-way ANOVA followed by a Tukey test for multiple comparisons. The statistical significance of observed differences between the means of two treatments was determined by a Student's *t*-test. The significance level for all statistical tests was $P < 0.05$.

Results

In situ experiments

Bolus injections of either 100 (Fig. 5.1A) or 500 (Fig. 5.1B) pmol kg⁻¹ Ang II did not affect the basal secretion rate of either noradrenaline or adrenaline from PCV preparations of *A. rostrata*. On the other hand, the subsequent injection of carbachol (10⁻⁵ mol kg⁻¹) following either Ang II treatment elicited a temporary increase in the secretion rate of noradrenaline and adrenaline (Fig 5.1A & B). While bolus injections of either 100 or 500 pmol kg⁻¹ Ang II did not affect the secretion rate of noradrenaline from PCV preparations of *O. mykiss*, they elicited dose-dependent increases in the secretion rate of adrenaline (Fig 5.1C & D). The subsequent injection of carbachol (10⁻⁵ mol kg⁻¹) in the trout PCV preparations elicited significant increases in the

Figure 5.1 Time course of changes in catecholamine secretion rates (pmol min^{-1}), noradrenaline (unfilled bars) and adrenaline (filled bars), in *in situ* head kidney perfusion preparations of *Anguilla rostrata* (A & B) and *Oncorhynchus mykiss* (C & D) administered with either 100 ($N=6$; A & C) or 500 ($N=6$; B & D) pmol kg^{-1} angiotensin II and followed with a bolus injection of carbachol ($10^{-5} \text{ mol kg}^{-1}$; A-D). The solid and dashed lines indicate when the angiotensin II and carbachol injections were given to the preparations, respectively. An asterisk denotes a significant difference from the -1 min value. A † symbol denotes a significant difference from the 15 min value. A ‡ symbol denotes a significant difference between angiotensin II doses for a given time and species ($P<0.05$). Values are means + 1 S.E.M. Note the different Y-axis scales for panels A-B and C-D.

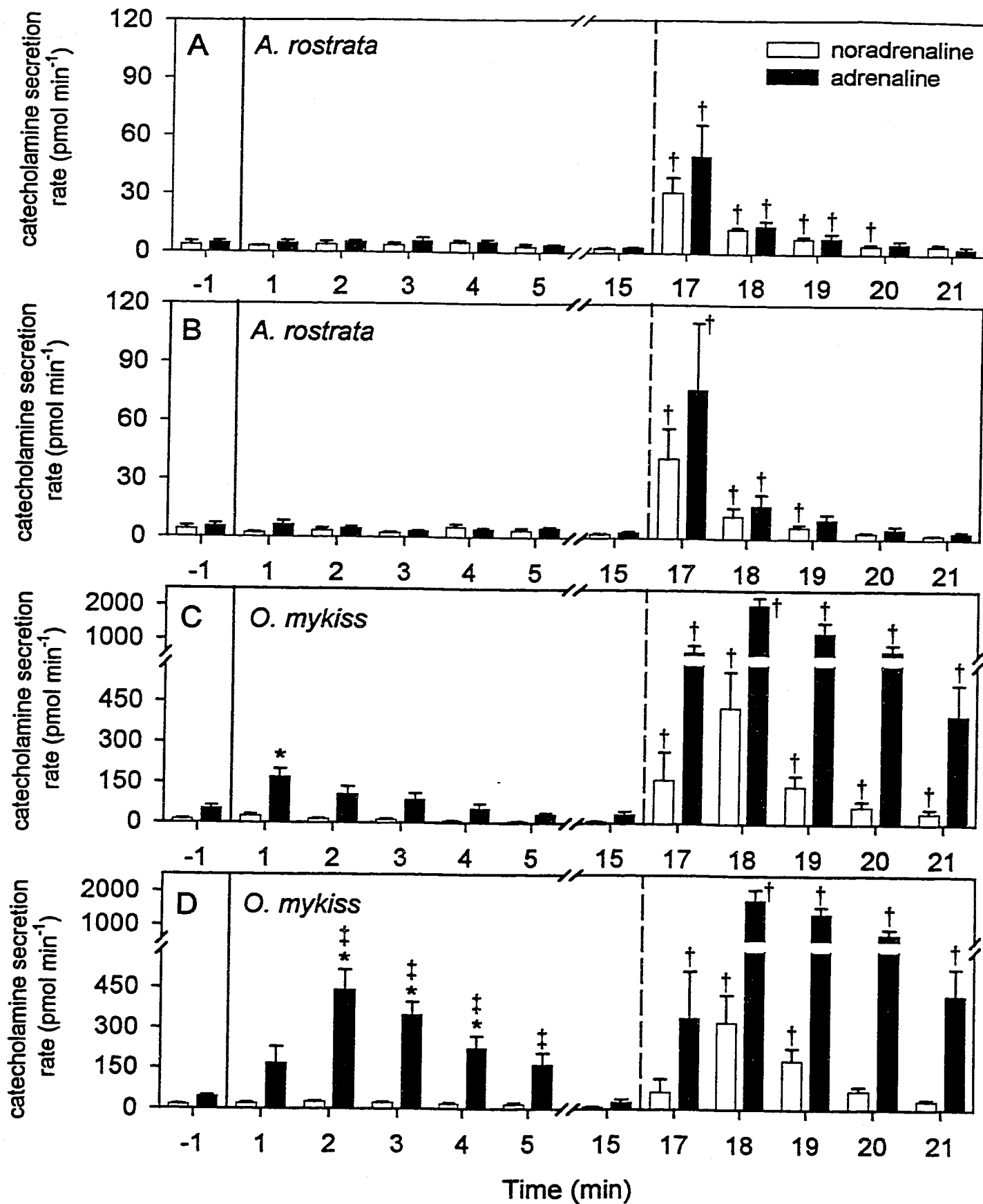


Figure 5.1

secretion rate of both catecholamines (Fig. 5.1C & D). Overall, the maximum carbachol-elicited increase in the secretion rate of noradrenaline (Nor) and adrenaline (Adr) from PCV preparations of *O. mykiss* (Nor: 432 ± 133 pmol min⁻¹; Adr: 2054 ± 233 pmol min⁻¹) were markedly higher than those recorded from preparations of *A. rostrata* (Nor: 41 ± 15 pmol min⁻¹; Adr: 76 ± 34 pmol min⁻¹).

In vivo experiments

Series 1: the effects of Ang II on plasma catecholamines

In *A. rostrata*, bolus injections of either saline, 100, or 500 pmol kg⁻¹ Ang II had no effect on the resting plasma adrenaline and noradrenaline concentrations (Table 5.1). Similarly, the injection of saline alone had no effect on the resting plasma catecholamine concentrations of *O. mykiss*. In contrast, bolus injections of 100 pmol kg⁻¹ Ang II in *O. mykiss* elicited a significant increase in plasma adrenaline, and the 500 pmol kg⁻¹ dose of Ang II elicited a significant increase in the circulating concentrations of both catecholamines. In *O. mykiss*, the Ang II-elicited increase in plasma adrenaline was dose-dependent. Overall, the resting plasma catecholamine concentrations of *A. rostrata* were significantly lower than those of *O. mykiss*.

Series 2: the vasopressor effects of Ang II with and without α -adrenoceptor blockade

While α -adrenoceptor blockade did not significantly change the resting P_{DA} of the American eels, prazosin treatment significantly lowered resting P_{DA} in the trout (Table 5.2). In both species, prazosin treatment was effective in blocking α -adrenoceptors. In response to a bolus injection of catecholamines, the increase in P_{DA} was reduced by 83 and 85% in *A. rostrata* and *O. mykiss*, respectively (Table 5.2).

Prior to prazosin treatment, bolus injections of Ang II resulted in dose-dependent increases in mean P_{DA} in *A. rostrata* and *O. mykiss* (Fig. 5.2A-D). In *A. rostrata*, the Ang II-

Table 5.1 The effects of a bolus injection of saline or angiotensin II on plasma adrenaline and noradrenaline concentrations (nmol l^{-1}) of *Anguilla rostrata* and *Oncorhynchus mykiss*.

| Treatment | N | Control | Time post-injection (min) | | | |
|--|---|----------|---------------------------|------------|------------|------------|
| | | | 1 | 2 | 3 | 5 |
| Plasma [adrenaline] of <i>Anguilla rostrata</i> | | | | | | |
| Saline | 6 | 0.6±0.1 | 0.8±0.1 | 0.5±0.1 | 0.4±0.2 | 0.7±0.1 |
| Ang II (100 pmol kg ⁻¹) | 6 | 0.5±0.2 | 0.5±0.2 | 0.6±0.2 | 0.6±0.2 | 0.5±0.2 |
| Ang II (500 pmol kg ⁻¹) | 6 | 0.6±0.2 | 0.7±0.2 | 0.6±0.3 | 0.7±0.4 | 0.5±0.3 |
| Plasma [noradrenaline] of <i>Anguilla rostrata</i> | | | | | | |
| Saline | 6 | 0.4±0.2 | 0.5±0.1 | 0.6±0.3 | 0.6±0.1 | 0.5±0.2 |
| Ang II (100 pmol kg ⁻¹) | 6 | 0.5±0.2 | 0.4±0.1 | 0.8±0.2 | 0.5±0.1 | 0.6±0.1 |
| Ang II (500 pmol kg ⁻¹) | 6 | 0.6±0.2 | 0.8±0.0 | 0.5±0.1 | 0.5±0.1 | 0.6±0.3 |
| Plasma [adrenaline] of <i>Oncorhynchus mykiss</i> | | | | | | |
| Saline | 6 | 1.6±0.5‡ | 1.8±0.6 | 1.7±0.6 | 1.9±0.6 | 1.9±0.5 |
| Ang II (100 pmol kg ⁻¹) | 6 | 1.6±0.3‡ | 2.1±0.8 | 12.5±3.0* | 12.8±1.1* | 5.7±1.5* |
| Ang II (500 pmol kg ⁻¹) | 6 | 1.3±0.2‡ | 15.2±7.6 | 40.3±5.9*† | 68.4±9.6*† | 38.9±6.4*† |
| Plasma [noradrenaline] of <i>Oncorhynchus mykiss</i> | | | | | | |
| Saline | 6 | 1.4±0.3‡ | 1.3±0.3 | 1.5±0.4 | 1.7±0.5 | 1.5±0.4 |
| Ang II (100 pmol kg ⁻¹) | 6 | 1.2±0.1‡ | 1.2±0.2 | 2.1±0.4 | 1.4±0.3 | 1.3±0.2 |
| Ang II (500 pmol kg ⁻¹) | 6 | 1.5±0.4‡ | 4.1±1.5 | 14.8±3.5* | 9.7±2.3* | 4.6±1.0* |

Values are means ± 1 S.E.M. Ang, angiotensin. Control values are the basal pre-injection plasma catecholamine concentrations. * Significantly different from control value for a given treatment. † Significant difference between Ang II doses at given time. ‡ Significantly different from control value of *A. rostrata* for a given treatment ($P < 0.05$).

Table 5.2 The effects of a bolus injection of catecholamines¹ on the dorsal aortic blood pressure of intact control and prazosin-treated *Anguilla rostrata* and *Oncorhynchus mykiss*.

| Treatment | N | P_{DA} (cm H ₂ O) | |
|-------------------|---|--------------------------------|------------------|
| | | <i>A. rostrata</i> | <i>O. mykiss</i> |
| Control | 6 | | |
| Basal values | | 29.7±1.3 | 35.7±1.7 |
| Post-injection | | 42.6±1.5* | 53.7±1.5* |
| Change from basal | | 12.9±0.7 | 18.0±1.9‡ |
| Prazosin | 6 | | |
| Basal values | | 26.3±1.7 | 28.1±2.7† |
| Post-injection | | 28.5±1.9* | 30.8±2.9* |
| Change from basal | | 2.2±0.7# | 2.7±0.5# |

1 – The catecholamine injection consisted of a 0.1 ml kg⁻¹ dose of 2.5×10⁻⁵ mol l⁻¹ noradrenaline bitartrate and adrenaline bitartrate diluted to a final volume of 100 µl with saline.

Values are means ± 1 S.E.M. * Significantly different from basal value for a given treatment. † Significantly different from basal value of the control treatment. # Significantly different from the change from basal value of the control treatment. ‡ Significantly different from the change from basal value of *A. rostrata* for a given treatment ($P < 0.05$).

Figure 5.2 Time course of changes in mean dorsal aortic pressure (P_{DA} , cm H₂O) in *Anguilla rostrata* (A & B) and *Oncorhynchus mykiss* (C & D) administered with either 100 ($N=6$; A & C) or 500 ($N=6$; B & D) pmol kg⁻¹ angiotensin II before (filled circle) and after (unfilled circle) treatment with the α -adrenoceptor antagonist, prazosin. The arrow at 0 min indicates when the bolus injection of angiotensin II was administered. Insets A-D are the angiotensin II-elicited maximum mean increases in P_{DA} before (filled bar) and after (unfilled bar) prazosin treatment. An asterisk denotes a significant difference from the control 0 min P_{DA} value for a given treatment. A § symbol denotes a significant difference from the control 0 min P_{DA} value of the 'after prazosin' treatment. The ‡ and # symbols denote significant differences in the increase in P_{DA} between the two Ang II doses for a given species and between the two fish species for a given dose, respectively. A † symbol denotes a significant difference from the increase in P_{DA} before prazosin treatment ($P<0.05$). Values are means \pm 1 S.E.M.

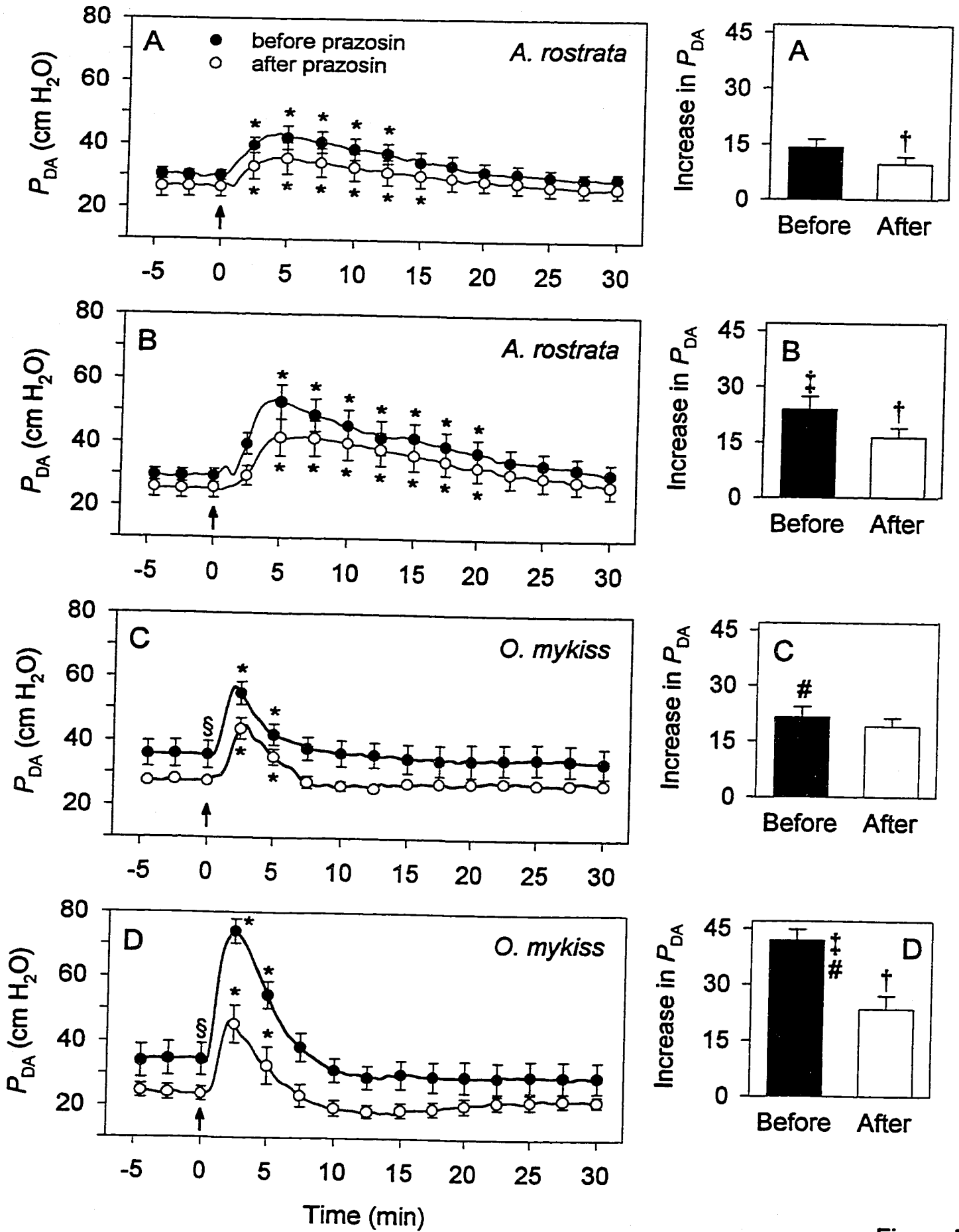


Figure 5.2

elicited increase in P_{DA} peaked approximately 4-5 min after the injection of the vasoactive hormone and remained elevated for 12.5 (100 pmol kg⁻¹ Ang II) to 20 min (500 pmol kg⁻¹ Ang II; Fig 5.2A-B). In contrast, peak P_{DA} occurred approximately 2-2.5 min after the injection of Ang II in *O. mykiss* and P_{DA} remained elevated for only 5 min before returning to resting levels (Fig. 5.2C-D). For a given Ang II dose the increase in P_{DA} was significantly greater in the trout than in the eel. In comparison to the control condition prior to α -adrenoceptor blockade, the Ang II-elicited increases in P_{DA} after prazosin treatment in *A. rostrata* were significantly reduced by 32 and 31% following the 100 and 500 pmol kg⁻¹ Ang II doses, respectively. In *O. mykiss*, while prazosin treatment did not significantly affect the increase in P_{DA} following the 100 pmol kg⁻¹ Ang II dose, it reduced the Ang II-elicited increase in P_{DA} by 44% in the 500 pmol kg⁻¹ treatment.

Series 3: the effects of hypotension on blood pressure, plasma catecholamines and Ang II

In the control treatments, intravenous injection of saline and repeated blood sampling had no effect on resting P_{DA} (Fig. 5.3), plasma adrenaline, noradrenaline (Fig. 5.4), and Ang II (Fig. 5.5) concentrations in either *A. rostrata* or *O. mykiss*.

In the American eel, the smooth muscle relaxant papaverine elicited a rapid decrease in P_{DA} (from 28.8±1.0 to 12.2±0.7 cm H₂O after 22.5 min) followed by a gradual recovery back to baseline conditions 85 min after the treatment (Fig. 5.3A). In rainbow trout, while papaverine also elicited a rapid decrease in blood pressure (from 32.2±3.0 to 12.2±4.2 cm H₂O after 12 min), P_{DA} was fully recovered after 20 min and then was significantly increased above baseline conditions for a further 20 min period before gradually returning to baseline values (Fig 5.3B). Papaverine treatment elicited increases in plasma adrenaline concentrations in both species (Fig. 5.4A & C). However, the increase in plasma adrenaline observed in the eel (from 0.9±0.2 to

9.5±1.9 nM after 20 min; Fig. 5.4A) in response to the hypotensive stress was markedly less than the increase recorded in the trout (from 1.6±0.6 to 546.5±138.6 nM after 20 min; Fig. 5.4C). In addition, while papaverine treatment had no effect on the circulating concentrations of plasma noradrenaline in *A. rostrata* (Fig. 5.4B), this treatment was accompanied by a significant increase in plasma noradrenaline in *O. mykiss* (from 1.7±0.7 to 101.2±27.5 nM after 10 min; Fig. 5.4D). Administering papaverine also resulted in increases in plasma Ang II concentrations in both species (Fig. 5.5). In the trout, the increase in plasma Ang II concentrations occurred earlier and was greater (from 126±29 to 4279±1207 pM after 20 min; Fig. 5.5B) than the increase observed in the eel (from 82±16 to 1040±289 pM after 60 min; Fig. 5.5A).

Intravenous injection of papaverine in prazosin-treated eels elicited a rapid decrease in P_{DA} (from 28.7±1.1 to 12.9±0.5 cm H₂O after 11.5 min) followed by a gradual recovery back to baseline conditions 75 min after the treatment (Fig. 5.3C). In prazosin-treated rainbow trout, papaverine injection elicited a rapid decrease in blood pressure (from 30.8±2.0 to 9.7±1.2 cm H₂O after 11 min; Fig. 5.3D) and, although gradual, an incomplete recovery in P_{DA} over the following 90 min (Fig. 5.3D). In both fish species, the combined prazosin + papaverine treatment was also associated with increases in plasma adrenaline, noradrenaline, and Ang II concentrations (Fig. 5.4 & 5.5). Overall, as observed in the papaverine treatment above, the increases in the circulating concentrations of plasma catecholamines and Ang II were much more pronounced in *O. mykiss* than in *A. rostrata*.

Over a 24 hour period, while bretylium treatment alone did not significantly change the resting P_{DA} of *A. rostrata*, this treatment significantly lowered the resting blood pressure of *O. mykiss* (from 31.6±1.7 to 25.9±1.7 cm H₂O; Fig. 5.3F). In addition, relative to the basal values of the control treatments, bretylium-treated eel and trout had slightly higher plasma adrenaline

and noradrenaline concentrations under resting conditions (Fig. 5.4).

Papaverine treatment in bretylium-treated eels produced a rapid decrease in P_{DA} (from 29.3 ± 1.5 to 10.7 ± 1.2 cm H₂O after 20.5 min) followed by a gradual recovery back to baseline conditions 85 min after the treatment (Fig. 5.3E). In bretylium-treated trout, administering papaverine also resulted in a rapid decrease in blood pressure (from 25.9 ± 1.6 to 10.3 ± 2.0 cm H₂O after 11 min). However, in trout, P_{DA} was fully recovered after 25 min and was significantly increased transiently above baseline conditions (60 min) before returning to basal values (Fig. 5.3F). As with the other papaverine treatments, the combined bretylium + papaverine treatment was characterized by significant increases in plasma catecholamines and Ang II concentrations, and these were more pronounced in the rainbow trout than in the American eel (Fig. 5.4 & 5.5).

Overall, in *A. rostrata*, the three papaverine treatments resulted in similar decreases in P_{DA} and were characterized by similar recovery profiles back to baseline conditions (Fig. 5.3A, C & E). In *O. mykiss*, while the different papaverine treatments also elicited hypotensions of equivalent magnitude, the rate and extent to which blood pressure recovered back to control conditions varied significantly according to the different treatments (Fig. 5.3B, D & F). Also, in either species and with very few exceptions ($t = 60$ and 90 min; Fig. 5.4B), at any given time during recovery from the hypotensive stress there was no significant difference between the three papaverine treatments in the plasma adrenaline and noradrenaline concentrations (Fig. 5.4). In *A. rostrata*, with the exception of a single sampling time ($t = 10$ min; Fig. 5.5A), there were no significant differences in the plasma Ang II concentrations between the three papaverine treatments. Finally, in the three groups of rainbow trout treated with the smooth muscle relaxant, although the increases in plasma Ang II were similar during the first 20 min after the

Figure 5.3 Time course of changes in mean dorsal aortic pressure (P_{DA} , cm H₂O) in *Anguilla rostrata* (A, C, & E) and *Oncorhynchus mykiss* (B, D, & F) administered with either (A-F) saline ($N=6$; unfilled circle), (A & B) the smooth muscle relaxant papaverine (10 mg kg⁻¹; $N=6$; filled circle), (C & D) papaverine (10 mg kg⁻¹) after pre-treatment with the α -adrenoceptor antagonist, prazosin ($N=6$; filled circle), or (E & F) papaverine (10 mg kg⁻¹) after pre-treatment with the adrenergic neuron blocking agent, bretylium ($N=6$; filled circle). The time during which papaverine was injected is shown by a gray box in graphs A-F. For a given treatment, an asterisk denotes a significant difference from the 0 min control value. A † symbol denotes a significant difference from the control treatment for a given species and time ($P<0.05$). Values are means \pm 1 S.E.M.

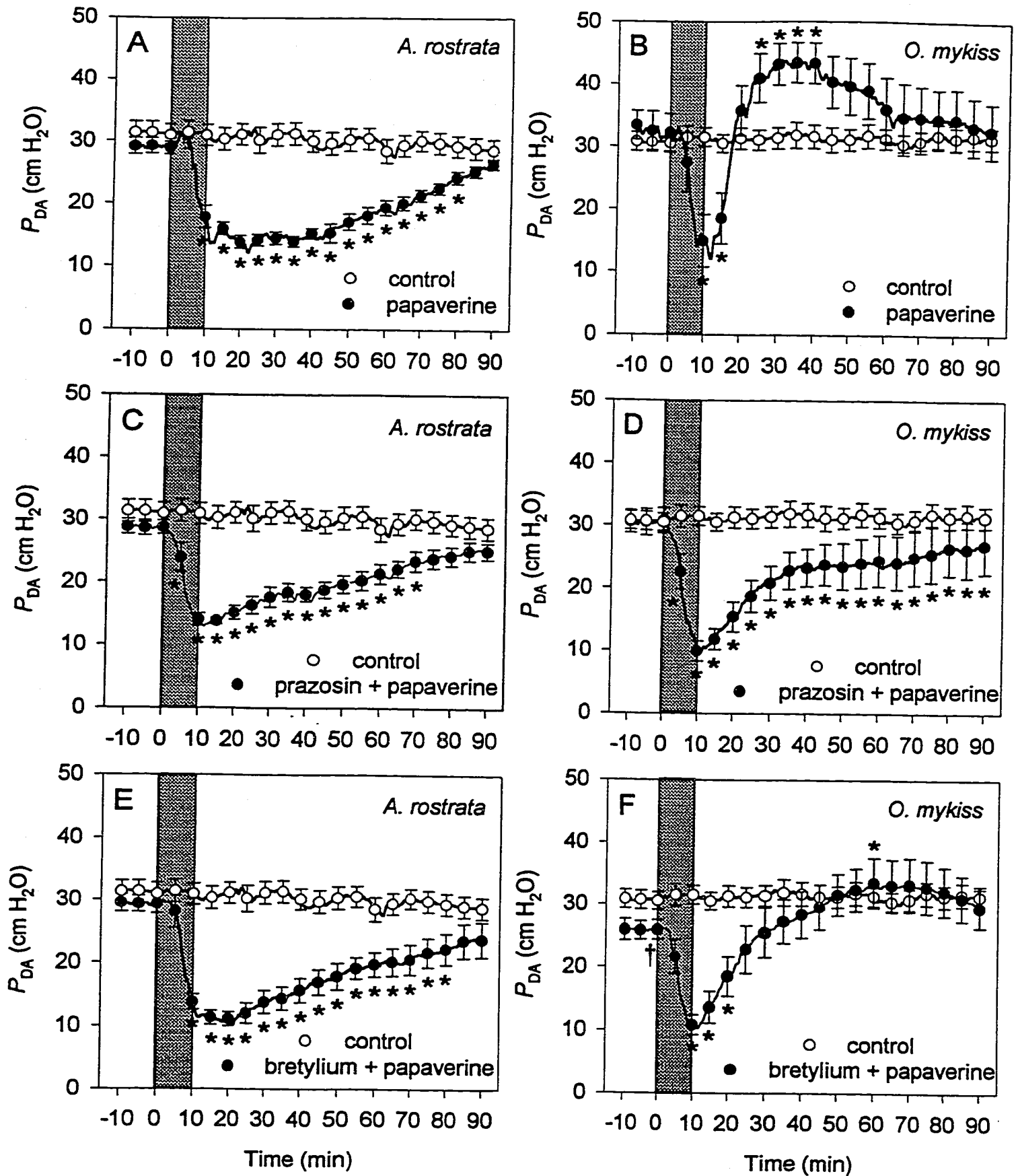


Figure 5.3

Figure 5.4 Time course of changes in plasma adrenaline (Adr; A & C) and noradrenaline (Nor; B & D) concentrations (nM) in *Anguilla rostrata* (A & B) and *Oncorhynchus mykiss* (C & D) administered with either saline ($N=6$; white bar), the smooth muscle relaxant papaverine (10 mg kg^{-1} ; $N=6$; gray bar), papaverine (10 mg kg^{-1}) after pre-treatment with the α -adrenoceptor antagonist, prazosin ($N=6$; black bar), or papaverine (10 mg kg^{-1}) after pre-treatment with the adrenergic neuron blocking agent, bretylium ($N=6$; cross-hatched bar). For a given treatment, an asterisk denotes a significant difference from the 0 min control value. Treatments that do not share a common letter for a given time and parameter are significantly different from each other ($P<0.05$). Values are means + 1 S.E.M. Note the different Y-axis scales for panels A-B and C-D.

