The corpuscles of Stannius contain essential elements of the renin-angiotensin system for the regulation of blood flow in freshwater North American eels, *Anguilla rostrata* LeSueur.

by

Donald H. Zhang

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Zoology
University of Toronto

ABSTRACT

The corpuscles of Stannius contain essential elements of the renin-angiotensin system for the regulation of blood flow in freshwater North American eels, *Anguilla rostrata* LeSueur.

Master of Science, 1999

Donald H. Zhang

Department of Zoology, University of Toronto.

These experiments examine how elements of the renin-angiotensin system (RAS) may modulate dorsal aortic blood flow (DABF) and caudal venous blood flow (CVBF) in a model teleost fish, the freshwater North American eel (*Anguilla rostrata* LeSueur). The experiments also examine the theory that the corpuscles of Stannius (CS) are part of the RAS in freshwater eels and that they may secrete renin. Blood flow rates were measured in the dorsal aorta (DA) and the caudal vein (CV) of free-swimming, conscious freshwater (FW) eels, using surgically implanted Doppler flow probes. DABF and CVBF increased in (FW) eels in a dose-dependent manner following i.v. injections of [Asn^1, Val^5, Gly^9]-Angiotensin I (ANG I), [Asn^1, Val^5]-Angiotensin II (ANG II) and [Val^4]-Angiotensin III (ANG III). Doses were given in 5 ng increments, ranged from 5 -50 ng.kg bw^{-1}. A minimum effective dose for ANG I and ANG II was 5 ng.kg bw^{-1}; for ANG III, 10 ng.kg bw^{-1}. In most cases, both DABF and CVBF flow increased during the first 2 minutes and remained elevated for 20-50 minutes (min) before decreasing to the pre-injection rates. [Sar^1, Val^5]-ANG II (Sarile), the AT_1 And AT_2 receptor

antagonist, completely blocked the increases in DABF and CVBF in responses to ANG II. Losartan, the mammalian AT_1 antagonist, and PD 123319, the mammalian AT_2 antagonist, both blocked partially, the increased DABF and CVBF which followed injections of ANG II. Failure of both Losartan and PD123319 to completely abolish the flow response to ANG II and CS-EXT suggest strongly that the blood flow responses to angiotensins is governed by more than one Angiotensin II receptor subtype in the eel.

An i.v. injection of an extract of 2.5 mg fresh corpuscles of Stannius (CS-EXT), presumed to contain renin, was followed by an immediate and sustained elevation in DABF and CVBF which lasted for approximately 30 min. A similar increase in DABF and CVBF followed the i.v. injection of 150 ng.kg bw^{-1} of human renin substrate (hRS). An extract of posterior kidney (PK-EXT) had no effect on either DABF or CVBF. Flow responses to CS-EXT and hRS were blocked completely by a prior i.v. injection of 1 mg.kg bw^{-1} of the mammalian renin inhibitor, Pepstatin A. Pepstatin A did not block the flow responses to either ANG I or ANG II. Intravenous injection of 1 mg.kg bw^{-1} of Captopril, the mammalian angiotensin-converting enzyme (ACE) inhibitor, completely abolished the DABF and CVBF responses to CS-EXT, hRS and ANG I, while the flow responses to ANG II was unaffected.

These findings in conjunction with other works on freshwater eels lead to the conclusion that angiotensins act centrally or via catecholamine release from the peripheral sympathetic nervous system or chromaffin cells of the anterior head kidney to increase cardiac output (CO). Moreover, my experiments have

shown that eel CS contain renin or a renin-like substance that is a component of an eel RAS.

ACKNOWLEDGEMENTS

I thank my supervisor, Dr. D. G. Butler, for the kindness and the patience he has shown me throughout the course of my training. His enthusiasm and commitment to the art and discipline of research has been both inspiring and edifying. I will greatly miss our long and scholarly discussions that have left indelible imprints on my growing awareness of the world.

To Alia Cadinouche, Gavin Oudit, Sunny Pak, Colleen Roe and Roham Zandevakili, I extend to you my sincerest gratitude for your friendship and support. I am especially indebted to Sunny, for her tireless and invaluable editorial feedback, to Colleen, for introducing me to the Doppler technique, and to Roham, for his vast computer expertise. Many thanks to Gavin and Alia, for making me feel welcomed and accepted in the lab.

I further extend my appreciation to Dr. R. Stephenson for the use of his Doppler Flowmeter, and to Dr. D. Jackson and Kristie Ciruna for their statistical counseling. I am also indebted to Diana Powell and Rosanna Soo for their assistance in revising my thesis. Many thanks to Eric Lin, Sonia McKenzie, Kim Gallant, Terry Hill, Elizabeth Tudor-Mulroney and Steve Smith for making my graduate experience a more enjoyable one.

To my family, words cannot express the full extent of my gratitude for your unfaltering love and support. Thank you for tolerating me during the often difficult and circuitous course of my training.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

α	Alpha
ACE	Angiotensin converting enzyme
ANG I	Angiotensin I
ANG II	Angiotensin II
ANG III	Angiotensin III
ANOVA	Analysis of variance
Asn	Asparagine
Asp	Aspartate
°C	Degree centigrade
cm	Centimeter
CO	Cardiac output
CRAS	Corpuscular renin-angiotensin system
CS	Corpuscles of Stannius
CS-EXT	Corpuscles of Stannius extract
CV	Caudal vein
CVBF	Caudal venous blood flow
DA	Dorsal aorta
DABF	Dorsal aortic blood flow
EGM	Extraglomerular mesangial cells
g	Gram
Gly	Glycine
hRS	Human renin-substrate
i.d.	Inner diameter
i.v.	Intravenous
i.m.	Intramuscular
JG	Juxtaglomerular cells
JGA	Juxtaglomerular apparatus
KDa	Kilodalton
Kg bw	Kilogram body weight
Khz	Kilohertz
l	Litre
µg	Microgram
MD	Macula densa
mg	Miligram
min	Minute
ml	Mililitre
mm	Milimetre
MS222	Methane tricanesulphonate
MW	Molecular weight
n	Sample size
ng	Nanogram
NaCl	Sodium chloride

o.d.	Outer diameter
pg	Picogram
P	Attained significant level
P_{DA}	Dorsal aortic blood pressure
PE	Polyethylene
PK-EXT	Posterior kidney extract
r	Correlation coefficient
RAS	Renin-angiotensin system
RS	Renin substrate
rm	repeated measures
SEM	Standard error of the mean
Val	Valine
VR	Venous return

INTRODUCTION

The corpuscles of Stannius (CS) are putative endocrine glands that may regulate cardiovascular function in teleostean fishes, in concert with the Renin-angiotensin system (RAS). Chester-Jones first described a rapid decline in blood pressure in European eels, *Anguilla anguilla*, following extirpation of the paired glands (Chester-Jones *et al.* 1966). Subsequently, he showed that extracts of the CS contain a powerful renin-like pressor substance, in the anaesthetized rat bioassay. More recently, the CS have been shown to contain angiotensin I (ANG I) and angiotensin II (ANG II), in a wide variety of teleost species (Hasegawa *et al.* 1984a, Takemoto *et al.* 1983, Yamada & Kobayashi 1987). The present study was undertaken to determine whether the CS in a model teleost fish, the North American eel, *Anguilla rostrata* LeSueur, contain physiologically significant renin-like activity. In these experiments, the effects of an extract of CS on the regulation of blood flow were investigated in conscious eels, with the aid of chemical antagonists of the RAS.

(A) The RAS in mammals

Brown and Séquard have been credited with the discovery of the RAS over a century ago, in 1892. Since its discovery, the vast majority of research on the RAS has been limited to studies on mammals. In mammals, the RAS is an intricate enzymatic cascade comprising 7 essential elements: 1) The enzyme renin, an aspartyl released by the juxtaglomerular cells (JG) of renal afferent and efferent arterioles, 2) the protein angiotensinogen, an α_2 globulin renin-substrate produced by the liver, 3) angiotensin-

converting enzyme (ACE), a dipeptidyl carboxypeptidase occurring mostly in the lung endothelium, and 4) angiotensinases that degrade the active hormones of the RAS into smaller, predominately inactive peptides. The active products of the RAS consist of angiotensin (ANG) peptides of varying lengths (7-10 amino acids), including 5) the ANG I decapeptide, 6) the ANG II octapeptide and 7) the ANG III heptapeptide. ANG II is considered the most potent of these peptides, in terms of its pressor effects and its primary function in the regulation of body fluid homeostasis .

Traditionally, the juxtaglomerular apparatus (JGA) was considered essential for the proper functioning of the RAS. Yet only birds and mammals have intact JGA's, even though species from every vertebrate class are dependent on the RAS (Nishimura 1980a, b). In birds and mammals, juxtaglomerular (JG) cells containing renin and prorenin granules are found in both the afferent and the efferent arterioles of the unit nephron. Apposed to one side of the afferent arteriole are specialized epithelial cells of the renal tubule called macula densa cells (MD). The MD act as sodium sensors which respond to drops in tubular luminal sodium concentration, by stimulating renin secretion from JG cells. Accessory cells associated with the JGA include extraglomerular mesangial cells (EGM) which are interspersed between the MD and the glomerulus. A second cell type, known as peripolar cells (Ryan *et al.* 1979), can be found encircling the origin of the glomerular tuft, although, their function is hitherto unknown.

(B) Evolution of the RAS

Comparative studies on the RAS began in the late 1960s, and it became rapidly apparent that all vertebrate classes have a RAS despite an incomplete or altogether absent JGA (Edwards 1940, Capelli *et al.*1970, Sokabe *et al.*1969). Studies on the RAS of fishes, amphibians and reptiles necessarily focus on the physiological and the biochemical features of the RAS, rather than on the presence of the JGA (Nishimura *et al.* 1973). Incubation of renal extract with homologous plasma produces angiotensins in select species from every vertebrate class, from primitive jawless fishes to mammals. In addition, incubation of renal extracts with homologous plasma, under the condition of angiotensinase inhibition, produce ANG II-like pressor substance in virtually every animal model examined (see review by Sokabe & Ogawa 1974).

A preponderance of research strongly suggests that the RAS is an evolutionarily ancient system whose ancestry may be traced as far back as invertebrates. ANG II-like substances have been isolated in the nervous systems of the leech, *Theromyzon tessulatum*, (Verger-Bocquet *et al.* 1992, Salzet *et al.* 1992, 1993), the earthworm, *Eisenia foetida* (Kobayashi & Takei 1996a) and the amphioxus, *Branchiostoma belcheri* (Uemura *et al.* 1994). In invertebrates, ANG II-like substances are thought to subserve a neuromodulatory function, whereas in vertebrates, it plays a pivotal role in cardiovascular regulation as well as fluid and electrolyte homeostasis. Hence, an evolutionary transition in the function of ANG II may have occurred from that of a neuromodulator to a regulator of cardiovascular function.

The presence of cells with renin-containing granules that have similar staining and chemical properties to their mammalian counterpart, is an *a priori* requirement for

the existence of a RAS within a vertebrate class. On the basis of this criteria, the RAS seems to have evolved first in fishes (Nishimura 1987). Primitive bony fishes, teleosts, lungfishes, amphibians, and reptiles all have granulated cells resembling mammalian JG cells in the small arteries and the arterioles of the kidney. In fishes, up to 6 different types of JG cell distributions have been described along the renal artery and arterioles (Krishnamurthy & Bern 1969). Vasopressor substances with similar biological properties to mammalian angiotensins have been formed from kidney extracts of lower vertebrates including primitive bony fishes, modern bony fishes, amphibians, reptiles, and birds (Sokabe *et al.* 1969: Nishimura *et al.* 1973).

(C) The RAS in Fishes

i. Cyclostomes Studies on the cyclostome RAS have focused on 3 species: the lamprey, *Lampetra japonica*, the hagfish, and the myxinoids *Paramyxine atami* and *Myxine glutinosa* (Nishimura *et al.* 1970, Oguri *et al.* 1970). Cyclostomes do not appear to have a RAS, since they lack granulated JG cells (Sokabe *et al.* 1969). Nevertheless, exogenous ANG II can stimulate vasoconstriction, drinking behaviour and mineralocorticoid release in the hagfish (Carroll & Opdyke 1982). Similarly, renal extracts of the lamprey, incubated with canine renin-substrate, produce ANG II-like pressor substances (Henderson *et al.* 1981). Most recently, Takei and Rankin have isolated ANG I from the lamprey (Kobayashi & Takei 1996b). In light of these new findings, more detailed studies on the histological basis of the RAS in cyclostomes may be warranted.

ii. Holocephalans There is a small number of studies on the RAS in holocephalans. In the rabbitfish, *Chimaera monstrosa* (Oguri 1978, 1980) and the ratfish, *Hydolagus colloeo* (Nishimura *et al.* 1973, Oguri 1978), cells similar to mammalian JG cells have been detected in the afferent arteriole, near the glomerulus. Incubation of ratfish kidney extract with homologous plasma produced a pressor substance similar to ANG II (Nishimura 1985), however, the existence of a JGA is doubtful since both species lack MD.

iii. Elasmobranchs Early histological studies failed to detect JG cells in elasmobranchs (see reviews by Sokabe & Ogawa 1974, Wilson 1984, Nishimura 1985). Of the species examined, renin could not be detected in kidney extracts (Bean, 1942, Nishimura *et al.* 1970, Nishimura, 1985). However, these studies relied on the old Bowie's staining method, and with the development of the toludine-blue staining technique as well as more sensitive assays for renin, the presence of the RAS in elasmobranches has since been clearly validated. Granulated JG cells have been identified in 4 species of elasmobranchs (Lacy & Reale 1990) along with the MD, EGM and the peripolar cells.

ANG II has also been detected in the brain, kidney, pituitary and rectal glands of the nurse shark, *Gynclymostoma cirratum*. In the spiny dogfish, *Squalus acanthias*, exogenous ANG I and ANG II elicits pronounced pressor responses. Furthermore, the effect of ANG I was blocked by the ACE inhibitor, SQ 20881 (Opdyke & Holcombe 1976). Direct intravenous injection of dogfish, *Scyliorhinus canicula*, renal extract or the injection of renal extract incubated with rat renin substrate, produced an ANG II-like pressor effect in nephrectomized rats (Henderson *et al.* 1981). In the dogfish and spiny

dogfish, exogenous ANG II elicits both pressor and dipsogenic responses and stimulates the release of mineralocorticoids (Hazon & Henderson 1985, Hazon *et al.* 1989, O'Toole *et al.* 1990). ANG II stimulates the secretion of 1 α-hydroxycorticosterone from the isolated and perfused interrenal gland of the dogfish (Armour *et al.* 1993). Plasma levels of ANG II in the nurse shark range between 30 to 80pg/ml (Galli-Phillips, 1991), and these levels increase significantly following hemorrhage or transfer from freshwater to 25% seawater.

Most recently, ANG I from the dogfish, *Triakis scyllia*, has been isolated and fully sequenced with startling results(Takei *et al.* 1993b). As in mammals, isoleucine occurs in the fifth position of the amino acid sequence of dogfish ANG I, a finding which may have important phylogenetic implications. In addition, dogfish is unique among vertebrates since its ANG I contains proline occurs in the third position, instead of valine.

iv. Sarcopterygians and ancient bony fishes In the kidneys of crossopterygians, *coelacanthodi* and *Latimeria chalumnae*, Bowie stain failed to detect JG granules (Nishimura & Ogawa, 1973), however, renin-activity was present in their kidney extracts (Nishimura *et al.* 1973). In dipnoan fishes, granulated cells resembling JG cells have been identified in the tunica media of renal arteries. Renal renin activity has been found in the kidney extracts of the dipnoan, *Protopterus aethiopicus*, and *Lepidosiren paradoxa* (Ogawa *et al.* 1972, Nishimura *et al.* 1973), and plasma renin activity has been detected in a third species, *Neoceratodus fosteri* (Blair-West et al, 1977).

To date, studies on the holostean RAS have been limited to only two species; the bowfin, *Amia calva* and the longnosed garpike, *Lepisosteus osseus*. Although granulated JG cells have only been identified in the garpike (Ogawa *et al.* 1972, Nishimura *et al.* 1973), kidneys of both species contain significant renin-activity (Nishimura *et al.* 1973). In the bowfin, experimental evidence suggests that the cardiovascular system is regulated by a RAS (Butler *et al.* 1995). Exogenous ANG II was found to increase arterial blood pressure, a response that can be blocked by the ACE inhibitor Captopril. In the bowfin, ANG II and its receptors may have evolved somewhat independently since exogenous eel-[Asn^1, Val^5, Gly^9]-ANG II and bowfin-[Asp^1, Val^5, Gly^9]-ANG II have identical pressor effects in the bowfin(Takei *et al.* 1998).

In 4 species of chondrosteans, the Bowie's stain method failed to detect granulated JG cells (Ogawa *et al.* 1972, Nishimura *et al.* 1973, Krishnamurthy & Bern 1969). Nevertheless, kidney extracts from *Polypterus senegalus* show renin activity, suggesting the presence of a chondrostean RAS (Nishimura *et al.* 1973). Of the sarcopterygian fishes examined thus far, there is no evidence of a JGA. Structures relevant to the JGA, including the MD and the EGM have not been detected (Nishimura *et al.* 1973, Sokabe & Ogawa 1974, Lagios 1974).

v. Modern bony fishes (Teleosteans) The teleostean RAS is the most extensively studied among fishes. Granulated JG cells have been observed in numerous species of teleosts using the Bowie's stain method and the periodic acid Schiff stain method (Sokabe & Ogawa, 1974, Oguri & Sokabe, 1968, Krishnamurthy & Bern, 1969). In

teleosts, there is no evidence of the MD (Krishnamurthy & Bern, 1969), or EGM cells in teleosts (Sokabe & Ogawa, 1974, Ogawa & Oguri 1978).

Both plasma renin activity and renin-substrate have been detected in the toadfish, *Opsanus tau* (Nishimura, 1980). Such studies provide strong indirect evidence for a renin or a renin-like enzyme in teleosts. In the European eel, *A. anguilla*, and the rainbow trout, *Oncorhynchus mykiss*, exogenous ANG II increases plasma cortisol levels (Borriraja *et al.* 1973, Arnold-Reed & Balment 1994). Exogenous ANG II can also increase water intake in teleosts (Perrott & Balment 1985, Perott *et al.* 1992, Hirano *et al.* 1978, Takei *et al.* 1979b, Hirano & Hasegawa 1984).

ANG I has been sequenced in 4 species of teleosts: the aglomerular goosefish, *Lophius litulon* (Hayashi *et al.* 1978), the chum salmon, *Oncorhynchus keta* (Takemoto *et al.* 1983), the Japanese eel, *A. japonica* (Hasegawa *et al.* 1983) and the North American eel, *A. rostrata* (Khosla *et al.* 1985). Unlike tetrapods, teleost ANG I contains the amino acid asparagine (Asn), instead of aspartate (Asp) in position one (Table 1). In the North American eel, [Asn^{1}-Arg^{2}-Val^{3}-Tyr^{4}-Val^{5}-His^{6}-Pro^{7}-Phe^{8}-Gly^{9}-Leu^{10}] ANG I predominates (Khosla *et al.* 1985). However, a small proportion of eel ANG I contains Asp in position 1, perhaps due to the enzymatic interconversion of Asn to Asp (Hayashi *et al.* 1978, Takemoto *et al.* 1983, Hasegawa *et al.* 1983a, Khosla *et al.* 1985). Position nine appears to be variable in teleosts and it may be occupied by Asn, as in the chum salmon (Takemoto *et al.* 1983), histadine as in the goosefish (Hayashi *et al.* 1978), or glycine as in the eel (Hasegawa *et al.* 1983, Khosla *et al.* 1985).