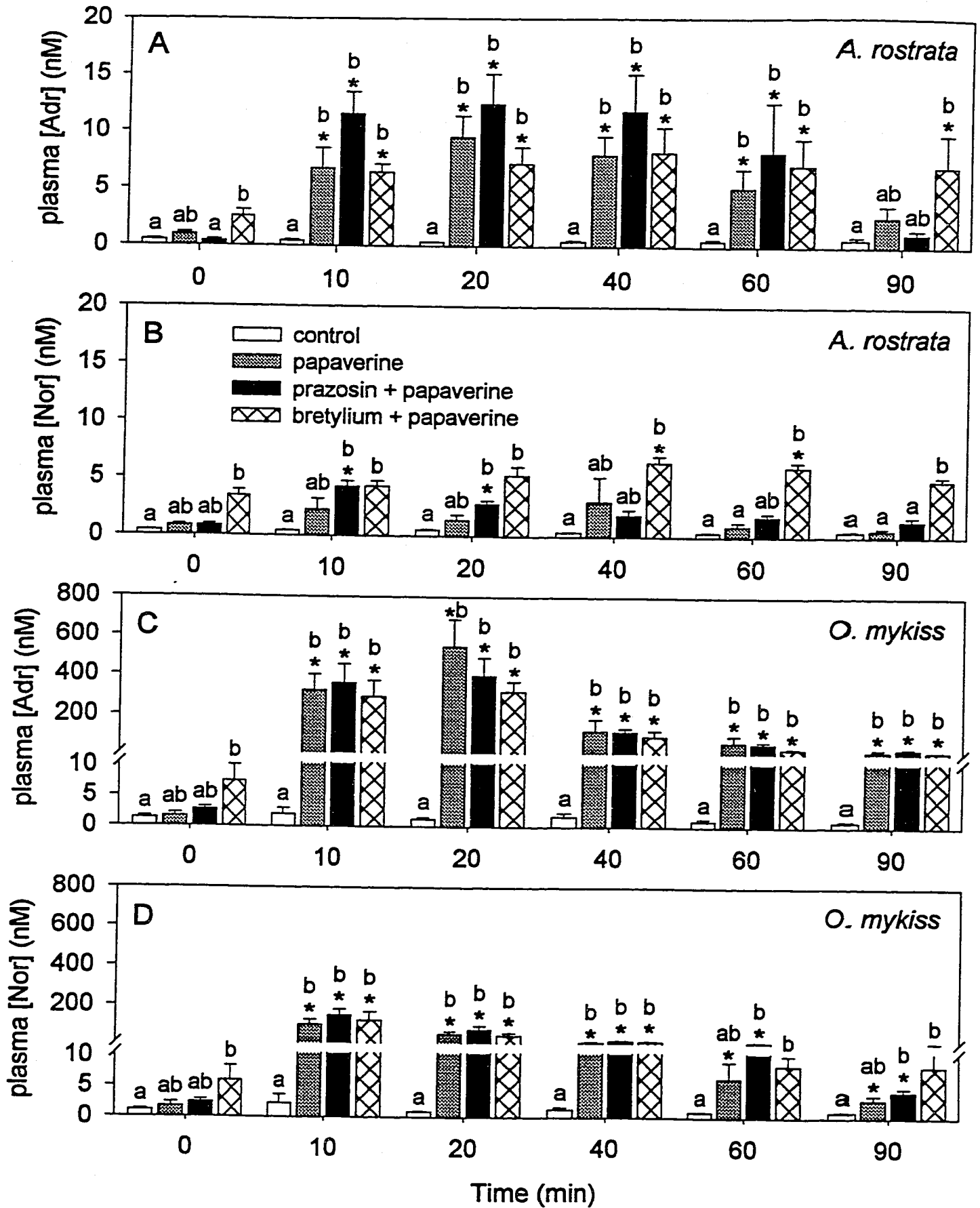


Figure 5.4

Figure 5.5 Time course of changes in plasma angiotensin II concentrations (pM) in *Anguilla rostrata* (A) and *Oncorhynchus mykiss* (B) administered with either saline ($N=6$; white bar), the smooth muscle relaxant papaverine (10 mg kg^{-1} ; $N=6$; gray bar), papaverine (10 mg kg^{-1}) after pre-treatment with the α -adrenoceptor antagonist, prazosin ($N=6$; black bar), or papaverine (10 mg kg^{-1}) after pre-treatment with the adrenergic neuron blocking agent, bretylium ($N=6$; cross-hatched bar). For a given treatment, an asterisk denotes a significant difference from the 0 min control value. Treatments that do not share a common letter for a given time are significantly different from each other ($P<0.05$). Values are means \pm 1 S.E.M.

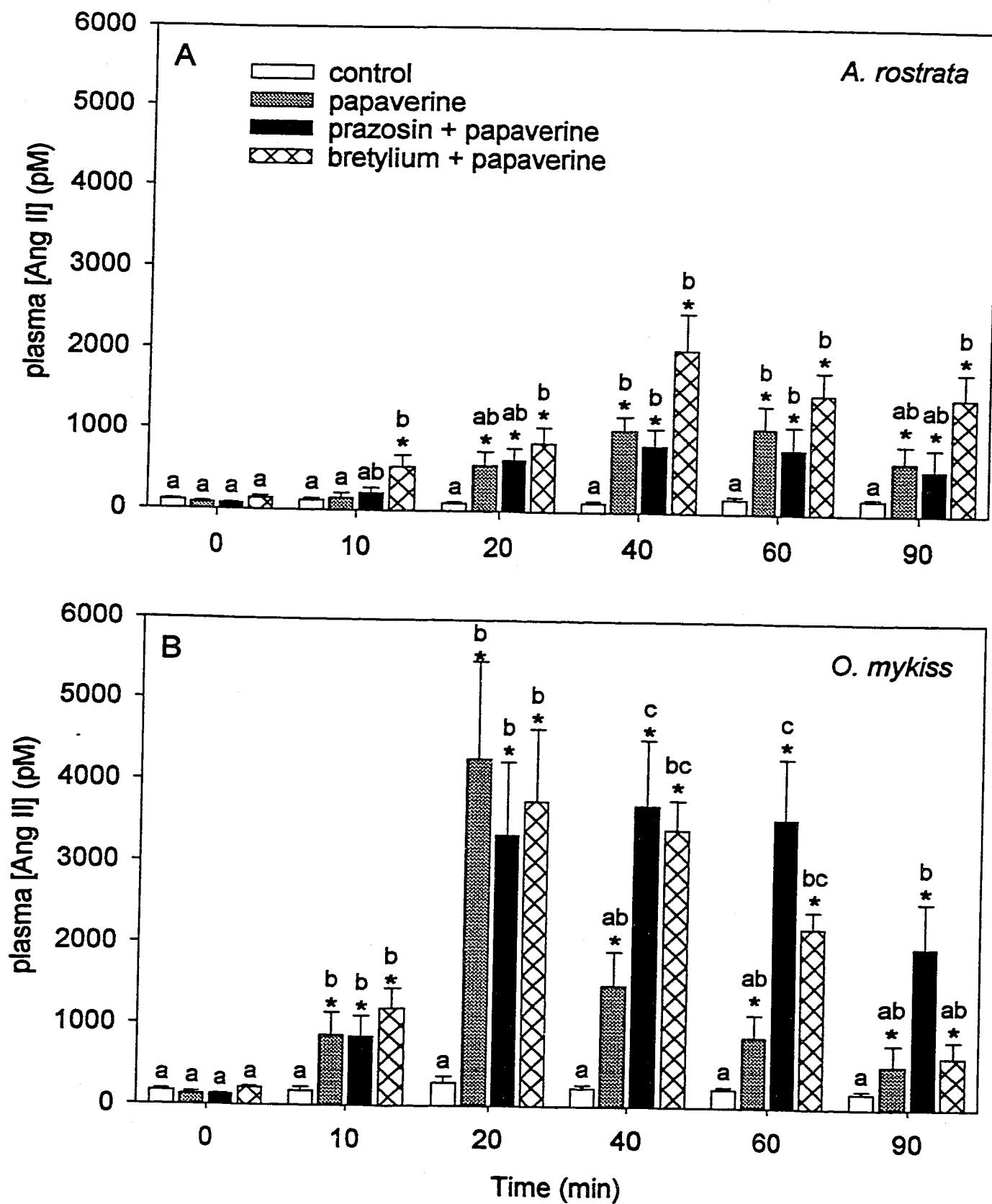


Figure 5.5

papaverine injection, Ang II concentrations were subsequently highest in the prazosin + papaverine treatment, intermediate in the bretylium + papaverine treatment, and lowest in the papaverine treatment (Fig. 5.5B).

Discussion

The present study demonstrates that homologous [Asn¹, Val⁵]-Ang II does not influence catecholamine release from the chromaffin tissue of *A. rostrata*, and confirms that Ang II is a potent secretagogue of humoral catecholamines in *O. mykiss*. Moreover, while a portion of the pressor responses elicited by exogenous Ang II injections can be mediated by the SNS in both *O. mykiss* and *A. rostrata*, interactions between endogenous Ang II and the SNS during a hypotensive stress are only apparent in *O. mykiss*. Although both circulating catecholamines and sympathetic nerves play important roles in the compensatory response to hypotension in *O. mykiss*, sympathetic nerves and α -adrenoceptor-mediated effects of humoral catecholamines are not essential contributors to blood pressure regulation in hypotensive *A. rostrata*.

In situ perfused PCV preparations have previously been used to investigate the control of catecholamine release from the chromaffin tissue of *A. rostrata* and *O. mykiss* (Reid and Perry 1994, 1995; Reid *et al.* 1996; Al-Kharrat *et al.* 1997; Bernier and Perry 1997; Abele *et al.* 1998; McKendry *et al.* 1999). In both species, the primary source of circulating catecholamines are the chromaffin cells located in the walls of the anterior region of the PCV and in the surrounding head kidney tissue (Hathaway and Epple 1989; Reid and Perry 1994). Although Ang II did not affect the secretion rate of either adrenaline or noradrenaline in *A. rostrata*, the suitability of the eel preparations to investigate the effects of potential secretagogues was confirmed by the stimulatory effects of carbachol on catecholamine secretion. Relative to the catecholamine

secretion rates of *O. mykiss*, the significantly lower carbachol-elicited catecholamine secretion rates in *A. rostrata* are characteristic of the previously described differential ability of these two species to respond to cholinergic stimulation (Reid and Perry 1994; McKendry *et al.* 1999). In *O. mykiss*, the dose-dependent stimulatory effect of Ang II on adrenaline secretion confirms the results of previous *in situ* experiments (Bernier and Perry 1997). On the other hand, and in contrast to previous results (Bernier and Perry 1997), Ang II did not affect the secretion rate of noradrenaline in the *in situ* PCV preparations of rainbow trout. Because Ang II was previously shown to preferentially stimulate adrenaline over noradrenaline secretion in rainbow trout (Bernier and Perry 1997; Bernier *et al.* 1999b), the above results may simply reflect intrinsic differences in the sensitivity of the chromaffin tissue to Ang II.

Exogenous injections of homologous Ang II also were without any effect on the circulating concentrations of plasma catecholamines in chronically cannulated and free swimming *A. rostrata*. In contrast and as previously observed (Bernier and Perry 1999), identical injections of Ang II in the trout were associated with a dose-dependent increase in plasma adrenaline and with a much smaller increase in plasma noradrenaline. Taken together, results from the *in situ* and *in vivo* Ang II injections suggest that the chromaffin tissue of *A. rostrata*, unlike that of *O. mykiss*, may be unresponsive to physiological concentrations of Ang II. Similar differences have been noted for mammals and include species differences for the presence of Ang II receptors in the adrenal medulla (Livett and Marley 1993).

Although exogenous injections of Ang II elicit a pressor response in both species, there are significant differences in the cardiovascular effects of Ang II between *A. rostrata* and *O. mykiss*. Relative to the response in *O. mykiss*, the smaller Ang II-mediated vasopressor response in *A. rostrata* has a slower onset and is longer lasting. While rapid changes in systemic vascular

resistance (R_S) and slower longer lasting changes in cardiac output (Q) contribute to the Ang II-elicited vasopressor response in both species, changes in R_S are a more important contributor in *O. mykiss* (Bernier and Perry 1999) than in *A. rostrata* (Oudit and Bulter 1995a). Similarly, although a portion of the Ang II-elicited vasopressor response is mediated indirectly via the SNS in both *O. mykiss* and *A. rostrata* (this study; Chan and Chow 1976; Nishimura *et al.* 1978; Nishimura 1985b; Olson *et al.* 1994; Oudit and Butler 1995a; Bernier and Perry 1999), the nature of the interaction between Ang II and the SNS differs between the two species. In *A. rostrata*, because Ang II does not stimulate humoral catecholamine release, the indirect SNS-mediated vasopressor action of Ang II must take place via an interaction with sympathetic nerves. In contrast, there is evidence that at least a portion of the cardiovascular effects of exogenous Ang II in trout can be attributed to increased levels of plasma adrenaline (Bernier and Perry 1999). Moreover, whereas α -adrenoceptor blockade has no effect on the Ang II-mediated increase in R_S in *A. rostrata* (Oudit and Butler 1995a), the Ang II-mediated increase in R_S is significantly reduced by a similar treatment in *O. mykiss* (Bernier and Perry 1999). In fact, in *A. rostrata*, it appears that changes in Q are responsible for the indirect SNS-mediated vasopressor action of Ang II (Oudit and Butler 1995a).

Administration of the smooth muscle relaxant papaverine elicited an acute reduction in blood pressure, and as evidenced by the increases in plasma Ang II levels, a recruitment of the RAS in both *A. rostrata* and *O. mykiss*. While these results are confirmatory (Tierney *et al.* 1995b; Bernier *et al.* 1999b), the parallel design of this experiment revealed significant differences between eel and trout in blood pressure recovery from a hypotensive stress. Following papaverine treatment, even though P_{DA} fell by approximately 60% in both species, recovery of blood pressure in *O. mykiss* took less than a quarter of the time than that required by

A. rostrata, and was characterized by a significant elevation of P_{DA} above resting conditions. Previous experiments have provided evidence for the importance of an RAS recruitment to blood pressure recovery in hypotensive teleosts (Perrott and Balment 1990; Tierney *et al.* 1995b; Bernier *et al.* 1999b). In *A. anguilla* (Tierney *et al.* 1995b) and *O. mykiss* (Bernier *et al.* 1999b), preventing the formation of Ang II with an angiotensin-converting enzyme (ACE) inhibitor significantly hinders recovery from a hypotensive stress. However, given a similar degree of hypotension, our results show that RAS recruitment in *O. mykiss* is faster and more pronounced than in *A. rostrata*. Under normotensive conditions, although blockade of the RAS with an ACE inhibitor lowers resting blood pressure in *O. mykiss* (Olson *et al.* 1997b; Bernier *et al.* 1999b), a similar treatment has no effect on P_{DA} in fresh water *A. anguilla* (Tierney *et al.* 1995b). Hence, while the RAS plays a tonic role in maintaining resting blood pressure in *O. mykiss*, this may not be the case in fresh water *A. rostrata*. Differences in the role that the RAS plays in regulating resting P_{DA} between both species presumably reflects on the basal activity of their RAS. In return, differences in basal RAS activity may explain, at least in part, the differential recruitment of the RAS in hypotensive *A. rostrata* and *O. mykiss* and their marked differences in blood pressure recovery.

The hypotensive properties of papaverine, in addition to stimulating the RAS, resulted in an increase in the circulating concentrations of plasma catecholamines in both *O. mykiss* and *A. rostrata*. However, as with the recruitment of the RAS, there was a marked difference in the magnitude of the papaverine-elicited increase in plasma catecholamines between the two teleost species. In rainbow trout, since ACE blockade prior to papaverine treatment significantly attenuates the hypotension-associated increase in plasma catecholamine levels (Bernier *et al.* 1999b), Ang II is thought to play an important role in mediating the pronounced recruitment of

humoral catecholamines during a hypotensive stress. In contrast, because we found no evidence that exogenous Ang II can stimulate catecholamine release in *A. rostrata*, the RAS, an important mediator of catecholamine release in hypotensive trout, is unlikely to play a role in the American eel. Because bretylium treatment prior to papaverine injection did not attenuate the hypotension-associated increase in plasma catecholamines, it is also improbable that sympathetic nerves were the source of the relatively small increase in plasma catecholamines observed in the papaverine-treated eels. In *A. rostrata*, in response to a hypotensive stress, while a reflexive neuronal stimulation of the chromaffin tissue via pre-ganglionic cholinergic fibers may stimulate catecholamine release, a variety of other mechanisms may also contribute to the control of humoral catecholamine release (Reid *et al.* 1995; Abele *et al.* 1998). However, given that the fish from the prazosin + papaverine and the papaverine treatments had similar catecholamine levels throughout the hypotensive stress, α -adrenoceptor mediation of catecholamine release (Abele *et al.* 1998) is unlikely to be a contributing mechanism in either the eel or the trout.

Although plasma catecholamine levels increased in both fish species following papaverine treatment, our results suggest that while the α -adrenoceptor-mediated effects of humoral catecholamines significantly contribute to blood pressure recovery in *O. mykiss* they do not play an essential role in the eel. In *A. rostrata*, relative to fish treated solely with papaverine, α -adrenoceptor blockade prior to papaverine treatment did not impede blood pressure recovery. Hence, since changes in Q are the primary contributor to the vasopressor response elicited by exogenous catecholamines in *A. rostrata* (Chan and Chow 1976; Oudit and Butler 1995b), our results suggest that the plasma catecholamine increases of papaverine-treated eels did not enhance cardiac performance via α -adrenoceptor-mediated mechanisms. At least they did enhance cardiac performance to the extent that such α -adrenoceptor-mediated cardiac changes

could not be compensated for by other cardiovascular adjustments in the eels treated with an α -adrenoceptor antagonist. Meanwhile, although there is some controversy as to whether the direct cardiac actions of catecholamines are mediated by α - (Chan and Chow 1976; Oudit and Butler 1995b) or β -adrenoceptors (Yasuda *et al.* 1996), it is possible that the hypotension-elicited increases in plasma catecholamines had significant physiological actions via cardiac β -adrenoceptors (Yasuda *et al.* 1996). In contrast to the eel, blockade of α -adrenoceptors prior to papaverine treatment prevented P_{DA} recovery in *O. mykiss* (this study; Bernier *et al.* 1999b). The observation that blockade of the sympathetic nerves with bretylium did not impede P_{DA} recovery to the same extent as α -adrenoceptor blockade, suggest that humoral catecholamines significantly contribute to cardiovascular control during a hypotensive stress in rainbow trout. This finding is corroborated by the previous observation that exogenous supplementation of plasma catecholamine concentrations prevents the chronic hypotensive effects of RAS blockade in papaverine-treated trout (Bernier *et al.* 1999b). Furthermore, plasma catecholamine levels of equivalent or smaller magnitude than those achieved in the papaverine-treated trout in this study, have previously been shown to contribute to cardiovascular control in *O. mykiss* by elevating P_{DA} , R_S , and Q (Gamperl *et al.* 1994a; Bernier and Perry 1999).

The compensatory response to hypotension in *A. rostrata* and *O. mykiss* also involves a differential contribution of the sympathetic nerves. In *A. rostrata*, P_{DA} recovery following the hypotensive stress was identical between the bretylium + papaverine and the papaverine treatments. Hence, despite the observations that a portion of the vasopressor response to exogenous Ang II injections may be mediated by sympathetic nerves (this study; Nishimura *et al.* 1978; Nishimura 1985b; Oudit and Butler 1995a) and that the RAS is recruited following papaverine treatment (this study; Tierney *et al.* 1995b), there is no evidence that sympathetic

nerves play an essential role in the compensatory response to hypotension in the American eel. In *O. mykiss*, bretylium treatment prior to papaverine did not prevent full P_{DA} recovery as the α -adrenoceptor treatment did. However, it annulled the rapid overshoot in blood pressure which characterized recovery in the papaverine treatment. Therefore, in *O. mykiss*, sympathetic nerves appear to be primarily involved in the short-term response to blood pressure regulation that immediately follows the hypotensive insult. Whether or not a portion of the SNS involvement in P_{DA} recovery results from the stimulatory effects of Ang II on sympathetic nerve endings (Reid 1992) can not be ascertained in the current study. However, because α -adrenoceptor blockade or bretylium treatment inhibit approximately 40% of the vasoconstrictory effect of Ang II in a perfused dorsal aorta preparation of *O. mykiss* (Olson *et al.* 1994), it appears likely that endogenous Ang II mediates a portion of its cardiovascular effects via an enhancement of sympathetic neurotransmission. Finally, under normotensive conditions, as previously observed by Hipkins *et al.* (1986) in *A. australis*, bretylium treatment did not decrease resting blood pressure in *A. rostrata*. In contrast, we corroborated the observation that chronic bretylium treatment significantly lowers resting P_{DA} in *O. mykiss* (Smith 1978). Therefore, while the SNS plays an important role in maintaining resting blood pressure in the trout (Wood and Shelton 1975; Wood and Shelton 1980; Xu and Olson 1993), there is no evidence for a sympathetic vasoconstrictor tone to the systemic vasculature of resting eel.

In summary, we have observed marked differences between *A. rostrata* and *O. mykiss* in their regulation of blood pressure during a hypotensive stress. These species-specific responses to hypotension can be explained, at least in part, by differential involvement of the RAS, the SNS, and their interactions in the homeostatic regulation of blood pressure. In *O. mykiss*, the compensatory response to hypotension involved an acute recruitment of the RAS, and a

significant involvement of sympathetic nerves and humoral catecholamines to blood pressure restoration. The contribution of humoral catecholamines to blood pressure regulation in the trout is primarily mediated by the stimulatory effect of Ang II on catecholamine release from the chromaffin tissue. In contrast, although the compensatory response to hypotension in *A. rostrata* also involves a recruitment of the RAS, Ang II does not elicit catecholamine release from the chromaffin tissue, and α -adrenoceptor-mediated catecholamine effects or sympathetic nerves do not play an essential role in blood pressure recovery.

CHAPTER 6.

**Cardiovascular control via angiotensin II and circulating catecholamines
in the spiny dogfish, *Squalus acanthias***

Abstract

The contributions of circulating angiotensin II and catecholamines to cardiovascular control in the spiny dogfish were investigated by monitoring the effects of exogenous and endogenous dogfish [Asn¹, Pro³, Ile⁵]-Ang II (dfAng II) on plasma catecholamine levels and blood pressure regulation. Bolus intravenous injections of dfAng II (30-1200 pmol kg⁻¹) elicited dose-dependent increases in plasma adrenaline and noradrenaline concentrations, caudal artery pressure (P_{CA}), and systemic vascular resistance (R_S), and a decrease in cardiac output (Q). Similar injections of Ang II in dogfish pre-treated with the α -adrenoceptor antagonist yohimbine (4 mg kg⁻¹) also elicited dose-dependent increases in plasma catecholamine levels yet the cardiovascular effects were abolished. Dogfish treated with yohimbine were hypotensive and had elevated levels of plasma Ang II and catecholamines. Intravenous injection of the smooth muscle relaxant papaverine (10 mg kg⁻¹) elicited a transient decrease in P_{CA} and R_S , and increases in plasma Ang II and catecholamine levels. In dogfish first treated with lisinopril (10⁻⁴ mol kg⁻¹), an angiotensin converting enzyme inhibitor, papaverine treatment caused a more prolonged and greater decrease in P_{CA} and R_S , an attenuated increase in plasma catecholamines, and no change in plasma Ang II. By itself, lisinopril treatment had little effect on P_{CA} , and no effect on R_S , plasma Ang II or catecholamines. In yohimbine-treated dogfish, papaverine treatment elicited marked decreases in P_{CA} , R_S , and Q , and increases in plasma Ang II and catecholamines. Among the 3 papaverine treatments, there was a positive linear relationship between plasma Ang II and catecholamine concentrations, and the cardiovascular and hormonal changes were most pronounced in the yohimbine + papaverine treatment. Therefore, under resting normotensive conditions, while Ang II does not appear to be involved in cardiovascular control, catecholamines

play an important role. However, following a hypotensive stress, Ang II indirectly contributes to cardiovascular control by a dose-dependent stimulation of catecholamine release.

Introduction

Throughout the vertebrates, the adrenergic and renin-angiotensin systems (RAS) play important roles in cardiovascular homeostasis. However, comparative studies have revealed that the mode of action of either the RAS or the adrenergic system, and the degree of interaction between the two, varies considerably within the vertebrate lineage (Morris and Nilsson 1994; Kobayashi and Takei 1996). In elasmobranchs, some of the major visceral arteries receive adrenergic innervation (Nilsson *et al.* 1975), but sympathetic nerves are absent from the heart (Short *et al.* 1977) and do not appear to play a significant role in the control of the systemic circulation (Opdyke *et al.* 1972; Holcombe *et al.* 1980). Catecholamine injections, in contrast, elicit systemic vasoconstriction in elasmobranchs (Capra and Satchell 1977b; Kent and Pierce 1978; Opdyke *et al.* 1982), and there is pharmacological evidence suggesting that circulating catecholamines may be important for maintaining basal cardiac activity and systemic vascular resistance (Opdyke *et al.* 1972; Short *et al.* 1977). Hence, although direct evidence is scarce, the adrenergic contribution to cardiovascular control in elasmobranchs appears to be primarily humoral (Butler and Metcalfe, 1988), unlike the situation in most vertebrates (Morris and Nilsson 1994).

While previously debated (see Olson 1992; Henderson *et al.* 1993; Kobayashi and Takei 1996 for reviews), the presence of a RAS in elasmobranchs was recently confirmed by the isolation of angiotensin I from the dogfish, *Triakis scyllia* (Takei *et al.* 1993). Evidence for an involvement of the RAS in the cardiovascular control of elasmobranchs is both direct and indirect.

As in other vertebrates, homologous and heterologous angiotensin II (Ang II), the active product of the RAS, have vasopressor activity in dogfish (Opdyke and Holcombe 1976; Khosla *et al.* 1983; Hazon *et al.* 1995; Tierney *et al.* 1997). Also, in response to hypotensive conditions, there is evidence that the pressor attributes of Ang II may contribute to blood pressure regulation (Hazon *et al.* 1989). In teleosts (Bernier and Perry 1999) and mammals (Butler *et al.* 1994), a varying portion of Ang II-mediated vasopressor activity can be attributed to an interaction with the adrenergic system. In elasmobranchs, however, there are conflicting reports regarding the nature of the interaction between the RAS and the adrenergic system in cardiovascular control. In the spiny dogfish (*Squalus acanthias*), the response to heterologous Ang II is blocked by α -adrenoceptor antagonists (Opdyke and Holcombe 1976; Carroll and Opdyke 1982; Khosla *et al.* 1983). Furthermore, exogenous teleost Ang II elicits an increase in plasma catecholamines (Opdyke *et al.* 1981b), and functional Ang II vascular receptors appear to be absent (Carroll 1981; Opdyke *et al.* 1982). Hence, in *S. acanthias*, the pressor response to heterologous Ang II appears to be mediated solely by circulating catecholamines. Conflicting results, however, were reported in the lesser spotted dogfish (*Scyliorhinus canicula*), where α -adrenoceptor blockade did not alter the magnitude of the vasopressor response to homologous Ang II (Tierney *et al.* 1997). Furthermore, in the Japanese dogfish (*Triakis scyllia*), there is *in vitro* evidence for the presence of functional Ang II vascular receptors (Hamano *et al.* 1998).