Table 1. Primary structure of ANG I from select vertebrate species

Species	Amino acid Sequence										Reference
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	
Mammals											
Human, pig, horse, sheep	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Akagi et al., 1992
rat, mouse, rabbit, guinea pig	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Fernley et al., 1986
Ox	Asp				Val				His		Akagi et al., 1992
Birds											
Fowl (*Gallus domesticus*)	Asp				Val				Ser		Nakayama et al., 1973
Quail (*Coturnix coturnix japonica*)	Asp				Val				Ser		Takei and Hasegawa, 1990
Reptiles											
Snake (*Elaphe climocophora*)	Asx				Val				Ser		Nakayama et al., 1977
Turtle (*Pseudemys scripta*)	Asp				Val				His		Hasegawa et al., 1984
Alligator (*Alligator mississipiensis*)	Asp				Val				Ala		Takei et al., 1993a
Amphibians											
Bullfrog (*Rana catesbeiana*)	Asp				Val				Asn		Hasegawa et al., 1983a
Teleost Fishes											
Salmon (*Oncorhynchus keta*)	Asn				Val				Asn		Takemoto et al., 1983
Eel (*Anguilla japonica*)	Asn				Val				Gly		Hasegawa et al., 1983b
Eel (*A. rostrata*)	Asn				Val				Gly		Khosla et al., 1985
Goosefish (*Lophius litulon*)	Asn				Val				His		Hayashi et al., 1978
Elasmobranch Fishes											
Dogfish (*Triakis syllia*)	Asn		Pro		Ile				Gln		Takei et al., 1993b

Along with the well-established pressor effects of exogenous ANG I and ANG II, investigators have further explored the mechanisms underlying the regulation of the teleost RAS. Hemorrhage is a potent stimulator of plasma renin activity in the rainbow trout, *O. Mykiss* (Bailey & Randall 1981). Acute hemorrhage has been found to increase plasma ANG II levels in the Japanese eel, *Anguilla japonica* (Takei *et al.* 1988). Therefore a putative baroreceptor mechanism, one that senses decreases in arterial blood pressure is thought to control the activation of the RAS in teleosts. Environmental changes that can lead to extracellular fluid loss also stimulates the teleost RAS. In the tilapia, *Tilapia mossambica,* JG cells increase in size and number following transfer to seawater (Krishnamurthy & Bern 1973). Plasma renin activity can also increase in teleosts following seawater transfer (Henderson *et al.* 1976, Sokabe *et al.* 1973, Jackson *et al.* 1977, Arillo *et al.* 1981). In eels, transfer to seawater has been found to increase plasma ANG II levels(Takei *et al.* 1988).

In mammals, the extrarenal RAS is an area of active investigation (see review by Johnson 1990). The synthesis of renin has been found to occur locally in many tissues and organs, such as the brain, ovaries, blood vessels and the heart. The local activation of the RAS and the consequent formation of ANG II may facilitate both endocrine and paracrine functions. In teleosts, the CS and perhaps the ovaries have been implicted as important sources of extrarenal-renin (Sokabe *et al.* 1970, Mandich & Massari 1994).

The corpuscles of Stannius (CS) of teleost fishes

(A) RAS and the CS

Since the discovery of the CS in 1839 by Stannius (see Pang & Schreibman 1986), many theories have been proposed that have linked the CS to osmoregulation in teleostean fishes. Rasquin (1956) observed that the activity and the size of the CS increased in relation to the salinity of the water. Until the discovery of the interrenals, the CS were thought to be the adrenocortical homologue of teleost fishes (De Smet 1962, Sandor *et al.* 1966, Youson *et al.* 1976). The CS were also thought to be the parathyroid glands of teleost fishes, until this theory was also abandoned owing to a lack of strong supporting evidence (Parsons *et al.* 1978, Shoumura *et al.* 1983, Lopez *et al.* 1984b, Milet *et al.* 1984). It has since been shown that the CS, at least in teleosts, contain a powerful renin-like pressor substance (Chester-Jones, 1966, Sokabe *et al.* 1970). The CS also contain stanniocalcin (Lafeber *et al.* 1988a,b, Wagner *et al.* 1988a, 1993), a 56 kDa glycoprotein (So & Fenwick 1977, 1979) that can suppress gill Mg^{2+} - Ca^{2+} activated ATPase (Ma & Copp 1978) to cause hypercalcemia.

In 1966, Chester-Jones was the first investigator to describe a link between the RAS and the CS, in teleosts. Using the rat bioassay, he demonstrated that CS extracts from freshwater eels, *A. anguilla*, produced a powerful, angiotensin-like pressor substance. He also showed that removal of the CS (stanniectomy) caused a rapid and profound decline in the blood pressure of the stanniectomized eel (Chester-Jones *et al.* 1966). Four years later, these findings were confirmed by studies on 3 additional teleost species (Sokabe 1970). Histological comparisons between the granulated cells of the CS and JG cells of the kidney reveal numerous structural similities (Sokabe & Ogawa

1974). Both ANG I and ANG II have been isolated from the CS of Japanese eels, *A. japonica*, chum salmon, *O. keta*, Japanese goosefish, *L. litulon*, rainbow trout, *O. mykiss* (Ogawa 1982, Takemoto *et al.* 1983, Hasegawa *et al.* 1984b, Yamada & Kobayashi 1987).

Most recently, in the freshwater North American eel, *A. rostrata*, stanniectomy led to a 45% reduction in dorsal aortic blood flow and a consequent 15% reduction in dorsal aortic blood pressure (Butler & Oudit 1995). Stanniectomy can also lead to hypercalcemia, hypocalcemia, hypernatremia, hyperkalemia and hypophosphatemia (see reviews by Hirano 1989). In a rarely cited study, Ogawa (1968) showed that the dramatic changes in plasma electrolyte levels following stanniectomy in goldfishes, *Carassius auratus*, could be corrected simply with i.v. injections of ANG II. Thus, it has been proposed that stanniectomy disrupts plasma electrolyte levels by impairing the RAS, leading to alterations in normal blood flow to osmoregulatory organs in the peripheral circulation, such as the gills, kidney and skin (Ogawa 1968, Butler *et al.* 1995, Butler & Cadinouche 1995).

(B) Distribution of the CS

The CS are small, ovoid endocrine glands unique to teleostean and holostean fishes. Embryonically, the CS may arise from the pronephric, mesonephric or opisthonephric ducts (Garrett 1942, Ford 1959, Belsare 1973a, Krishnamurthy 1967). A number of important generalizations may be made with regard to the anatomical distribution of the CS. In the more primitive holosteans, such as the bowfin and garpike, the CS tend to be small, numerous and widely distributed throughout the kidney mass (Garett 1942,

Bauchot 1953, De Smet 1962, Youson & Butler 1976). In teleosts, such as the eel, the CS tend to be larger, and occurring as a single pair embedded in the ventral surface of the posterior mesonephric kidney (Bauchot 1953). There are notable exceptions to this general trend. For instance, only one corpuscle is present in the ancient *Notopterus notopterus* (Belsare 1973a), whereas in the relatively modern salmon, up to 14 corpuscles have been reported (Krishnamurthy 1976).

(C) Vascularization of the CS

The cells of the CS are arranged in strands and lobules that are separated by connective tissue septa which also support the vascular and the nervous elements (Krishnamurthy & Bern 1969). The CS is supplied by an extensive network of blood vessels that can adapt to accommodate changing metabolic demands (Johnson 1972, Wendelaar Bonga *et al.* 1977, Bhattacharya & Butler 1978). In the species examined thus far, there appears to be considerable variation in the blood supply to the CS. In the mullet, *Mugli cephalus*, branches from the renal arteries and the cardinal vein supply the glands (Johnson 1972). In the stickleback, *Gasterosteus aculeatus*, the CS receives blood, mainly from segmental veins of the dorsal musculature (Wendelaar Bonga *et al.* 1977). Regardless of the species, all the glandular cells of the CS recieve a rich vascular supply, reflecting perhaps an important secretory function of the cells of the CS.

(D) Innervation of the CS

The CS are richly innervated by both sympathetic and parasympathetic efferents, which run parallel to the blood vessels supplying the glandular tissues (Heyl 1970, Wendelaar Bonga *et al.* 1977). However, there is no evidence of direct synaptic contact between the nerve fibres and the cells of the CS (Heyl 1970, Krishnamurthy & Bern 1971, Wendelaar Bonga *et al.* 1977, Bhattacharya & Butler 1978). Instead, the nerves are confined to the interlobular connective tissue septa, where they terminate on adjacent blood vessels and presumably modulate local blood flow. Fluorimetric techniques show that these fibres can be either cholinergic or adrenergic; carrying the neurotransmitters acetylcholine, noradrenaline or serotonin (Unsicker *et al.* 1977).

(E) Cell Types of the CS

The CS of teleostean fishes contain 2 distinct secretory cell types, the type-1 cells and type-2 cells (Wendelaar-Bonga & Greven 1975, Wendelaar-Bonga *et al.* 1977). An additional neurosecretory cell type has been identified in the CS of the white sucker, *Catastomus commersoni* (Marra et al. 1998). The structure of type-1 cells resemble that of other cells active in the synthesis and secretion of polypeptides, such as cells of the exocrine pancreas. The type-1 cell is ovoid, contain large nucleus with a pronounced nucleolus, extensive granular endoplasmic reticulum, large Golgi bodies and characteristic large, round, densely-staining secretory granules. In all species examined, the majority of CS cells are of the type-1 cytology (Oguri 1966, Fujita & Honma 1967, Carpenter & Heyl 1974, Cohen *et al.* 1975). In the bowfin, toadfish and a

number of other species (Youson & Butler 1978, Wendelaar Bonga & Greven 1975, Bhattacharya & Butler 1978), the CS is composed almost exclusively of type-1 cells.

The other cell type, type-2 cells, tend to be narrower, with smaller, irregularly shaped granules, sparsely distributed endoplasmic reticulum and fewer golgi bodies (Wendelaar Bonga & Greven 1975, Wendelaar Bonga *et al.* 1977). There is evidence that the two cell types come from a single lineage, whereby their histologicals differences may reflect different stages of development or different states of activity (Kaneko *et al.* 1992, Bhattacharya & Butler 1978, Youson & Butler 1976, Bhattacharya *et al.* 1978). It has even been suggested that type-2 cells may represent type-1 cells that are undergoing programmed cell death, or apotosis (Wyllie *et al.* 1980).

Pharmacological inhibition of the RAS

The RAS plays a major role in the regulation of blood pressure, as well as fluid and electrolyte homeostasis. It is a closed-loop, negative-feedback system responding to volume or sodium depletion and to factors modifying renal blood flow. Two key enzymes in the RAS cascade are the highly specific aspartyl protease, renin, and by contrast, the highly non-specific ACE, a dipeptidyl-carboxypeptidase. Renin hydrolyses the globular plasma protein, angiotensinogen, to release the decapeptide, ANG I. ANG I is subsequently converted to the powerful vasoconstrictive octapeptide, ANG II by ACE. ANG II is hydrolyzed further by aminopeptidases to become ANG III, and other relatively inactive peptides.

In theory, pharmacological interference with the RAS is possible at every step in the formation or action of ANG II. However, at present, drugs have been developed to

block the RAS at only 3 steps. Firstly, renin inhibitors selectively block the formation of ANG I from renin-substrate. Secondly, ACE inhibitors prevent the formation of ANG II from ANG I. Thirdly, ANG II receptor antagonists inhibit the cellular responses to ANG II. ANG II analog receptor-antagonists such as [Sar^1, Ile^8]-ANG II (Sarile) have helped to clarify the role of the RAS in normal as well as pathological models. More recently, nonpeptide ANG II antagonists such as Losartan, have gained rapid and widespread acceptance clinically and experimentally. Interestingly, the biosynthesis, secretion and maturation of renin are potential sites of inhibition that have not yet been explored.

Given the intense medical interest in the RAS, much of the research on the biochemical properties of the RAS and its interaction with chemical inhibitors has focused primarily on mammalian models. To date, the amino acid sequence of renin has only been determined for the rat, mouse and human (Hirose & Murakami 1992). In recent years, tremendous strides have been made in our understanding of the biological and the biochemical properties of the RAS in mammals. However, the role of the RAS and the effect of RAS inhibitors on the cardiovascular regulation of teleost fishes have not been examined in depth.

(A) Inhibition of renin

Renin belongs to the aspartyl protease family of enzymes that includes pepsin, prochymosin and penicillopepsin. Renin is distinct from other aspartyl protease due to its high specificity for its substrate, angiotensinogen. Renin occurs naturally in both active and inactive forms (Lumbers 1971). In the hog kidney, 3 forms of renin have been isolated (Inagami & Murakami 1977): a 42-kDa active renin, a 61-kDa prorenin

and a 140-kDa inactive renin. In humans, it is estimated that between 50 to 60 % of circulating renin is in the form of prorenin or inactive renin (Boyd 1977). Renin-binding protein, located in the renal tubules of rats, has been implicated in the interconversion of active and inactive renin (Ikemoto *et al.* 1982).

Angiotensinogen, or renin substrate, is a 58 kDa hepatic glycoprotein that has been isolated in humans, as well as a handful of other mammals (Schiffrin & Genest 1983). In mammals, the ANG I decapeptide is liberated from the N-terminal of angiotensinogen by renin, through the hydrolytic cleavage of a leucine-valine bond called the "scissile" bond (Tewsbury *et al.* 1981). As is the case with renin, there also appears to be variable forms of angiotensinogen (Murakai *et al.* 1984). In humans, variations in the primary structure of angiotensinogen are thought to be the result of different pathways of post-translational processing and of noncovalent interactions between the newly secreted angiotensinogen molecule and other circulating moieties (Campbell *et al. 1985*). Two enzymes, a cardiac chyme and tonin, an enzyme found in the mouse submaxillary gland, can synthesize ANG II directly from renin substrate, without the participation of ACE (Erdos 1976, Urata *et al.* 1990).

Prior to the development of ACE inhibitors and nonpeptide ANG II receptor antagonists, numerous attempts have been made to synthesis renin inhibitors for therapeutic applications. Five different categories of renin inhibitors are described in the literature: phospholipid inhibitors, renin antibody inhibitors, angiotensinogen-analogue inhibitors, prorenin inhibitors and the most widely studied renin inhibitor, Pepstatin A. Membrane-derived phospholipid preinhibitors, such as PE-140, were the first renin inhibitors to receive serious consideration (Sen *et al.* 1969a, b). PE-140 blocked the

pressor effects of exogenous renin and lowers blood pressure in dogs (Antonaccio 1982, Davis *et al.* 1974). Specific antibodies to renin were also considered as candidates for renin inhibitors. Initial findings showed that the antibodies can inhibit renin activity both *in vitro* and *in vivo* (Ondetti *et al.* 1982, Kohler *et al.* 1975). Chemically modified angiotensinogens have also shown promise as renin inhibitors. Modifications are made to the "scissile" bond, thereby preventing renin from carrying out the proteolytic release of ANG I (Burton *et al.* 1975, Poulsen *et al.* 1973). Alternatively, fragments of prorenin from the mouse submaxillary-gland have also been found to have inhibitory effects on renin (Panthier *et al.* 1982). Unfortunately, none of these four strategies have proven to be effective in the long term.

In 1971, a bacterially derived peptide, Pepstatin A, was introduced as a nonspecific inhibitor of acid proteases, such as renin (Marciniszyn *et al.* 1976, Aoyagi *et al.* 1971). Of the five different classes of renin inhibitors, Pepstatin A is the most effective. Pepstatin A blocks the pressor effects of exogenous renin and renin substrate, without interfering with the actions of ANG I or ANG II. The other major physiological effects of renin, attributable to the *de novo* synthesis of ANG I and ANG II, can also be blocked by pepstatin A. Currently, Pepstatin A is one of the only renin inhibitors that are commercially available. The effect of Pepstatin A on the RAS has not been examined in fishes.

(B) Inhibition of ACE

ACE is a large, 150 to 206-kDa peptidyl carboxypeptidase that is found in the lung, kidney and plasma of mammals (Peach 1977). Unlike renin, ACE has a wide substrate

specificity (Ehlers *et al.* 1989, Erdos & Skidgel 1987), enabling it to hydrolyze ANG I, bradykinin, enkephalins, neurotensin, substance P as well as LHRH (see review by Erdos 1976). Recently, 2 forms of ACE have been identified. One occurs in the endothelium of the lung and kidney, and the other in germinal cells of the testes (Ehler & Riordan 1990, Beldent *et al.* 1993, Hooper 1990). In the RAS, ACE is responsible for the final proteolytic step in the formation of ANG II. ACE cleaves the two C-terminal amino acids from ANG I, to form the ANG II octapeptide.

Although ANG I does not stimulate vascular smooth muscle contraction, it can act directly on the central nervous system, adrenal cortex, adrenal medulla and the kidney (Buckley 1972, Swanson et al, 1973, Saruta *et al.* 1972, Itskowitz & McGriff 1974). The discovery of ANG I-specific binding sites support these findings (Goodfriend *et al.* 1972). ANG I has a highly conserved structure. Between species, variations in its primary structure is restricted almost exclusively to positions 1, 5 and 9 (Table 1). Among mammals, the ANG I of ox is unique because it contains valine, instead of isoleucine in the fifth position (Akagi *et al.* 1982). This amino acid configuration of ANG I is characteristic of submammalians, not of mammalians.

ACE inhibitors such as captopril, mask the catalytic properties of ACE by competitively binding to its active site (Ehlers & Riordan 1989, 1991). In mammals, ACE-inhibition decreased plasma ANG II and aldosterone levels (Johnson *et al.* 1979, Hulthen 1978), causing total peripheral resistance and arterial blood pressure to decline (Lund-Johansen *et al.* 1984). Similar findings have also been reported in nonmammalian species. Butler *et al.* (1995) showed that in the bowfin, *A. calva*, captopril can attenuate the pressor response to exogenous ANG I.

(C) Inhibition of ANG II Receptors

ANG II has been implicated in the pathogenesis of various cardiovascular disorders that affect millions of people(Peach 1977). Synthetic ANG II analogs, which act as competitive antagonists of ANG II receptors, have proven to be clinically and experimentally relevant (Khosla *et al.* 1974). Such compounds, including [Sar^1, Ile^8]-ANG II (Sarile) and [Sar^1, Ala^8]-ANG II (Saralasin) inhibit the actions of ANG II at the receptor level (Brown *et al.* 1983, Timmermans *et al.* 1992) and has been shown to bind all ANG II receptor subtypes, ubiquitously (Khosla *et al.* 1974). Hence, these compounds are ideally suited for inhibiting the biological actions of ANG II and for identifying its specific sites of action.

The recent development of specific, nonpeptide ANG II receptor antagonists, in particular Losartan, PD123319, PD123177 and CGP42112A have enabled researchers to characterize different subtypes of ANG II receptors on the basis of their affinity for these compounds. Losartan (Dup 753) binds the AT_1 receptor subtype (Timmermans *et al.* 1993), while CGP42112A, PD123177 and PD123319 bind preferentially to receptors of the AT_2 subtype (Chiu *et al.* 1989, Bumpus *et al.* 1991). Since AT_1 receptors mediate all the known actions of ANG II, their inhibition by Losartan can block all the effects of ANG II on vascular smooth muscle, the kidney, and the adrenal cortex (Timmermans *et al.* 1993). Although studies have been undertaken to identify AT_1 receptors in nonmammalian species, the physiological role of AT_1 receptors in nonmammalians has yet to be established. The role of AT_2 receptors, in all species, is unclear (Saavedra & Timmermans 1994). In mammals, the use of AT_2 receptor antagonists such as

PD123177, PD123319 and CGP42112A do not affect any of the actions of ANG II (Chiu *et al.* 1989, Whitebread *et al.* 1989). A study on the effects of AT_1 and AT_2 inhibition, in a piscine species, has not been previously undertaken.