Whether these discrepancies in the mode of action of Ang II in elasmobranchs reflect species differences, Ang II dosage differences, or to important substitutions in the amino acid sequence of homologous versus heterologous Ang II (Takei *et al.* 1993) are not known. Moreover, although there is evidence that Ang II may play a role in cardiovascular control during a hypotensive stress (Hazon *et al.* 1989; Galli and Kiang 1990), the direct involvement and mode

of action of either circulating Ang II or catecholamines under such conditions have yet to be assessed.

The objectives of this study, therefore, were to determine whether the pressor response to homologous Ang II in *S. acanthias* is mediated by plasma catecholamines, and to assess the relative contributions of circulating Ang II and catecholamines to cardiovascular control during normotensive and hypotensive conditions.

Materials and methods

Experimental animals

Spiny dogfish, *Squalus acanthias*, of either sex weighing between 1130 and 2355 g (experimental $N = 30$) were collected by net during trawl by local fisherman and transported to holding facilities at Bamfield Marine Station (Bamfield, British Columbia). The dogfish were kept under natural photoperiod in a 75,000 liter opaque circular tank provided with aerated full-strength sea water at 11°C. They were fed twice weekly with herring and used for experimentation within 4 weeks of capture.

Surgical procedures

Dogfish were immersed in an aerated anaesthetic solution of ethyl-*m*-aminobenzoate (0.1 g l⁻¹; MS-222; Syndel, Vancouver, B.C.) and transferred to an operating table where the gills were irrigated continuously with the same anaesthetic solution. A lateral incision was made in the caudal peduncle to expose and cannulate (PE 50; Clay Adams) both the caudal vein and the caudal artery in the anterograde direction. While the arterial cannula allowed caudal artery blood pressure (P_{CA}) measurements, the caudal vein cannula permitted injections and repeated blood sampling. Both cannulae were filled with heparinized (100 i.u. ml⁻¹ sodium heparin; Sigma

Chemical, St. Louis, MO) dogfish saline (500 mmol l⁻¹ NaCl). In addition, the pericardial cavity was exposed with a ventral midline incision and the pericardium was dissected to expose the conus arteriosus. To enable measurement of cardiac output (Q), a 3S or 4S ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY) was placed non-occlusively around the conus. Lubricating jelly was used with the perivascular flowprobe as an acoustic couplant. Silk sutures were used to close the ventral and caudal peduncle incisions, and to anchor the cardiac output probe lead and the cannulae to the skin. After surgery, dogfish were placed into individual flow-through opaque acrylic or wooden boxes and left to recover for 24 hours before experimentation.

Experimental protocol

Series 1: the effects of exogenous dfAng II on cardiovascular function and plasma catecholamine levels

Dogfish were monitored during an initial period of 30 to 60 min to assess the stability of P_{CA} and Q traces. Upon stabilisation, control baseline parameters were recorded for 5 min, after which dogfish were given a bolus injection (0.5 ml) of homologous [Asn¹, Pro³, Ile⁵]-Ang II (dfAng II; Peptide Institute Inc., Osaka) through the caudal vein cannula over a period of 30 sec. Cardiovascular responses to the injection were monitored continuously, and following recovery of cardiovascular variables to control baseline levels for a 1 hour period, a second dose of dfAng II was injected. Repeating this protocol, five doses of dfAng II (30, 100, 300, 600, 1200 pmol kg⁻¹) and saline were randomly and sequentially tested on each fish ($N = 6$). Each injection was followed by 0.4 ml of saline to clear the cannula.

Using the same experimental protocol as above, three doses of dfAng II (30, 300, and 1200 pmol kg⁻¹) and saline were tested in a second group of dogfish ($N = 6$), first treated with the α -adrenoceptor blocker, yohimbine (RBI, Natick, MA). Alpha-adrenergic blockade was achieved

by slowly (over a 5 min period) injecting a 4 mg kg^{-1} dose of yohimbine 60 min prior to experimentation. Yohimbine was dissolved in $200 \mu\text{l}$ of ethanol and diluted in saline (4 mg ml^{-1}) prior to injection (1 ml kg^{-1}). Before yohimbine treatment and after the three Ang II doses, the effectiveness of the α -adrenergic blockade was assessed by injection of a catecholamine cocktail (0.1 ml kg^{-1}) prepared in dogfish saline and consisting of $1 \times 10^{-4} \text{ mol l}^{-1}$ adrenaline bitartrate and noradrenaline bitartrate (Arterenol, Sigma).

In both groups of fish, a blood sample (0.35 ml) was taken during the 5 min control period before each dfAng II dose to assess basal plasma catecholamines. Four additional blood samples (0.35 ml) were then taken 1, 2.5, 5, and 10 min after each Ang II injection to assess the effects of dfAng II on circulating plasma catecholamine concentrations. Each blood sample, which was replaced by an equivalent volume of saline, was collected in a 1.5 ml microcentrifuge tube, and centrifuged immediately at $10,000 \text{ g}$ for 15 sec. The separated plasma was combined with $10 \mu\text{l}$ of a 5% EDTA / 10 % sodium bisulphite solution, quick frozen in liquid nitrogen, and stored at -80°C for later analysis of catecholamines.

Series 2: the effects of hypotension on circulating Ang II and catecholamine concentrations

To investigate the relative contributions of circulating catecholamines and Ang II to cardiovascular control in hypotensive *S. acanthias*, three separate groups of fish were given one of the following treatments: papaverine; lisinopril + papaverine; or yohimbine + papaverine.

Once the stability of P_{CA} and Q traces had been established, control baseline cardiovascular parameters were recorded for 10 min and fish ($N = 6$) were then injected (0.375 ml kg^{-1}) over the subsequent 10 min period with the smooth muscle relaxant, papaverine (10 mg kg^{-1} ; RBI). The papaverine injection was followed by 0.4 ml of saline to clear the caudal vein cannula, and the cardiovascular effects of this hypotensive treatment were then monitored continuously for

the following 50 min. A blood sample (0.5 ml) was taken immediately before the papaverine injection, as well as 10, 15, 20, 25, 30, 40, 50, and 60 min after the beginning of the injection for subsequent analysis of catecholamines and [Asn¹, Pro³, Ile⁵]-Ang II. Each blood sample was replaced by an equivalent volume of saline, and plasma was obtained by centrifugation and treated as above (see *Series 1*) prior to being frozen and stored at -80°C.

In the lisinopril + papaverine or yohimbine + papaverine treatments, the cardiovascular effects of papaverine were assessed one hour after dogfish received an injection of either the angiotensin converting enzyme (ACE) inhibitor, lisinopril (Sigma), or yohimbine (see series 1). Blockade of the RAS was achieved by injecting a 10⁻⁴ mol kg⁻¹ dose of lisinopril (1 ml kg⁻¹) over a 2 min period. In both treatments, the cardiovascular effects of the blockers were monitored, and blood samples (0.5 ml) were taken before (0 and 5 min) and after (5, 10, 15, and 20 min) injection of either lisinopril or yohimbine to assess their effects on circulating concentrations of catecholamines and dfAng II.

In the lisinopril + papaverine treatment, the effectiveness of ACE blockade was assessed by comparing the cardiovascular effects of a bolus injection of 1000 pmol kg⁻¹ [Ans¹, Val⁵, Asn⁹]-Ang I (salmon Ang I; Sigma) given before and after lisinopril. Salmon Ang I was used to test the effectiveness of ACE blockade because of the unavailability of dogfish Ang I at the time of experimentation.

Analytical procedures

Caudal artery pressure (P_{CA}) was measured with a UFI model 1050BP (UFI, Morro Bay, CA) pressure transducer that was calibrated against a static water column. Mean blood pressure was calculated as: (systolic pressure + diastolic pressure) / 2. The perivascular flow probes used

to measure Q were connected to a Transonic T106 small animal blood flow meter (Transonic Systems Inc., Ithaca, NY). These probes were pre-calibrated in the factory and verified in the laboratory by pump perfusion of the heart of an immersed euthanized fish with saline at known flow rates. Both P_{CA} and Q signals were recorded with a data acquisition system (Biopac System Inc., Goleta, CA) and collected at 0.04 sec intervals using Acknowledge III™ (Biopac System Inc.) data acquisition software. Systemic vascular resistance (R_S) was calculated as mean P_{CA} divided by Q (i.e. $R_S = P_{CA} / Q$), heart rate (f_H) was derived from the caudal artery pressure pulse trace, and stroke volume (V_S) was calculated as Q divided by f_H (i.e. $V_S = Q / f_H$).

Plasma catecholamines (adrenaline and noradrenaline) were determined on alumina-extracted plasma samples (0.2 ml) using high-pressure liquid chromatography (HPLC) with electrochemical detection (Bernier and Perry 1997). The extracted samples were passed through an Ultratechsphere ODS-C18 5 μ m column (HPLC Technology Ltd), using a catecholamine and metanephrine mobile phase (Chromosystems, Munich, Germany). The separated amines were integrated using the Star Chromatography software program (version 4.0, Varian, Walnut Creek, CA). Concentrations were calculated relative to appropriate standards and with 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations. Plasma Ang II concentrations were determined on extracted plasma samples and measured by radioimmunoassay (Bernier *et al.* 1999b). The antibody used in this assay exhibited 73.4% cross-reactivity with dogfish [Asn¹, Pro³, Ile⁵]-Ang II. The dilution curve of immunoreactive Ang II in extracted *S. acanthias* plasma was parallel to the standard curve of [Asn¹, Pro³, Ile⁵]-Ang II. The intra- and inter-assay coefficients of variation were 5.5% and 8.1%, respectively.

Statistical analyses

Data are presented as mean values \pm one standard error of the mean (S.E.M). The statistical significance of the observed effects of an injection within a group was tested using a one-way repeated-measures analysis of variance (ANOVA). Dunnett's *post-hoc* multiple-comparison test was used to compare the pre-injection control data point with values at subsequent times. Following a given dfAng II injection, differences between the control and maximum plasma catecholamine levels were analysed using a Student's paired *t*-test. Among the various dfAng II injections within a treatment, differences in the effects on cardiovascular variables, or differences in the maximum increase of a catecholamine, were determined using one-way ANOVA followed by a Tukey test for multiple comparisons. The statistical significance of observed differences between the means of two treatments was determined by a Student's *t*-test. For a given parameter and time, differences among the 3 papaverine treatments were assessed with a one-way ANOVA followed by Dunnett's multiple-comparisons test to compare the lisinopril + papaverine and the yohimbine + papaverine treatments to the control papaverine treatment. The significance level for all statistical tests was $P < 0.05$.

Results

Series 1: the effects of exogenous dfAng II on cardiovascular function and plasma catecholamine levels

Bolus injections of dfAng II between 30 and 1200 pmol kg⁻¹ elicited increases in P_{CA} and R_S , and decreases in Q , f_H , and V_S (Table 6.1; Fig. 6.1A-E). While the dfAng II-elicited increase in P_{CA} was dose-dependent between 30 and 300 pmol kg⁻¹, the increase in R_S was dose-dependent between 30 and 1200 pmol kg⁻¹ (Table 6.1). The dfAng II injections also elicited dose-dependent

increases in plasma noradrenaline (100 to 1200 pmol kg⁻¹; Fig. 6.2A) and plasma adrenaline (30 to 1200 pmol kg⁻¹; Fig. 6.2B) levels. For a given Ang II dose (30 to 1200 pmol kg⁻¹), the increase in plasma adrenaline was significantly greater (2-2.7 times) than the increase in noradrenaline (Fig. 6.2A-B).

In comparison to the control group, fish treated with the α -adrenergic antagonist yohimbine had significantly lower resting mean P_{CA} and R_s , similar Q (Table 6.2; Fig. 6.1A-C), and higher basal plasma noradrenaline and adrenaline concentrations (Fig. 6.2A-B). Relative to the pre-injection control condition, yohimbine treatment reduced the pressure response and the increase in R_s to a bolus injection of catecholamines by 81 and 93%, respectively, and abolished the reduction in Q (Table 6.2). Although bolus injections of dfAng II (30 to 1200 pmol kg⁻¹) had no significant effect on any of the measured cardiovascular parameters in the yohimbine-treated dogfish (Table 6.1; Fig. 6.1A-E), they elicited dose-dependent (30 to 1200 pmol kg⁻¹) increases in plasma catecholamine levels (Fig. 6.2A-B). As in the control group, the increases in plasma adrenaline were significantly greater (2-2.5 times) than the increases in noradrenaline (Fig. 6.2A-B).

Injection of saline alone had no effect on the resting cardiovascular parameters or on the basal plasma catecholamine concentrations of either the control or yohimbine-treated dogfish (Table 6.1, Fig. 6.2A-B).

Series 2: the effects of hypotension on circulating Ang II and catecholamine concentrations

Intravenous injection of papaverine elicited rapid and transient decreases in P_{CA} (47%) and R_s (39%), and no significant change in Q (Fig. 6.3A-C), f_H , or V_s (data not shown). Papaverine treatment elicited sustained increases in plasma adrenaline, noradrenaline and Ang II concentrations (Fig. 6.3D-E).

Lisinopril administration produced a small and transient decrease in P_{CA} (17%), and no significant change in R_S , Q , plasma catecholamines, or plasma Ang II (Fig. 6.4A-E). In comparison to control fish, lisinopril treatment abolished the changes in P_{CA} , R_S , and Q associated with a bolus injection of [Asn¹, Val⁵, Asn⁹]-Ang I (Table 6.3) thus confirming ACE inhibition. Papaverine injection in lisinopril-treated fish elicited rapid and sustained decreases in P_{CA} (64%) and R_S (56%), and no significant change in Q (Fig. 6.4F-H), f_H , or V_S (data not shown). The papaverine-elicited decreases in P_{CA} and R_S were significantly greater in the lisinopril-treated dogfish than in the control papaverine group. Papaverine injection in lisinopril-treated fish also produced a transient increase in plasma adrenaline, a small and sustained increase in plasma noradrenaline, and had no effect on plasma Ang II concentrations (Fig. 6.4I-J).

Yohimbine administration produced marked and sustained decreases in P_{CA} (56%) and R_S (54%), no significant change in Q , and increases in plasma catecholamine and Ang II concentrations (Fig. 6.5A-E). Papaverine injection in yohimbine-treated fish resulted in further decreases in P_{CA} (47%), and R_S (27%), as well as decreases in Q (24%; Fig. 6.5F-H), and V_S , but no significant change in f_H (data not shown). In comparison to the pre-yohimbine baseline cardiovascular variables, the combined yohimbine + papaverine treatment decreased P_{CA} and R_S by 72 and 61%, respectively. The papaverine-elicited decreases in P_{CA} and R_S in the yohimbine-treated dogfish were significantly greater than in the control papaverine group. Papaverine injection in α -adrenoceptor blocked dogfish also resulted in plasma catecholamine and Ang II concentrations that were significantly higher than in the control papaverine group (Fig. 6.5I-J).

Table 6.1 Absolute changes in cardiovascular parameters¹ following bolus injection of saline or [Asn¹, Pro³, Ile⁵]-Ang II in control or yohimbine-treated spiny dogfish, *Squalus acanthias*.

| Parameter | Saline | [Asn ¹ ,Pro ³ ,Ile ⁵]-Ang II dose (pmol kg ⁻¹) | | | | |
|-------------------------|-------------------------|--|---------------------------|--------------------------|--------------------------|--------------------------|
| | | 30 | 100 | 300 | 600 | 1200 |
| Control treatment (6) | | | | | | |
| P_{CA} | +4.5±0.6 ^a | +10.4± 2.3 ^{*ab} | +14.6± 0.7 ^{*bc} | +19.5±1.4 ^{*c} | +21.9±1.9 ^{*c} | +21.3±2.7 ^{*c} |
| R_S | +0.2±0.0 ^a | +0.8±0.2 ^{*ab} | +1.2±0.2 ^{*abc} | +1.9±0.3 ^{*bcd} | +2.2±0.3 ^{*cd} | +2.5±0.4 ^{*d} |
| Q | +1.7±0.6 ^a | -4.8±1.1 ^{*b} | -5.6±1.6 ^{*b} | -6.7±1.6 ^{*b} | -7.3±1.7 ^{*b} | -8.1±1.3 ^{*b} |
| f_H | +0.8±0.6 ^a | -3.7±1.1 ^{*b} | -3.3±0.8 ^{*b} | -3.5±0.8 ^{*b} | -3.7±1.0 ^{*b} | -4.7±0.8 ^{*b} |
| V_S | +0.08±0.06 ^a | -0.12±0.05 ^{ab} | -0.25±0.05 ^{*b} | -0.26±0.06 ^{*b} | -0.29±0.05 ^{*b} | -0.32±0.06 ^{*b} |
| Yohimbine treatment (6) | | | | | | |
| P_{CA} | +1.7±0.7 | +1.7±0.9 | | +1.2±0.3 | | +2.5±1.4 |
| R_S | +0.1±0.0 | +0.1±0.0 | | +0.1±0.0 | | +0.1±0.0 |
| Q | +2.7±0.5 | +4.3±2.0 | | +2.0±1.2 | | +1.1±0.4 |
| f_H | +0.2±0.5 | +0.8±0.5 | | -0.8±0.9 | | -0.7±1.0 |
| V_S | +0.02±0.07 | -0.09±0.07 | | -0.01±0.08 | | +0.07±0.09 |

Values are means ± 1 S.E.M. Values in parentheses represent N values.

* Significantly different from resting value for a given injection. Means that do not share a common letter for a given parameter and treatment are significantly different from each other ($P < 0.05$).

¹ - P_{CA} , mean caudal artery pressure (cm H₂O); R_S , systemic resistance (cm H₂O ml⁻¹ min⁻¹ kg⁻¹); Q , cardiac output (ml min⁻¹ kg⁻¹); f_H , heart rate (beats min⁻¹); V_S , stroke volume (ml kg⁻¹ beat⁻¹)

Figure 6.1 (A) Mean caudal artery pressure (P_{CA} , cm H₂O), (B) systemic vascular resistance (R_s , cm H₂O ml⁻¹ min⁻¹ kg⁻¹), (C) cardiac output (Q , ml min⁻¹ kg⁻¹), (D) heart rate (f_H , beats min⁻¹), and (E) stroke volume (V_s , ml kg⁻¹ beat⁻¹) in intact control ($N=6$; filled circles) and yohimbine-treated ($N=6$; unfilled circles) Pacific spiny dogfish, *Squalus acanthias*, given a bolus injection of 300 pmol kg⁻¹ homologous [Asn¹, Pro³, Ile⁵]-Ang II. The dashed line in each graph indicates the time of Ang II injection. An asterisk denotes a significant difference from the resting value immediately preceding the dashed line ($P<0.05$). Values are means \pm 1 S.E.M.

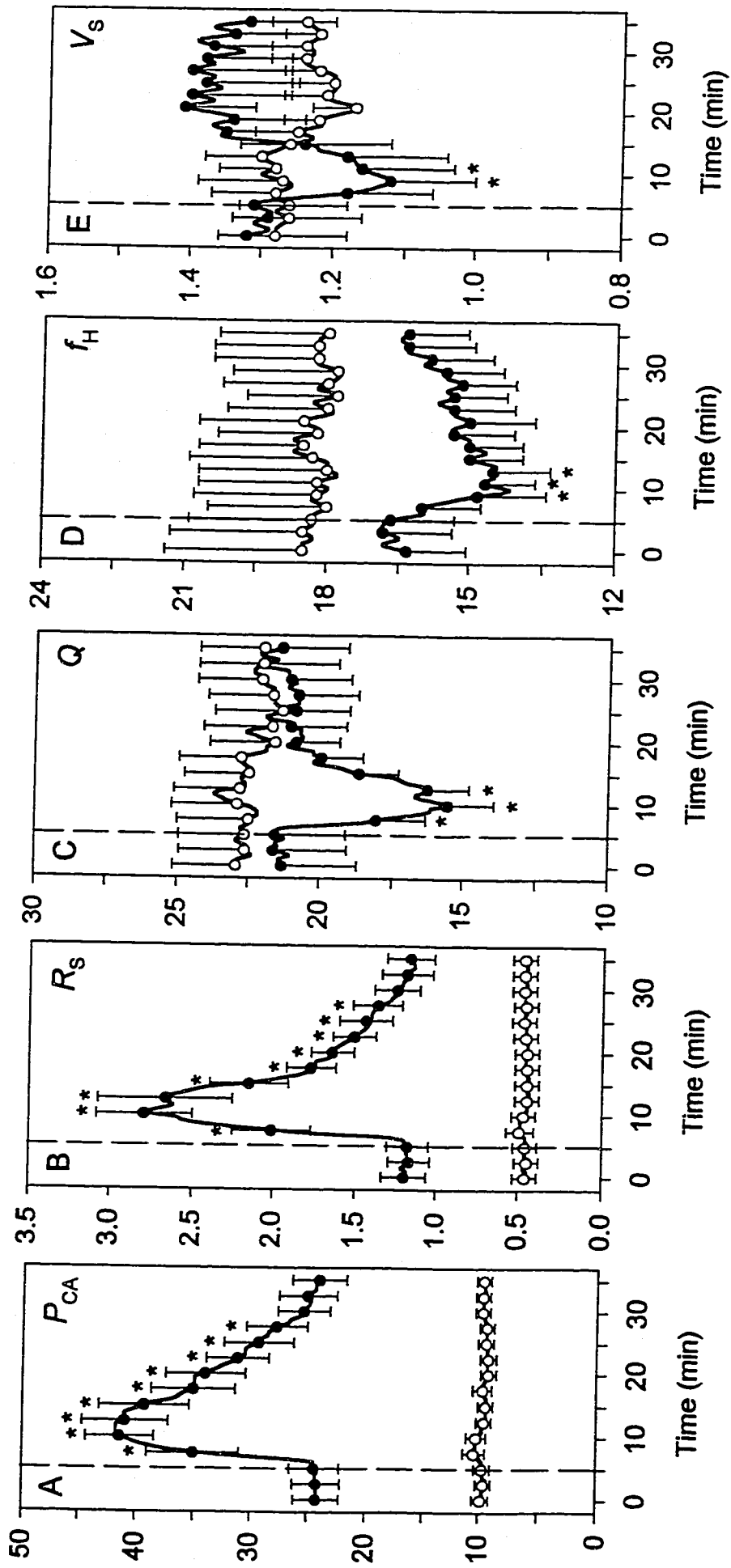


Figure 6.1

Figure 6.2 Effects of bolus injections of either saline or homologous [Asn¹, Pro³, Ile⁵]-Ang II on plasma (A) noradrenaline and (B) adrenaline concentrations in intact control (N=6) and yohimbine-treated (N=6) Pacific spiny dogfish, *Squalus acanthias*. The unfilled and black bars indicate the basal catecholamine concentrations prior to the injection in the control and yohimbine-treated dogfish, respectively. The diagonal- and cross-hatched bars indicate the maximum catecholamine concentrations in response to the injection in the control and yohimbine-treated dogfish, respectively. An asterisk denotes a significant difference from the basal value for a given dose and treatment. Means that do not share a common letter for a given parameter and treatment are significantly different from each other. † denotes a significant difference from the corresponding value in the control treatment for a given dose ($P < 0.05$). Values are means \pm 1 S.E.M.

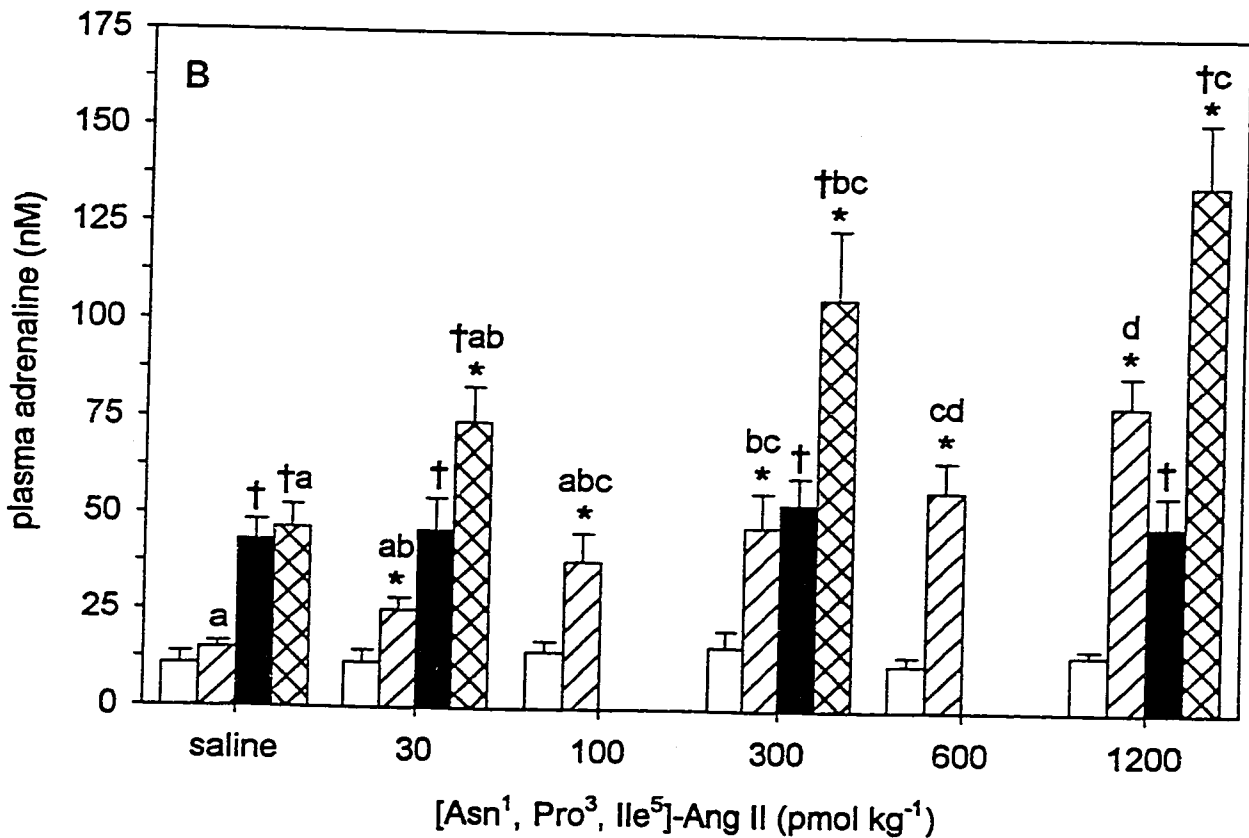
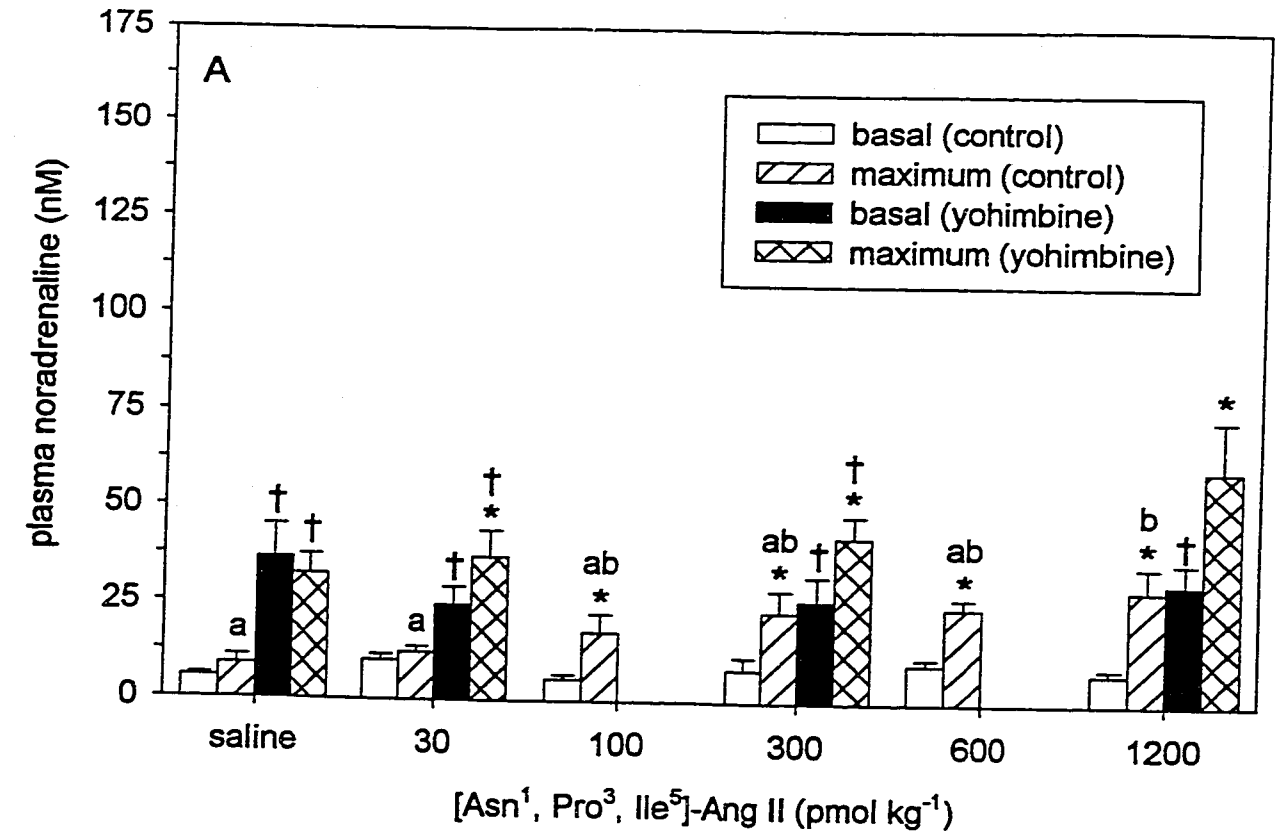


Figure 6.2

Table 6.2 The effects of a bolus injection of catecholamines¹ on the cardiovascular variables² of intact control and yohimbine-treated spiny dogfish, *Squalus acanthias*.

| Treatment | <i>N</i> | <i>P</i> _{CA} | <i>R</i> _S | <i>Q</i> |
|-------------------|----------|------------------------|--|--|
| | | (cm H ₂ O) | (cm H ₂ O ml ⁻¹ min ⁻¹ kg ⁻¹) | (ml min ⁻¹ kg ⁻¹) |
| Control | 6 | | | |
| Basal values | | 27.5±2.7 | 1.1±0.1 | 26.1±3.3 |
| Post-injection | | 40.3±3.3* | 3.1±0.6* | 15.8±3.5* |
| Change from basal | | 12.8±1.9 | 1.9±0.5 | -10.2±2.1 |
| Yohimbine | 6 | | | |
| Basal values | | 11.7±1.9† | 0.5±0.1† | 23.0±2.7 |
| Post-injection | | 14.2±2.5* | 0.7±0.1* | 19.9±1.5 |
| Change from basal | | 2.5±1.0# | 0.1±0.0# | -3.1±1.9# |

1 – The catecholamine injection contained 10 nmol kg⁻¹ body wt noradrenaline bitartrate and adrenaline bitartrate.