OBJECTIVE

The purpose of this investigation is to test the hypothsis that the CS, in the North American eel (*Anguilla rostrata* LeSueur), is an important source of renin-like enzyme activity. The effect of i.v. injections of CS extract (CS-EXT) on blood flow in the dorsal aorta and the caudal vein will be explored. Furthermore, pharmacological antagonists of the RAS will be tested in the eel, to determine their effects on the dorsal aortic and caudal venous blood flow responses to CS-EXT. In addition, this is the first study to test the effects of subtype specific, nonpeptide antagonists of ANG II receptors, in fishes.

MATERIALS AND METHOD

Experimental animals

Female North American eels, (*Anguilla rostrata* LeSueur), weighing 800-1200 g were collected in October 1996, from the St. Lawrence River near Quebec City by Pecheries Gingras, DuPont, St. Nicholas, Quebec, Canada, and shipped by air to Toronto, Ontario. They were delivered to the Department of Zoology, animal care facilities, where they were held in aerated plastic holding tanks (25 fish per 500L capacity tank), supplied with aerated, dechlorinated, Toronto tap water (Na^+, 0.45; Cl^- 0.95; K^+ 0.02; Ca^{2+}, 0.98; Mg^{2+}, 1.59 mmol l^{-1}) at 11.0 $\pm$ 1.0 ° C . Eels were held under a 12 : 12 h light-dark cycle and fed worms *ad libium*. Eels were selected at random for experiments that started in March 1997.

Experimental set-up and surgical procedures

Eels were selected at random and placed individually in Plexiglas observation chambers (13.8 x 12.0 x 114.5 cm) supplied with aerated, dechlorinated tap water (12.0 $\pm$ 0.5^c C). Eels were adapted to the observation chambers for at least 3 days before the experiment started.

(A) Insertion of Doppler flow probes

Eels were anaesthesized in a solution of methane tricanesulphonate (MS222) in tap water (1 g l^{-1}). When movement of the operculum ceased, the eel was deemed to be unconscious, and it was then wrapped carefully in a wet cotton towel and placed on its

back on an enamel surgical tray. A ventro-medial incision into the coelomic cavity was started near the anterior border of the liver, and extended 5-6 cm posteriorly. The edges of the body wall were retracted and the liver was deflected to the right to expose the dorsal aorta. Next, the dorsal aorta was carefully dissected free of connective tissue, care being taken not to break the fine intersegmental arteries supplying the body wall. The dorsal aorta varied in diameter according to the size of the eel, so in each case, it was fitted with a probe bearing a cuff diameter of 2.6, 2.8, or 3.0 mm. Signal transmission was improved if ultrasonic transmission gel (Aquasonic 100, Parker Labs., New Jersey) was smeared on the contact surface between the transducer crystal of the probe and the wall of the artery. Pieces of sterile Gelfoam measuring 3 mm X 5 mm were packed against the outside of the cuffed vessel to provide support and to reduce any bleeding. The lead coming from the flow probe was loosely stitched to the body wall to hold it in the correct position. Then the cut edges of the body wall were pulled together with size 3-0 Chinese braided silk sutures. Finally, the skin was sutured with size 3-0 stainless steel wire.

The caudal vein was also fitted with an appropriately sized Doppler probe. Access to the caudal vein was made through an 5-cm ventro-medial incision through the body wall along the anterior border of the caudal fin. The edges of the body wall were retracted to expose the caudal vein. The vein was stripped free of connective tissue; great care being taken to not tear the segmental veins draining into it. Application of gel was followed by insertion of a suitable sized Doppler cuff which was tied in place in much the same manner as the dorsal aortic cuff. The surgical area was packed with small, 3 x 5 cm pieces of Gelfoam sponge. Then the cut edges of the body

wall were pulled together with size 3-0 Chinese silk sutures. Finally, the skin was sutured with size 3-0 stainless steel wire.

When the surgery was completed, the eel was rinsed with tap water to remove any residual anaesthetic. It was then given a 2-3 ml injection of Ampicillin (100 mg. ml^{-1} i.m.). When the eel was able to swim again, it was transferred to an observation chamber that was covered with a black plastic sheet. 1 ml of Ampicillin (100 mg. ml^{-1} i.v.) was deliver i.v. daily via the caudal vein catheter for the duration of the experimental period. Each eel was allowed to rest for 3-4 days before experiments commenced, to allow its cardiovascular parameters to return to normal. There was no evidence of infection during the next four days and the wound healed well with no apparent leakage or discomfort for the eel.

(B) Collecting the corpuscles of Stannius and posterior kidney tissue

Each eel was randomly selected from stock, anaesthetized with MS 222 (1.0g. l^{-1}) and placed on a wet cotton towel. It was rolled over onto its side and a 5 cm incision through the body wall was made a few mm below the lateral line and parallel to it, immediately above the ventral fin. The cut edges were retracted to expose the edge of the posterior portion of the kidney. The bladder was lifted away from the ventral surface of the kidney by blunt dissection, with no evident bleeding. The oval, ivory-coloured CS, measuring approximately 2-3 mm in diameter and weighing approximately 6 mg, could now be seen as embedded in the surface of the kidney, at the point where the right posterior cardinal vein emerges from the kidney surface. Both corpuscles were easily excised and then placed on ice, where they were immediately transferred to a –50 °C

refrigeration compartment. The bladder was carefully lowered back into place before the edges of the body wall muscle were sutured together with size 3-0 Chinese braided silk. Following this, the skin was sutured with 3-0 stainless steel wire. Finally the eel was given a 2-3 ml injection of Ampicillin (100 $mg.ml^{-1}$ i.m.) and returned to a 400 liter glass aquarium supplied with constantly flowing aerated dechlorinated tap water (12.5 ± 0.5 °C), until it was used for additional blood flow experiments.

Samples of posterior kidney tissue adjacent to the corpuscles were removed from five eels. The tissue was frozen immediately, later to be thawed, weighed and extracted on the day of the experiment. The posterior kidney extracts were prepared using the same method used to prepare extracts of the CS. Afterward, the eel was killed with an overdose of MS 222.

(C) Insertion of drug delivery catheter

A 3 cm incision was made 3 mm below and parallel to the lateral line near the tip of the caudal fin. Muscle tissue was gently plied apart and retracted to expose a 2 cm length of caudal vein. It was freed of connective tissue and bone of the neurohaemal arch. A heparinized polyethylene catheter (PE-10, Clay Adams) was inserted into the caudal vein and pushed 10-12 cm anteriorly. The catheter was then tied in place with 5-0 braided silk sutures before the incision was closed with 5-0 braided silk sutures. This left a trailing length of approximately 20 cm. The end of the catheter was heat-sealed. The catheter was used later for the delivery of all the drugs and hormones without disturbing the eel during the experiments. It was flushed regularly with 0.4 ml heparin-solution to prevent the formation of blood clots.

(D) Calibration of Doppler flow probes

A postmortem calibration of dorsal aortic and caudal venous flow probes was performed *in situ*. This allowed the experimenter to calculate the absolute values for blood flows (*Butler & Oudit 1995, Oudit & Butler 1995*). At the end of the flow experiment, the eel was killed using a 5 ml injection of Somnotol (sodium pentobarbital, 65 $mg.ml^{-1}$ i.v.). The ventral incision was re-opened to gain access to the Doppler flow probe on the dorsal aorta. First, the dorsal aorta was exposed and catheterized 5-10 mm anterior to the probe with PE-100 (i. d. 0.86 mm, o. d. 1.5 mm) polyethylene tubing for blood perfusion. It was placed close to the probe so as to minimize leakage through the segmental, as well other arteries. Then the catheter was tied in place with 3-0 silk sutures. An outflow catheter, also PE-100, was inserted into the dorsal aorta posterior to the probe and positioned 5 mm behind it. Then the catheter was tied in place with 3-0 silk sutures. The Doppler flow probe on the caudal vein was prepared for calibration in a similar manner. There was no sign of hemorrhaging in the region of the probe, nor of coagulated blood or other obstructive tissues on the contact surface between the probe cuff and the blood vessels. Connective tissue growth surrounding the Doppler probes provided additional stability for the duration of the experiments and the calibrations.

Freshly collected, heparinized eel blood was infused using an infusion pump (Harvard Apparatus Infusion Pump), over a range of flow rates that encompassed the minimum and the maximum values for each eel (Butler & Oudit 1995a, b). Flow rates were analyzed by linear regression analysis to provide calibration curves for the dorsal

aortic probe and the caudal venous probe. These plots confirmed that proper acoustic coupling was maintained during the calibration procedure.

Drugs and peptides

Eel Angiotensin I = [Asn^1, Val^5, Gly^9]-ANG I (MW=1200.7); Eel Angiotensin II = [Asn^1, Val^5]-ANG II (MW=1030.5); Eel Angiotensin III = [Val^4]-ANG III (MW=916.5); Human renin substrate tetradecapeptide = [1-14]-hRS (MW=1758.9), Pepstatin A (MW= 685.9) and Sarile = [Sar^1, Ile^8]-ANG II (MW=967.6) were all supplied by Penninsula Laboratories (Belmont, CA). Captopril (SQ 14 225, MW = 217.28) was supplied by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). The ANG II receptor blockers were supplied as follows: Losartan potassium (DuP 753, MK 954 MW=461), Dupont Merck Research and Development, Wilmington, DE.; PD123319 (MW=749.4), Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI. Methane tricainesulfonate (MS 222), Sigma Chemical Corp., St. Louis, MO.; Somnotol (Sodium pentobarbital in isotonic saline), MTC Pharmaceuticals, Cambridge, Ont., Canada; Ampicillin sodium, Novopharm, Toronto, Ont., Canada; Heparin solution, Hepalean-Organon Tekita, Toronto, Ont., Canada. All of the drug and peptide injections were made with 0.9% NaCl.

Blood flow experiments: Experimental protocol

Series I: Response to components of the RAS

Experiment 1*:* [Asn^1, Val^5, Gly^9]-ANG I. 5, 10, 20, and 50-ng kg bw^{-1} doses of [Asn^1, Val^5, Gly^9]-ANG I were injected i.v. in 0.2 ml of 0.9% NaCl to test the effect on dorsal aortic and caudal venous blood flows (n=5). These test doses were not injected until flow rates had stabilized and remained at baseline levels for 45 minutes. The caudal venous drug delivery catheter was flushed with 0.2 ml of 0.9% NaCl after each peptide injection to ensure that all the peptide reached the bloodstream. When blood flow had returned to normal, after the effect of the peptide had subsided, the animals were injected with a second 0.50 ml of 0.9% NaCl solution to show that volume loads had not affected blood flows. This basic experimental protocol was used also for experiments 2, 3, 4 and 5 which are described in this section. Following each experiment, the animals were given a one-day recovery period.

Experiment 2*:* [Asn^1, Val^5]-ANG II This experiment measured the effect of 5, 10, 20, and 50 ng kg bw^{-1} doses of [Asn^1, Val^5]-ANG II, in 0.2 ml of 0.9% NaCl, on dorsal aortic and caudal venous blood flows (n=5).

Experiment 3: Human Renin Substrate (hRS) This experiment measured the effect of 50, 100, 150 ng kg bw^{-1} hRS, in 0.2 ml of 0.9% NaCl, on dorsal aortic and caudal venous blood flows (n=5).

Experiment 4: [Val^4]-ANG III This experiment measured the effect of 5, 10, 20, 50 ng kg bw^{-1} [Val^4]-ANG III, in 0.2 ml of 0.9% NaCl, on dorsal aortic and caudal venous blood flows (n=5).

Experiment 5: Eel corpuscle of Stannius extract (CS-EXT) This experiment measured the effect of extracts of 0.50, 1.25, 2.50 mg of eel corpuscles of Stannius (CS-EXT) .kg bw^{-1}, delivered in 0.2 ml of 0.9% NaCl, on dorsal aortic and caudal venous blood flows (n=5).

Experiment 6: Eel posterior kidney extract (PK-EXT) This experiment measured the effect of an extract of 5 mg of eel posterior kidney (PK-EXT).kg bw^{-1}, delivered in 0.2 ml of 0.9% NaCl, on dorsal aortic and caudal venous blood flows (n=5).

Series II: Response to RAS antagonists

Experiment 1: **Effect of Pepstatin A on the blood flow response to [Asn^1, Val^5, Gly^9]-ANG I** This experiment measured the effect of 1 mg kg bw^{-1} dose of Pepstatin A, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to 50 ng kg bw^{-1} dose of ANG I i.v. Dorsal aortic and caudal venous blood flows were measured continuously. The drug delivery catheter was flushed with 0.2 ml of 0.9% NaCl after each injection to ensure that all the peptide or antagonist reached the bloodstream. Eels were injected with 0.50 ml saline solution (0.9% NaCl) to measure the effect of volume loads on blood flow. 55 minutes later, eels were injected

with 50 ng kg bw^{-1} dose of [Asn^1, Val^5, Gly^9]-ANG I. After a further 45 minutes, when the flow rates had returned to preinjection levels, the eels were given a 1 mg kg bw^{-1} dose of Pepstatin A. Eel was injected with 50 ng kg bw^{-1} dose of [Asn^1, Val^5, Gly^9]-ANG I 10 minutes later. Flow rates were measured for an additional 40 minutes, until rates had returned to preinjection levels. The basic experimental protocol for this experiment was also used for experiments 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 on this section (n=5).

Experiment 2: Effect of Pepstatin A on the blood flow response to [Asn^1, Val^5]-ANG II This experiment measured the effect of a 1 mg kg bw^{-1} dose of Pepstatin A, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 50 ng kg bw^{-1} dose of [Asn^1, Val^5]-ANG II (n=5).

Experiment 3: Effect of Pepstatin A on the blood flow response to human renin substrate (hRS) This experiment measured the effect a of 1 mg kg bw^{-1} dose of Pepstatin A, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 150 ng kg bw^{-1} dose of hRS (n=5).

Experiment 4: Effect of Pepstatin A on the blood flow response to extract of corpuscles of Stannius (CS-EXT) This experiment measured the effect of a 1 mg kg bw^{-1} dose of Pepstatin A, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 2.50 mg kg bw^{-1} of CS-EXT(n=5).

Experiment 5: Effect of Captopril on the blood flow response to [Asn^1, Val^5, Gly^9]-ANG I This experiment measured the effect of a 1 mg · kg bw^{-1} dose of Captopril, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 50 ng kg bw^{-1} dose of [Asn^1, Val^5, Gly^9]-ANG I (n=5).

Experiment 6: Effect of Captopril on the blood flow response to [Asn^1, Val^5]-ANG II This experiment measured the effect of a 1 mg kg bw^{-1} dose of Captopril, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 50 ng kg bw^{-1} dose of [Asn^1, Val^5]-ANG II(n=5) .

Experiment 7: Effect of Captopril on the blood flow response to human renin substrate (hRS) This experiment measured the effect of a 1 mg kg bw^{-1} dose of Captopril, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to 150 ng kg bw^{-1} dose of hRS (n=5).

Experiment 8: Effect of Captopril on the blood flow response to extract of corpuscles of Stannius (CS-EXT) This experiment measured the effect of 1 mg kg bw^{-1} dose of Captopril, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to 2.50 mg kg bw^{-1} dose of CS-EXT (n=5).

Experiment 9: Effect of [Sar^1, Ile^8]-ANG II (Sarile) on the blood flow response to [Asn^1, Val^5]-ANG II This experiment measured the effect of a 50 μg . kg bw^{-1} dose of

Sarile, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 50 ng kg bw^{-1} dose of [Asn^1, Val^5]-ANG II (n=5).

Experiment 10: Effect of [Sar^1, Ile^8]-ANG II (Sarile) on the blood flow response to extract of the corpuscles of Stannius (CS-EXT) This experiment measured the effect of a 50 μg . kg bw^{-1} dose of Sarile, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 2.50 mg · kg bw^{-1} dose of CS-EXT (n=5).

Experiment 11: Effect of Losartan on the blood flow response to [Asn^1, Val^5]-ANG II This experiment measured the effect of a 5 mg kg bw^{-1} dose of Losartan, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 50 ng kg bw^{-1} dose of [Asn^1, Val^5]-ANG II (n=5).

Experiment 12: Effect of Losartan on the blood flow response to extract of corpuscles of Stannius (CS-EXT) This experiment measured the effect of a 5 mg · kg bw^{-1} bw dose of Losartan, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 2.50 mg kg bw^{-1} dose of CS-EXT(n=5) .

Experiment 13: Effect of PD123319 on the blood flow response to [Asn^1, Val^5]-ANG II This experiment measured the effect of a 5 mg · kg bw^{-1} dose of PD123319, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 50 ng kg bw^{-1} dose of [Asn^1, Val^5]-ANG II (n=5).

Experiment 14: Effect of PD123319 on the blood flow response to extract of corpuscles of Stannius (CS-EXT) This experiment measured the effect of a 5 mg kg bw^{-1} dose of PD123319, delivered i.v. in 0.5ml of 0.9% NaCl, on the dorsal aortic and caudal venous blood flow response to a 2.50 mg kg bw^{-1} dose of CS-EXT (n=5).

RESULTS

Calibration curves and velocity profiles

Dorsal aortic and caudal venous calibration curves for three freshwater eels are shown in Figure 1. Both curves have similar slopes and there was a strong positive linear correlation between the Doppler shift (KHz) and the blood perfusion rate in $ml.min^{-1}.kg\ bw^{-1}$. Dorsal aortic and caudal venous blood flows were then obtained by interpolation. Velocity profiles across the dorsal aorta and caudal vein (2.8 mm probe) of freshwater eels were obtained by making small increments in the range outputs which determined the distance at which the blood velocity was measured (0-10 mm) (Butler & Oudit 1995). During the present experiments, the range was adjusted accordingly so as to measure the peak mean blood flow in both the dorsal aorta and caudal vein.

(A) ANG I, ANG II or ANG III and blood flow

This experiment compared the blood flow responses (BFR) in freshwater eels to a range of doses of each of three angiotensins including [Asn^1,Val^5,Gly^9]-ANG I (ANG I), [Asn^1,Val^5]-ANG II (ANG-II), and [Val^4]-ANG III (ANG III). The repeated measures ANOVA showed that whereas ANG I and ANG II groups both produced significant increases in caudal venous blood flow (CVBF) and dorsal aortic blood flow (DABF), while the ANG III group had a more modest effect when compared with the saline injected control group (Figure 2).

CVBF increased significantly following the i.v. injection of only 5 $ng.kg\ bw^{-1}$ of ANG I or ANG II (Figure 2) but it was not until the dose reached 10 $ng.kg\ bw^{-1}$ of each

Figure 1. Caudal venous (○) and dorsal aortic (●) calibration curves for freshwater North American eels, *A. rostrata* ($n = 3$). Linear regression lines:

Y = Doppler shift (Khz), X = Blood flow rate ($ml.min^{-1}.kg\ bw^{-1}$);

CV: $Y = 0.08X + 0.11$, $r = 0.98$, $p < 0.0001$,

DA: $Y = 0.08X + 0.0004$, $r = 0.99$, $p < 0.0001$.