Values are mean ± 1 S.E.M.

* Significantly different from basal value for a given treatment. † Significantly different from basal value of the control treatment. # Significantly different from the change from basal value of the control treatment ($P < 0.05$).

2 – *P*_{CA}, mean caudal artery pressure; *R*_S, systemic resistance; *Q*, cardiac output.

Figure 6.3 Effects of an intravenous injection of the smooth muscle relaxant, papaverine (10 mg kg⁻¹) on (A) mean caudal artery pressure (P_{CA} , cm H₂O), (B) systemic vascular resistance (R_s , cm H₂O ml⁻¹ min⁻¹ kg⁻¹), (C) cardiac output (Q , ml min⁻¹ kg⁻¹), (D) plasma catecholamines (nM), and (E) plasma [Asn¹, Pro³, Ile⁵]-Ang II (pM) in Pacific spiny dogfish, *Squalus acanthias* (N=6). The time during which papaverine was injected is shown by a gray box in graphs A, B, and C. An asterisk denotes a significant difference from the control value immediately preceding the papaverine injection (10-min) for a given parameter ($P < 0.05$). Values are means \pm 1 S.E.M.

Papaverine injection

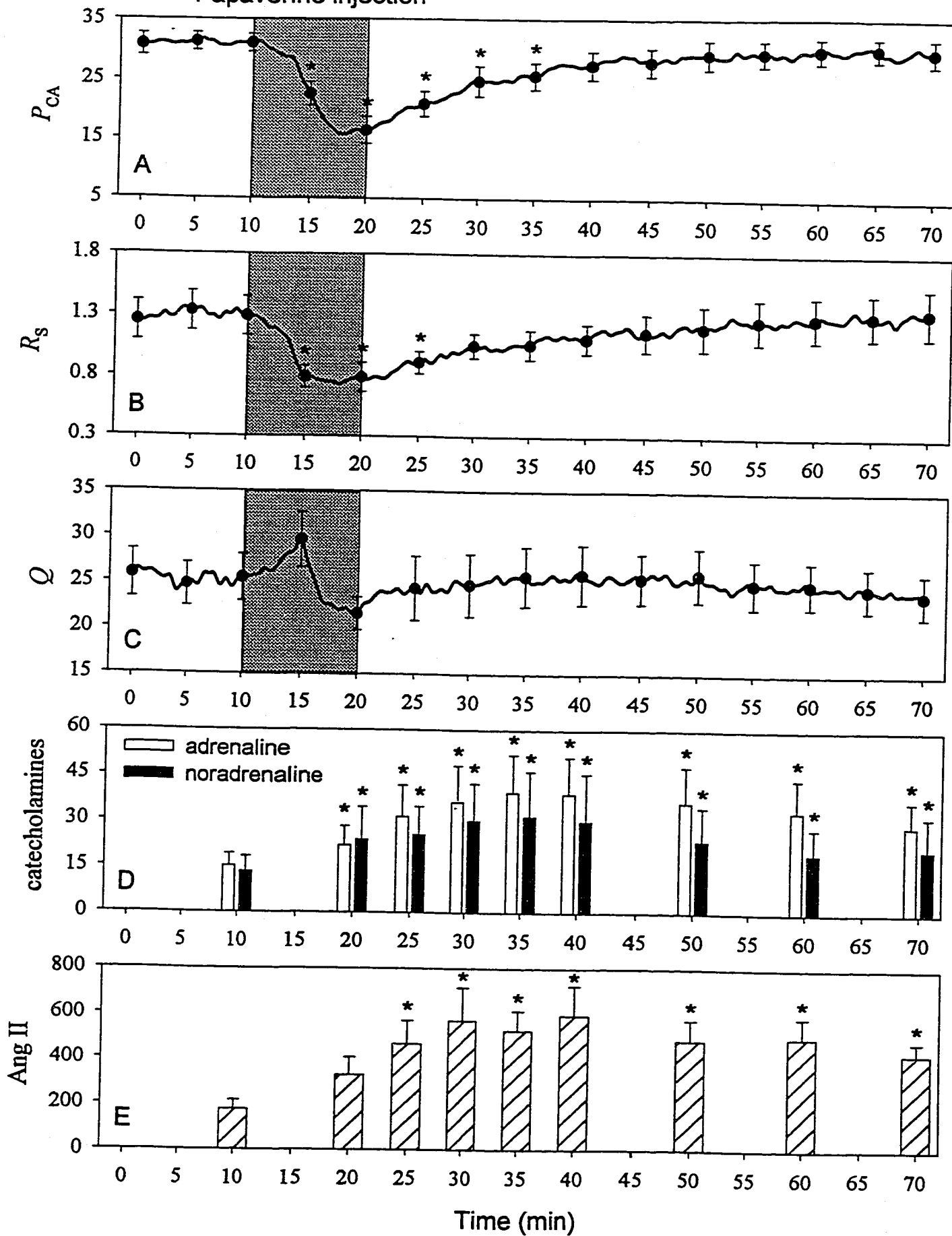


Figure 6.3

Figure 6.4 Effects of an intravenous injection of the angiotensin-converting-enzyme inhibitor, lisinopril (A-E; 10^{-4} mol kg^{-1}) followed by an intravenous injection of the smooth muscle relaxant, papaverine (F-J; 10 mg kg^{-1}) on (A & F) mean caudal artery pressure (P_{CA} , cm H_2O), (B & G) systemic vascular resistance (R_s , cm H_2O ml^{-1} min^{-1} kg^{-1}), (C & H) cardiac output (Q , ml min^{-1} kg^{-1}), (D & I) plasma catecholamines (nM), and (E & J) plasma [Asn¹, Pro³, Ile⁵]-Ang II (pM) in Pacific spiny dogfish, *Squalus acanthias* ($N=6$). The time during which lisinopril was injected is shown by a gray box in graphs A, B, and C. The time during which papaverine was injected is shown by a gray box in graphs F, G, and H. An asterisk denotes a significant difference from the control value immediately preceding the papaverine injection (10-min) for a given parameter. † denotes a significant difference from the corresponding value in the papaverine treatment (Fig. 6.3) for a given parameter. # denotes a significant difference from the control value immediately preceding the lisinopril injection (5-min) for a given parameter ($P<0.05$). Values are means \pm 1 S.E.M.

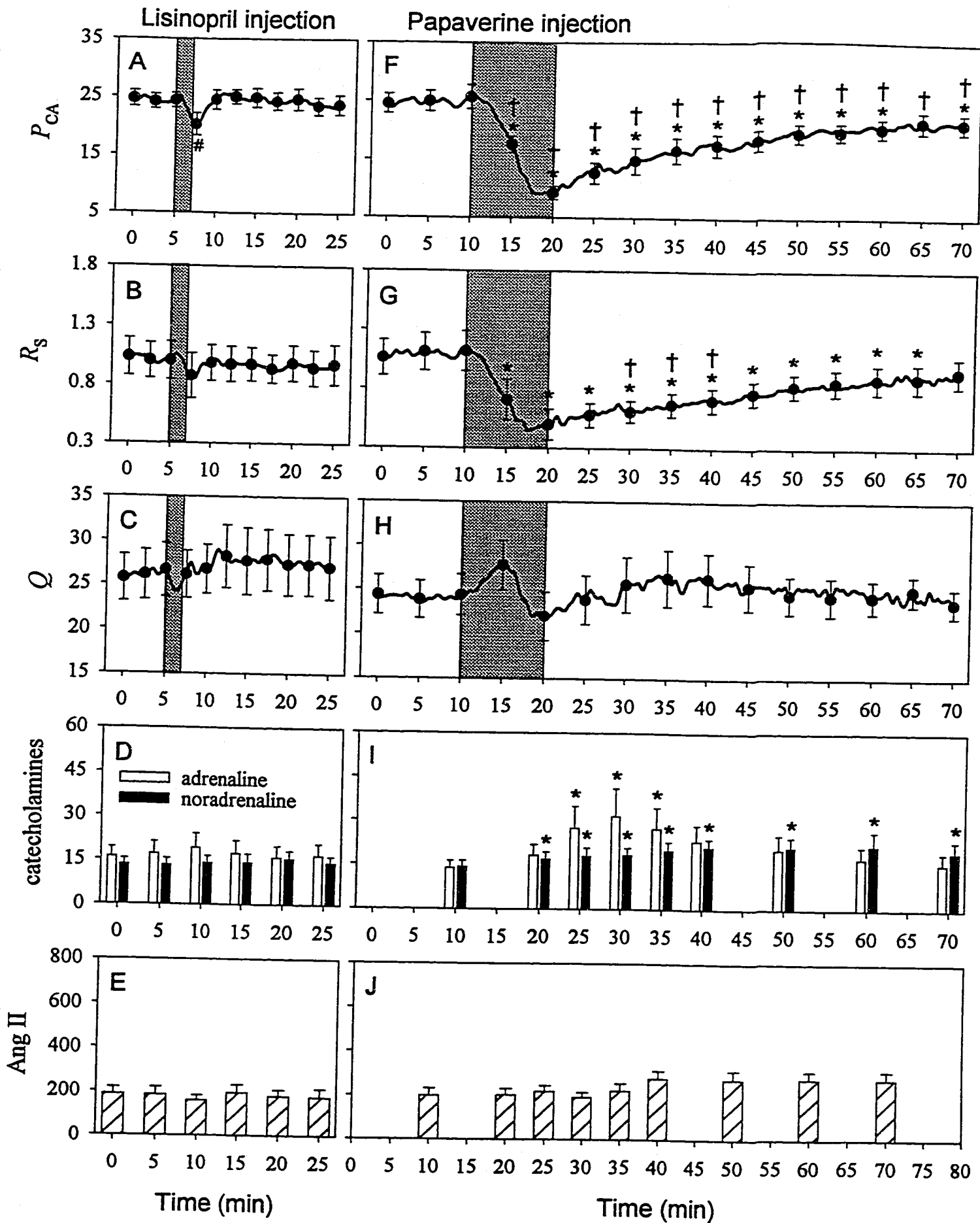


Figure 6.4

Table 6.3 The effects of a bolus injection of [Asn¹,Val⁵,Asn⁹]-Ang I¹ on the cardiovascular variables² of intact control and lisinopril-treated spiny dogfish, *Squalus acanthias*.

| Treatment | <i>N</i> | <i>P</i> _{CA} | <i>R</i> _S | <i>Q</i> |
|-------------------|----------|------------------------|--|--|
| | | (cm H ₂ O) | (cm H ₂ O ml ⁻¹ min ⁻¹ kg ⁻¹) | (ml min ⁻¹ kg ⁻¹) |
| Control | 6 | | | |
| Basal values | | 24.7±1.1 | 1.0±0.1 | 28.1±3.3 |
| Post-injection | | 29.5±1.8* | 1.3±0.2* | 25.1±2.9* |
| Change from basal | | 4.8±0.7 | 0.3±0.1 | -3.0±0.8 |
| Lisinopril | 6 | | | |
| Basal values | | 23.9±1.5 | 1.0±0.1 | 25.7±2.5 |
| Post-injection | | 25.5±1.4 | 1.1±0.1 | 25.7±2.6 |
| Change from basal | | 1.7±0.9# | 0.1±0.0# | 0.0±0.5# |

1 – The angiotensin injection contained 1000 pmol kg⁻¹ body wt [Asn¹,Val⁵,Asn⁹]-angiotensin I.

Values are mean ± 1 S.E.M.

* Significantly different from basal value for a given treatment. # Significantly different from the change from basal value of the control treatment ($P < 0.05$).

2 – *P*_{DA}, mean caudal artery pressure; *R*_S, systemic resistance; *Q*, cardiac output.

Figure 6.5 Effects of an intravenous injection of the α -adrenoceptor antagonist, yohimbine (A-E; 4 mg kg⁻¹) followed by an intravenous injection of the smooth muscle relaxant, papaverine (F-J; 10 mg kg⁻¹) on (A & F) mean caudal artery pressure (P_{CA} , cm H₂O), (B & G) systemic vascular resistance (R_s , cm H₂O ml⁻¹ min⁻¹ kg⁻¹), (C & H) cardiac output (Q , ml min⁻¹ kg⁻¹), (D & I) plasma catecholamines (nM), and (E & J) plasma [Asn¹, Pro³, Ile⁵]-Ang II (pM) in Pacific spiny dogfish, *Squalus acanthias* ($N=6$). The time during which yohimbine was injected is shown by a gray box in graphs A, B, and C. The time during which papaverine was injected is shown by a gray box in graphs F, G, and H. An asterisk denotes a significant difference from the control value immediately preceding the papaverine injection (10-min) for a given parameter. † denotes a significant difference from the corresponding value in the papaverine treatment (Fig. 6.3) for a given parameter. # denotes a significant difference from the control value immediately preceding the yohimbine injection (5-min) for a given parameter ($P<0.05$). Values are means \pm 1 S.E.M.

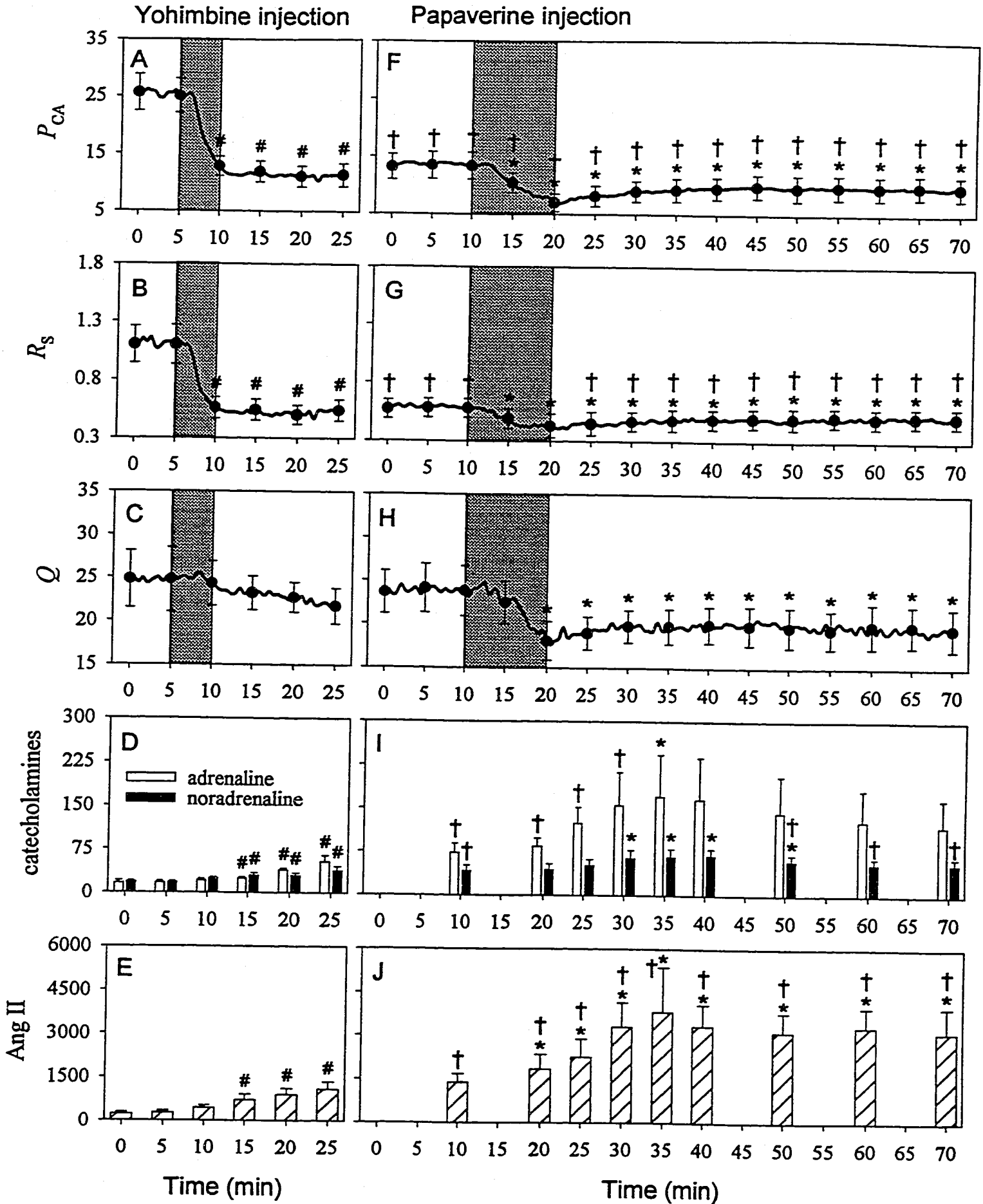


Figure 6.5

The overall relationship between plasma [Asn¹, Pro³, Ile⁵]-Ang II and plasma catecholamine (adrenaline + noradrenaline) concentrations at each sampling time in the papaverine, lisinopril + papaverine, and yohimbine + papaverine treatments is shown in figure 6.6. This relationship ($r^2 = 0.61$) is significant ($P < 0.001$), and can be described by the following linear equation: [catecholamines] = 0.047[Ang II] + 34.57, where [catecholamines] is in nM and [Ang II] is in pM.

Discussion

The present study demonstrates that the cardiovascular effects of homologous Ang II in the spiny dogfish, *Squalus acanthias*, are mediated entirely by catecholamines. In addition, we have simultaneously assessed, for the first time, plasma Ang II, plasma catecholamines and cardiovascular parameters during normotensive and hypotensive conditions. This approach has provided evidence that catecholamines play a tonic role in the regulation of the systemic vascular resistance in elasmobranchs, and that the renin-angiotensin system can indirectly contribute to the humoral adrenergic system during hypotensive conditions.

The effects of exogenous dfAng II on cardiovascular function and plasma catecholamine levels

In support of previous observations using a variety of heterologous Ang I and Ang II peptides (Opdyke and Holcombe 1976; Khosla *et al.* 1983), we found no evidence that physiological doses of homologous dfAng II can directly affect the primary contributors to blood pressure regulation, R_s and Q . In fact, the dose-dependent dfAng II-elicited pressor response appears to be mediated by an increase in R_s that can be ascribed to a dose-dependent increase in humoral catecholamines. α -adrenoceptor blockade, in addition to eliminating the vasopressor effects of exogenous dfAng II, was characterized by a drop in R_s despite a five-fold increase in

Figure 6.6 Correlation between plasma [Asn¹, Pro³, Ile⁵]-Ang II and total plasma catecholamines in Pacific spiny dogfish, *Squalus acanthias*, before and after receiving an intravenous injection of either papaverine alone (filled square), an injection of lisinopril followed by papaverine (unfilled circle), or an injection of yohimbine followed by papaverine (unfilled triangle). Linear regression relationship ($r^2 = 0.61$) and its 95% confidence interval are indicated by the solid and dotted lines, respectively.

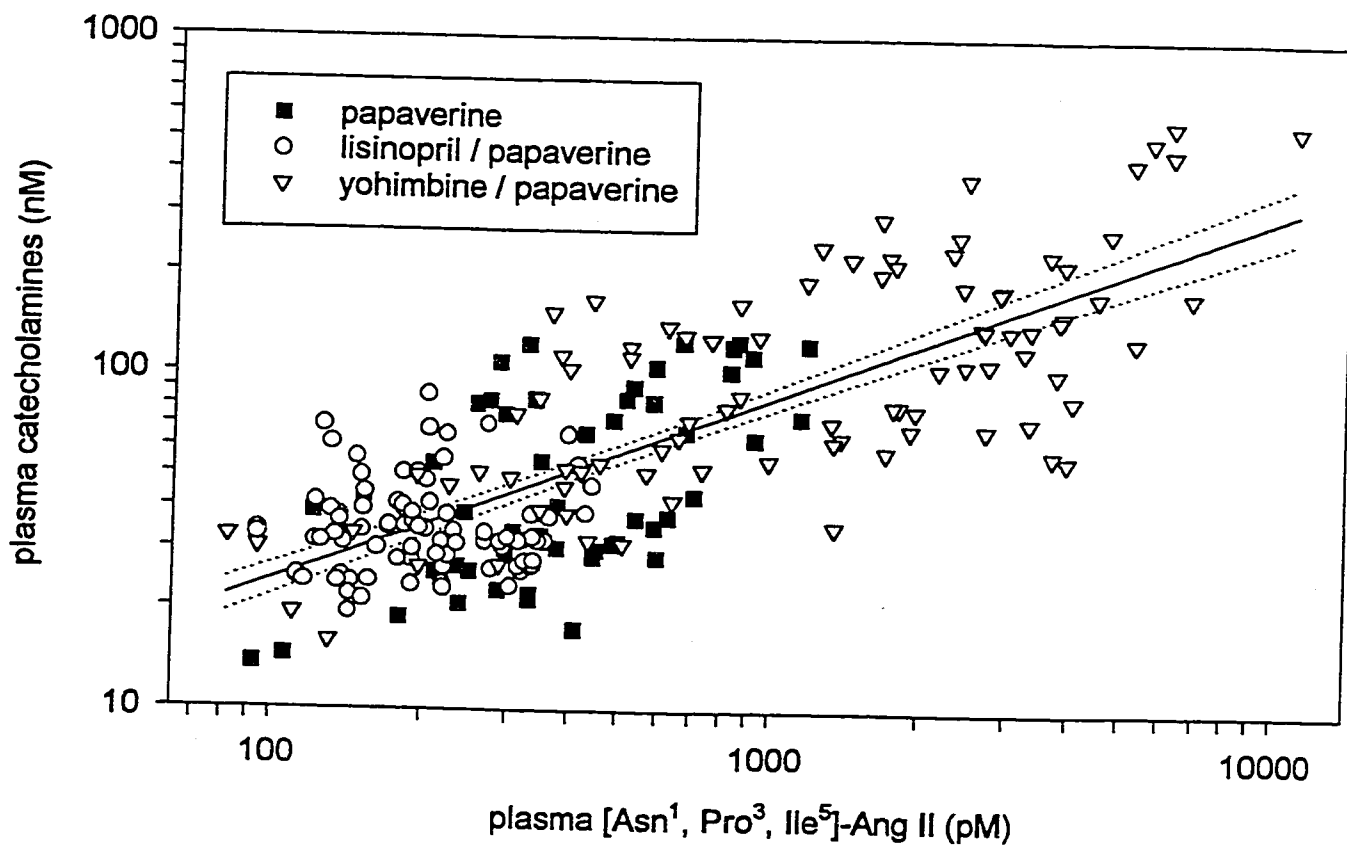


Figure 6.6

plasma Ang II concentrations. Hence in *S. acanthias*, independent of dosage or amino acid sequence, the cardiovascular effects of exogenous Ang II are mediated indirectly via a stimulation of catecholamine release and their subsequent interaction with the α -adrenoceptors of the vasculature.

These results are in sharp contrast with the direct and adrenergically-independent dfAng II-induced pressor response observed in *S. canicula* (Tierney *et al.* 1997). Although the amino acid sequence of dogfish angiotensin is unique (Takei *et al.* 1993), differences between the sequence of homologous and heterologous angiotensins cannot account for the discrepant results obtained in *S. canicula* (Tierney *et al.* 1997) versus *S. acanthias* (Opdyke and Holcombe 1976; Khosla *et al.* 1983). Similarly, Ang II dosage differences cannot explain the different mode of action of Ang II in *S. canicula* and *S. acanthias*. Although the original studies in *S. acanthias* used supraphysiological doses of Ang II (Opdyke and Holcombe 1976; Khosla *et al.* 1983), in the present study we used physiological doses similar to those used by Tierney *et al.* (1997) in *S. canicula*. Hence, differences in the mode of action of Ang II in *S. acanthias* and *S. canicula* may reflect true species differences. For example, whereas blood pressure recovered within 15 minutes after an injection of phentolamine in *S. canicula* (Tierney *et al.* 1997), P_{CA} and R_S remained depressed for hours in yohimbine-treated *S. acanthias* despite an increase in plasma Ang II. Further investigations into the pharmacology of α -adrenoceptors, and experiments aimed at determining the presence or absence of functional vascular Ang II receptors in both species, may help to resolve whether Ang II has a single or dual mode of action in elasmobranchs.

Although the failure of heterologous Ang II to directly constrict blood vessels in *S. acanthias* has previously been interpreted as evidence that Ang II vascular receptors are absent in dogfish (Opdyke and Holcombe 1978; Carroll 1981), there is recent evidence to the contrary

(Hamano *et al.* 1998). Whereas a dose of 780 000 pM of teleost Ang II failed to constrict isolated rings from the coeliac artery in *S. acanthias* (Carroll 1981), a dose of 100 000 pM of dfAng II elicited α -adrenoceptor-independent vasopressor activity on the same vessel in *T. scyllia* (Hamano *et al.* 1998). Given the known differences in the vasopressor activity of homologous versus heterologous Ang II in dogfish (Takei *et al.* 1993; see also below), these results suggest that the failure of previous experiments to demonstrate Ang II vascular receptor in *S. acanthias* may be explained by their selection of peptide dosage. While the results of Hamano *et al.* (1998) provide evidence for functional Ang II vascular receptors in **large arteries** of dogfish, our results do not provide any physiological evidence for the involvement of Ang II receptors in the **resistance vessels** of *S. acanthias*.

Although homologous and heterologous angiotensins appear to have the same indirect cardiovascular effects in *S. acanthias*, the important amino acid sequence differences between these peptides (Takei *et al.* 1993) are responsible for differences in their vasopressor activity. As previously observed in *Triakis scyllia* with dfAng I (Takei *et al.* 1993), homologous dfAng II had a much greater vasopressor activity than heterologous Ang II in *S. acanthias* (Opdyke and Holcombe 1976; Khosla *et al.* 1983). Conversely, the dose-response relationship for the vasopressor effects of dfAng I in *T. scyllia* (Takei *et al.* 1993), dfAng II in *S. canicula* (Tierney *et al.* 1997) and dfAng II in *S. acanthias* are remarkably similar.

In contrast to the equal potency of teleost, tetrapod, and mammalian Ang II agonists in stimulating catecholamine release in rainbow trout (Bernier and Perry 1997), it appears that the specific amino acid sequence of the Ang II injected in dogfish may impact on its ability to elicit catecholamine release. In elasmobranchs, the catecholamine contents of the most important site of catecholamine storage, the axillary bodies, are composed of approximately 80% noradrenaline

and 20% adrenaline (Abrahamsson 1979). Moreover, storage granules of both catecholamines have been identified and these appear in separate cells (Coupland 1971). In *S. acanthias*, since Ang II elicits catecholamine release in the presence of the ganglionic blocker hexamethonium, the stimulatory effects of Ang II on the chromaffin tissue are thought to be direct (Opdyke and Holcombe 1976). However, while Opdyke *et al.* (1981b) reported a preferential increase in plasma noradrenaline over adrenaline in response to supraphysiological doses of heterologous Ang II (4800 pmol kg⁻¹), opposite effects were obtained with physiological doses (30-1200 pmol kg⁻¹) of homologous dfAng II in this study. Given the differential haemodynamic responses to adrenaline and noradrenaline in *S. acanthias* (Capra and Satchell 1977b), the preferential stimulation of adrenaline by homologous Ang II may be relevant to the role of the RAS in cardiovascular control (i.e. elevation of blood pressure and blood volume). For example, in dogfish, adrenaline is a more potent vasopressor than noradrenaline whereas the latter is a more potent vasodilator (Capra and Satchell 1977b). The preferential increase in plasma adrenaline over noradrenaline with dfAng II in *S. acanthias* is similar to the stimulatory effects of homologous Ang II in teleosts (Carroll and Opdyke 1982; Bernier and Perry 1997, 1999) and in mammals (Butler *et al.* 1994).

Series 2: the effects of hypotension on circulating Ang II and catecholamine concentrations

The transient hypotensive effects of papaverine in *S. acanthias* concur with the previously observed effects of this smooth muscle relaxant in *S. canicula* (Hazon *et al.* 1989) and in a number of teleost species (Nishimura *et al.* 1979; Tierney *et al.* 1995b; Bernier *et al.* 1999b). The significant and prolonged increases in plasma Ang II concentration following this hypotensive stress provide the first direct evidence that papaverine treatment truly stimulates the RAS in elasmobranchs. These results also corroborate the preliminary observations of Galli and Kiang

(1990) showing that a 30% haemorrhage can increase immunoreactive [Val⁵]-Ang II in the nurse shark, *Ginglymostoma cirratum*. Because blockade of the RAS exacerbates and prolongs the hypotensive effects of papaverine in *S. acanthias*, as in *S. canicula* (Hazon *et al.* 1989), the papaverine-elicited increase in plasma Ang II must play an important role in cardiovascular control during a hypotensive stress. On the other hand, since various ACE inhibitors either have no cardiovascular effect (Opdyke and Holcombe 1976; Hazon *et al.* 1989) or only a small and brief hypotensive effect (this study), the RAS does not appear to be involved in the cardiovascular control of elasmobranchs during normotensive conditions. In contrast, the RAS plays a tonic anti-depressor role in teleosts and ACE blockade chronically reduces blood pressure (Olson 1992; Tierney *et al.* 1995b; Bernier *et al.* 1999b). Furthermore, while lisinopril treatment has no effect on circulating catecholamines in *S. acanthias*, it is associated with a significant decrease in the basal circulating concentration of adrenaline in *Oncorhynchus mykiss* (Bernier *et al.* 1999b).

Because the humoral adrenergic system appears to be an essential intermediate for the involvement of Ang II in cardiovascular control (see *Series I*; Opdyke and Holcombe 1976; Khosla *et al.* 1983), the cardiovascular effects of the RAS in hypotensive *S. acanthias* are likely mediated through an increase in plasma catecholamines. Prior to this study, however, the effects of a hypotensive stress on plasma catecholamine levels in elasmobranchs had not been assessed. The significant correlation between plasma Ang II and catecholamine concentrations following the different papaverine treatments suggests that the RAS may play a central role in controlling the recruitment of the adrenergic system in response to hypotension. However, although lisinopril completely blocked the papaverine-elicited increase in plasma Ang II, ACE blockade was associated only with a partial reduction of the overall increase in plasma catecholamines following the hypotensive treatment. Hence, while a portion of the increase in plasma catecholamines may

be attributed to an elevation in plasma Ang II, other secretagogues may also be involved in stimulating catecholamine release during a hypotensive stress in dogfish. The presence of cholinergic and non-cholinergic neuronal pathways innervating the chromaffin cells of the axillary bodies (Abrahamsson 1979; Opdyke *et al.* 1983; Reid *et al.* 1995) offers a variety of potential mechanisms for the control of catecholamine release in response to stress. Because treatment of dogfish with the ganglionic blocker hexamethonium results in a significant reduction in blood pressure, it appears likely that at least part of the vasomotor tone provided by circulating catecholamines is controlled by cholinergic neuronal activity (Holcombe *et al.* 1980). Also, in addition to Ang II, potassium (Opdyke *et al.* 1983), urotensin II (Conlon *et al.* 1996c), and C-type natriuretic peptides (CNP; McKendry *et al.* 1999) are other non-cholinergic secretagogues that have been shown to stimulate catecholamine release in elasmobranchs. While some of these catecholamine secretagogues may also be involved in cardiovascular regulation (Conlon *et al.* 1996c; McKendry *et al.* 1999), their specific involvement during a hypotensive stress remains to be ascertained.

In elasmobranchs, the location of the axillary bodies, in the venous blood immediately behind the heart (Abrahamsson 1979), has often been associated with their potential to act as a source of adrenergic cardiac control (for references, see Morris and Nilsson 1994). Although the cardiac responses to catecholamine injections are variable (Capra and Satchell 1977b; Morris and Nilsson 1994), catecholamines generally elicit β -adrenoceptor-mediated positive inotropic and chronotropic effects on the isolated heart of *S. acanthias* (Capra and Satchell 1977a). In this study, however, despite the elevated circulating catecholamine levels associated with a variety of treatments, we did not observe any stimulatory effects *in vivo* on the heart of *S. acanthias*. While the potential stimulatory effects of the dfAng II-elicited increase in plasma catecholamines might

have been masked by vasopressor-mediated reflexive (Lutz and Wyman 1932) or passive inhibition of the heart, the Ang II-elicited increase in plasma catecholamines in the yohimbine-treated dogfish was also without any cardiac effect. By itself, Ang II does not affect the contractility of the isolated dogfish heart (Opdyke *et al.* 1982). Furthermore, the significant increases in plasma catecholamines elicited by either yohimbine or papaverine treatment were not associated with any change in either f_H or V_S . In fact, in the combined yohimbine + papaverine treatment, although the post-papaverine circulating concentration of plasma adrenaline reached almost 200 nM, V_S and Q decreased significantly in parallel with the drops in R_S and P_{CA} . Presumably, this reduction in V_S results from a decrease in venous return. In rainbow trout, in contrast, either α -adrenoceptor blockade or the imposition of hypotensive stressors is characterised by a significant increase in Q (Bernier *et al.* 1999b). Hence, in dogfish, while circulating catecholamines may be important in maintaining resting cardiac activity (Short *et al.* 1977), they do not appear to be involved in increasing Q either at rest or following a hypotensive stress. On the other hand, the marked vasodepressor actions of yohimbine and phentolamine (Opdyke *et al.* 1972; Holcombe *et al.* 1980) in normotensive *S. acanthias* suggest that circulating catecholamines are an important mediator in the control of basal systemic vascular resistance.

In our ongoing attempt to understand the importance of, and the mechanisms that characterize, the interaction between the RAS and the adrenergic system in the cardiovascular control of fishes, this study provides direct evidence that both systems are essential to cardiovascular homeostasis in hypotensive elasmobranchs. Although the RAS does not appear to be involved in the minute-to-minute regulation of resting blood pressure, the circulating concentrations of plasma Ang II increase in proportion to the intensity of hypotension. However, in *S. acanthias*, irrespective of whether the increase in plasma Ang II has an endogenous or

exogenous origin, the cardiovascular effects of homologous Ang II are mediated indirectly through an interaction with the adrenergic system. A strong correlation between the circulating concentrations of plasma Ang II and catecholamines suggests that the nature of this interaction primarily involves the humoral component of the adrenergic system. Plasma catecholamines, in addition to their tonic role in maintaining basal cardiac activity and vascular resistance, can be recruited by Ang II and other secretagogues during a hypotensive stress and play a key role in the regulation of systemic vascular resistance. On the other hand, the ability of plasma catecholamines to enhance cardiac activity in dogfish appears to be limited.

CHAPTER 7.**Control of Catecholamine and Serotonin Release from the
Chromaffin Tissue of the Atlantic Hagfish**

Abstract

An *in situ* saline-perfused systemic heart / posterior cardinal vein preparation of the Atlantic hagfish (*Myxine glutinosa*) was used to assess (1) the ability of the chromaffin tissue to release catecholamines in response to adrenocorticotrophic hormone (ACTH; $7.5 \text{ U}\cdot\text{kg}^{-1}$), serotonin ($250 \text{ nmol}\cdot\text{kg}^{-1}$), carbachol ($100 \text{ }\mu\text{mol}\cdot\text{kg}^{-1}$), [ASN¹-VAL⁵]angiotensin II (Ang II; $100 \text{ nmol}\cdot\text{kg}^{-1}$), histamine ($0.3 - 300 \text{ }\mu\text{mol}\cdot\text{l}^{-1}$), and a high $[\text{K}^+]$ saline ($60 \text{ mmol}\cdot\text{l}^{-1}$), (2) whether serotonin is co-released with the catecholamines of the chromaffin tissues, and (3) the potential modulatory effects of NECA, an adenosine receptor agonist, and DPSPX, an adenosine receptor antagonist, on catecholamine release.

Bolus injections of either ACTH, serotonin, or carbachol, or perfusion with the high $[\text{K}^+]$ saline, all elicited the release of both adrenaline and noradrenaline. Pre-treatment with the serotonergic receptor antagonist methysergide, or the ganglionic receptor blocker hexamethonium, abolished the serotonin- and carbachol-mediated catecholamine releases, respectively. Neither receptor antagonist affected the ACTH-mediated catecholamine release. Bolus injections of Ang II, or perfusion with a range of histamine concentrations, two potent secretagogues in other vertebrates, did not elicit catecholamine secretion in hagfish.

While injections of Ang II or perfusion with the high $[\text{K}^+]$ saline both elicited the release of serotonin, treatments with either ACTH, carbachol, or histamine did not. Hence, co-release of catecholamines and serotonin was elicited by non-specific cell membrane depolarization using K^+ , but not by the specific secretagogues assessed in this study.

The adenosine receptor agonist, NECA, and antagonist, DPSPX, significantly modified the secretory responses elicited by ACTH, serotonin, and carbachol. The results suggest that

adenosine may inhibit catecholamine release induced by serotonin or carbachol, while stimulating ACTH-induced release.

Although the contribution of the different secretagogues identified in this study has yet to be explored *in vivo*, our results suggest that the control of catecholamine and serotonin release from the aneural chromaffin tissue of the Atlantic hagfish can be achieved through hormonal and / or paracrine means.

Introduction

In response to an acute physiological challenge, vertebrates release the catecholamine stress hormones adrenaline and noradrenaline (Axelrod and Reisine 1984; Randall and Perry 1992) into the circulation. The primary control mechanism of catecholamine release in most vertebrates is through pre-ganglionic sympathetic fibers (Randall and Perry 1992; Edwards and Jones 1993). A notable exception to this general pattern is found in hagfish. The principle catecholamine storing tissue of hagfish, the chromaffin cells of the systemic heart, are not innervated (Green 1902; Augustinsson *et al.* 1956). So, in principle, the control of catecholamine secretion in these primitive vertebrates should be entirely through non-cholinergic mechanisms.

Hagfish store large quantities of catecholamines in their systemic and portal hearts, and posterior cardinal vein (Östlund 1954; Augustinsson *et al.* 1956; Östlund *et al.* 1960; Euler and Fange 1961; Perry *et al.* 1993). The morphology and pharmacology of these catecholamine-storing cells are similar to the chromaffin cells of the mammalian adrenal medulla (Östlund *et al.* 1960; Bloom *et al.* 1961). The chromaffin cells of hagfish also contain the key enzymes of catecholamine biosynthesis (Jonsson 1983; Reid *et al.* 1995). Despite these homologies with the

chromaffin cells of other vertebrates, recent efforts investigating possible *in vivo* mechanisms of catecholamine release in hagfish, were unable to identify specific secretagogues (Perry *et al.* 1993).

Previous studies have shown that catecholamines are released during severe hypoxia in hagfish (Perry *et al.* 1993; Bernier *et al.* 1996). However, perfusion of *in situ* preparations with anoxic or acidic saline failed to elicit catecholamine release (Perry *et al.* 1993). Similarly, *in situ* elevation of plasma K^+ concentration within the physiological range also failed to stimulate catecholamine secretion (Perry *et al.* 1993). Although the endogenous catecholamines of the hagfish hearts may be important for the regulation of cardiac function (Bloom *et al.* 1961; Axelsson *et al.* 1990; Johnsson *et al.* 1996), and studies have shown that regulation of the heart is sensitive to the increase in blood pressure observed during hypoxia (Jensen 1961; Axelsson *et al.* 1990; Forster *et al.* 1992), catecholamine secretion appears to be insensitive to changes in perfusion pressure (Perry *et al.* 1993). Carbachol, a general cholinergic receptor agonist, has been shown to stimulate catecholamine secretion. However, because there is no evidence that the chromaffin cells are innervated, this is not likely to be an important mechanism *in vivo* (Perry *et al.* 1993).

Several other lines of evidence suggest the involvement of hormones and neuromodulators in catecholamine secretion in hagfish. Injections of Atlantic cod (*Gadus morhua*) pituitary extracts elicited a marked release of catecholamines from *in situ* preparations of *Myxine glutinosa* (Perry *et al.* 1993). There is immunohistochemical evidence that the systemic and portal hearts of hagfish contain serotonin (Reid *et al.* 1995), a secretagogue of catecholamine release in rainbow trout (Fritsche *et al.* 1993). Histamine, a potent secretagogue in mammals (Burgoyne 1991), is also stored in the hagfish hearts (Augustinsson *et al.* 1956). Indirect evidence suggests that

angiotensin II (Ang II), a secretagogue throughout vertebrates, may stimulate catecholamine secretion in *Myxine glutinosa* (Carroll and Opdyke 1982). Finally, *in vivo* treatment of hypoxic *Eptatretus stouti* with the adenosine receptor antagonist, theophylline, elicited a marked increase in the circulating concentration of plasma adrenaline (Bernier *et al.* 1996). Thus, results from these various studies suggest that adrenocorticotrophic hormone (ACTH), serotonin, Ang II, histamine, and adenosine, may be involved in the control of catecholamine release in hagfish.

The goals of this study, therefore, were to a) determine if any of the potential secretagogues listed above do indeed stimulate catecholamine release in *Myxine glutinosa*, b) assess whether serotonin is co-released with the catecholamines of the chromaffin tissues, and c) investigate the potential modulatory effects of adenosine on catecholamine release.

Materials and methods

Experimental animals

Atlantic hagfish (*Myxine glutinosa*) weighing between 26 and 72 g (mean mass = 40.6 ± 1.4 g; experimental $N = 65$) were obtained from Huntsman Marine Science Centre (St. Andrews, New Brunswick). They were maintained indoors in a large fiberglass tank supplied with recirculated artificial sea water (temperature 5-6°C). The salinity of the water was kept between 27 and 29 ‰ by replacing a portion of the recirculated water as required. The tank was kept covered and the animals were not fed throughout the holding period (from July to November).

In situ experiments

To assess the ability of the chromaffin tissue to release catecholamines in response to potential secretagogues, the *in situ* saline-perfused systemic heart / posterior cardinal vein (PCV) preparation of Perry *et al.* (1993) was used with the following modifications. Hagfish were

anaesthetized for 30 min in a solution of 2.5 g l^{-1} MS222 (tricaine methanesulphonate) in sea water. The PCV (inflow vessel) and the ventral aorta (outflow vessel) were cannulated with polyethylene (PE 60) tubing stretched to fit the vessels. The cannulated hagfish were immersed in a bath of sea water without anaesthetic and perfused with aerated hagfish saline (pH 8.1; Axelsson *et al.* 1990). The sea water bath and the saline were placed on ice and kept cool ($6\text{--}8^{\circ}\text{C}$) throughout the perfusions. In all experiments, the *in situ* preparations were perfused for 20 min prior to sample collection. After this stabilization period, two pre-treatment samples were taken at one minute intervals. The perfusion saline was then altered in accordance with the treatments described below over a period of one min, and seven post-treatment samples were collected 1, 2, 3, 4, 5, 7.5, and 10 min after the intervention. All samples were collected in pre-weighed 1.5 ml microcentrifuge tubes over a period of 50 sec and immediately frozen in liquid N_2 . The frozen samples were weighed and stored at -80°C until analysis of catecholamine content (within 7 days). In series 1, 2, and 3 (see below), each sample also was analyzed for serotonin content.

Series 1: The effects of hagfish saline on catecholamine and serotonin release

Hagfish were given a bolus injection ($300 \mu\text{l}$) of hagfish saline after the stabilization period, and the response was monitored ($N = 6$). The total perfusion period of each preparation was 30 min.

Series 2: The effects of ACTH, serotonin, Ang II, carbachol, and elevated $[\text{K}^+]$ on catecholamine and serotonin release

After the 20 min stabilization period and collection of the two pre-treatment samples, a bolus injection ($300 \mu\text{l}$) of either: a) 7.5 U kg^{-1} of porcine adrenocorticotrophic hormone (ACTH 1-39, Sigma Chemicals, Cat. # A-6303), b) 250 nmol kg^{-1} of serotonin oxalate salt (Sigma

Chemicals, Cat. # H-7877), c) 100 nmol·kg⁻¹ of [ASN¹-VAL⁵]angiotensin II (Sigma Chemicals, Cat. # A-6402), d) 100 μmol·kg⁻¹ of the cholinergic receptor agonist carbachol (Research Biochemicals International, Cat. # C-107) was administered to the preparation via a valve in the inflow catheter and the post-treatment samples were collected. All drugs were prepared daily in hagfish saline. After collecting the post-treatment samples from the first drug injection, the preparation was perfused for a second stabilization period of 20 min. After this time, two new pre-treatment samples were collected, a second drug was administered and the response monitored. Using this protocol, the 4 drugs were randomly tested on each of 10 hagfish. This experimental protocol was chosen to minimize the number of animals. Once the responses of the 4 drugs were assessed, the preparation was perfused for a fifth and final 20 min stabilization period. Once the two pre-treatment samples were collected, the perfusate reservoir was switched to a hagfish saline containing 60 mmol·l⁻¹ KCl. This protocol is known to provoke a non-specific depolarization of the chromaffin cells and a marked release of catecholamines (Perry *et al.* 1993). Therefore, it was used to assess the viability of the preparations. Overall, the total perfusion period of each preparation was 150 min.

Series 3: The effects of histamine on the release of catecholamines and serotonin

After the 20 min stabilization period and collection of the two pre-treatment samples, the perfusate reservoir was switched to a hagfish saline containing a [histamine] of 0.3 μmol·l⁻¹ and perfused for 10 min. Once the post-treatment samples were collected and a new stabilization period had elapsed, this procedure was repeated three more times with saline solutions containing histamine concentrations of 3, 30, and 300 μmol·l⁻¹ (N=4). Overall, the total perfusion period of each preparation was 120 min. In 4 other hagfish, after the stabilization period, the preparations were perfused with a hagfish saline containing a single [histamine] of 300 μmol·l⁻¹.

Series 4: The effects of methysergide on catecholamine release

The stimulatory effects of serotonin and ACTH on catecholamine release were assessed with the serotonergic receptor antagonist, methysergide (methysergide maleate, RBI Cat. # M-137), added to the perfusion saline (final concentration = 10^{-5} mol l⁻¹). After the 20 min stabilization period and the collection of the pre-treatment samples, a bolus dose of serotonin (250 nmol kg⁻¹) was injected into the infusion cannula. After sampling and re-stabilization, pre-treatment samples were taken and a bolus injection of ACTH (7.5 U kg⁻¹) was administered. Overall the total perfusion period of each of these preparation was 60 min ($N = 8$).

Series 5: The effects of hexamethonium on catecholamine release

The stimulatory effects of carbachol and ACTH on catecholamine release were assessed with the cholinergic antagonist, hexamethonium (hexamethonium dichloride, RBI Cat. # H-132), added to the perfusion saline (final concentration = 10^{-3} mol l⁻¹). The protocol used in this series was identical to the one described for series 4. In each preparation, the effects of carbachol (100 μ mol kg⁻¹) were assessed first, and then after a second period of stabilization, the effects of ACTH (7.5 U kg⁻¹) were assessed ($N = 8$).

Series 6: Assessing the modulatory effects of NECA on catecholamine release

The stimulatory effects of ACTH, serotonin, and carbachol on catecholamine release were assessed with the adenosine receptor agonist, NECA (5'-(*N*-ethylcarboxamido) adenosine, RBI Cat. # A-104), added to the perfusion saline (20 mg l⁻¹). NECA is a potent adenosine receptor agonist with nearly equal affinity at A₁ and A₂ receptors (Bruns *et al.* 1986). Using the protocol and the dosages described in series 2, ACTH, serotonin, and carbachol were sequentially and randomly tested on each hagfish ($N = 10$). Overall, the total perfusion period of each of these preparation was 90 min.

Series 7: Assessing the modulatory effects of DPSPX on catecholamine release

The stimulatory effects of ACTH, serotonin, and carbachol on catecholamine release were assessed with the adenosine receptor antagonist, DPSPX (1,3-dipropyl-8-*p*-sulfophenylxanthine, RBI Cat. # A-022), added to the perfusion saline (20 mg l⁻¹). DPSPX is a potent adenosine receptor antagonist with slight selectivity for A₁ over A₂ receptors (Daly *et al.* 1985). The experimental conditions of this series were similar to series 6 (*N* = 15).

Analytical procedures

Perfusate adrenaline (Adr) and noradrenaline (Nor) levels were determined on alumina-extracted saline samples (200 µl) using high performance liquid chromatography (HPLC) with electrochemical detection (Woodward 1982). The HPLC incorporates a Varian Star 9012 solvent delivery system (Varian Chromatography Systems, Walnut Creek, CA) coupled to a Princeton Applied Research 400 electrochemical detector (EG & G Instruments Corporation, Princeton, NJ). The extracted samples were passed through an Ultratechsphere ODS-C18-5µm column (HPLC Technology LTD), using a catecholamine and metanephrine mobile phase (Chromsystems, Munich, Germany). The separated amines were integrated with the Star Chromatography software program (version 4.0, Varian). Concentrations were calculated relative to appropriate standards, and with 3, 4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard in all determinations.

Perfusate serotonin levels were determined on 100 µl aliquots of non-extracted saline using the same HPLC system and column as above, and with a Cat-A-Phase II mobile phase (Scientific Products & Equipment, Concord, Canada). Calculations of concentrations were based on a linear standard curve, with a detection limit of 1 nmol l⁻¹.

Statistical analyses

All data are presented as mean \pm one standard error. The statistical significance of observed effects of a given drug injection within a group were tested by one-way repeated measures ANOVA. Dunnett's test was used to compare the second pre-treatment sample mean (the pre-treatment sample taken immediately before alterations to the perfusion saline) with values at subsequent and previous times. For a given drug, a paired t-test was used to compare the second pre-treatment sample mean (pre-treatment secretion) with the mean of the maximum post-treatment sample values (maximum secretion). The statistical significance of observed differences between the means of several treatments were tested by one-way ANOVA. The significance level for all statistical tests was $P < 0.05$.

Results

Series 1: The effects of hagfish saline on catecholamine and serotonin release

Bolus injections of saline did not significantly affect the basal secretion of either noradrenaline or adrenaline (Fig. 7.1). Baseline serotonin secretion was also unaffected by hagfish saline injections.

Series 2: The effects of ACTH, serotonin, Ang II, carbachol, and elevated $[K^+]$ on catecholamine and serotonin release

Bolus injections of either ACTH, serotonin, or carbachol resulted in a significant release of both Nor and Adr (Figs. 7.1, 7.2). Although the mean adrenaline secretion rate did not significantly increase in response to serotonin and carbachol injections (Figs. 7.2F, 7.2G), the maximum adrenaline secretion rate did (Fig. 7.1B). This is because the maximum secretion rate

Figure 7.1 (A) Noradrenaline and (B) adrenaline secretion rates of *in situ* preparations of Atlantic hagfish injected with either physiological saline (control conditions, $N = 6$), $7.5 \text{ U}\cdot\text{kg}^{-1}$ of porcine ACTH ($N = 10$), or $250 \text{ nmol}\cdot\text{kg}^{-1}$ of serotonin ($N = 10$), or $100 \text{ }\mu\text{mol}\cdot\text{kg}^{-1}$ of carbachol ($N = 10$), or $100 \text{ nmol}\cdot\text{kg}^{-1}$ of $[\text{ASN}^1\text{-VAL}^5]\text{Ang II}$ ($N = 10$), or exposed to saline solutions containing either a [histamine] of $300 \text{ }\mu\text{mol}\cdot\text{l}^{-1}$ ($N = 8$), or a $[\text{K}^+]$ of $60 \text{ mmol}\cdot\text{l}^{-1}$ ($N = 10$). The open bars indicate the catecholamine secretion rates prior to the treatments listed above. The solid bars indicate the maximum catecholamine secretion rates in response to those treatments. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment ($P < 0.05$). Values are means \pm 1 SEM.