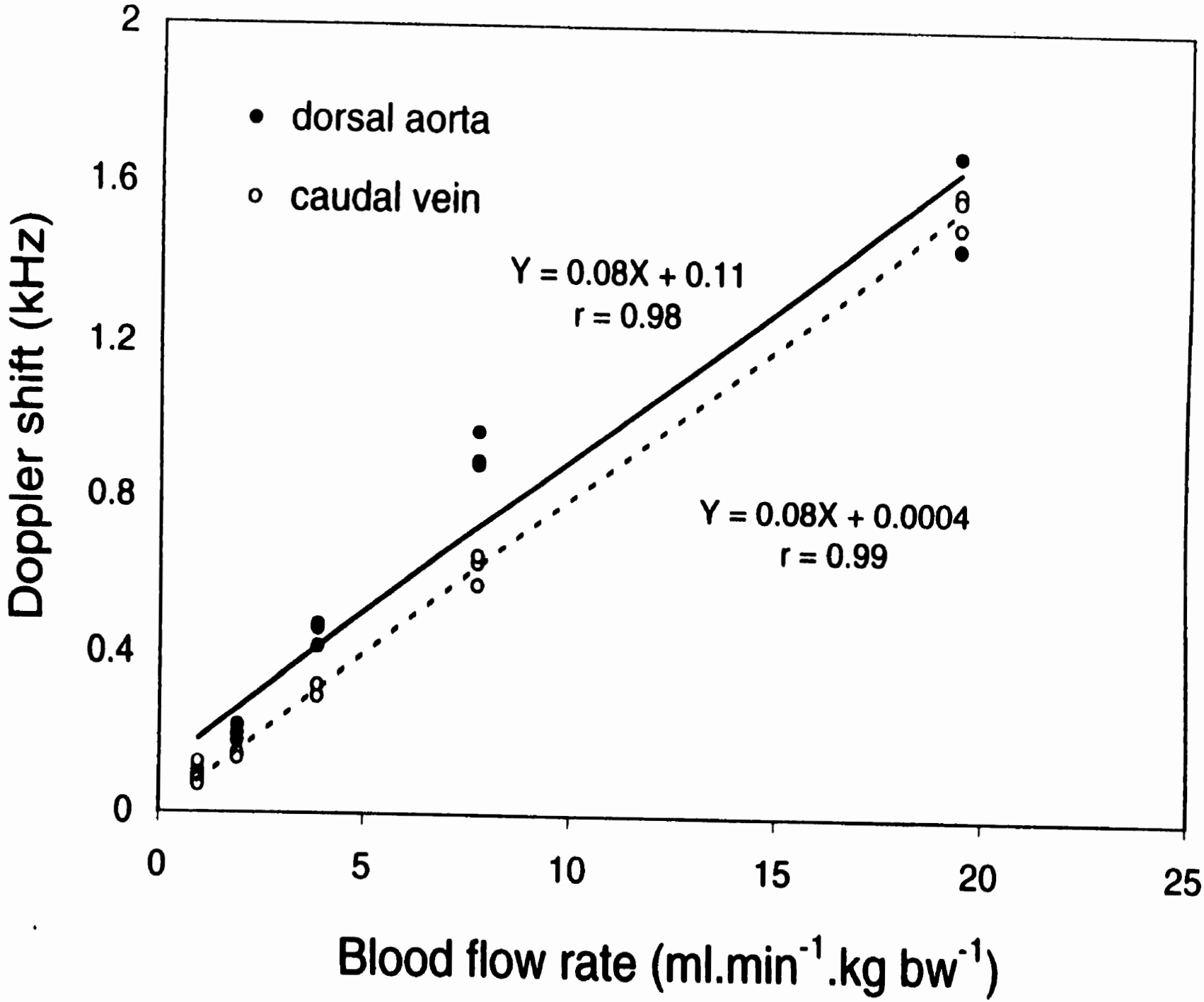
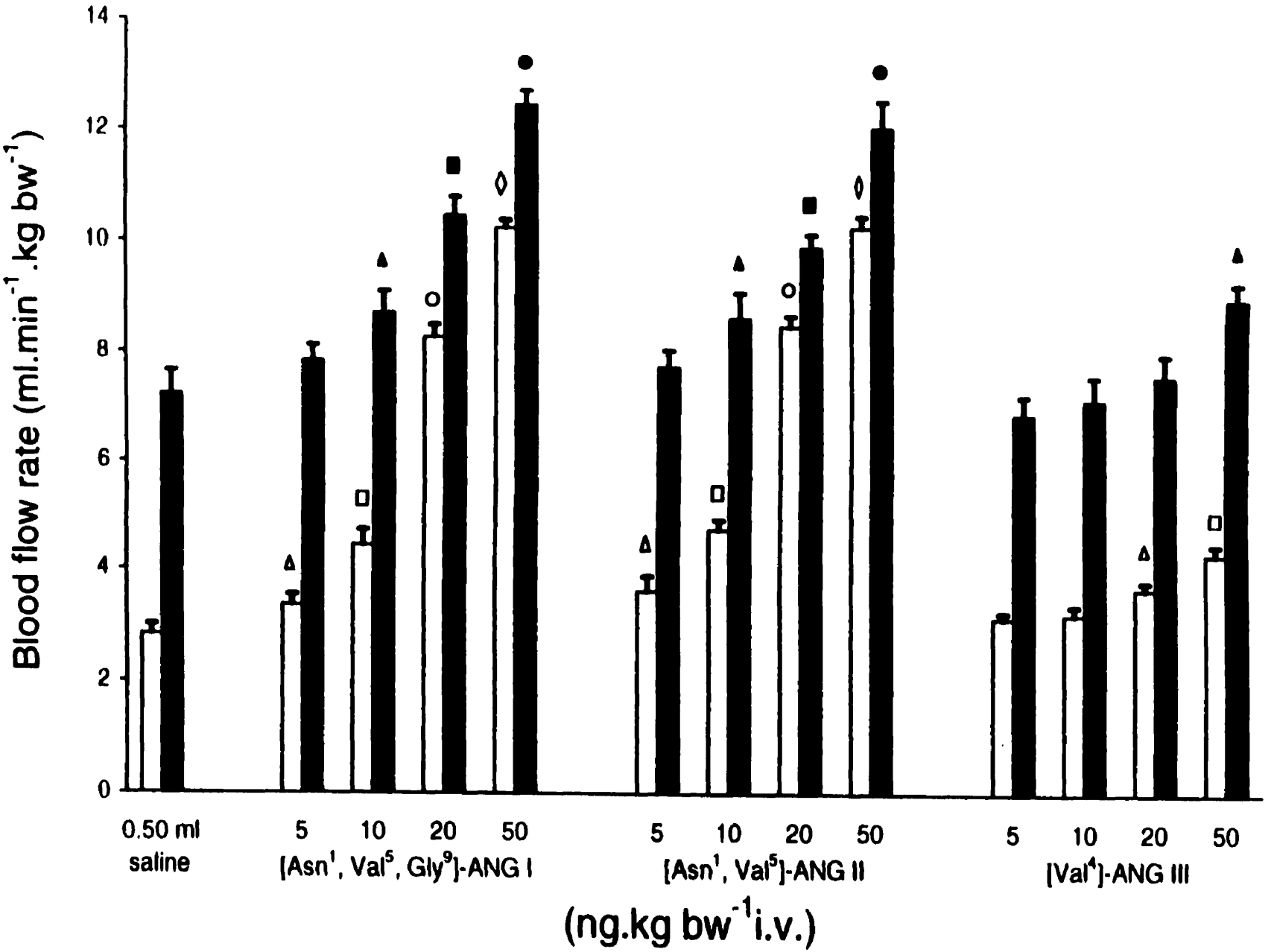

dorsal aorta
caudal vein
Y = 0.08X + 0.11
r = 0.98
Y = 0.08X + 0.0004
r = 0.99
Doppler shift (kHz)
Blood flow rate (ml.min-1.kg bw-1)
0
0.4
0.8
1.2
1.6
2
0
5
10
15
20
25

Figure 2. CVBF (▭) and DABF (▬), in freshwater North American eels, *A. Rostrata*, in response to i.v. injection of 5, 10, 20 or 50 ng. kg $bw^{-1}$of [Asn^1, Val^5, Gly^9]-ANG I, [Asn^1, Val^5]-ANG II and [Val^4]-ANG III. **CVBF response to ANG I or ANG II:** ◇ $P < 0.05$ compared with saline control, 5, 10, 20 ng. kg bw^{-1} peptide; ○ $P < 0.05$ compared with saline control, 5, 10 ng. kg bw^{-1} peptide; □ $P < 0.05$ compared with saline control and 5 ng. kg bw^{-1}. △ $P < 0.05$ compared with saline control. **DABF response to ANG I or ANG II:** ● $P < 0.05$ compared with saline control, 5, 10, 20 ng. kg bw^{-1} peptide; ■ $P < 0.05$ compared with saline control, 5 and 10 ng. kg bw^{-1} (for ANG I and ANG II). ▲ $P < 0.05$ compared with saline control and 5 ng. kg bw^{-1}. **CVBF response to ANG III:** □ $P < 0.05$ compared with saline control, 5, 10, 20 ng. kg bw^{-1} peptide. △ $P < 0.05$ compared with saline control, 5, 10, ng. kg bw^{-1} peptide. **DABF response to ANG III:** ▲ $P < 0.05$ compared with saline control, 5, 10, 20 ng. kg bw^{-1} peptide; ANOVA, Duncan's Multiple Range Test. Values are mean + SEM; n = 5.


Blood flow rate (ml.min^{-1}.kg bw^{-1})
14
12
10
8
6
4
2
0
0.50 ml saline
5
10
20
50
[Asn^1, Val^5, Gly^9]-ANG I
5
10
20
50
[Asn^1, Val^5]-ANG II
5
10
20
50
[Val^4]-ANG III
(ng.kg bw^{-1} i.v.)

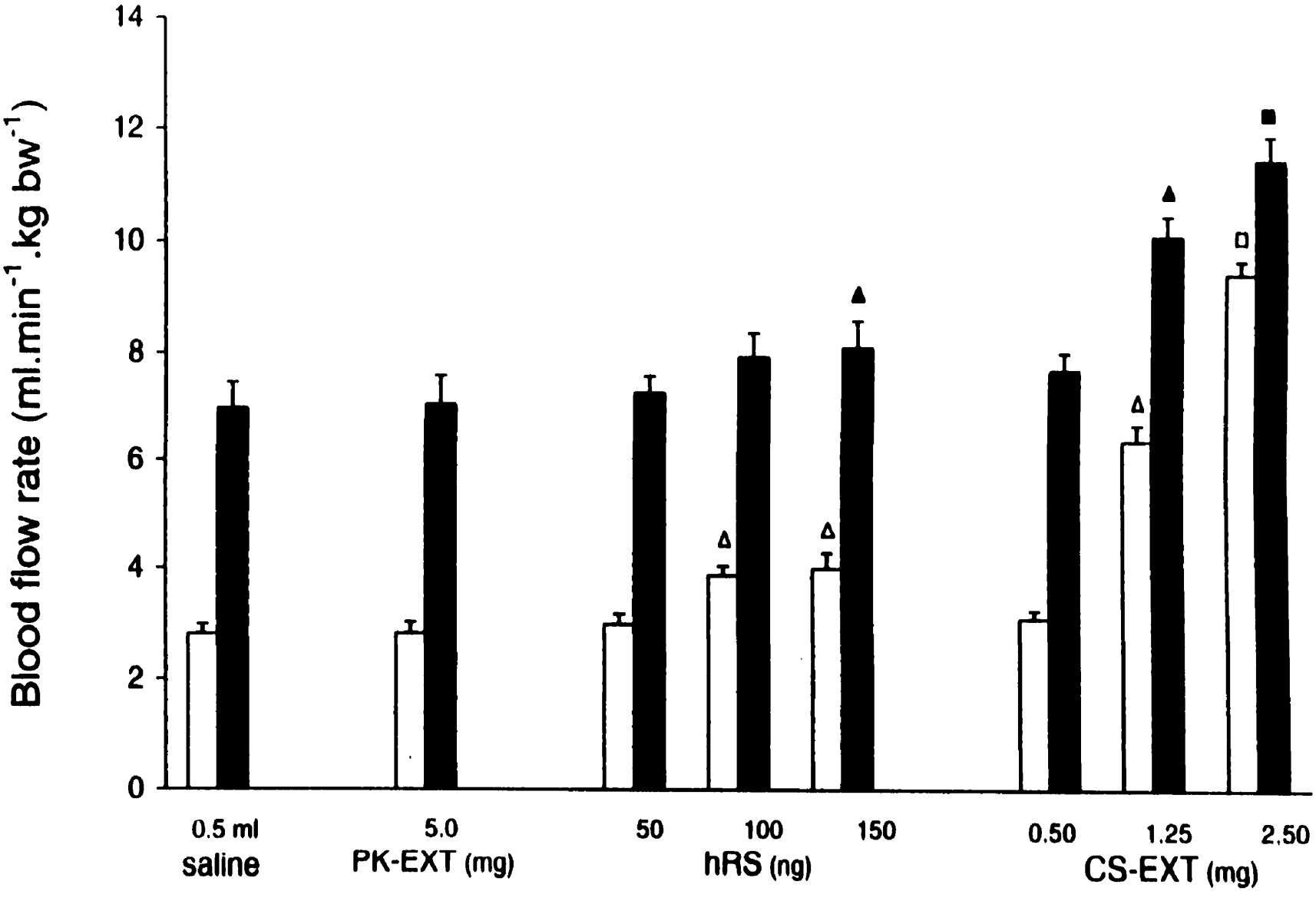
peptide that there was a significant increase in DABF. ANG III stood alone insofar as it required 20 ng .kg bw^{-1} of the peptide to increase significantly CVBF and 50 ng.kg bw^{-1} to increase DABF (Figure 2). In both the ANG I and ANG II groups there was a dose dependent increase in both CVBF and DABF.

(B) Tissue extracts and blood flow

Posterior kidney extracts (PK-EXT), human renin substrate or human angiotensinogen 1-14 (hRS) and extracts of corpuscles of Stannius (CS-EXT) were each tested for their effect on CVBF and DABF in freshwater eels. An i.v. injection of 5 mg of PK-EXT had no measurable effect on either CVBF or DABF compared with saline injected controls (Figure 3). An i.v. injection of 50 ng.kg bw^{-1} of hRS had no measurable effect on CVBF or DABF whereas 100 ng.kg bw^{-1} of the peptide was followed by a significant increase in CVBF but not DABF. At the highest dose (150 ng.kg bw^{-1}) both CVBF and DABF increased significantly compared with the saline injected controls (Figure 3).

Extracts of CS brought about important changes in flows. An i.v. injection of extract from 1.25 mg of tissue increased the CVBF from 2.80 $\pm$ 0.18 ml. min^{-1}.kg bw^{-1} to 6.38 $\pm$ 0.31 ml.min.kg bw^{-1} ($P < 0.05$) and the DABF from 6.99 $\pm$ 0.47 ml.min.kg bw^{-1} to 10.14 $\pm$ 0.36 ml.min.kg bw^{-1} ($P < 0.05$). The highest dose of extract of 2.5 mg of tissue i.v. increased further both the CVBF and DABF to levels which were significantly greater than the flows observed in both the saline injected controls and the 1.25 mg. kg bw^{-1} CS-EXT group.

Figure 3. CVBF (▭) and DABF (▬), in freshwater North American eels, *A. rostrata*, in response to i.v. injection of 50, 100 or 150 ng. kg bw^{-1} of human renin-substrate (hRS) and 0.5, 1.25 and 2.5 mg. kg bw^{-1} extract of CS (CS-EXT) expressed in units. kg bw^{-1}.**CVBF response to hRS**: Δ $P < 0.05$ compared with saline control, posterior kidney extract injection (PK-EXT) and 50 ng. kg bw^{-1} hRS. **DABF response to hRS**: ▲ $P < 0.05$ compared with saline control, PK-EXT, 50 and 100 ng. kg bw^{-1} hRS. **CVBF response to CS-EXT**: □ $P < 0.05$ compared with saline control, PK-EXT, 0.5 and 1.25 mg. kg bw^{-1} extract; Δ $P < 0.05$ compared with saline control, PK-EXT, 0.5 mg. kg bw^{-1} extract. **DABF response to CS-EXT:** ■ $P < 0.05$ compared with saline control, PK-EXT, 0.5 and 1.25 mg. kg bw^{-1} extract; ▲ $P < 0.05$ compared with saline control, PK-EXT, 0.5 mg. kg bw^{-1} extract; ANOVA, Duncan's Multiple Range Test. Values are mean + SEM; n = 5.


Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml
saline
5.0
PK-EXT (mg)
50
100
150
hRS (ng)
0.50
1.25
2.50
CS-EXT (mg)

(C) Pepstatin A and the blood flow responses to ANG I, ANG II, hRS or CS-EXT

Figure 4 shows that CVBF and DABF both increased significantly in freshwater eels following an i.v. injection of 50 ng.kg bw^{-1} of ANG I. Peak blood flow rate (BFR) in the CV and DA were 10.32 ± 0.23 and 12.44 ± 0.49 ml.kg bw^{-1} respectively. The onset of peak CVBF and DABF occurred 8 and 9 minutes after the injection of ANG I and remained elevated for 28 and 18 minutes before returning to the preinjection rates. These patterns in flow were not affected measurably by the prior i.v. injection of 1 mg.kg bw^{-1} of Pepstatin A (Figure 4). The Pepstatin A experiment was repeated using 50 ng.kg bw^{-1} of ANG II (Figure 5). Again, there was a clear and significant increase in both CVB and DABF both before and after an i.v. injection of 1 mg.kg bw^{-1} of Pepstatin A. These results were not unexpected since Pepstatin A is an effective renin inhibitor in mammals.

Next, 150 ng.kg bw^{-1} of hRS were injected i.v. which led to smaller, though statistically significant ($P<0.05$) increases in both CVBF and DABF. Both CVBF and DABF were increased significantly within 3 minutes after the hRS was injected. Peak flow rates in the CV and DA occurred 6 and 7 minutes after hRS injection and were 4.0 ±0.29 and 8.1±.49 ml.min^{-1}.kg bw^{-1} respectively (Figure 6). The form of the flow responses were similar to those observed following injection of ANG I and ANG II. The i.v. injection of 1 mg. kg bw^{-1} of Pepstatin A completely blocked the increased CVBF and DABF that had previously followed the injection of hRS (Figure 6). Flow rates remained at the preinjection level.