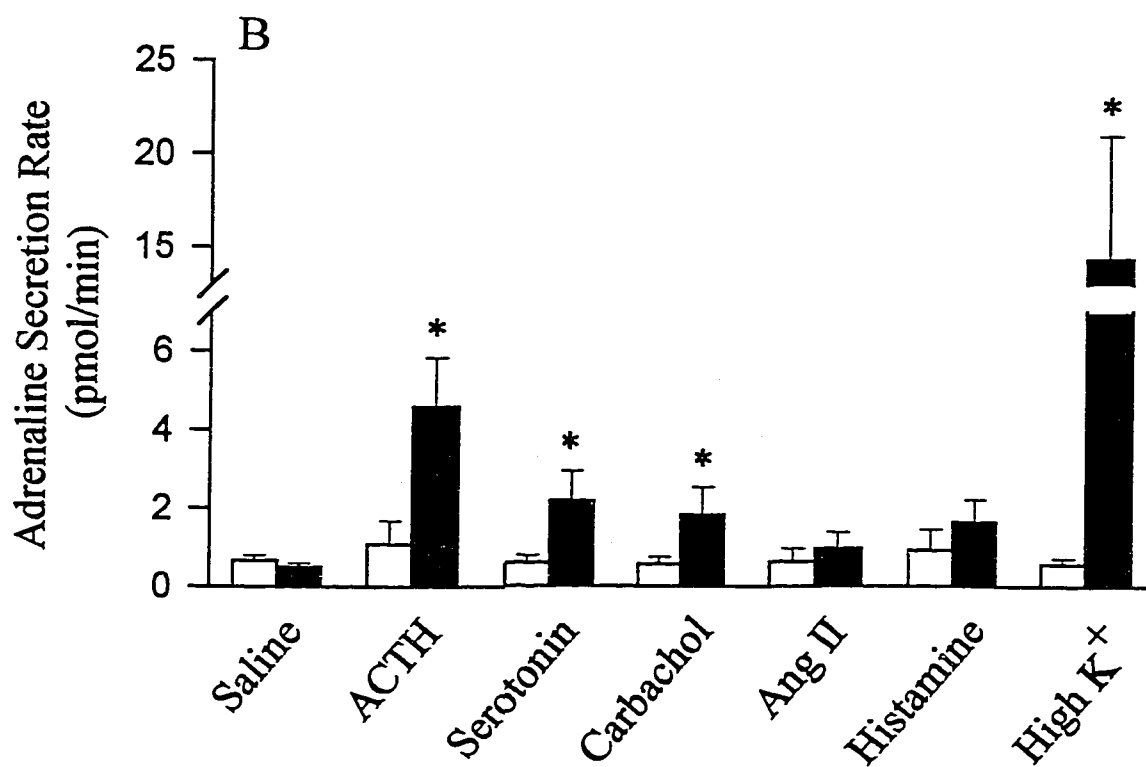
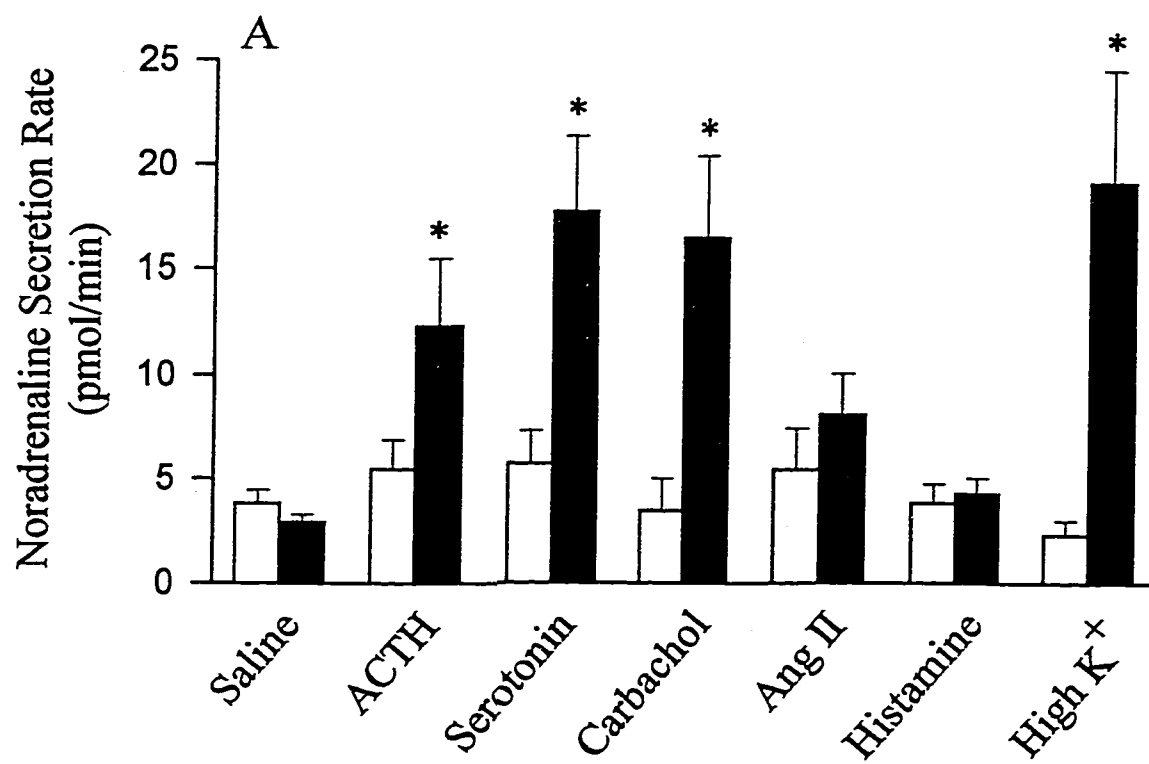


Figure 7.1

Figure 7.2 Noradrenaline (A-D) and adrenaline (E-H) secretion rates of *in situ* preparations of Atlantic hagfish injected with either ACTH, or serotonin, or carbachol, or exposed to a saline solution containing a high $[K^+]$. Values to the left of the hatched bar in each plot are the pre-treatment catecholamine secretion rates and values to the right are the secretion rates in response to the treatments listed above. An asterisk denotes a significant difference from the time -1 control value for a given treatment ($P < 0.05$). Values are means \pm 1 SEM. For further details on the treatments and the N values see the legend of Fig. 7.1.

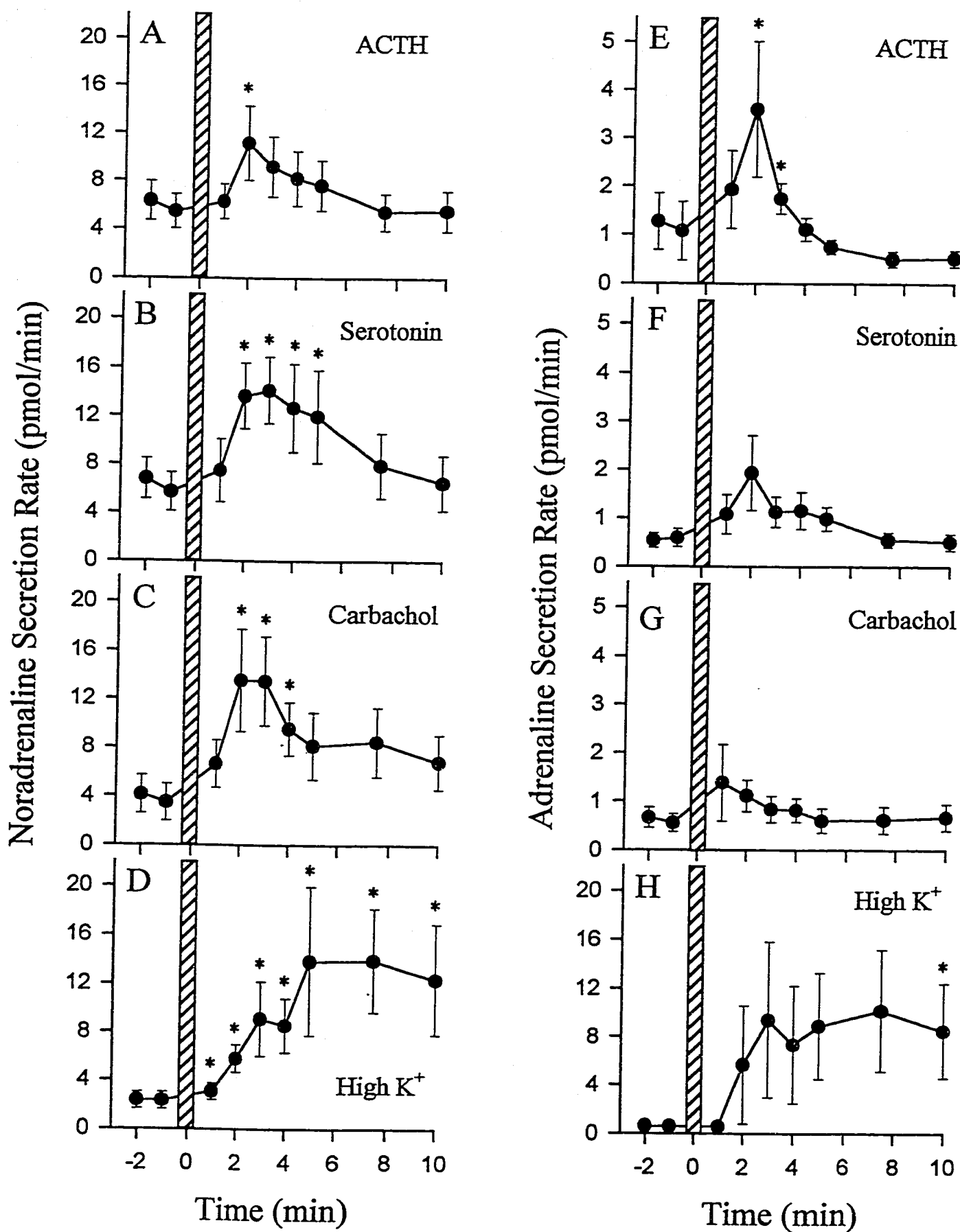


Figure 7.2

values account for temporal differences in the release process between preparations, whereas the mean secretion rate values do not. Ang II injections did not significantly affect the secretion rate of either noradrenaline or adrenaline (Fig. 7.1).

Comparison of the results obtained from the random administration of the four potential secretagogues on each *in situ* preparation shows that these results are independent from the order in which these compounds were given. Also, perfusing the *in situ* systemic heart / PCV preparations with the high $[K^+]$ saline stimulated the release of both catecholamines (Figs. 7.1, 7.2), and therefore confirmed the viability of the preparations.

Noradrenaline was the predominant catecholamine released in all the treatments, either before or after the injection of drugs, or the changes in the $[K^+]$ of the saline (Figs. 7.1, 7.2). The perfusate noradrenaline/adrenaline secretion rate ratio decreased significantly from the basal ratio in response to ACTH injections, increased in response to serotonin and carbachol injections, and remained unchanged in response to Ang II, and high $[K^+]$ saline (Table 7.1).

The high $[K^+]$ saline perfusion treatment, and the addition of Ang II to the preparations, both elicited an increase in the secretion rate of serotonin (Figs. 7.3A, 7.3B). ACTH or carbachol treatment had no significant effects on the serotonin secretion rate (Figs. 7.3C, 7.3D). Only 16 % of all the control samples had measurable amounts of serotonin. All the other control samples were assigned the HPLC detection limit value of 1 nmol l^{-1} .

Series 3: The effects of histamine on catecholamine and serotonin release

Perfusing the *in situ* systemic heart / PCV preparations with a hagfish saline containing a [histamine] of $0.3 - 300 \text{ } \mu\text{mol l}^{-1}$ did not stimulate catecholamine secretion above the basal secretion rate (Fig. 7.1, only data for the $300 \text{ } \mu\text{mol l}^{-1}$ treatment are shown). Noradrenaline was the predominant catecholamine released in all the treatments, and the perfusate

Table 7.1 The effects of ACTH, serotonin, carbachol, Ang II, histamine, and high $[K^+]$ saline on the ratio of noradrenaline to adrenaline secretion rate from the hagfish (*Myxine glutinosa*) *in situ* systemic heart/posterior cardinal vein preparation

| Treatment | Basal Nor/Adr secretion rate | Maximal Nor/Adr secretion rate |
|--------------------------|---------------------------------|-----------------------------------|
| ACTH (10) | 4.21 ± 1.50 | 1.95 ± 0.74* |
| Serotonin (10) | 5.74 ± 2.52 | 12.03 ± 5.13* |
| Carbachol (10) | 3.66 ± 1.00 | 9.07 ± 2.58* |
| Ang II (10) | 4.57 ± 1.84 | 3.17 ± 1.09 |
| Histamine (8) | 8.20 ± 3.06 | 6.90 ± 1.65 |
| High $[K^+]$ saline (10) | 8.10 ± 2.23 | 4.85 ± 1.42 |

Values are the ratio of Nor to Adr secretion rate levels. The basal values are the samples taken immediately before application of the treatment indicated. The maximal values are the maximum secretion rates recorded after treatment application.

Means ± 1 S.E.M.; *N* values are indicated in parentheses.

* indicates a significant difference from basal value ($P < 0.05$; paired *t*-test).

ACTH, adrenocorticotrop hormone; Ang II, $[Asn^1-Val^5]$ angiotensin II; Nor, noradrenaline; Adr, adrenaline.

Figure 7.3 Perfusate serotonin secretion rate of *in situ* preparations of Atlantic hagfish injected with either Ang II ($N=6$), or ACTH ($N=6$), or carbachol ($N=6$), or exposed to saline solutions containing either a high $[K^+]$ ($N=7$), or histamine ($N=6$). Values to the left of the hatched bar in each plot are the pre-treatment serotonin secretion rates and values to the right are the secretion rates in response to the treatments listed above. An asterisk denotes a significant difference from the time -1 control value for a given treatment ($P < 0.05$). Values are means \pm 1 SEM. For further details see the legend of Fig. 7.1.

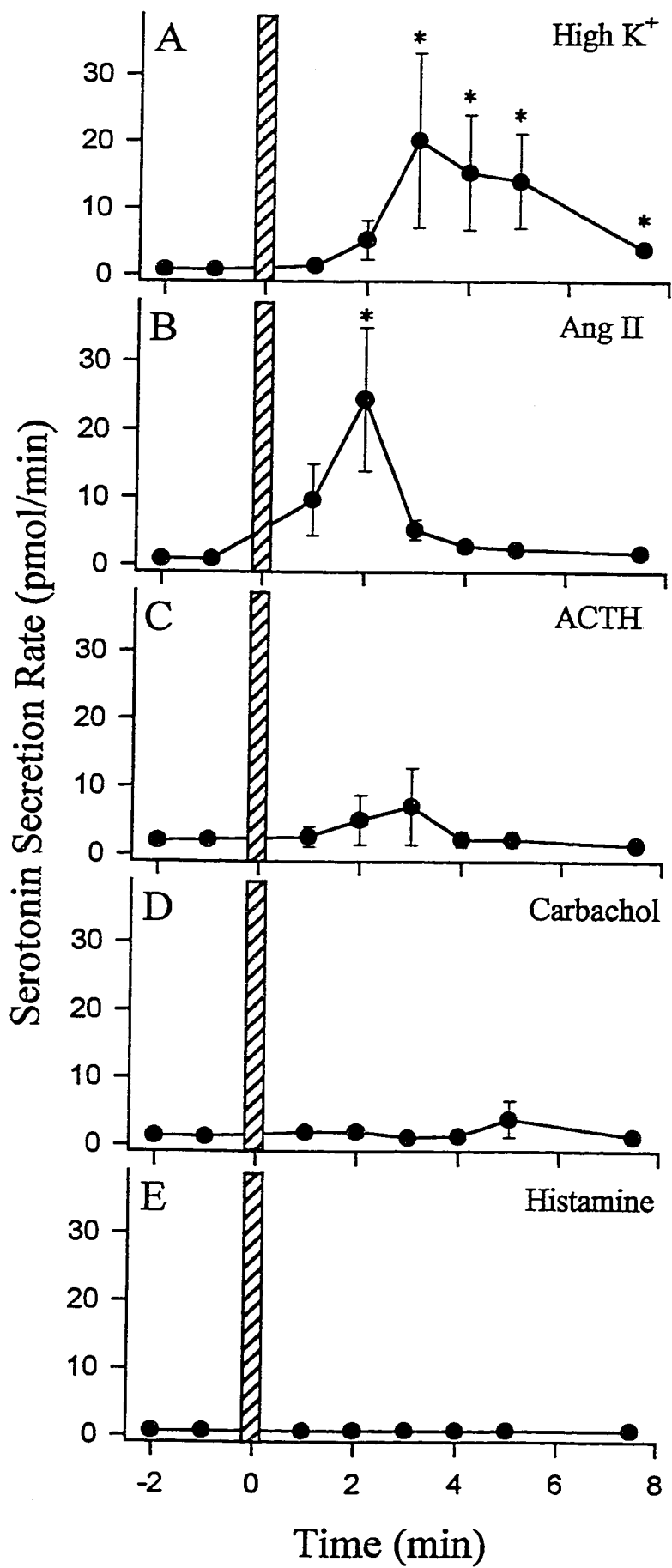


Figure 7.3

noradrenaline/adrenaline secretion ratio remained unchanged in response to histamine perfusions (Table 7.1). Histamine also did not affect the serotonin secretion rate of the preparations (Fig. 7.3E). In fact, although measurable average amounts of serotonin were detected in the saline of hagfish injected with all the other potential secretagogues tested, the concentration of serotonin was below the detection limit of HPLC in all the preparations perfused with histamine (Fig. 7.3).

Series 4: The effects of methysergide on catecholamine release

The presence of methysergide in the perfusion saline prevented the serotonin-induced increase in noradrenaline and adrenaline secretion rate, and had no effect on the ACTH-induced increase in both catecholamines (Figs. 7.4A, 7.4B).

Series 5: The effects of hexamethonium on catecholamine release

The presence of hexamethonium in the perfusion saline prevented the carbachol-induced increase in catecholamine secretion, and had no effect on the ACTH-induced catecholamine secretion (Figs. 7.5A, 7.5B).

Series 6 & 7: Assessing the modulatory effects of NECA and DPSPX on catecholamine release

While the presence of NECA in the perfusion saline of the *in situ* preparations decreased the serotonin- and carbachol-induced stimulation of noradrenaline secretion rate, the presence of DPSPX had no effect (Figs. 7.6A, 7.7A). NECA also abolished the serotonin- and carbachol-induced release of adrenaline, and relative to the effects of NECA, the addition of DPSPX to the perfusion fluid resulted in a significant increase in the release of adrenaline (Figs. 7.6B, 7.7B).

The ACTH-induced increase in noradrenaline secretion rate was unchanged by the presence of either the adenosine receptor agonist NECA, or the adenosine receptor antagonist DPSPX in the perfusion saline (Fig. 7.8A). On the other hand, the ACTH-induced increase in adrenaline secretion rate was increased by the presence of NECA in the perfusion saline, and

Figure 7.4 Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish injected with either 250 nmol·kg⁻¹ of serotonin ($N = 8$), or 7.5 U·kg⁻¹ of porcine ACTH ($N = 8$). The perfusate consisted of only saline in the groups represented by an open bar or by a diagonal-hatch bar. Methysergide (10^{-5} mol·l⁻¹), a serotonergic receptor antagonist, was added to the saline in the groups represented by a solid bar or by a cross-hatch bar. The open or solid bars also indicate the pre-treatment catecholamine secretion rates prior to the injections listed above. The bars with a diagonal- or cross-hatch also indicate the maximum catecholamine secretion rates in response to the injections. An asterisk denotes a significant difference between the control value and the maximum secretion rate for a given treatment ($P < 0.05$). Values are means \pm 1 SEM.

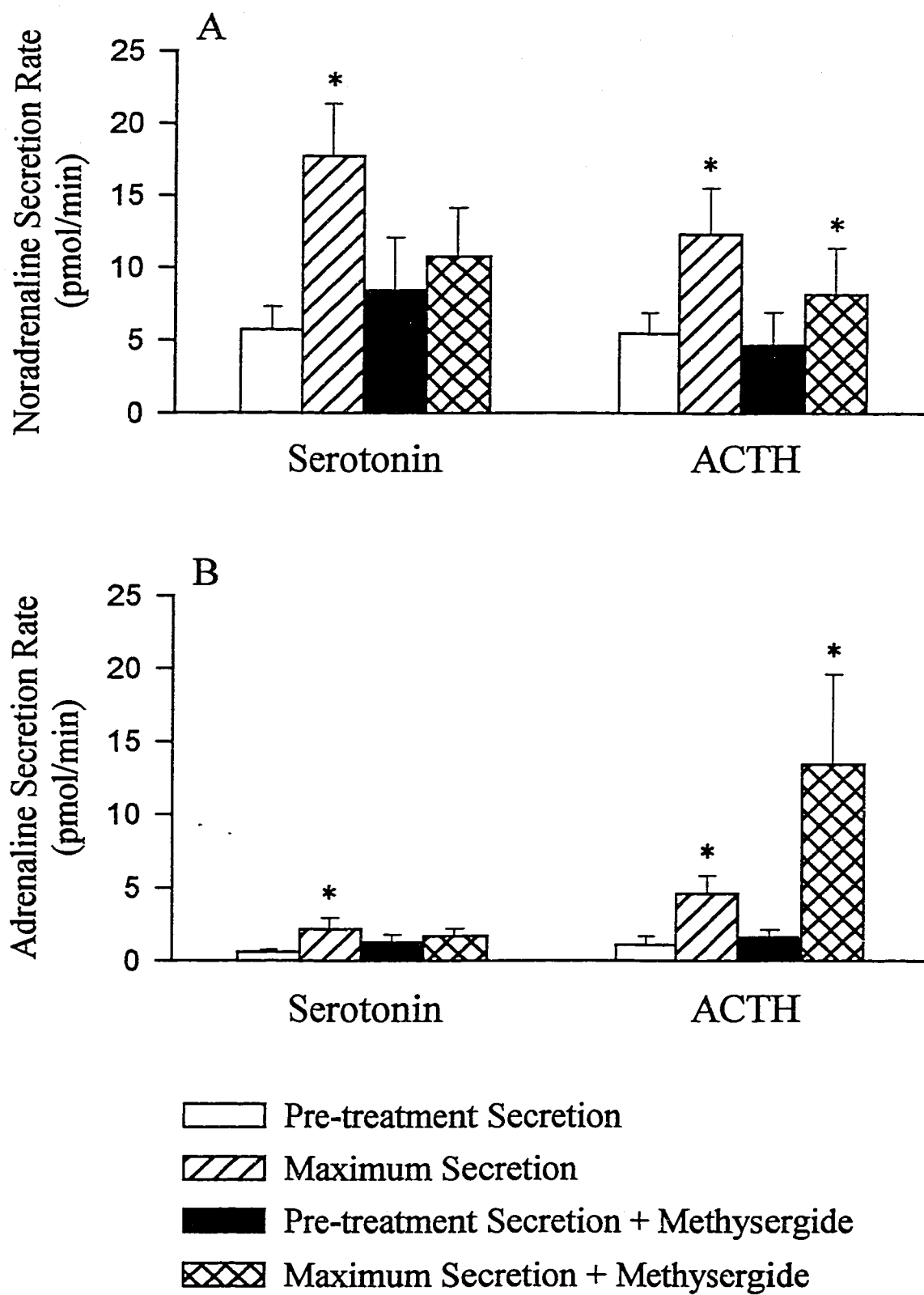


Figure 7.4

Figure 7.5 Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish injected with either, $100 \mu\text{mol}\cdot\text{kg}^{-1}$ of carbachol ($N = 8$), or $7.5 \text{ U}\cdot\text{kg}^{-1}$ of porcine ACTH ($N = 8$). The perfusate consisted of only saline in the groups represented by an open bar or by a diagonal-hatch bar. Hexamethonium ($10^{-3} \text{ mol}\cdot\text{l}^{-1}$), a ganglionic receptor blocker, was added to the saline in the groups represented by a solid bar or by a cross-hatch bar. The open or solid bars also indicate the pre-treatment catecholamine secretion rates prior to the injections listed above. The bars with a diagonal- or cross-hatch also indicate the maximum catecholamine secretion rates in response to the injections. An asterisk denotes a significant difference between the control value and the maximum secretion rate for a given treatment ($P < 0.05$). Values are means ± 1 SEM

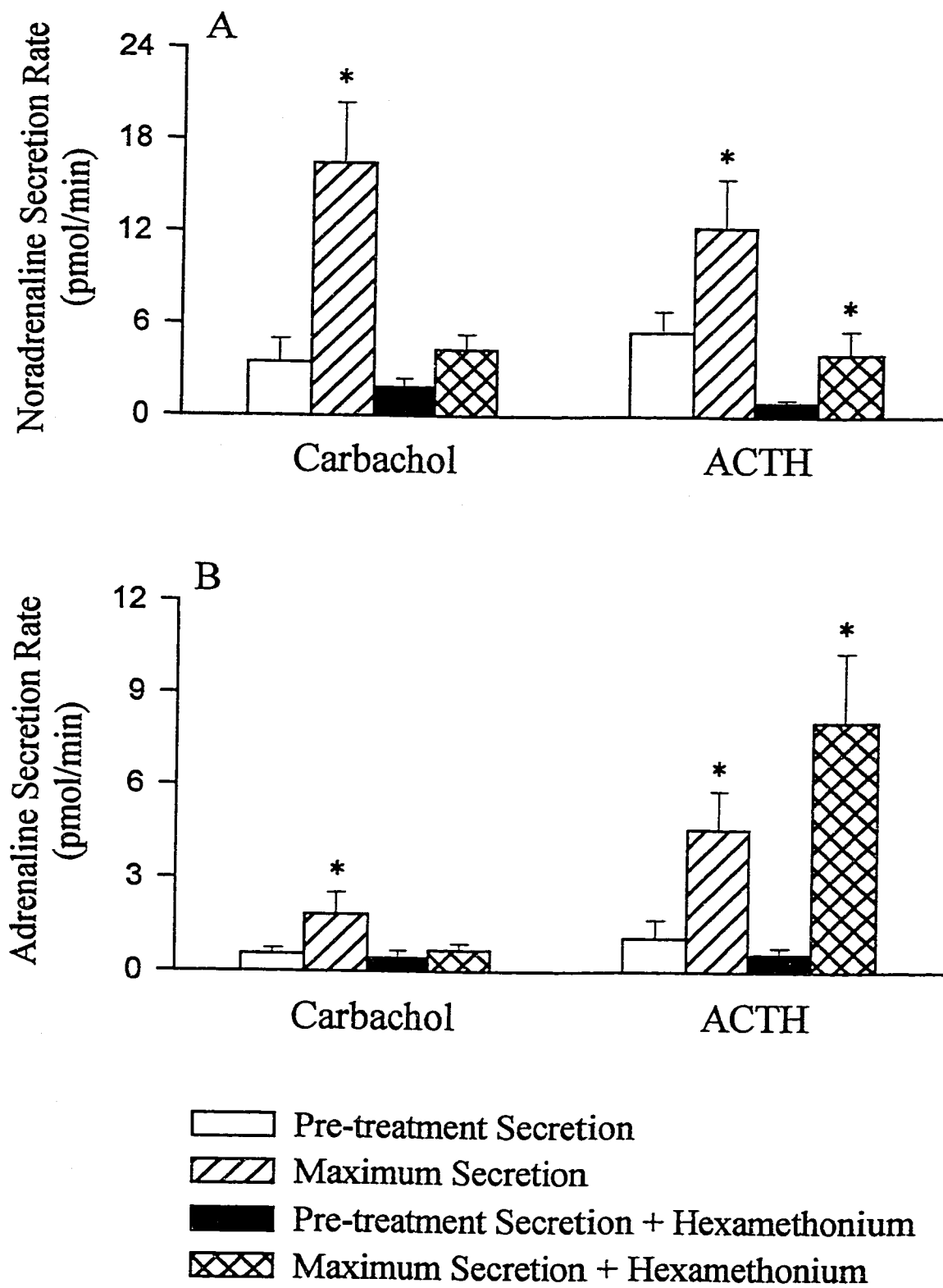


Figure 7.5

Figure 7.6 Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish injected with 250 nmol kg^{-1} of serotonin. The preparations were either perfused with saline alone (Serotonin, $N = 10$), saline containing the adenosine receptor agonist, NECA (Serotonin + NECA, $N = 10$), or saline containing the adenosine receptor antagonist, DPSPX (Serotonin + DPSPX, $N = 15$). The open bars indicate the pre-treatment catecholamine secretion rates prior to the injection of serotonin. The solid bars indicate the maximum catecholamine secretion rates in response to the injections. The diagonal-hatch bars indicate the averaged individual increase in catecholamine secretion rate in response to the injections. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment. Where letters are used to denote statistical differences, treatments which do not share a common letter for a given bar type are significantly different from each other ($P < 0.05$). Values are means \pm 1 SEM.