Figure 4. Effect of the mammalian renin antagonist pepstatin A on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 50 ng. kg bw^{-1} of [Asn^{1}, Val^{5}, Gly^{9}]-ANG I given before (time = 55) and after (time = 110) the i.v. injection of 1 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

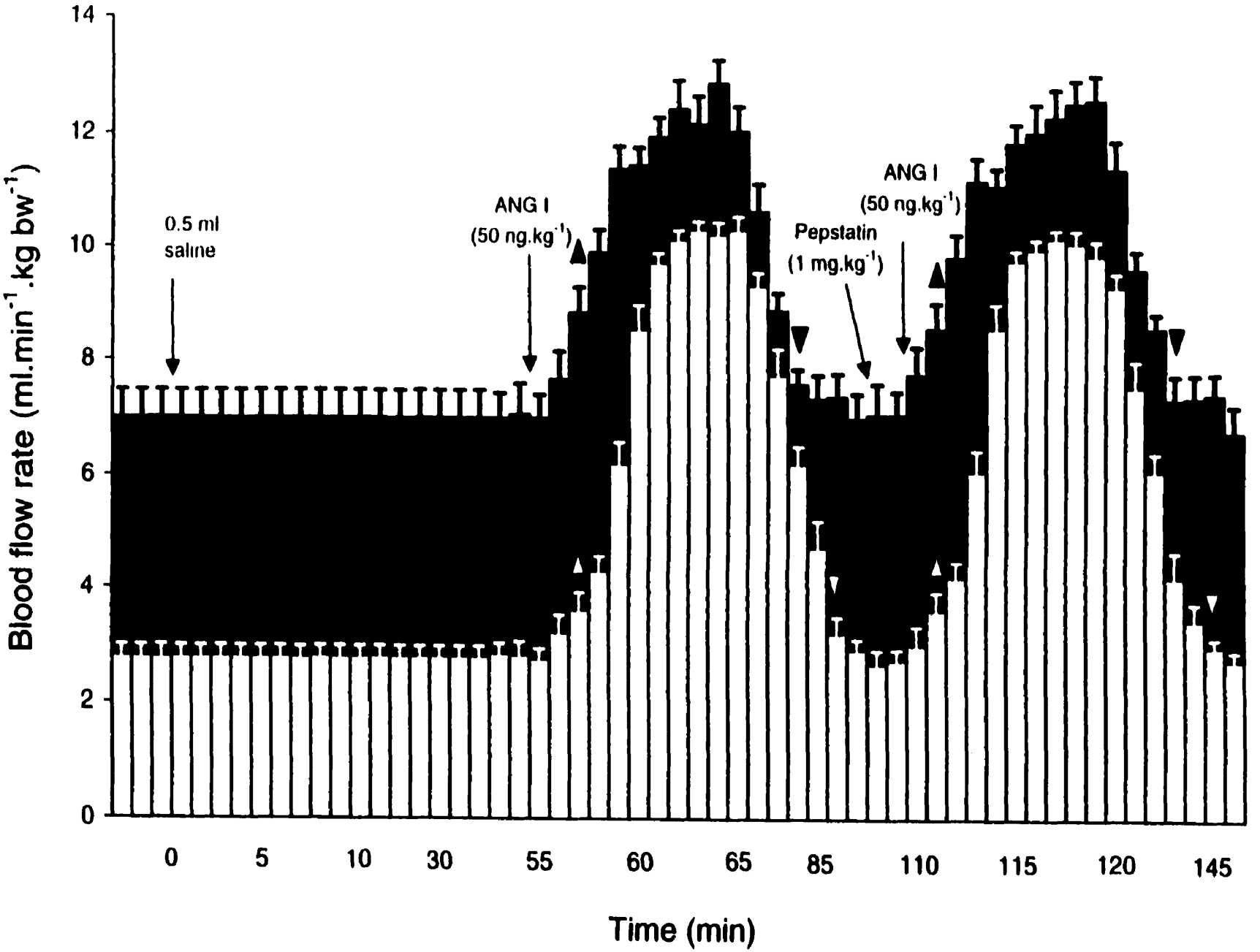

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
ANG I (50 ng.kg-1)
Pepstatin (1 mg.kg-1)
ANG I (50 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 5. Effect of the mammalian renin antagonist pepstatin A on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II given before (time = 55) and after (time = 110) the i.v. injection of 1 mg. kg bw^{-1} of antagonist (time = 100). **CVBF:** (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF:** (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

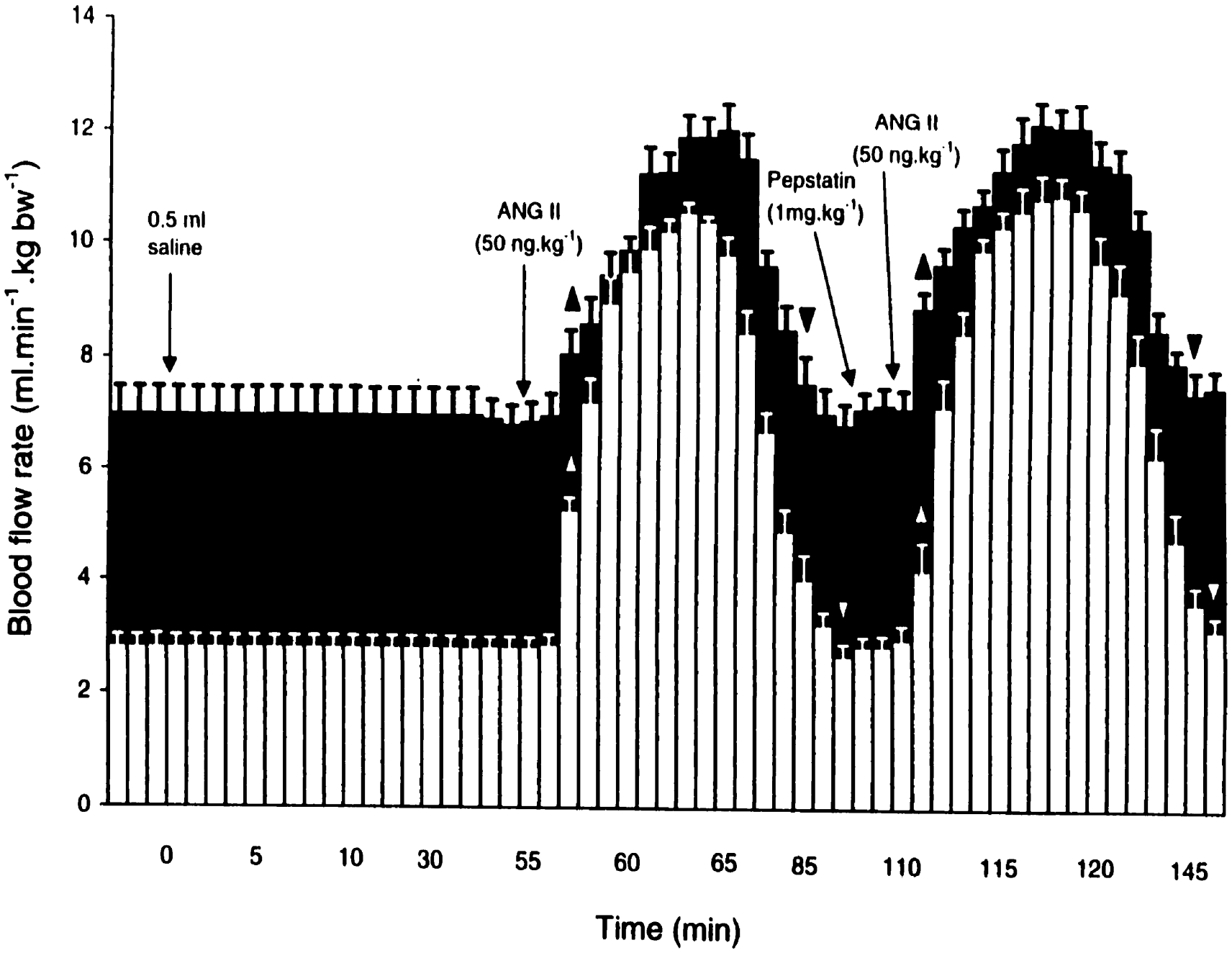

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
ANG II (50 ng.kg-1)
Pepstatin (1mg.kg-1)
ANG II (50 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 6. Effect of the mammalian renin antagonist pepstatin A on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 150 ng. kg bw^{-1} of hRS given before (time = 55) and after (time = 110) the i.v. injection of 1 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

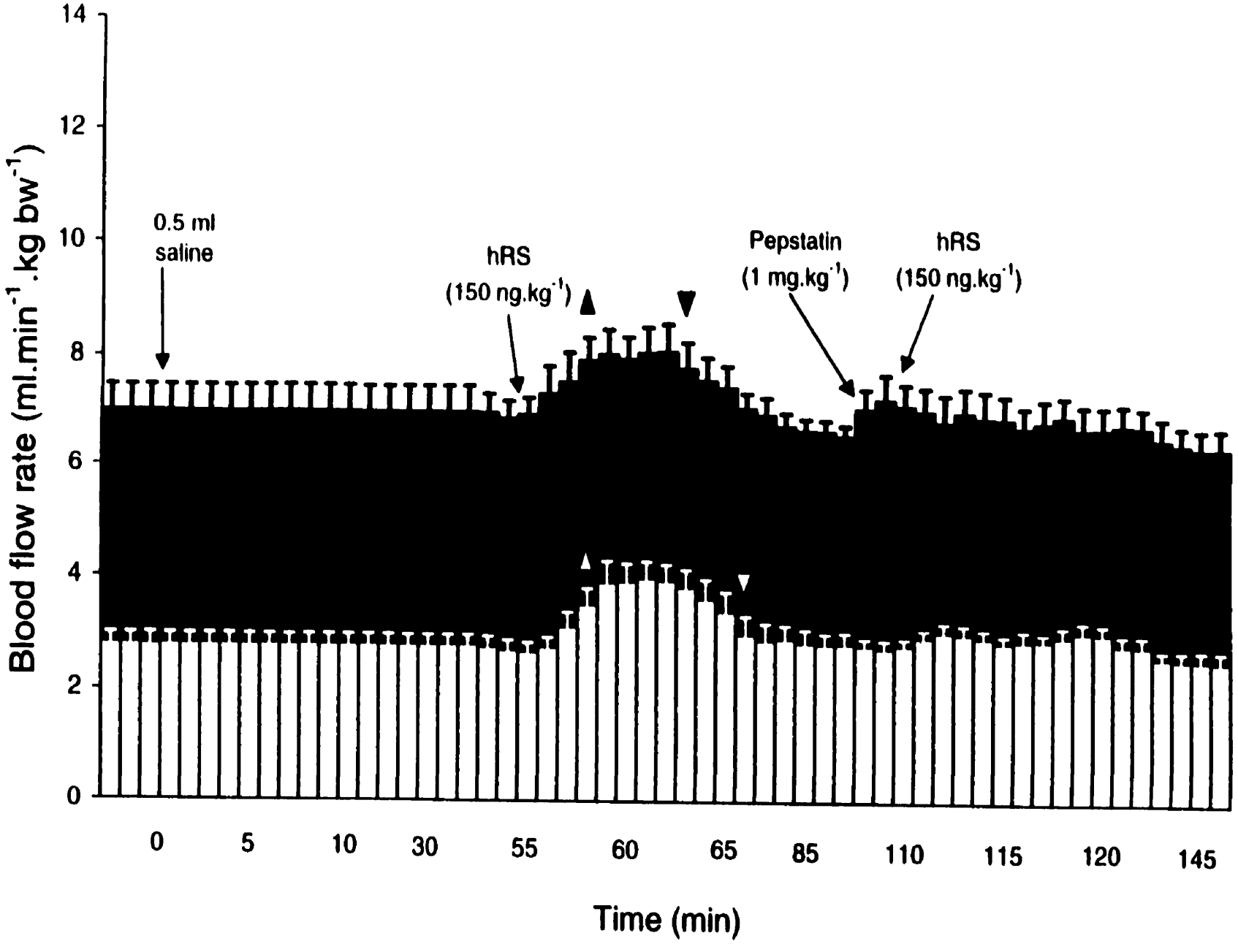

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
hRS (150 ng.kg-1)
Pepstatin (1 mg.kg-1)
hRS (150 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 7. Effect of the mammalian renin antagonist pepstatin A on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 2.5 mg. kg bw^{-1} of CS-EXT given before (time = 55) and after (time = 110) the i.v. injection of 1 mg, kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM; n = 5.

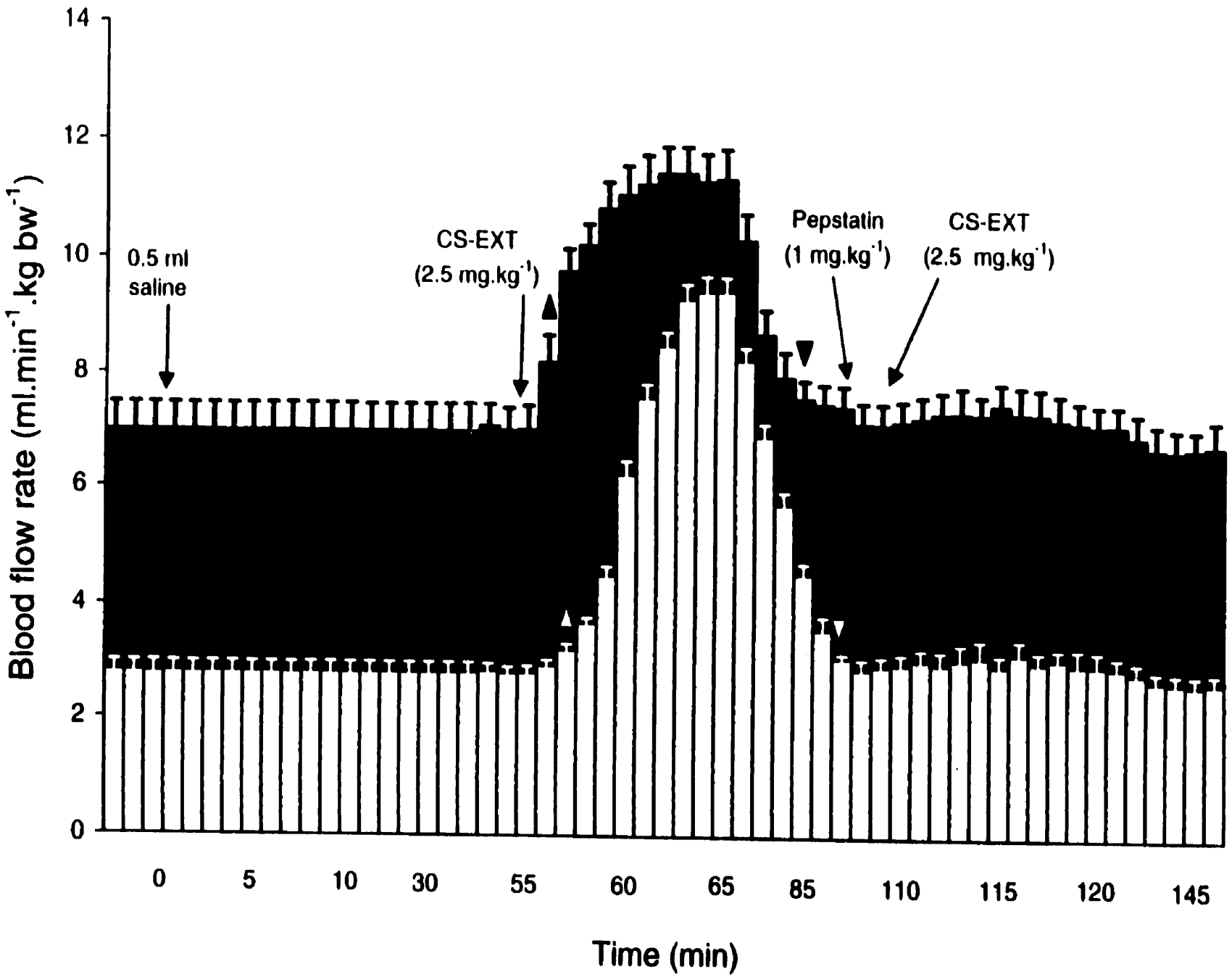

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
CS-EXT (2.5 mg.kg-1)
Pepstatin (1 mg.kg-1)
CS-EXT (2.5 mg.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 7 shows that an extract of 2.5 mg of CS injected i.v. was followed by rapid and significant increases in both CVBF and DABF. Peak flow rates were 9.5 ± 0.24 and 11.5 ±0.42 $ml.min^{-1}.kg\ bw^{-1}$ respectively within 10 and 7 minutes after the injection of CS-EXT. These significant increases in blood flow rates were completed blocked by an i.v. injection of 1 $mg.kg\ bw^{-1}$ of Pepstatin A. CVBF and DABF remained at preinjection rates. That implied that the renin-inhibitor had blocked the activity of renin or a renin-like material contained within the CS.

(D) Captopril and the blood flow responses to ANG I, ANG II, hRS or CS-EXT

Figure 8 illustrates a 266 % increase in CVBF resulting in a peak rate of 10.3 ± 0.22 $ml.min^{-1}.kg\ bw^{-1}$; ($P<0.05$) and a concomitant 84 % increase in DABF with a peak rate of 12.9 ± 0.39 $ml.min^{-1}.kg\ bw^{-1}$; ($P<0.05$) which occurred within approximately 9 minutes after an i.v. injection of 50 $ng.kg\ bw^{-1}$ of ANG I. An i.v. injection of 1 $mg.kg\ bw^{-1}$ of Captopril blocked completely the blood flow responses to a second i.v. injection of 50 $ng.kg\ bw^{-1}$ of ANG I (Figure 8). The experiment was repeated using ANG II instead of ANG I. Figure 9 shows that, after the first i.v. injection of 50 ng of ANG II, the CVBF and DABF rose rapidly and peaked at 10.6 ±0.16 (278% increase) and 12.3 ±.49 $ml.min^{-1}.kg\ bw^{-1}$ (75% increase), respectively within 8 and 10 minutes, then subsided. The i.v. injection of 1 $mg.kg\ bw^{-1}$ of Captopril did not block the flow responses to a second i.v. injection of 50 ng of ANG II because the angiotensin-converting enzyme (ACE) was bypassed (Figure 9). The onset of peak flow rates occurred with 7 (CVBF) and 10 (DABF) minutes after the injection of peptide; the peak flows were 10.5 ± 0.19 (275% increase) and 12.1 ± 0.44 $ml.min^{-1}.kg\ bw^{-1}$ (73% increase) for CVBF and DABF (Figure 9).

Figure 8. Effect of the mammalian ACE antagonist captopril on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 50 ng. kg bw^{-1} of [Asn^{1}, Val^{5}, Gly^{9}]-ANG I given before (time = 55) and after (time = 110) the i.v. injection of 1 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

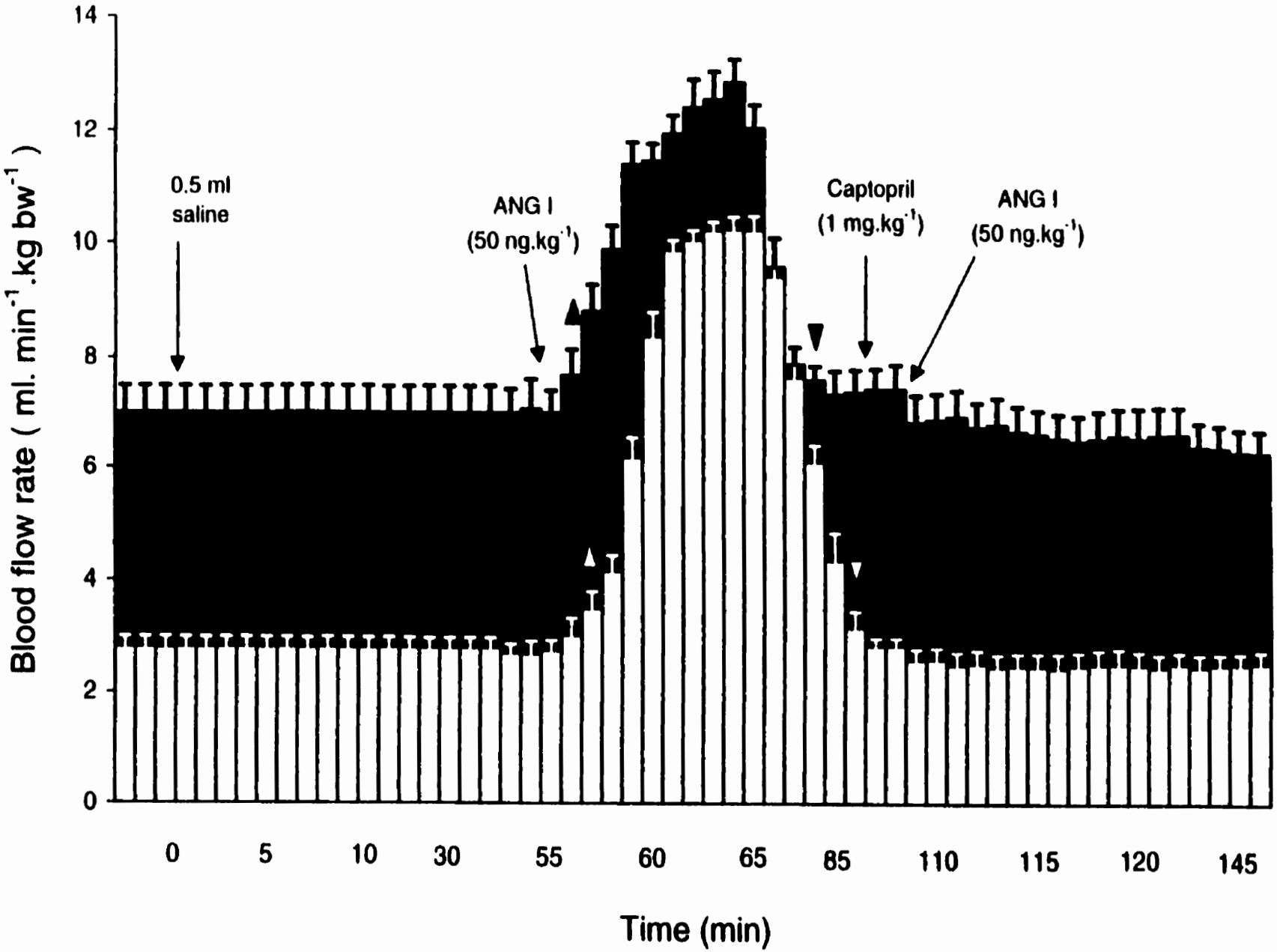

Blood flow rate (ml. min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml
saline
ANG I
(50 ng.kg-1)
Captopril
(1 mg.kg-1)
ANG I
(50 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 9. Effect of the mammalian ACE antagonist captopril on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II given before (time = 55) and after (time = 110) the i.v. injection of 1 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