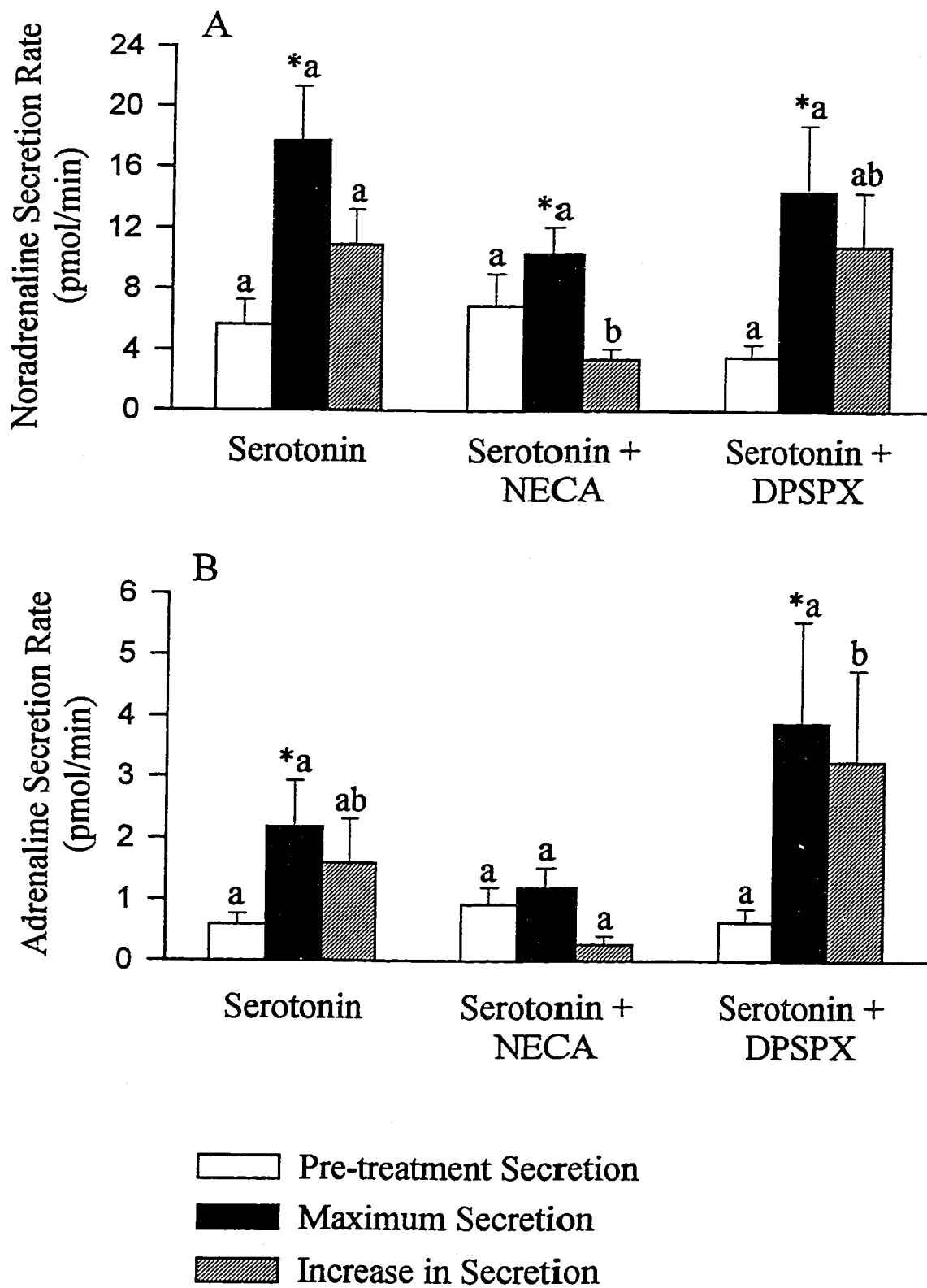


Figure 7.6

Figure 7.7 Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish injected with $100 \mu\text{mol}\cdot\text{kg}^{-1}$ of carbachol. The preparations were either perfused with saline alone (Carbachol, $N = 10$), saline containing the adenosine receptor agonist, NECA (Carbachol + NECA, $N = 10$), or saline containing the adenosine receptor antagonist, DPSPX (Carbachol + DPSPX, $N = 15$). The open bars indicate the pre-treatment catecholamine secretion rates prior to the injection of carbachol. The solid bars indicate the maximum catecholamine secretion rates in response to the injections. The diagonal-hatch bars indicate the averaged individual increase in catecholamine secretion rate in response to the injections. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment. P values of the paired t-test between the pre-treatment and the maximum secretion rate are shown to emphasize differences between treatments. Where letters are used to denote statistical differences, treatments which do not share a common letter for a given bar type are significantly different from each other ($P < 0.05$). Values are means \pm 1 SEM.

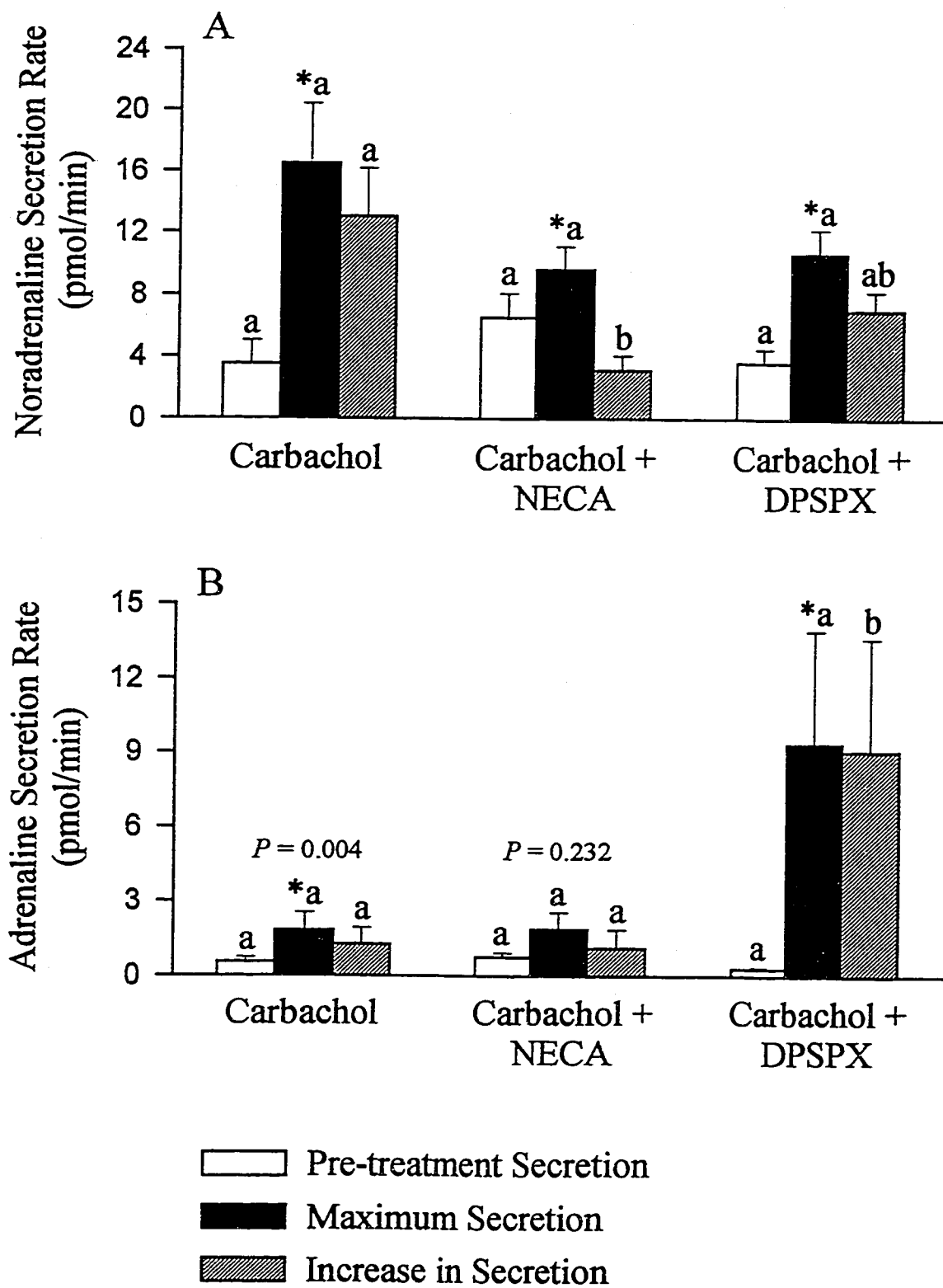


Figure 7.7

Figure 7.8 Noradrenaline (A) and adrenaline (B) secretion rates of *in situ* preparations of Atlantic hagfish injected with 7.5 U kg^{-1} of porcine ACTH. The preparations were either perfused with saline alone (ACTH, $N = 10$), saline containing the adenosine receptor agonist, NECA (ACTH + NECA, $N = 10$), or saline containing the adenosine receptor antagonist, DPSPX (ACTH + DPSPX, $N = 15$). The open bars indicate the pre-treatment catecholamine secretion rates prior to the injection of ACTH. The solid bars indicate the maximum catecholamine secretion rates in response to the injections. The diagonal-hatch bars indicate the averaged individual increase in catecholamine secretion rate in response to the injections. An asterisk denotes a significant difference between the pre-treatment value and the maximum secretion rate for a given treatment. Where letters are used to denote statistical differences, treatments which do not share a common letter for a given bar type are significantly different from each other ($P < 0.05$). Values are means $\pm 1 \text{ SEM}$.

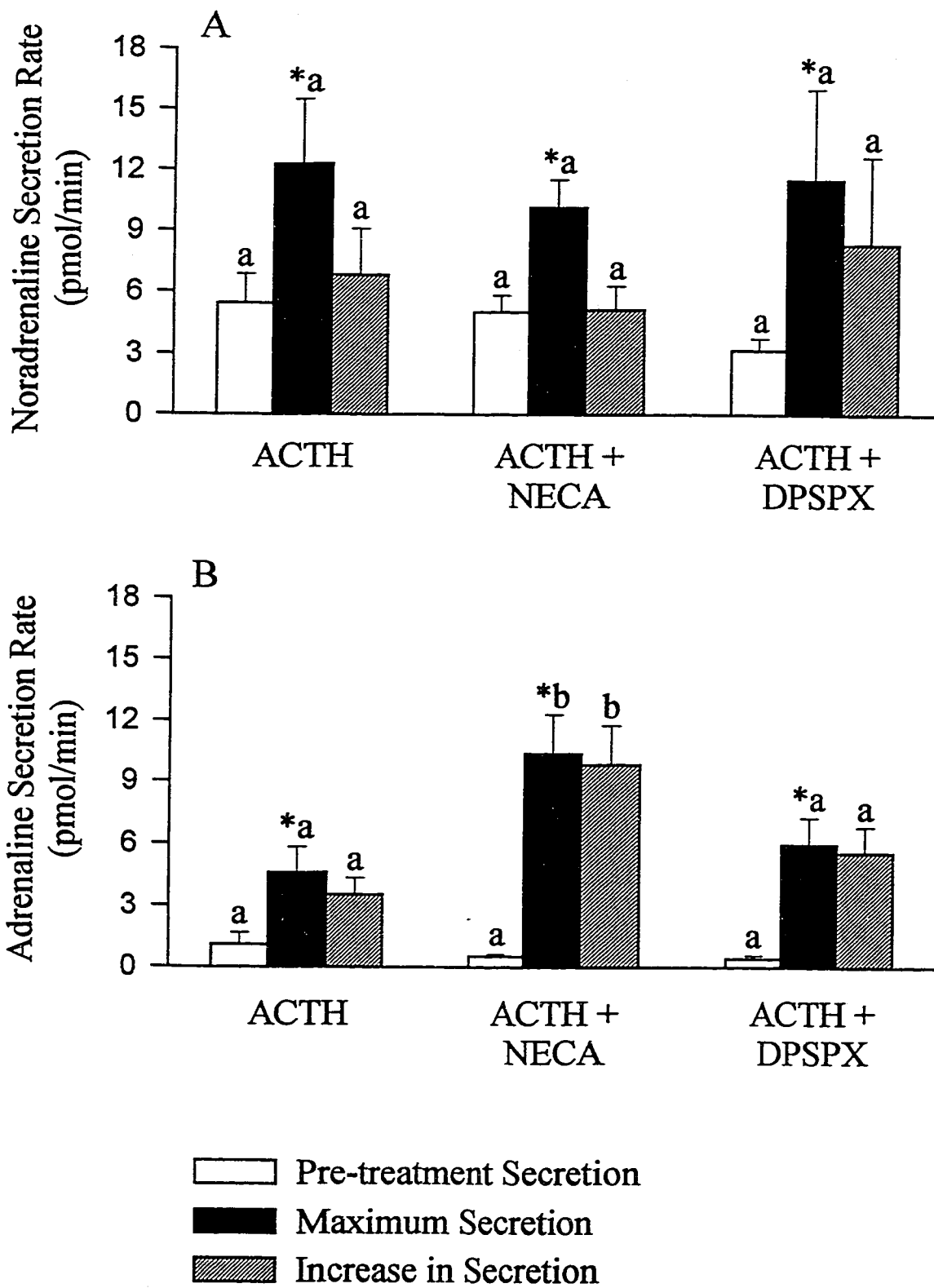


Figure 7.8

unchanged by the presence of DPSPX (Fig. 7.8B).

Overall, irrespective of the secretagogue used to elicit catecholamine secretion from the chromaffin tissue, NECA and DPSPX had greater modulatory effects on the adrenaline secretion rate than on the noradrenaline secretion rate.

Discussion

Secretagogues of catecholamine release

This study presents *in situ* evidence that ACTH, serotonin, and carbachol can stimulate catecholamine secretion in *Myxine glutinosa*. While ACTH and serotonin are known to stimulate catecholamine secretion in a few vertebrates (Feniuk *et al.* 1980; Chaouloff *et al.* 1992; Fritsche *et al.* 1993; Reid *et al.* 1996), they are the first non-cholinergic secretagogues of catecholamine release to be identified in hagfish. The stimulatory effects of carbachol reported in this study confirm earlier observations of Perry *et al.* (1993). Catecholamine secretion in response to cholinergic receptor agonists (Burgoyne 1991; Reid and Perry 1994), and to perfusion with a supra-physiological $[K^+]$ saline (Burgoyne 1991; Leboulenger *et al.* 1993; Reid and Perry 1994), are two properties that hagfish chromaffin cells appear to share with the chromaffin systems of other vertebrates. In contrast, the failure of Ang II to elicit catecholamine secretion in the *in situ* saline-perfused systemic heart / PCV preparation appears to be unique among vertebrates (Carroll and Opdyke 1982). Histamine, a potent secretagogue in higher vertebrates (Burgoyne 1991), is also without effect on catecholamine release in hagfish.

Adrenocorticotrophic Hormone

Using a preparation of *Myxine glutinosa* similar to the one used in this study, Perry *et al.* (1993) claimed that the most likely hormone responsible for catecholamine release in response to

a pituitary extract of *Gadus morhua* was ACTH. The stimulatory effects of ACTH on catecholamine release observed in this study support this claim.

The possible involvement of ACTH in mediating an acute stress response in hagfish is also supported by the ACTH-like activity of the pituitary gland of *Myxine glutinosa* (Buckingham *et al.* 1985), and by morphological, pharmacological (Fernholm and Olsson 1969), and immunohistochemical evidence (S.G. Reid, personal communication) for the presence of ACTH secreting cells in the pituitary of *Myxine glutinosa*. However, other immunohistochemical studies, also using antisera against mammalian ACTH in the adenohypophysis of hagfish, have failed to obtain positive results in either *Eptatretus burgeri* (Jirikowski *et al.* 1984) or in *Eptatretus stouti* (Nozaki 1985). Marked differences between the dose-response curves of hagfish pituitary extracts and mammalian ACTH in a cytochemical bioassay (Buckingham *et al.* 1985), and differences in the results obtained by the different immunohistochemical studies discussed above, suggest that hagfish ACTH may be structurally distinct from mammalian ACTH.

The only other vertebrate where ACTH is known to stimulate catecholamine secretion is the rainbow trout (Reid *et al.* 1996). Similar to the results reported in this study, injections of porcine ACTH elicited a significant secretion of both catecholamines in an *in situ* PCV preparation of the trout (Reid *et al.* 1996). However, while ACTH favors the secretion of noradrenaline over adrenaline in hagfish, the opposite was observed in trout (Reid *et al.* 1996). Moreover, injections of 40 or 200 mU of ACTH *in vivo*, as well as extracts of trout pituitary given *in situ*, caused an elevation of plasma adrenaline, but failed to increase noradrenaline levels in the trout (Reid *et al.* 1996). Although the noradrenaline to adrenaline storage ratio varies considerably between the different sites of catecholamine storage in the hagfish (Augustinsson *et al.* 1956; Östlund *et al.* 1960; Perry *et al.* 1993), and it is not known if one or all of these sites

release catecholamines in response to ACTH stimulation, differences in catecholamine storage between the two species (Perry *et al.* 1993; Reid and Perry 1994) may explain the differences in the pattern of catecholamine release elicited by ACTH. Among the three secretagogues identified in this study, ACTH elicited the largest secretion of adrenaline, and while carbachol and serotonin injections increased the noradrenaline to adrenaline ratio in the perfusate, ACTH stimulation decreased it.

In hagfish, as in trout (Reid *et al.* 1996), the stimulatory effects of ACTH on catecholamine release are unaffected by pre-treatment with the ganglionic blocker, hexamethonium, or by the serotonergic receptor antagonist, methysergide. These results suggest that ACTH is not exerting its effects by interacting with either serotonergic or cholinergic receptors.

Serotonin

As originally hypothesized by Reid *et al.* (1995), serotonin can directly elicit the release of catecholamines from the chromaffin tissue of hagfish. This serotonin-induced release of adrenaline and noradrenaline is abolished by the serotonergic receptor antagonist, methysergide. These results suggest that the stimulatory effects of serotonin are specifically mediated by serotonergic methysergide-sensitive receptors which are located on both adrenaline- and noradrenaline-chromaffin cells.

The stimulatory effects of serotonin on catecholamine release have previously been observed in rainbow trout (Fritsche *et al.* 1993). The dosage injected in the *in situ* hagfish preparations, $250 \text{ nmol}\cdot\text{kg}^{-1}$, also elicit the secretion of catecholamines in rainbow trout, under both *in vivo* and *in situ* conditions (Fritsche *et al.* 1993). While serotonin primarily elicits the secretion of noradrenaline in hagfish, the release of adrenaline appears to be favored in trout

(Fritsche *et al.* 1993). As with ACTH-elicited catecholamine secretion (see above), a likely explanation for these differences may be the known differences in catecholamine storage between the two species. For example, while the noradrenaline to adrenaline storage ratio of the PCV and the systemic heart of Atlantic hagfish are approximately 26 and 1, respectively (Perry *et al.* 1993), it is only 0.4 in the PCV of rainbow trout (Reid and Perry 1994). Results of experiments with methysergide suggest that the presence of serotonergic receptors are only on the adrenaline-chromaffin cells in rainbow trout, and that serotonin may also stimulate catecholamine release indirectly via the activation of neural pathways (Fritsche *et al.* 1993).

Carbachol

Perry *et al.* (1993) demonstrated that bolus injections of the cholinergic receptor agonist, carbachol, elicit dose-dependent increases in the release of catecholamines from the *in situ* systemic heart / PCV preparation of *Myxine glutinosa*. Results from this study confirm the stimulatory effects of carbachol on catecholamine release, and show that the response can be abolished by the cholinergic antagonist selective for nicotinic cholinceptors of ganglia, hexamethonium. Since carbachol is a non-specific cholinergic receptor agonist which stimulates both nicotinic and muscarinic receptors, these results suggest that the secretion from adrenaline- and noradrenaline-containing chromaffin cells of *Myxine glutinosa* is controlled exclusively by nicotinic receptors. In general, although the two subtypes of cholinergic receptors can mediate catecholamine secretion from chromaffin cells in vertebrates, their distribution is species-dependent (Parker *et al.* 1993). For example, while the control of catecholamine release in eels (*Anguilla rostrata*; Reid and Perry 1994) and cod (*Gadus morhua*; Nilsson 1983) is mediated only via nicotinic receptors, like the hagfish, the adrenaline-storing chromaffin cells of rainbow trout also appear to have functional muscarinic receptors (Fritsche *et al.* 1993). Since there is no

evidence that the chromaffin cells in hagfish are innervated (Green 1902; Augustinsson *et al.* 1956), the presence of nicotinic receptors on these cells may only reflect their common embryological origin as sympathetic neurons (Burgoyne 1991). However, although the autonomic nervous system is poorly developed in hagfish (Campbell 1970; Nilsson 1983), the possibility that the chromaffin tissues of the PCV are innervated can not be excluded, and it is possible that the nicotinic receptors of the PCV chromaffin cells play a physiological role *in vivo*.

Angiotensin

Whereas angiotensins have been shown to stimulate the secretion of catecholamines from elasmobranchs to mammals (Carroll and Opdyke 1982), [Asn¹,Val⁵]Ang II had no effect on the secretion rate of noradrenaline and adrenaline in the *in situ* systemic heart / PCV preparation of *Myxine glutinosa*. The impetus to investigate Ang II in this study arose from the observation that the pressor activity of mammalian angiotensin II (Ang II) could be abolished by adrenergic receptor blockade in hagfish (Carroll and Opdyke 1982). In light of these results, Carroll and Opdyke (1982) concluded that the pressor response of Ang II in hagfish was mediated entirely by catecholamines. However, although angiotensins elicit a pressor response in *Myxine glutinosa* (Carroll and Opdyke 1982), and angiotensin-converting enzyme-like activity has been measured in the liver and plasma of *Eptatretus stouti* (Lipke and Olson 1988), a complete renin-angiotensin system has not yet been identified in hagfish (Taylor 1977; Nishimura 1985a). Differences between the results obtained in this study and that of Carroll and Opdyke (1982) cannot be explained by differences in Ang II dosage. While Carroll and Opdyke (1982) used a dose of 1.91 nmol·kg⁻¹ of mammalian Ang II, this study used the much greater dose of 100 nmol·kg⁻¹ of teleost Ang II ([Asn¹,Val⁵]Ang II), the dose which causes maximal catecholamine secretion from the chromaffin tissue of rainbow trout (unpublished observation, N. Bernier and S. Perry).

Differences in the amino acid sequence of the Ang II used in the two studies also are not likely to explain the contradictory results, since among vertebrates diverse species respond to homologous and non homologous angiotensins (Olson 1992; Silldorff and Stephens 1992b). One possible explanation, is that the Ang II-induced pressor response observed in *Myxine glutinosa* by Carroll and Opdyke (1982) was mediated through actions of Ang II on the sympathetic nerve system (Wilson 1984a; Reid 1992). However, the adrenergic autonomic nerve fibers of the hagfish vasculature appear to be poorly developed (Nilsson 1983). Thus, although Ang II exerts a pressure response, there is no direct evidence for its' role in catecholamine release in hagfish.

Histamine

Although histamine elicits a substantial secretory response from the mammalian adrenal medulla and it is the most potent non-cholinergic secretagogue in bovine adrenal chromaffin cells (Burgoyne 1991), histamine failed to stimulate catecholamine secretion in the *in situ* systemic heart / PCV preparation of *Myxine glutinosa*. While there is chromatographic evidence that the systemic and portal hearts of *Myxine glutinosa* contain histamine (Augustinsson *et al.* 1956), intravascular injections of histamine yield only weak and inconsistent effects on the systemic and branchial vasculature, and are without effect on the activity of the heart in both *Myxine glutinosa* and *Polistotrema stouti* (Reite 1969). However, since the effects of histamine on the branchial blood vessels of *Myxine glutinosa* were similar to those obtained with adrenaline and noradrenaline in the same preparation, Reite (1969) suggested that these may be elicited either by direct stimulation of adrenergic receptors or indirectly by release of endogenously stored catecholamines. Results obtained in this study do not support the latter hypothesis. Since histamine elicits the release of catecholamines from *in vitro* perfused rat adrenals with an EC_{50} of $3 \mu\text{mol l}^{-1}$ (Borges 1994), perfusing the *in situ* hagfish preparation with saline solutions containing

histamine concentrations ranging from 0.3 to 300 $\mu\text{mol l}^{-1}$ should have been sufficient to elicit potential secretory activity from the chromaffin tissues.

Catecholamine release and the noradrenaline to adrenaline secretion ratios

The noradrenaline to adrenaline secretion ratios of Table 7.1 suggest that in hagfish, as in other vertebrates (Accordi 1991; Chritton *et al.* 1991; Reid and Perry 1994), noradrenaline and adrenaline are stored in different chromaffin cell types. While non-specific cell membrane depolarization with constant high $[\text{K}^+]$ perfusion did not alter the basal noradrenaline to adrenaline secretion ratio, injections of either ACTH, serotonin, or carbachol all had significant effects. Relative to the control conditions, the significant decrease in the noradrenaline to adrenaline ratio following ACTH injections suggest that ACTH-elicited secretion may arise primarily from adrenaline-storing cells. Similarly, the significant increase in the noradrenaline to adrenaline ratio following serotonin and carbachol injections suggest that catecholamine release elicited by these secretagogues may arise primarily from noradrenaline-storing cells. Meanwhile, the high $[\text{K}^+]$ saline was non-selective.

Secretagogues of serotonin release

The presence of serotonin in the systemic heart of *Myxine glutinosa*, as suggested by the immunohistochemical evidence of Reid *et al.* (1995), is confirmed by the perfusion results obtained in this study. Although serotonin was not detectable in most of the control *in situ* preparations perfused only with hagfish saline, perfusing these same preparations with a high $[\text{K}^+]$ saline elicited a marked release of serotonin. While the effects of a high $[\text{K}^+]$ saline on chromaffin cells are non-specific and do not involve the intervention of receptors (Burgoyne 1991), the serotonin secretion elicited by teleost Ang II ($[\text{Asn}^1, \text{Val}^5]\text{Ang II}$) in this study, like the Ang II-mediated catecholamine secretion in mammals (Marley *et al.* 1989), is probably mediated via

specific receptors. Since Ang II does not elicit catecholamine secretion in *Myxine glutinosa*, the effects of Ang II appear to be specific to the control of serotonin release. Moreover, known secretagogues of catecholamine release in hagfish, ACTH and carbachol, had no effect on the [serotonin] of the perfusate. Hence, although non-specific depolarization of chromaffin cells elicits the release of both catecholamines and serotonin, the secretagogues involved in the control of catecholamine and serotonin secretion may be different. These results also suggest that the serotonin-containing cells of the systemic heart may represent a different population of cells than the catecholamine-containing chromaffin cells.

Immunohistochemical evidence for the presence of serotonin in chromaffin cells has also been reported in several teleost species (Reid *et al.* 1995), in amphibians (Delarue *et al.* 1988), as well as in mammals (Brownfield *et al.* 1985; Holzwarth and Brownfield 1985). In rainbow trout, Fritsche *et al.* (1993) showed that serotonin is stored in high concentrations in the anterior region of the PCV within the head kidney. As in hagfish, Reid *et al.* (1995) have suggested that the catecholamine-storing chromaffin cells and the serotonin-storing chromaffin cells of trout may represent different populations. In contrast, the serotonin-containing cells present in the chromaffin tissues of the eel (*Anguilla anguilla*) and the cod (*Gadus morhua*) appear to be analogous to their catecholamine-storing cells (Reid *et al.* 1995). While carbachol induces the release of catecholamines in trout, it failed to elicit the release of the stored serotonin (Fritsche *et al.* 1993). Potential secretagogues of serotonin release in teleosts have yet to be identified.

Since the presence of angiotensins and a complete renin-angiotensin system has yet to be established in hagfish (see above), the functional significance of the Ang II-elicited serotonin release remains to be determined. Although our results suggest that serotonin ($250 \text{ nmol}\cdot\text{kg}^{-1}$ in $300 \text{ }\mu\text{l}$ of hagfish saline; estimated maximum concentration = $33.8 \text{ }\mu\text{mol}\cdot\text{l}^{-1}$) can elicit

catecholamine release, the presence of a lower concentration of serotonin in the perfusate following Ang II injections (maximum secretion = $24.7 \pm 7.9 \text{ nmol l}^{-1}$) failed to stimulate catecholamine secretion. In addition to a potential paracrine role in the stimulation of catecholamine secretion, the serotonin stored in the systemic heart and PCV of hagfish may have other physiological functions. This is supported by the observations that serotonin has inotropic and chronotropic effects on the systemic heart (Augustinsson *et al.* 1956), and potential vasoactive actions on the gill vasculature of *Myxine glutinosa* (Sundin *et al.* 1994).

Modulatory effects of adenosine on catecholamine release

The adenosine receptor agonist, NECA, and antagonist, DPSPX, significantly modified the secretory response elicited by ACTH, serotonin, and carbachol. Taken together, results obtained from the perfusions in the presence of the adenosine receptor agonist or antagonist, indicate that adenosine may inhibit the rates of catecholamine secretion induced by either serotonin or carbachol, and stimulate those induced by ACTH. Although adenosine and the adenosine analogue PIA (*N*⁶-L-phenylisopropyladenosine) have previously been shown to inhibit the catecholamine secretion elicited by acetylcholine and the nicotinic agonist DMPP (1,1-dimethyl-4-phenylpiperazinium), respectively, in isolated bovine chromaffin cells (Chern *et al.* 1987, 1992), the modulatory effects of NECA and DPSPX on carbachol-, serotonin-, and ACTH-induced catecholamine secretion observed in this study have not been previously reported.