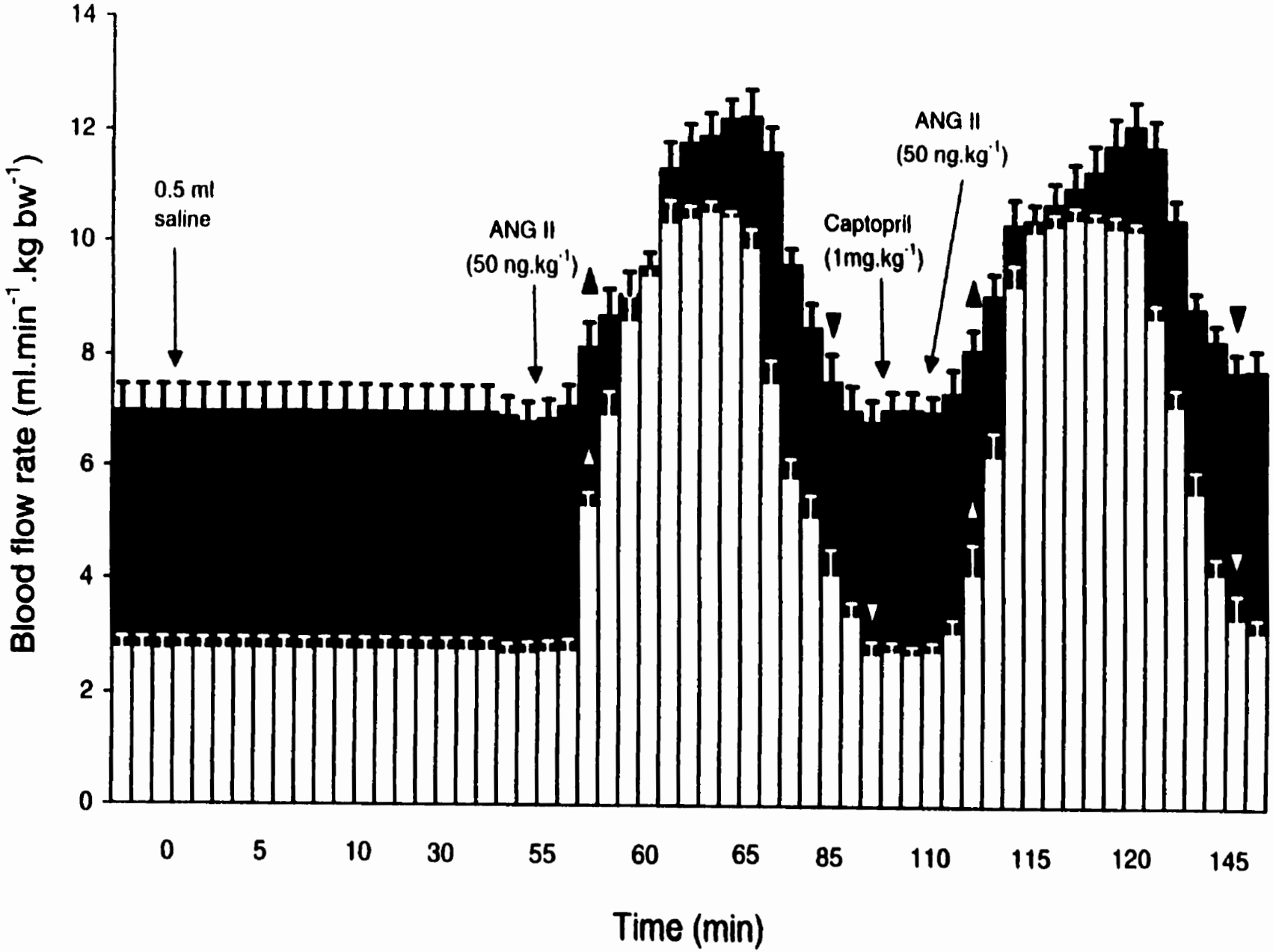

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
ANG II (50 ng.kg-1)
Captopril (1mg.kg-1)
ANG II (50 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 10. Effect of the mammalian ACE antagonist captopril on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 150 ng. kg bw^{-1} of hRS given before (time = 55) and after (time = 110) the i.v. injection of 1 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

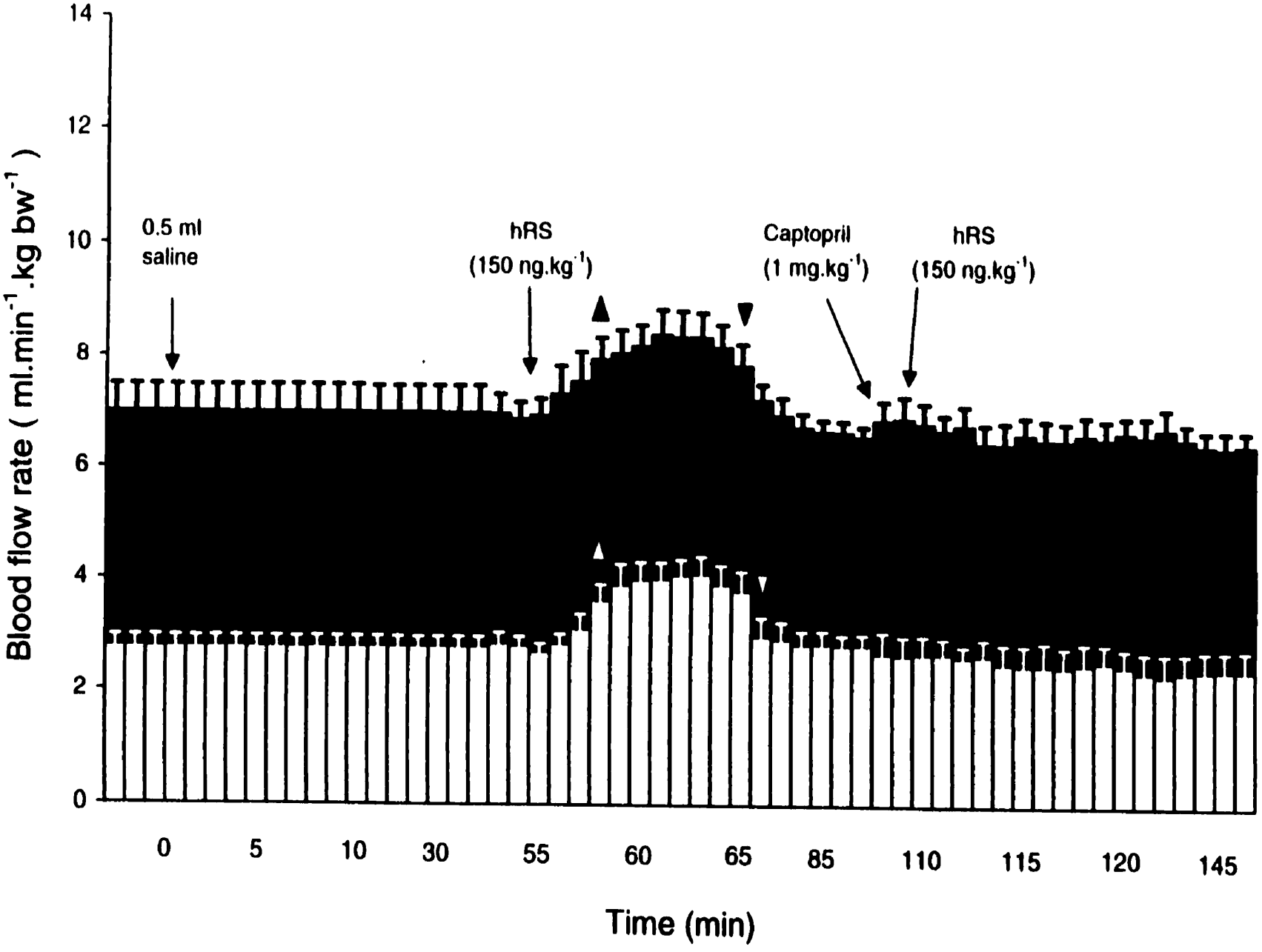

14
12
10
8
6
4
2
0
Blood flow rate (ml.min-1.kg bw-1)
0.5 ml
saline
hRS
(150 ng.kg-1)
Captopril
(1 mg.kg-1)
hRS
(150 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 11. Effect of the mammalian angiotensin-converting enzyme (ACE) antagonist captopril on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 2.5 mg. kg bw^{-1} of CS-EXT given before (time = 55) and after (time = 110) the i.v. injection of 1 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

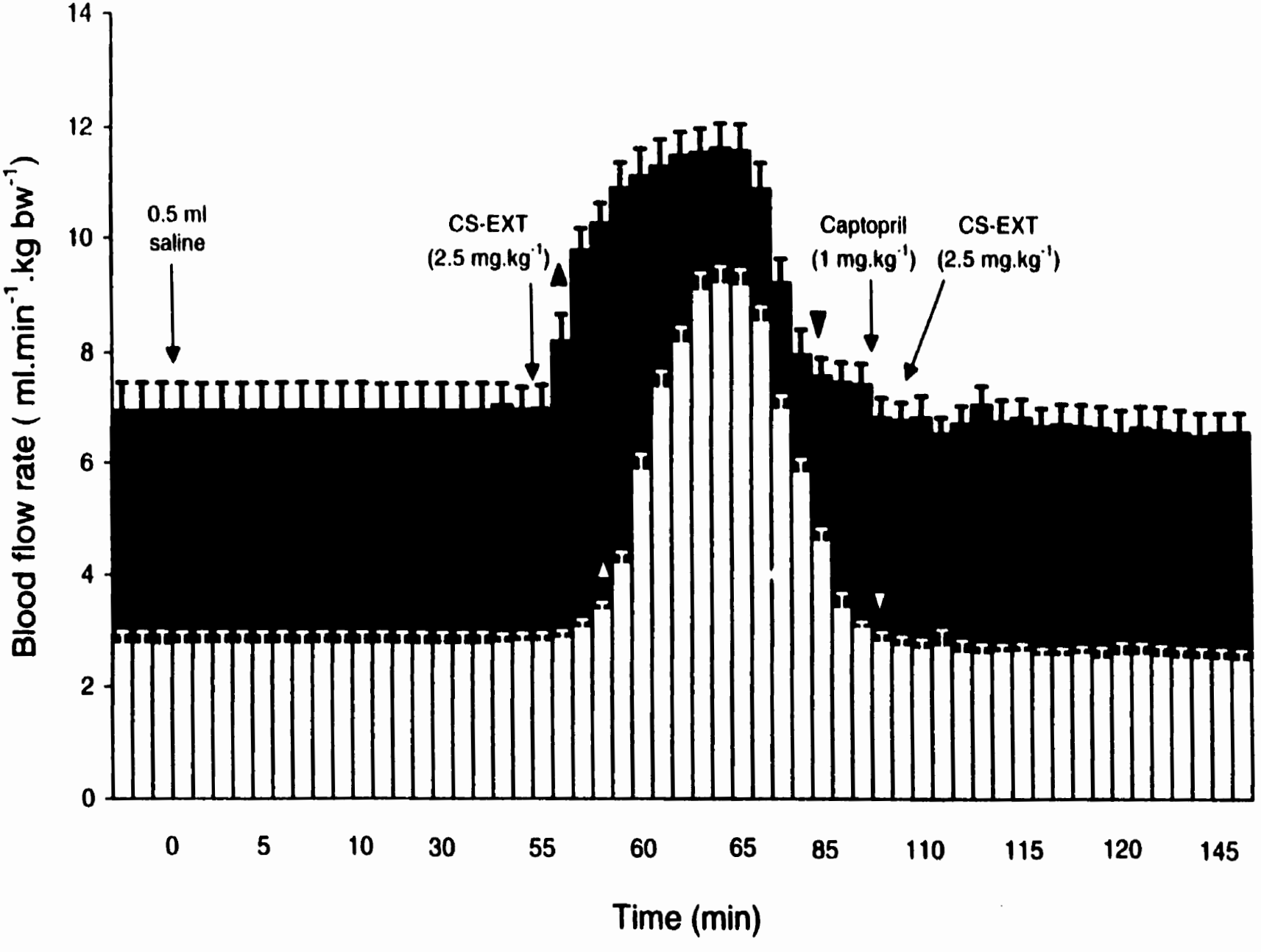

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
CS-EXT (2.5 mg.kg-1)
Captopril (1 mg.kg-1)
CS-EXT (2.5 mg.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 10 shows that there was a modest but significant increase ($P<0.05$)in both CVBF (47%) and DABF (20%) following an i.v. injection of 150 ng.kg bw^{-1} of hRS (human renin substrate), as observed in the Pepstatin A experiment (see Figure 6). BFR in the CV peaked at 4.1 ±0.31 $ml.min^{-1}.kg\ bw^{-1}$ and in the DA at 8.4 ±0.46 $ml.min^{-1}.kg\ bw^{-1}$ 8 and 6 min after the injection of hRS. However if the eel was given an i.v. injection of 1 mg.kg bw^{-1} of Captopril before the second dose of hRS, the flow responses were abolished (Figure 10). In the subsequent , but related experiment, an eel was given an i.v. injection of an extract of 2.5 mg of fresh CS (Figure 11). Peak flow rates in the CV and DA were 9.2 ± 0.26 and 11.6 ± 0.44 $ml.min^{-1}.kg\ bw^{-1}$ amounting to increases of 230% and 66% respectively. These peaks occurred about 9 min after injection of the CS-EXT (Figure 11) whereas CVBF and DABF remained above baseline rates for about 32 and 24 min. An i.v. injection of 1 mg.kg bw^{-1} of Captopril was followed by a second injection of an extract of 2.5 mg of fresh CS extract, but the flow response was completely abolished by the drug (Figure 11).

(E) [Sar^1, Ile^8]-ANG II (Sarile) and the blood flow responses to ANG II or CS-EXT

Figure 12 shows that both CVBF and DABF increased significantly after an i.v. injection of 50 ng.kg bw^{-1} of ANG II reaching peak flows of 10.3±0.21 and 11.8 ±.49 ml.min-1.kg bw^{-1} at 7 and 10 minutes after injection of the peptide. CVBF and DABF remained elevated for approximately 33 and 23 minutes respectively, before returning to pre-injection baseline rates (Figure 12). Eels were then injected i.v. with 50 μg.kg bw^{-1} of Sarile and, 10 minutes later, by a further 50 ng.kg bw^{-1} of ANG II. There was no

Figure 12. **Effect of the mammalian ANG II receptor antagonist Sarile on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II given before (time = 55) and after (time = 110) the i.v. injection of 50 ug. kg bw^{-1} of antagonist (time = 100). CVBF: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). DABF: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test.** Values are mean + SEM. n = 5.

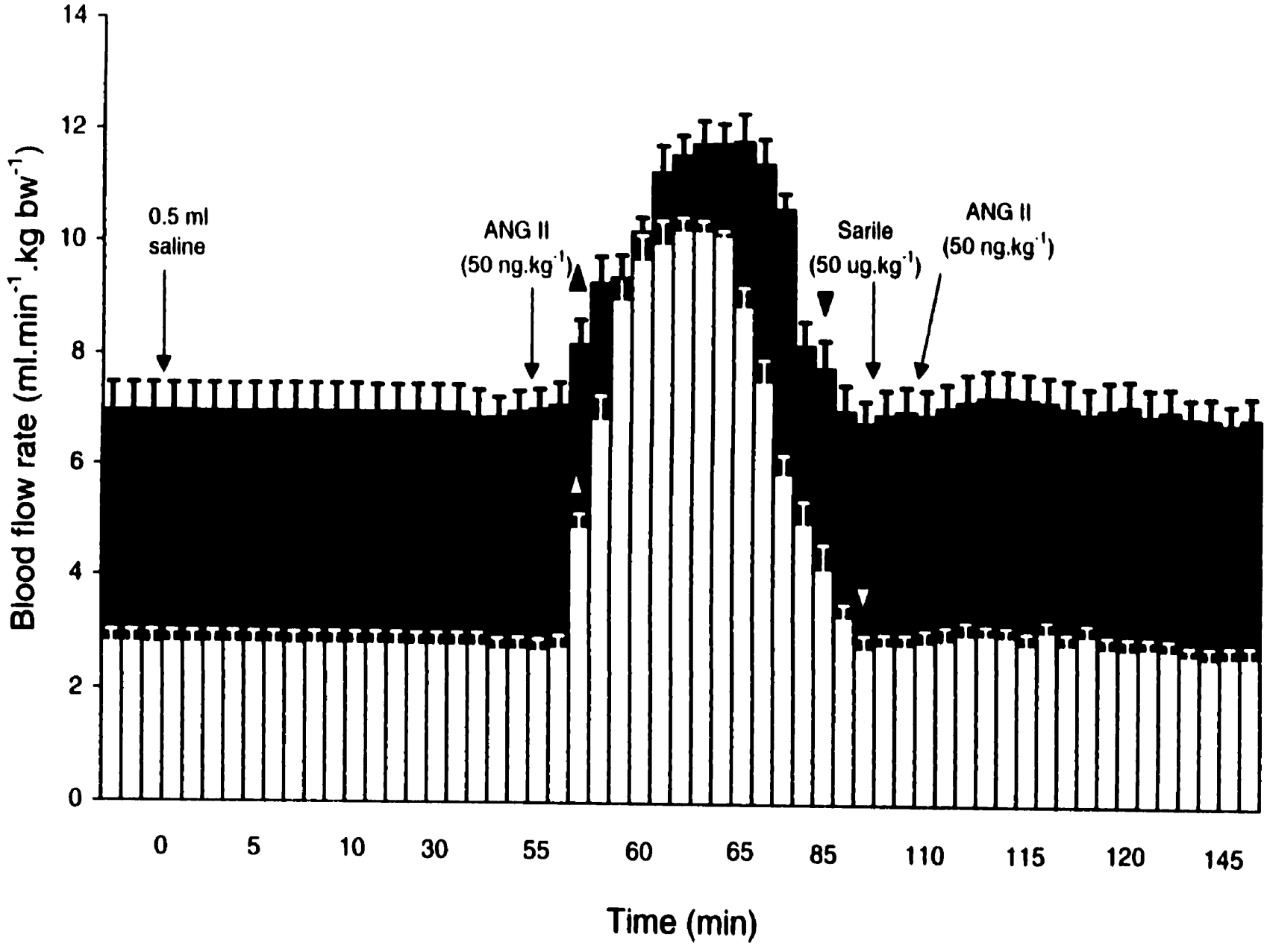
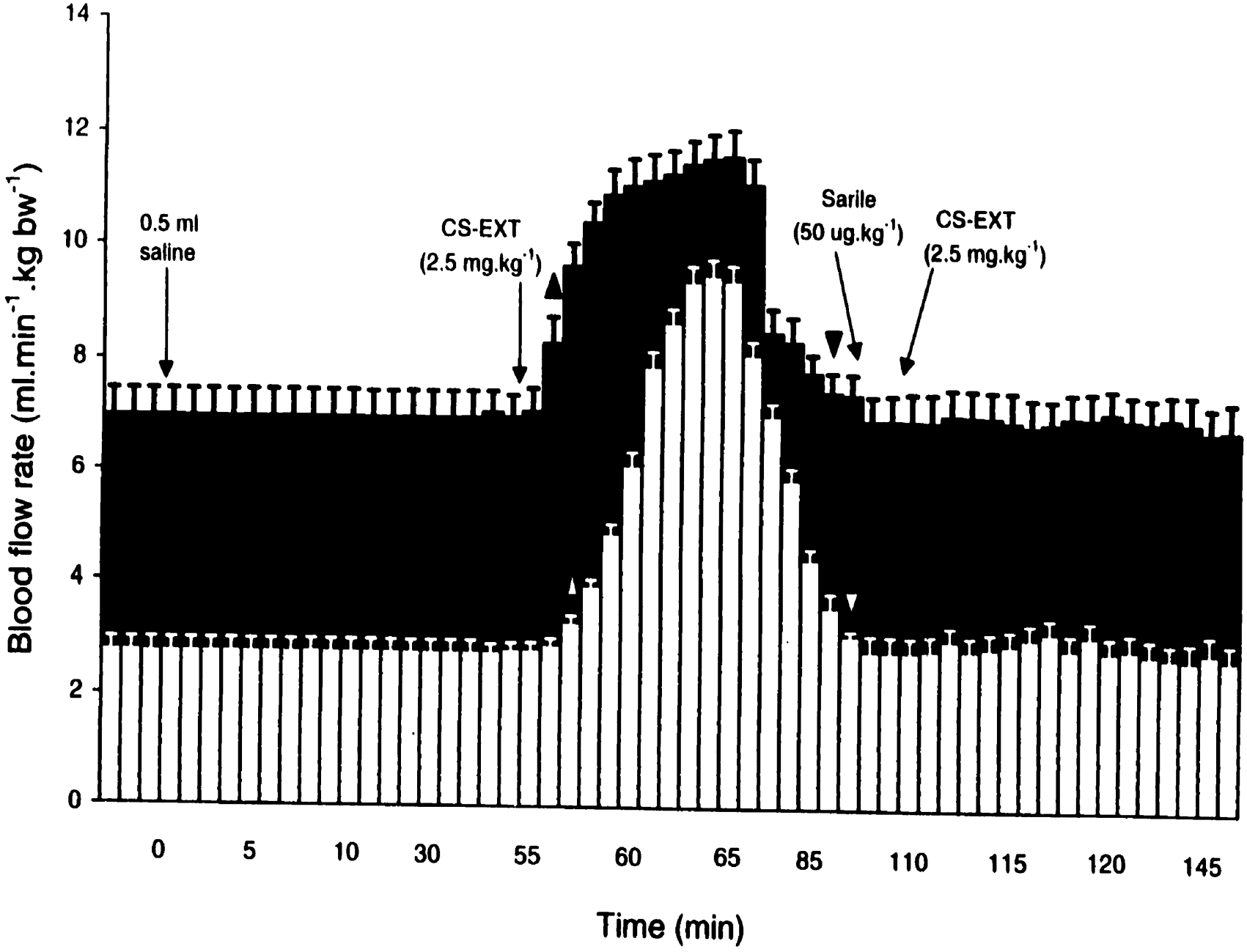

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
ANG II (50 ng.kg-1)
Sarile (50 ug.kg-1)
ANG II (50 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 13. Effect of the mammalian ANG II receptor antagonist [Sar^1, Ile^8]-ANG II (Sarile) on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 2.5 mg. kg bw^{-1} of CS-EXT given before (time = 55) and after (time = 110) the i.v. injection of 50 ug. Kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.


Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
CS-EXT (2.5 mg.kg-1)
Sarile (50 ug.kg-1)
CS-EXT (2.5 mg.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

subsequent increase in either CVBF or DABF which showed that Sarile had blocked completely the flow responses to ANG II (Figure 12).

Figure 13 illustrates an experiment in which freshwater eels are first injected i.v. with an extract of 2.5 mg of fresh CS. After about 10 min, CVBF had increased to peak rates of 9.5±0.26 (241% increase) and DABF to 11.6 ±.47 $ml.min^{-1}.kg\ bw^{-1}$ (66% increase). CVBF and DABF remained elevated for about 33 min and 29 min before subsiding to the pre-injection rates (Figure 13). There followed an i.v. injection of 50 $\mu g.kg\ bw^{-1}$ of Sarile which blocked completely the subsequent flow response to a second injection of extract of 2.5 mg of fresh CS (Figure 13).

(F) Losartan and the blood flow responses to ANG II or CS-EXT

An injection of 50 $ng.kg\ bw^{-1}$ of ANG II was again followed by increased flows in the CV and DA. The peak blood flow rate in the CV was 10.1 ± 0.08 $ml.min^{-1}.kg\ bw^{-1}$ and in the DA 12.4 ± 0.49 $ml.min^{-1}.kg\ bw^{-1}$ amounting to increases of 263% and 77% respectively compared with pre-injection flows (Figure 14). CVBF remained above baseline for 28 min; DABF for 23 min. The subsequent injection of the mammalian AT_1 blocker losartan (5 $mg.kg\ bw^{-1}$ i.v.) only partially inhibited the flow responses to a second injection of 50 $ng.kg\ bw^{-1}$ of ANG II. The peak CVBF was reduced to 5.9 ± 0.29 $ml.min^{-1}.kg\ bw^{-1}$ (112% increase above baseline) and the DABF to 9.0 ± 0.18 $ml.min^{-1}.kg\ bw^{-1}$ (29% above baseline). Here the peak response in the CV took 8 min to develop; in the DA , 10 min to develop. There was a difference in the duration of the increased flow rates. CVBF and DABF remained elevated for 27 min and 16 min respectively before returning to

Figure 14. Effect of the mammalian AT_1 receptor antagonist losartan on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II given before (time = 55) and after (time = 110) the i.v. injection of 5 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

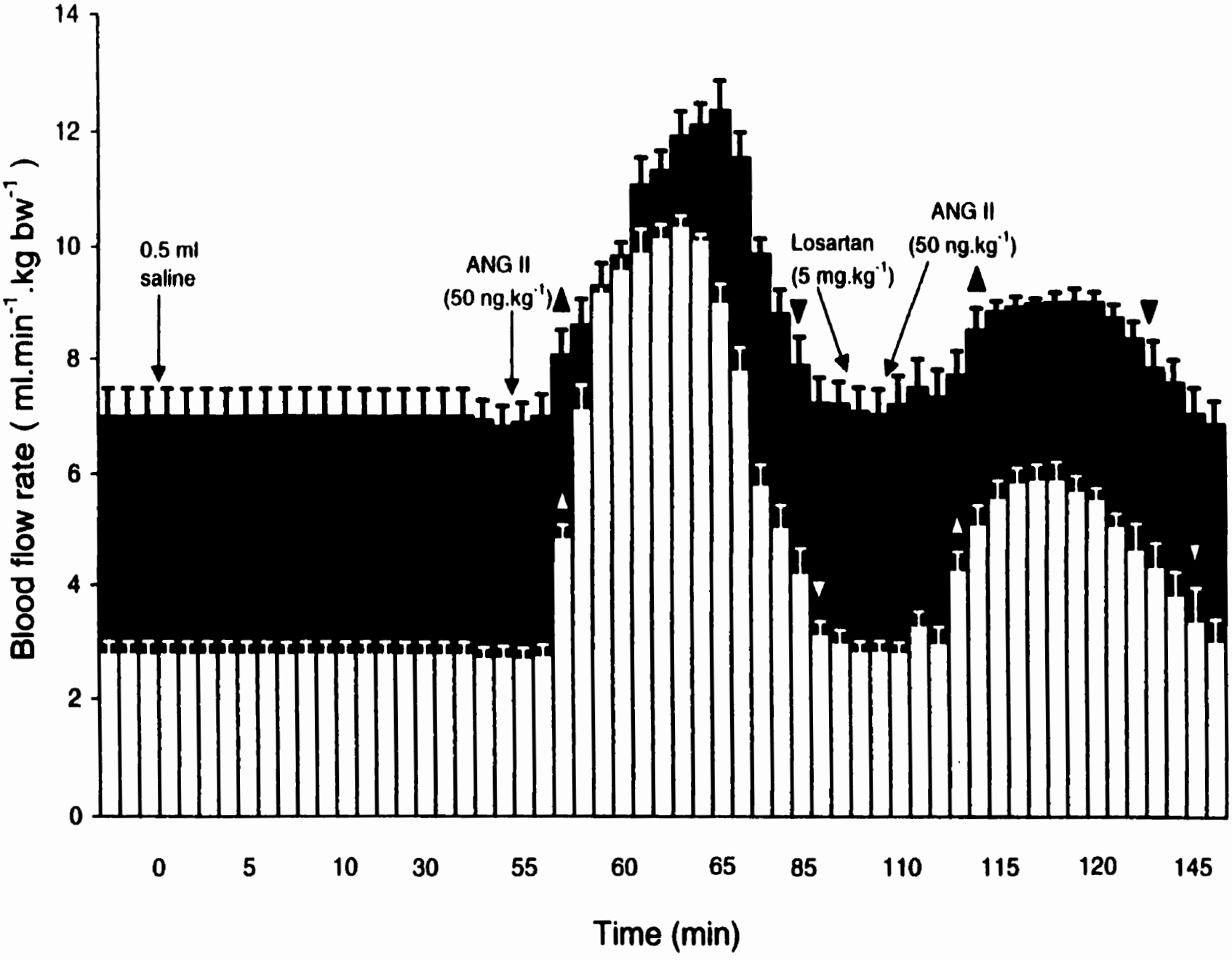
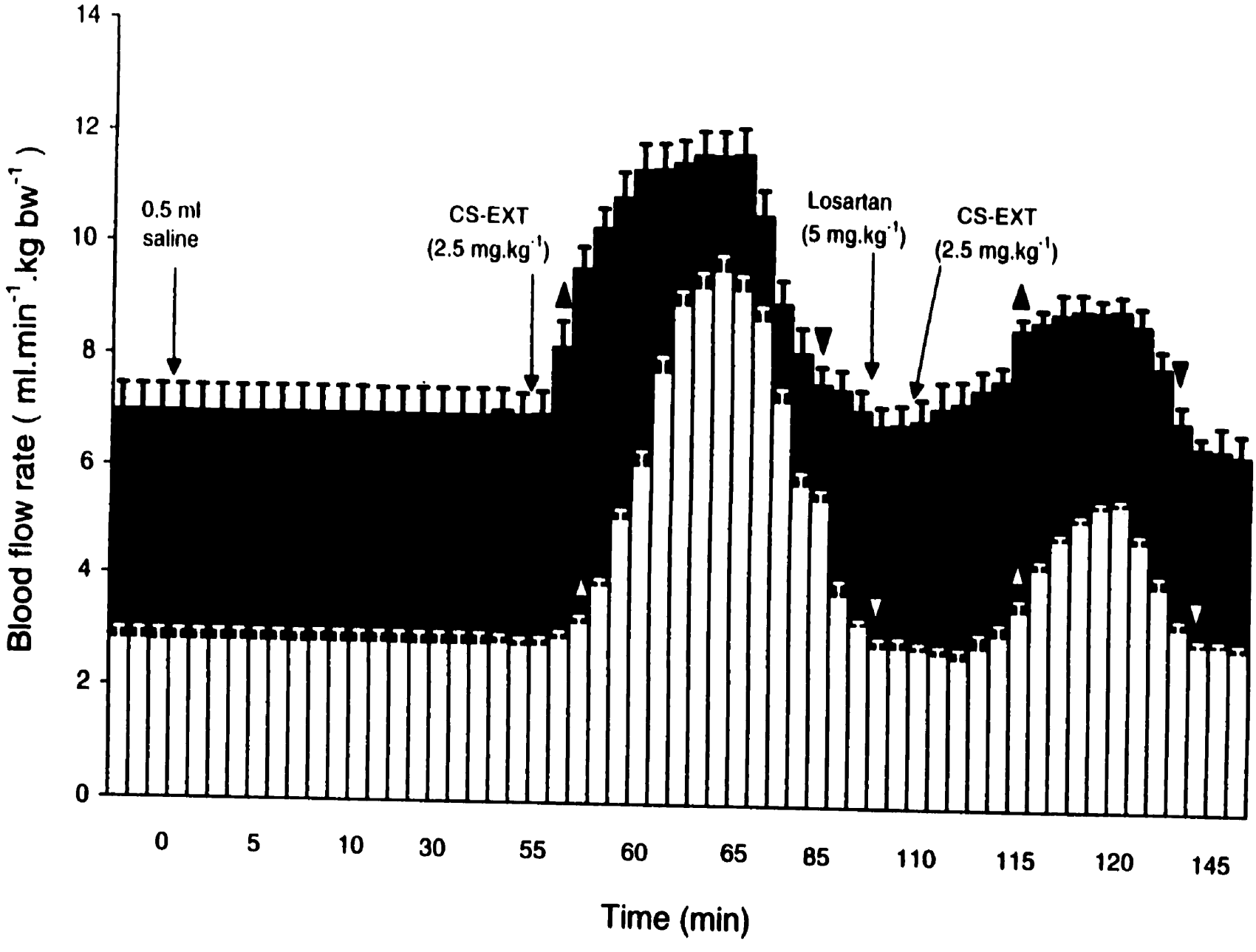

Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml
saline
ANG II
(50 ng.kg-1)
Losartan
(5 mg.kg-1)
ANG II
(50 ng.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

Figure 15. Effect of the mammalian AT_1 receptor antagonist losartan on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 2.5 mg. kg bw^{-1} of CS-EXT given before (time = 55) and after (time = 110) the i.v. injection of 5 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.


Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
CS-EXT (2.5 mg.kg-1)
Losartan (5 mg.kg-1)
CS-EXT (2.5 mg.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

baseline pre-injection rates. These experiments showed that losartan was not a completely effective AT_1 inhibitor in freshwater eels (Figure 14).

Figure 15 shows that the injection of an extract of 2.5 mg of CS i.v. was followed by a 244% increase in CVBF (9.6 ± 0.26 $ml.min^{-1}.kg\ bw^{-1}$) and a 67% increase in DABF (11.7 ± 0.47 $ml.min^{-1}.kg\ bw^{-1}$). CVBF and DABF remained elevated for 38 min and 24 min respectively before falling to the pre-injection flow rates. An injection of Losartan (5 $mg.kg\ bw^{-1}$ i.v.) reduced significantly the response to the second injection of extract of 2.5 mg of CS. CVBF increased to only 5.5 ± 0.09 $ml.min^{-1}.kg\ bw^{-1}$ (97 % above baseline) and DABF increased to only 9.0 ± 0.25 $ml.min^{-1}.kg\ bw^{-1}$ (29% above baseline). This amounts to a 43% reduction in the flow response in the CV and a 23% reduction in flow response in the DA (Figure 15).

(G) PD 123319 and the blood flow responses to ANG II or CS-EXT

Figure 16 illustrates the expected increases in CVBF and DABF following the injection of 50 $ng.kg\ bw^{-1}$ i.v. of ANG II. CVBF increased by 267% to 10.3 ±0.21 $ml.min^{-1}.kg\ bw^{-1}$ and DABF by 74% to 12.2 ±0.37 $ml.min^{-1}.kg\ bw^{-1}$. BFR remained elevated in the CV for 33 min and in the DA for 23 min before returning to the pre-injection rates. Treatment with the mammalian AT_2 blocker PD 123319 (5 $mg.kg\ bw^{-1}$ i.v.) reduced the duration and intensity of the BF responses to a second i.v. injection of 50 $ng.kg\ bw^{-1}$ of ANG II. CVBF increased by only 7.2 ± 0.35 $ml.min^{-1}.kg$ bw-1 (159%) and DABF by only 10.3 ± 0.28 $ml.min^{-1}.kg\ bw^{-1}$ (47%). The flows remained elevated for 33 min and 13 min respectively (Figure 16).

Figure 16. Effect of the mammalian AT_2 receptor antagonist PD123319 on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II given before (time = 55) and after (time = 110) the i.v. injection of 5 mg.kg bw^{-1} antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.