Adenosine, formed from the breakdown of ATP which is released in parallel with catecholamines (Douglas *et al.* 1965), is thought to have a physiological role in catecholamine secretion from mammalian chromaffin cells by acting as a negative feedback modulator of release from the adrenal medulla (Chern *et al.* 1987, 1992). At the cellular level, adenosine inhibits catecholamine secretion from bovine adrenal medullary cells by reducing agonist-evoked calcium

fluxes across the plasma membrane (Chern *et al.* 1987). *In vivo*, support for these modulatory attributes of adenosine on catecholamine secretion, comes from adenosine receptor blockade studies performed on hypotensive rats (Tseng *et al.* 1994), hypoxic rainbow trout and Pacific hagfish (*Eptatretus stouti*; Bernier *et al.* 1996). In *Eptatretus stouti*, whereas exposure to a PwO_2 of 10 torr for 60 min had no effect on the plasma adrenaline concentrations, it increased the latter by 3.8-fold in hypoxic hagfish pre-treated with the adenosine receptor antagonist theophylline (Bernier *et al.* 1996). In this study, the *in situ* effects of NECA and DPSPX on the pattern of catecholamine secretion induced by serotonin and carbachol agree with the inhibitory properties attributed to adenosine by Chern and his co-workers (Chern *et al.* 1987, 1992). On the other hand, the interactions between NECA and ACTH-evoked catecholamine release in *Myxine glutinosa* suggest that adenosine can also produce a positive feedback modulatory role. In the presence of forskolin, a drug which enhances the activity of adenylate cyclase and causes a marked rise in intracellular cAMP, adenosine, but not NECA, has been shown to enhance catecholamine secretion in isolated bovine chromaffin cells (Chern *et al.* 1988). Hence, although species differences may exist in the signal transduction systems of adenosine receptors, the modulatory effects of adenosine on catecholamine secretion may either be stimulatory or inhibitory and depend on the secretagogue involved.

Overall NECA and DPSPX had greater modulatory effects on the adrenaline secretion rate than on the noradrenaline secretion rate of the perfused chromaffin tissue. These observations are consistent with the *in vivo* results obtained in *Eptatretus stouti*, rainbow trout, (Bernier *et al.* 1996), and in rats (Tseng *et al.* 1994), and, together, they suggest that the modulatory effects of adenosine may be mostly aimed at the adrenaline-storing cells.

Conclusion

The *in situ* evidence presented in this study suggests that the chromaffin tissues of *Myxine glutinosa* store both catecholamines and serotonin, and that these hormones may be found in different populations of chromaffin cells. While both serotonin and ACTH stimulate catecholamine release, Ang II and histamine do not. In contrast, Ang II stimulates serotonin release, whereas ACTH and histamine have no effect. Hence, co-secretion of catecholamines and serotonin is not elicited by the specific secretagogues tested in this study. Our data also reveal that ACTH- and serotonin-elicited catecholamine release can be modulated by adenosine. Although the relative contributions of ACTH, serotonin, Ang II, and adenosine have yet to be explored in the overall control of catecholamine and serotonin release *in vivo*, our results suggest that this control can be achieved through hormonal and / or paracrine means.

CHAPTER 8

General Discussion

SUMMARY OF PRINCIPLE FINDINGS

CHAPTER 2: Angiotensins can elicit catecholamine release from the chromaffin tissue of *O. mykiss* via specific Ang II binding sites. The source of Ang II in the head kidney may be either from a local renal RAS or from the systemic RAS.

CHAPTER 3: A portion of the cardiovascular effects of Ang II in *O. mykiss* can be attributed to increased levels of plasma adrenaline. Physiological levels of plasma adrenaline can significantly influence cardiovascular function in *O. mykiss*.

CHAPTER 4: The RAS and humoral catecholamines play important roles in the compensatory response to an acute hypotensive stress in *O. mykiss*. The contribution of the RAS is largely indirect and relies on an Ang II-mediated secretion of catecholamines.

CHAPTER 5: Ang II does not elicit catecholamine release from the chromaffin tissue of *A. rostrata*. During a hypotensive stress, although the RAS is recruited in *O. mykiss* and *A. rostrata*, an essential involvement of sympathetic nerves and humoral catecholamines to blood pressure restoration is only apparent in *O. mykiss*.

CHAPTER 6: Humoral catecholamines play an important role in the cardiovascular control of *S. acanthias* under resting and hypotensive conditions. Ang II indirectly contributes to cardiovascular control during hypotension by eliciting catecholamine release, but does not appear to be involved in blood pressure regulation during rest.

CHAPTER 7: While serotonin, ACTH, and adenosine may play a role in the control of catecholamine release from the chromaffin tissue of *M. glutinosa*, there is no evidence that Ang II or histamine are involved.

DISCUSSION

Rationale for selection of α -adrenoceptor blocking agents

To determine the contribution of plasma catecholamines to the cardiovascular effects of elevated levels of Ang II in fish, 3 different α -adrenoceptor antagonists were utilized in this thesis.

In the initial studies conducted with rainbow trout (chapters 3 & 4), phenoxybenzamine was selected as an α -adrenoceptor antagonist based on preliminary experiments that compared the effectiveness of phenoxybenzamine (chapter 3) and phentolamine (2 mg kg^{-1}) in providing chronic (≥ 8 hours) α -adrenergic blockade against exogenous catecholamine injections. Phenoxybenzamine and phentolamine are both non-selective α -adrenoceptor antagonists that have similar affinities for α_1 and α_2 receptors. However, phenoxybenzamine is a non-competitive antagonist that irreversibly blocks α -adrenoceptors while phentolamine is a competitive antagonist that is extensively metabolized (Hoffman and Lefkowitz 1996). Although phentolamine has previously been used to investigate the contribution of the sympathetic nervous system to the cardiovascular effects of Ang II (Nishimura *et al.* 1978, 1985b; Oudit and Butler 1995a), relative to phenoxybenzamine, phentolamine was ineffective in providing the sustained blockade required for the chronic experiments of chapter 3.

Although phenoxybenzamine was an effective α -adrenoceptor antagonist in chapters 2 & 3, given the apparent discrepancy in the cardiovascular effects of phenoxybenzamine treatment in trout (Randall and Stevens, 1967; Wood and Shelton 1980; Olson and Duff, 1992; Xu and Olson 1993a; see also chapter 3 for discussion), several experiments carried out previously with phenoxybenzamine were repeated with

the competitive α_1 -adrenoceptor antagonist, prazosin (chapter 5). Overall, α -adrenoceptor blockade with either phenoxybenamine or prazosin had comparable effects on the cardiovascular of rainbow trout and gave conclusive evidence that the cardiovascular effects of Ang II are partly mediated by humoral catecholamines in this teleost species.

The selection of yohimbine as an α -adrenoceptor antagonist in *S. acanthias* (chapter 6) was based on preliminary experiments that assessed the effectiveness of yohimbine (4 mg kg^{-1}), prazosin (4 mg kg^{-1}), and phentolamine (3 mg kg^{-1}) in providing chronic (≥ 2 hours) α -adrenergic blockade against exogenous catecholamine injections. Relative to yohimbine, phentolamine and prazosin were both ineffective in providing the sustained blockade for chronic experiments in the spiny dogfish. Although yohimbine is a competitive α_2 -adrenergic antagonist in mammals (Hoffman and Lefkowitz 1996), this adrenoceptor antagonist is known to block the α -adrenoceptors that mediate contraction in the systemic vasculature of different fish species (Holmgren and Nilsson 1974; Wood and Shelton 1980).

Overall, results from this thesis and from a number of other studies suggest that the pharmacology of α -adrenoceptors in fish indicate a non-mammalian adrenergic receptor classification (for review, see Fabbri *et al.* 1998). In addition to the unexpected results obtained with some α -adrenoceptor antagonists in this thesis, several studies have shown that clonidine, methoxamine and phenylephrine, standard mammalian α -receptor agonists (Hoffman and Lefkowitz 1996), behave differently in fish than in mammals (Holmgren and Nilsson 1982; Fabbri *et al.* 1998).

Significance of Ang II as a secretagogue of humoral catecholamine release

An overview of the principle findings from this thesis (see above) suggests that while Ang II may be a potent secretagogue of humoral catecholamine release in some fish species, it does not appear to play a role in others. Similarly, in mammals, a role for Ang II in adrenal medullary physiology is species specific (Livett and Marley 1993). Although such inter-species differences may lead one to speculate about the physiological significance of Ang II as a secretagogue of humoral catecholamine release, it is important to keep in mind that the interactions between Ang II and the SNS are multifaceted.

In the Atlantic hagfish, *M. glutinosa*, despite the observations of Carroll and Opdyke (1982) that α -adrenoceptor blockade can fully inhibit the pressor response to teleost Ang II ([Asn¹,Val⁵]-Ang II), there is no evidence that [Asn¹,Val⁵]-Ang II can stimulate humoral catecholamine release in this species (chapter 7). Hence, even though the autonomic nervous system of cyclostomes is very poorly developed (Nilsson 1984), presumably Ang II-elicited pressor responses in hagfish are mediated by sympathetic nerves. Even though attempts at isolating Ang II from hagfish have previously failed (Y. Takei, personal communication), the presence of Ang II in lampreys and in the nervous system of invertebrates (for references see Kobayashi and Takei 1996) suggests that Ang II is likely to play a role in the nervous system of hagfish.

Similarly, there is no evidence that Ang II can elicit humoral catecholamine release in the American eel (chapter 5). While Ang II elicited pressor responses that could be partially inhibited by α -adrenoceptor blockade, it did not stimulate humoral catecholamine release. Therefore, as observed in the hagfish, the previously reported

interactions between Ang II and the SNS in eels (Chan and Chow 1976; Nishimura *et al.* 1978; Nishimura 1985b; Oudit and Butler 1995a) are most likely mediated by sympathetic nerves. In teleosts, there is *in situ* evidence that Ang II may facilitate catecholamine release from adrenergic nerve endings (Olson *et al.* 1994; Platzack 1995). However, it is noteworthy that during acute hypotensive conditions in *A. rostrata*, when endogenous Ang II is recruited, there does not appear to be any significant contribution of the SNS to cardiovascular control (chapter 5). Therefore, while α -adrenoceptors may mediate a significant percentage of the pressor effect of exogenous Ang II under normotensive conditions in *A. rostrata* (Nishimura *et al.* 1978; Nishimura 1985b; Oudit and Butler 1995a), the significance of this interaction may be masked by other cardiovascular adjustments during an acute hypotensive stress.

In contrast, results from this thesis suggest that homologous Ang II is a potent secretagogue of humoral catecholamine release in rainbow trout (chapters 2-5) and in spiny dogfish (chapter 6). In both species, I have provided evidence that Ang II plays an important role in the control of humoral catecholamine release during an acute hypotensive stress. Therefore, keeping in mind that humoral catecholamines are only released in significant amounts under conditions of acute stress (Randall and Perry 1992), these results suggest that at least in some species of fish, Ang II is a key contributor to the control of catecholamine release from the chromaffin tissue.

In addition to the stimulatory effects of Ang II on humoral catecholamine release during acute hypotension in rainbow trout, Ang II may chronically stimulate basal adrenaline release from the chromaffin tissue during normotensive conditions (chapter 4). Given the importance of circulating catecholamines for blood pressure regulation in *O.*

mykiss (see below), the chronic stimulatory effect of Ang II on adrenaline release may be an important component of the RAS function as an anti-drop regulator of blood pressure in this species. On the other hand, there is no evidence that the RAS of the spiny dogfish is involved in stimulating basal catecholamine secretion from the chromaffin tissue (chapter 6). Interestingly, there is also no evidence that the RAS is involved in maintaining resting blood pressure in dogfish (chapter 6; Opdyke and Holcombe 1976; Hazon *et al.* 1989).

The presence of a systemic and local renal RAS in *O. mykiss* (chapter 2; Brown *et al.* 1995) suggests that not all the chromaffin cells of the head kidney may be exposed to similar Ang II levels. In mammals, although the adrenal gland is endowed with a local RAS and contains the highest level of angiotensin that has been measured in any tissue (Livett and Marley 1993; Phillips *et al.* 1993), the physiological significance of this local RAS is unclear. In *O. mykiss*, whether a local renal RAS plays a tonic role in the control of catecholamine release, and whether the systemic and local RAS have a differential impact on the chromaffin cells of the head kidney warrant further consideration.

In addition to humoral catecholamine secretion, Ang II has a number of other actions on chromaffin cells in mammals (Livett and Marley 1993). The ability of Ang II to activate key enzymes of the Blaschko pathway and to increase the expression of the genes coding for these enzymes (Stachowiak *et al.* 1990; Goc and Stachowiak 1994), indicate that Ang II is involved in the short- and long-term regulation of catecholamine synthesis. Whether Ang II also influences the synthesis of catecholamines in fish has yet to be investigated. Such Ang II-mediated modulation of catecholamine synthesis may

take place under conditions where the RAS is chronically activated, for example during changes in environmental salinity (Kobayashi *et al.* 1980; Takei *et al.* 1988).

Given the marked inter-species differences observed throughout this thesis, it is difficult to generalize on what the overall significance of Ang II as a secretagogue of humoral catecholamine release in fish might be. However, my results do suggest that Ang II is more likely to stimulate humoral catecholamine release in fish species where blood pressure is promptly regulated during a hypotensive stress (e.g. *O. mykiss* and *S. acanthias*) than in species where it is not (e.g. *A. rostrata*).

Significance of Ang II and humoral catecholamines in cardiovascular control

Under normotensive conditions, as previously observed (Olson 1992), the RAS of rainbow trout is involved in the tonic regulation of blood pressure. Although this may be achieved in part by the constrictor effect of Ang II on the systemic resistance vessels (Olson *et al.* 1994), my results also suggest an indirect contribution via the chronic stimulatory effect of Ang II on basal adrenaline secretion from the chromaffin tissue (chapter 4). While basal circulating levels of catecholamines may not directly contribute to cardiovascular control (chapter 3), there is evidence that low levels of humoral catecholamines are necessary to maintain sympathetic neuronal tone in rainbow trout (Xu and Olson 1993b). Hence, in rainbow trout, the regulation of resting blood pressure may be achieved, at least in part, through interactions between the RAS, humoral catecholamines and sympathetic nerves.

While only the neuronal component of the SNS may directly contribute to maintaining resting blood pressure in *O. mykiss*, whether humoral catecholamines

contribute to cardiovascular control independently of sympathetic nerves during stress has been debated (Xu and Olson 1993b; Nilsson 1994). In chapter 3, by correlating *in vivo* cardiovascular function with circulating catecholamine concentrations, I provided evidence for the first time that relatively low levels of circulating adrenaline can significantly contribute to cardiovascular function in rainbow trout. Further evidence that humoral catecholamines contribute to cardiovascular control was provided by the observation that exogenous supplementation of plasma catecholamines concentrations prevents the chronic hypotensive effects of RAS blockade in papaverine-treated trout (chapter 4). In addition, following hypotensive stress, the slower rate of blood pressure recovery in α -adrenoceptor-treated trout than in bretylium-treated trout (chapter 5) suggests that both humoral catecholamines and adrenergic nerves play a significant role in the cardiovascular regulation of hypotensive *O. mykiss*.

Recruitment of the RAS is also an important component of the physiological response of *O. mykiss* to hypotensive stress. However, the contribution of Ang II to cardiovascular control is mostly indirect and involves an acute stimulation of humoral catecholamine release (chapters 4 & 5).

In sharp contrast to rainbow trout, neither humoral catecholamines, adrenergic nerves or Ang II appears to be involved in the regulation of blood pressure in normotensive fresh water eels (chapter 5; Hipkins *et al.* 1986; Tierney *et al.* 1995b). In *Anguilla anguilla*, however, transfer to seawater increases the activity of the RAS and under such conditions Ang II appears to play a role in the regulation of resting blood pressure (Tierney *et al.* 1995b). Although an important component of the physiological response of *A. rostrata* to a hypotensive stress is the recruitment of the RAS (chapter 5),

unlike the observations in *O. mykiss*, the cardiovascular effects of Ang II are not mediated by humoral catecholamines in the eel. In fact, the results of chapter 5 suggest that the α -adrenoceptor mediated pressor effects of Ang II in *A. rostrata* are not an essential component of cardiovascular control during acute hypotensive conditions.

Generally in teleosts, whether the individual and interactive contributions of Ang II and humoral catecholamines to blood pressure regulation are more similar to *O. mykiss* or to *A. rostrata* is not clear. In the only other teleost species where the involvement of the RAS and the SNS in cardiovascular control have been extensively investigated, *G. morhua*, the available data tentatively suggest an intermediate pattern. In the Atlantic cod, the RAS contributes to resting blood pressure regulation, is recruited during exercise-induced hyperaemia, and facilitates adrenaline release from sympathetic nerve endings (Platzack *et al.* 1993; Platzack 1995). Also, there is evidence that adrenergic nerves are more important than humoral catecholamines for blood pressure regulation during rest, exercise, and hypoxia (Nilsson 1994). However, interactions between Ang II and the control of humoral catecholamine release have yet to be investigated in the Atlantic cod. Furthermore, experiments examining the contribution of humoral catecholamines and Ang II during an acute hypotensive stress have not been performed in *G. morhua*. Another unknown is whether the stimulatory effects of Ang II on humoral catecholamines observed in *O. mykiss* also contribute to resting blood pressure regulation in other fish species. Therefore, considering that only a handful of teleosts have been investigated among a very large and diverse array of species (~21 000), generalizing about the possible involvement of Ang II and humoral catecholamines in blood pressure regulation among teleosts is a daunting task (Henderson and Deacon 1993; Nilsson

1994). Suffice it to say that blood pressure regulation is complex, and that Ang II, humoral catecholamines, adrenergic nerves, and other mechanisms not investigated here can play a significant role in at least some teleost species.

In spiny dogfish, unlike teleosts, my results have provided further evidence that humoral catecholamines play an important and direct role in maintaining resting blood pressure in elasmobranchs (chapter 6; Opdyke *et al.* 1972; Short *et al.* 1977; Butler and Metcalfe 1988). Ang II, while not involved in the control of resting blood pressure (chapter 6; Opdyke and Holcombe 1976; Hazon *et al.* 1989), significantly contributes to cardiovascular control during a hypotensive stress by stimulating catecholamine release (chapter 6). Therefore, in *S. acanthias*, humoral catecholamines appear to be essential in the regulation of blood pressure under both normotensive and hypotensive conditions. Ang II, on the other hand, only plays a supportive role during a hypotensive stress.

Overall, the capacity of either *O. mykiss*, *A. rostrata*, or *S. acanthias* to compensate for a drop in blood pressure is in part coupled to the involvement of Ang II and the SNS in blood pressure regulation. As exemplified by *A. rostrata*, regulation of blood pressure during hypotension primarily through the single involvement of the RAS may limit the ability of a fish to promptly regain homeostatic conditions. Additional recruitment of the neuronal (e.g. *O. mykiss*) and humoral (e.g. *O. mykiss* and *S. acanthias*) components of the SNS may enhance cardiovascular regulatory capabilities. Given the involvement of Ang II in the control of catecholamines from the chromaffin tissue, the significance of the contribution by humoral catecholamines to cardiovascular homeostasis will become increasingly important with the severity of the hypotensive stress.

Potential role of β -adrenoceptors in mediating the cardiovascular effects of Ang II

Throughout this thesis, results from several experiments have shown that a significant component of the cardiovascular effects of Ang II in *O. mykiss* and *S. acanthias* are mediated via the stimulatory effects of Ang II on catecholamine release from the chromaffin tissue. While the α -adrenoceptor component of the cardiovascular effects of Ang II were specifically investigated (chapters 3-6), a portion of the cardiovascular effects of circulating catecholamines in fish can also be mediated via β -adrenoceptors. Positive inotropic and to some extent chronotropic effects are mediated by β -adrenoceptors in the heart of most teleosts (Holmgren and Nilsson 1982) and in elasmobranchs (Nilsson and Holmgren 1988). The branchial vasculature of teleosts and elasmobranchs is also sensitive to circulating catecholamines (Wood and Shelton 1975; Holmgren and Nilsson 1982; Morris and Nilsson 1994). The primary effect of humoral catecholamines in the gills is a β -adrenoceptor-mediated vasodilation (Wood and Shelton 1975; Morris and Nilsson 1994). While the contribution of the heart to the cardiovascular effects of an Ang II-mediated release of catecholamines was investigated in *O. mykiss* (chapters 3 & 4) and *S. acanthias* (chapter 6), the potential contribution of branchial resistance (R_G) to the cardiovascular effects of Ang II is not known. Since R_G and R_S are in series in fish, a decrease in R_G leads to an increase in P_{DA} . Therefore, it is possible that an Ang II-mediated elevation in plasma catecholamines could decrease R_G and thereby contribute to the Ang II-elicited increase in P_{DA} . However, α -adrenoceptor blockade with phenoxybenzamine in *O. mykiss* reduced the pressor response to a bolus injection of catecholamines by 67%, abolished the increase in R_S and reduced the increase in Q by 42% (chapter 3). Because a portion of the remaining pressure response elicited by

exogenous catecholamines in these α -adrenoceptor blocked fish can be attributed to an increase in Q (chapter 3), these results suggest that the contribution of R_G to the pressor response of catecholamines may be small. Furthermore, α -adrenoceptor blockade was shown to prevent blood pressure restoration during an acute hypotensive stress and to acutely reduce R_S in *O. mykiss* (chapter 4) and *S. acanthias* (chapter 6). Therefore, while it may be argued that a catecholamine-mediated reduction in R_G during an acute hypotensive stress may prevent a complete cardiovascular collapse, the contribution of potential changes in R_G to the Ang II-mediated adrenergic response during a hypotensive stress appear to be relatively minor. Finally, because Ang II increased vascular resistance in a perfused gill preparation of *O. mykiss*, Ang II will not directly interfere with the systemic pressor effects of Ang II in the trout (Olson *et al.* 1994).

Significance of non-cholinergic secretagogues in the control of catecholamine release

Given the aneural nature of the chromaffin tissue in hagfish (see Bernier and Perry 1998b for review), the control of catecholamine release in these primitive fish is by definition non-cholinergic. However, although evidence suggests that the catecholamine stores found in the systemic and portal hearts are involved in the tonic control of cardiac function (Forster 1998), determining the nature of the mechanisms involved in the control of catecholamine release has proven to be challenging (Perry *et al.* 1993). Overall, by identifying potential secretagogues of catecholamine release in an *in situ* perfusion preparation of *M. glutinosa* (chapter 7), I have provided direction for future investigations into the control of catecholamine release and cardiac function in hagfish.

The observation that both serotonin and ACTH can elicit catecholamine release in *M. glutinosa* (chapter 7) concurs with similar *in situ* and *in vivo* results obtained in rainbow trout (Fritsche *et al.* 1993; Reid *et al.* 1996). In both species, however, the relative contribution of either serotonin or ACTH to the overall control of catecholamine release *in vivo* in response to specific stresses has not been explored. Also, whether serotonin or ACTH indiscriminately stimulate catecholamine secretion or only play a role under some specific physiological conditions is not known. In fact, with the exception of Ang II, the relative contribution and the physiological conditions under which most of the non-cholinergic secretagogues identified so far in fish are not known (Reid *et al.* 1998). Therefore, while current knowledge on the control of catecholamine release from the chromaffin tissue of fish suggests a complex process involving numerous non-cholinergic secretagogues (Reid *et al.* 1998), additional work is required to characterize the *in vivo* role of these secretagogues before their physiological significance can be elucidated.

CONCLUSION

This thesis primarily investigated the role of Ang II as a non-cholinergic secretagogue of humoral catecholamine release among an evolutionary diverse group of fish. Experimental evidence suggests that Ang II is a potent secretagogue of humoral catecholamine release in the teleost *Oncorhynchus mykiss* and the elasmobranch *Squalus acanthias*. In both species, Ang II plays a significant role in the control of catecholamine release during an acute hypotensive stress, and this interaction is an important physiological response for the maintenance of cardiovascular homeostasis. On the other hand, although previously suggested, Ang II does not appear to be a secretagogue of humoral catecholamine release in either the teleost *Anguilla rostrata* or the hagfish *Myxine glutinosa*. Therefore, while a number of potential non-cholinergic secretagogues may be involved in the control of catecholamine release among fish, the results from this thesis point to the need for a more comprehensive and comparative approach in order to further our understanding of this complex process.

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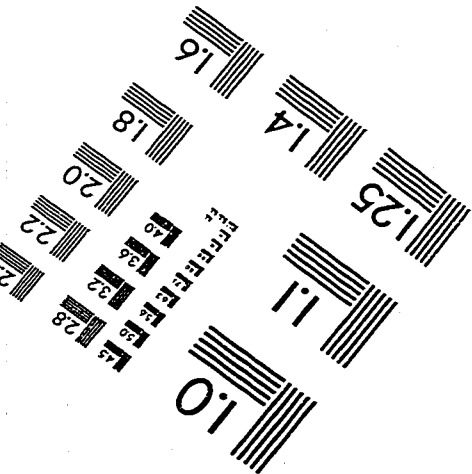
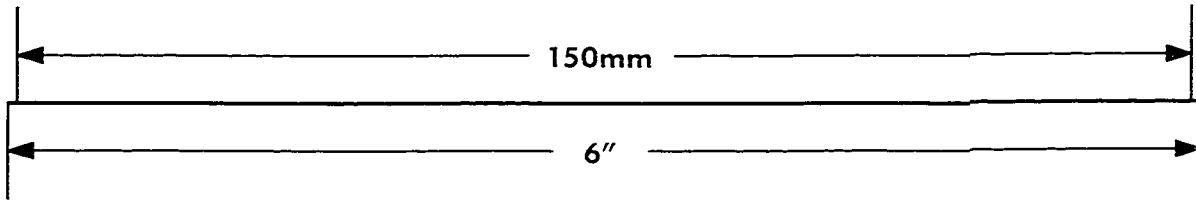
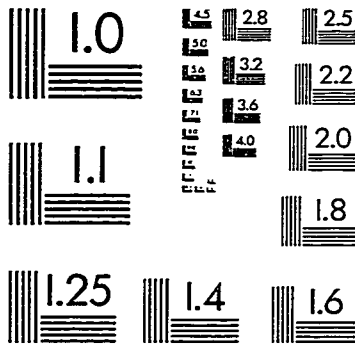
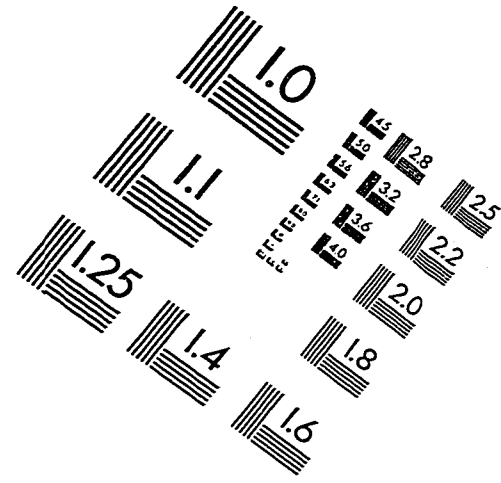
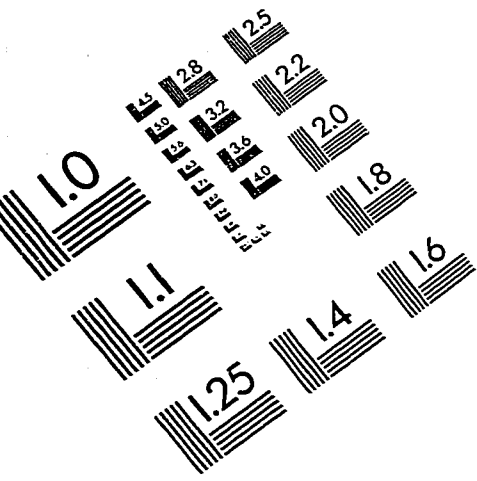
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IMAGE EVALUATION TEST TARGET (QA-3)



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