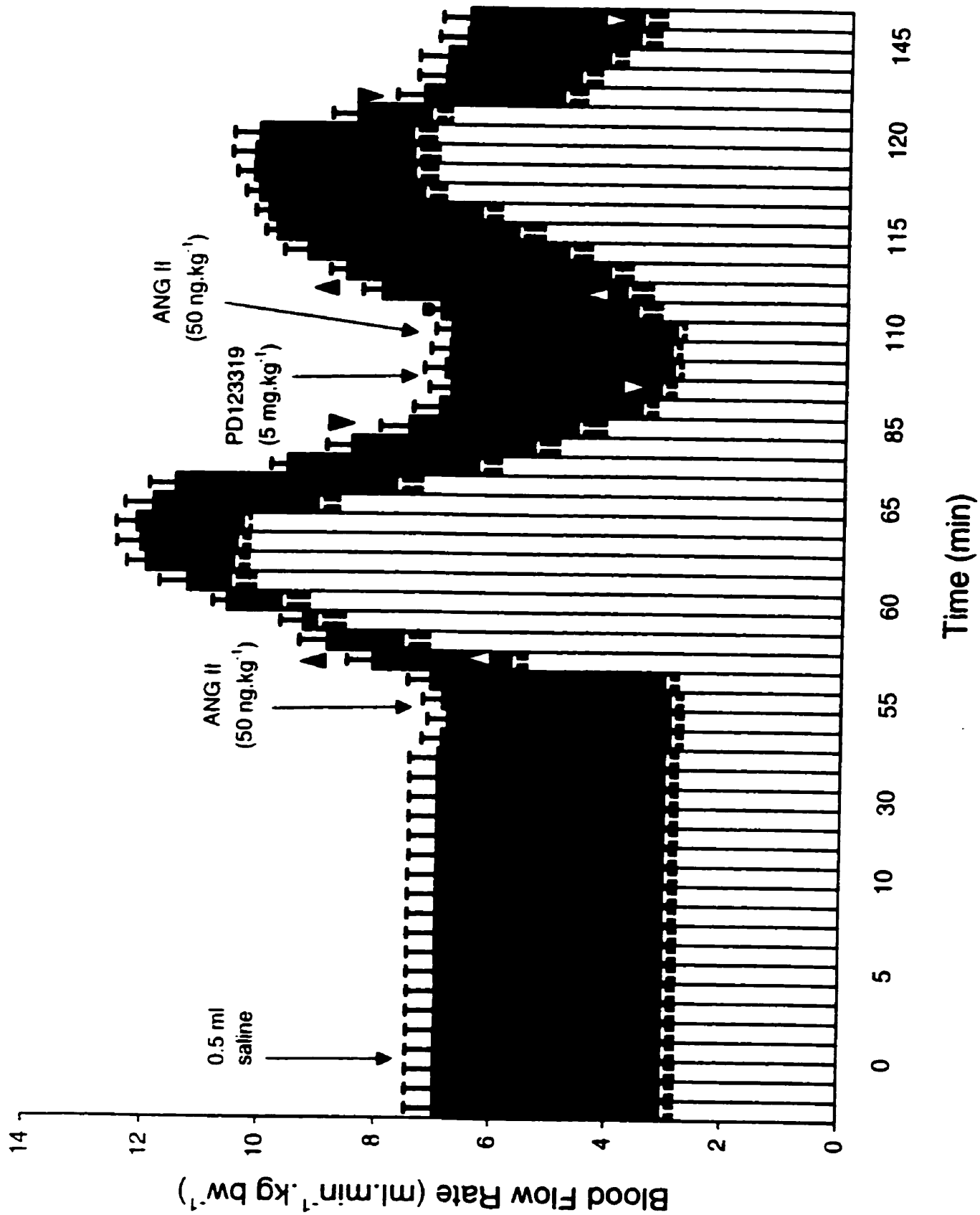
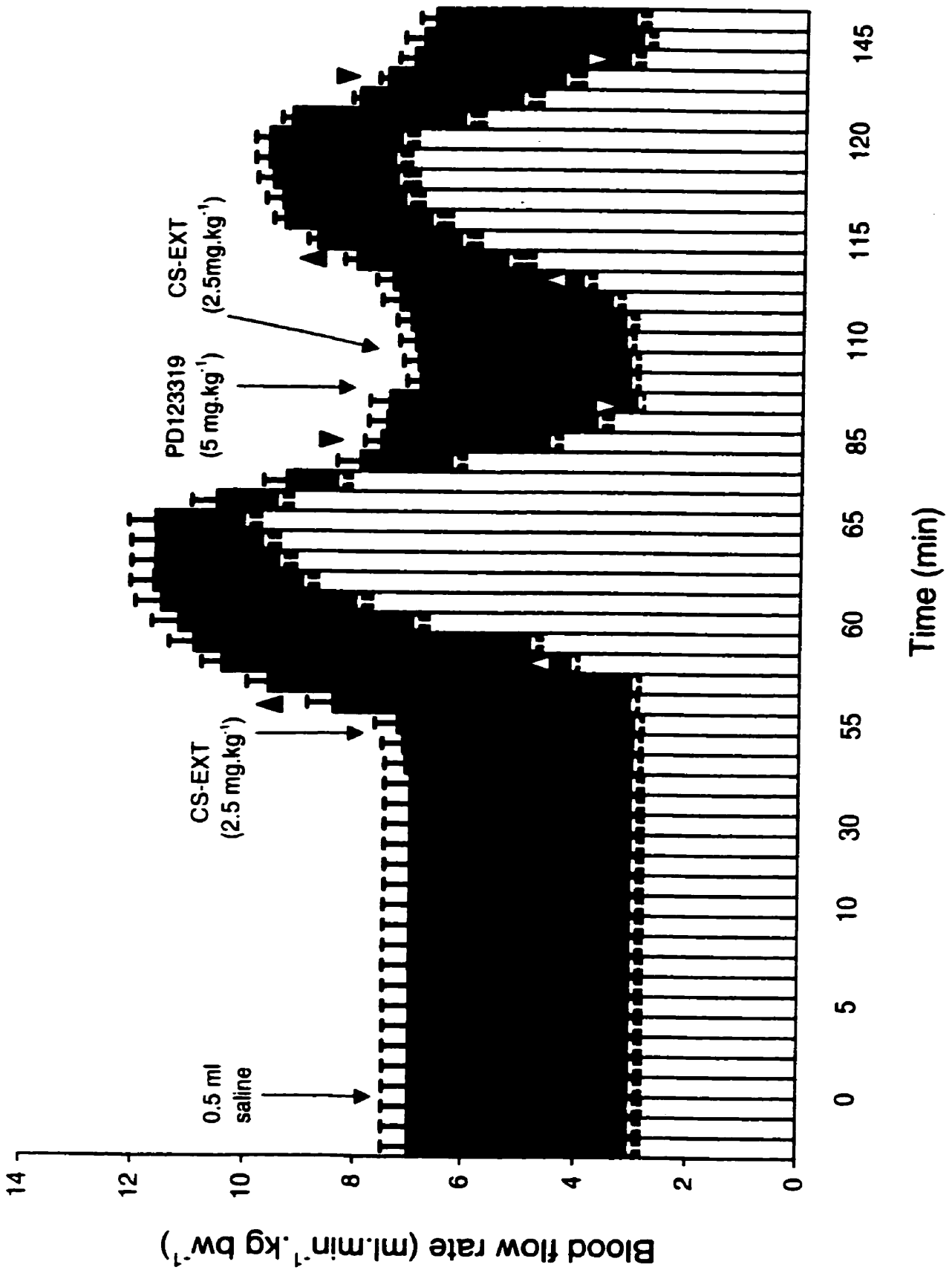

Blood Flow Rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)
0.5 ml saline
ANG II (50 ng.kg-1)
PD123319 (5 mg.kg-1)
ANG II (50 ng.kg-1)

Figure 17. Effect of the mammalian AT_2 receptor antagonist PD123319 on the temporal changes in CVBF (▭) and DABF (▬) in freshwater North American eels, *A. rostrata*, after 2 i.v. injections of 2.5 mg. kg bw^{-1} of CS-EXT given before (time = 55) and after (time = 110) the i.v. injection of 5 mg. kg bw^{-1} of antagonist (time = 100). **CVBF**: (△ to ▽) $P < 0.05$ compared with the preinjection values (time = 1 to 50). **DABF**: (▲ to ▼) $P < 0.05$ compared with the preinjection values (time = 1 to 50); ANOVA, Duncan's Multiple Range Test. Values are mean + SEM. n = 5.


Blood flow rate (ml.min-1.kg bw-1)
14
12
10
8
6
4
2
0
0.5 ml saline
CS-EXT (2.5 mg.kg-1)
PD123319 (5 mg.kg-1)
CS-EXT (2.5mg.kg-1)
0
5
10
30
55
60
65
85
110
115
120
145
Time (min)

The i.v. injection of an extract of 2.5 mg of CS was followed by significant and prolonged increases in CVBF and DABF. At its peak, CVBF had increased by 250% to 9.8 ± 0.24 ml.min^{-1}.kg bw^{-1} and the DABF had increased by 66% to 11.6 ±0.43 ml.min^{-1}.kg bw^{-1}. The BFR in the CV remained elevated for 32 min whereas the BFR in the DA remained elevated for 24 min before returning to the pre-injection level (Figure 17).

An i.v. injection of 5 mg. kg bw^{-1} of the mammalian AT_2 blocker PD 123319 reduced significantly the responses to a second i.v. injection of an extract of 2.5 mg of fresh CS tissue. CVBF reached a peak rate of 7.1 ±0.25 ml.min^{-1}.kg bw^{-1} (154% increase) and DABF a peak rate of 9.7 ± 0.24 ml.min^{-1}.kg bw^{-1} (an increase of 39%). Flow rates in the CV remained elevated for 22 and 16 minutes respectively before returning to the pre-injection rates (Figure 17). Overall, PD 123319 only partially blocked the flow responses to components of the RAS.

DISCUSSION

The effects of ANG I and ANG II on blood flow

The RAS has been thoroughly examined in over 100 different species of teleost fishes (Nishimura 1985, Sokabe & Ogawa 1974). ANG I, a decapeptide, has been isolated and sequenced following extraction from the plasma of chum salmon, O. *keta* (Takemoto et al. 1983), goosefish, *L. litulon* (Hayashi et al. 1978), Japanese eel, *A. japonica* (Hasegawa et al. 1983) and North American eel, *A. rostrata* (Khosla et al. 1985). ANG I is a biosynthetic intermediate of the RAS and has no known physiological function (Hirano & Hasegawa, 1984). Angiotensin converting enzyme (ACE) which, in fishes is located primarily in the gills and kidney (Nishimura 1985), rapidly converts ANG I to ANG II by cleaving a dipeptide from the C-terminal end of the molecule. Thus formed, ANG II has powerful direct and indirect effects on cardiovascular function in teleosts as well as higher vertebrates (Olson et al. 1989, Polanc et al. 1990).

ANG II is the principal effector peptide of the RAS in teleost fishes, wherein it exerts a powerful vasopressor effect (Nishimura & Sawyer 1976, Churchill *et al.* 1979, Zuker & Nishimura 1981, Carroll & Opdyke 1982, Perrott & Balment 1990). ANG II is also known to increase cardiac output through its chronotropic and inotropic effects which lead to an increase in arterial blood flow and peripheral vasoconstriction in freshwater North American eels (Oudit & Butler 1995). ANG II also stimulates thirst (Carrick & Balment 1983, Balment & Carrick 1985) during adaptation of euryhaline fishes to seawater. ANG II is converted to ANG III by the cleavage of the N-terminal amino acid asparagine (Asn)

and its conversion to a heptapeptide. The conversion is achieved by the action of peptidases known to be abundant in the kidneys and gills (Olson 1992).

In the present study, approximate physiological doses of the peptides, ranging from 5 to 50 ng. kg bw^{-1} were used. Due to the rapid, peripheral conversion of ANG I to ANG II (Nishimura 1985), the effects of these peptides on flow were nearly identical. For instance, a 5 ng. kg bw^{-1} dose of either [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II caused a significant increase in venous blood flow. The increase in the rate of arterial blood flow became significant at the 10 ng. kg bw^{-1} dose (Figure 2). The responses were higher at the 20 ng. kg bw^{-1} dose and the highest at the 50 ng. kg bw^{-1} dose. Because the range of doses did not exceed 50 ng. kg bw^{-1}, a true curvilinear dose-response relationship could not be established. However, I experiments show a strong positive correlation between the dose of peptide delivered and the resultant blood flow response. These results are consistent with earlier studies that showed that i.v. injection of [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II were followed by significant pressor responses in North American eels (Nishimura & Sawyer 1976, Nishimura *et al.* 1978). Oudit and Butler (1995) were the first investigators to demonstrate that the increase in arterial blood flow was the main contributor to the ANG II mediated pressor response in freshwater North American eels. They found that [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II has stimulatory effects on heart rate and stroke volume which increases arterial blood flow (cardiac output), and leads subsequently to higher arterial blood pressure. Although blood pressure was not measured in our experiments, the increase in arterial blood flow, following i.v. injection of [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II may have also caused an increase in arterial blood pressure.

Figure 1 shows the dorsal aortic and caudal venous calibration curves for three freshwater eels. All of the regression lines have nearly identical slopes showing the highly significant positive linear correlation between the blood flow rate and corresponding Doppler shifts. Dorsal aortic and caudal venous blood flows were determined by interpolation from these curves. Velocity profiles across the dorsal aorta and caudal veins (2.8 mm probe) were determined by making small serial increments in the range output which allowed us to determine the optimal distance at which blood velocity could be measured. As in earlier experiments (Butler & Oudit 1995, Oudit & Butler 1995), both the dorsal aortic and caudal venous flow profiles were parabolic, indicating that flow was laminar in both vessels. During the experiments, the range was adjusted so that the peak mean blood flow was always measured. Similar velocity profiles have been observed in the pulmonary arteries of green turtles, *Chelonia mydas* (West 1989) and in the ventral and dorsal aortae of freshwater eels (Butler & Oudit 1995). In summary I was fully confident that Doppler flow probe measurements in my experiments gave valid estimates of blood flow velocities.

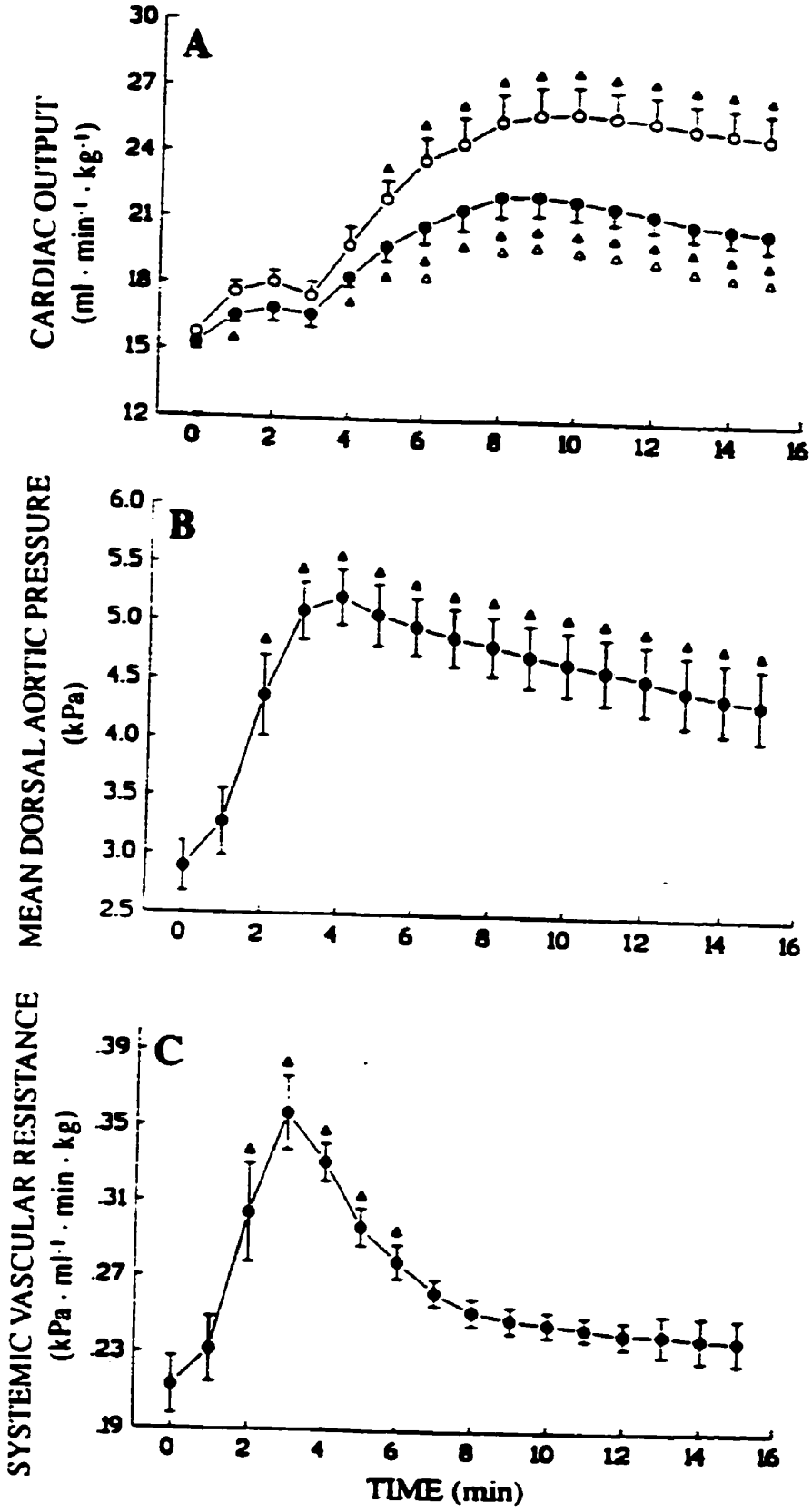
The Relationship between cardiac output, dorsal aortic blood pressure and dorsal aortic and caudal venous blood flows

Butler and Oudit (1995) have studied the relationship between cardiac output (CO), dorsal aortic blood flow (DABF) and dorsal aortic blood pressure (P_{DA}) in intact freshwater eels before and after removal of the corpuscles of Stannius (CS), which they presumed to contain renin or a renin-like substance. Three weeks after removal of the CS, there followed a 100% increase in estimated branchial shunting wherein the blood

bypassed the gills and flowed directly to the dorsal aorta, and also a 25% decrease in cardiac output. DABF decreased by about 45% one week after CSX and remained there for the duration of the experiment. P_{DA} fell by 15% within four days after the glands were removed and gradually climbed back into the normal range by the 12th day. These findings implied that the CS release a renin or a renin-like substance which led to further experiments by Oudit and Butler (1995). Their hypothesis was that the end-product of the corpuscular RAS (CRAS) is ANG II and that this peptide regulates cardiovascular function in freshwater eels.

CO, DABF and P_{DA} were measured simultaneously in conscious freshwater eels following i.v. injections of a series of graded doses of 25, 50, 100 and 150 ng.kg bw^{-1} of [Asn^{1}, Val ^{5}] Angiotensin II (ANG II) (Figure 18). CO increased gradually at all doses but the increase was disproportionately large at the lower doses (Figure 18A). For example, 25 ng.kg bw^{-1} increased the CO by 4 ml.min^{-1}.kg bw^{-1} whereas a six-fold increase in dose (150 ng.kg bw^{-1}) increased the CO by only 8.2 ml.min^{-1}.kg bw^{-1}. Muscarinic receptor blockade with atropine potentiated these responses to graded doses of ANG II implying that cholinergic innervation of the eel heart initially downregulates CO via a baroreceptor reflex. ANG II also produced a dose-related increase in P_{DA} (Oudit & Butler 1995) which has been observed in other studies on freshwater eels (Nishimura *et al.* 1978, Hirano & Hasegawa 1984). At a dose of 100 ng.kg bw^{-1} of ANG II, CO increased slightly within the first min but leveled off for a further two min suggesting a reflex inhibition of cardiac frequency. However, there was

Figure 18. Temporal changes in **CO** (A) [control (n = 8; ●) and atropine treated (n = 6; O)], mean $\mathbf{P_{DA}}$ (**B**; n = 6), and $\mathbf{R_{sys}}$ (**C**; n = 6) in freshwater North American eels *Anguilla rostrata*, after intravenous injection of 100 ng/kg of [Asn^1, Val^5]-ANG II. Dorsal aortic blood flow followed the same trend as the changes in **CO**. ▲ $P < 0.01$ compared with the preinjection value (time = 0); Δ $P < 0.05$ compared with atropine treated. Values are mean ± SE. (Adapted with permission from Oudit and Butler, 1995)


A
CARDIAC OUTPUT
(ml · min⁻¹ · kg⁻¹)
30
27
24
21
18
15
12
0
2
4
6
8
10
12
14
16
B
MEAN DORSAL AORTIC PRESSURE
(kPa)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
C
SYSTEMIC VASCULAR RESISTANCE
(kPa · ml⁻¹ · min · kg)
.39
.35
.31
.27
.23
.19
TIME (min)

a rebound, as CO climbed rapidly reaching a peak within 9 min and remaining elevated for 15 min when the experiment ended (Figure 18B). In contrast the P_{DA} increased rapidly from the outset, peaked at 4 min and drifted downward slowly. By 15 min it had fallen to about 50% of the peak P_{DA}. Peak systemic vascular resistance coincided with the peak P_{DA} and fell rapidly thereafter so that it was only slightly above baseline for the last half of the 15 min observation period (Figure 18C). In summary, Oudit and Butler (1995) have shown that the increased CO due to increased stroke volume and cardiac frequency are the driving force for the increased P_{DA} which follow elevated circulating levels of [Asn^1, Val^5]-ANG II. Nevertheless, these conclusions have been drawn without measurements of dorsal aortic blood flows or returning blood flows through the large caudal vein. If caudal venous flow increased substantially, then according to the Frank Starling Law, the increased filling pressure of the eel atrium (end diastolic volume) would lead to yet a further increase in cardiac output, superimposed on the direct action of ANG II on heart rate and stroke volume. Thus I have expanded on the work of Butler and Oudit (1995) and Oudit and Butler (1995) by measuring the dorsal aortic blood flow and caudal venous blood flow in freshwater eels following the i.v. injection of a series of doses 5, 10, 20 and 50 ng.kg bw^{-1} of [Asn^1, Val^5, Gly^9]-ANG I, [Asn^1, Val^5]-ANG II and [Val^4]-ANG III (Figure 2). The amount of hormone, as low as 5 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II, required to elicit the observed cardiovascular responses, are some of the lowest reported to date. Therefore, the measurement of blood flow in response to peptides, may be useful as a sensitive bioassay for testing elements of the RAS such as ANG I, II and III or other vasoactive peptides such as 8-arginine vasotocin.

The effects of ANG III on blood flow

Figure 2 shows that DABF increased significantly following injections of 10, 20 and 50 ng of [Asn^1, Val^5, Gly^9]-ANG I and that all four doses increased CVBF. This showed that peripheral conversion of [Asn^1, Val^5, Gly^9]-ANG I to [Asn^1, Val^5]-ANG II was rapid and that each dose of this biologically inactive intermediate was as effective as an equal dose of exogenous ANG II (figure 2). Again, with [Asn^1, Val^5]-ANG II a 5 ng.kg bw^{-1} dose did not significantly increase DABF. These flow responses to doses of [Asn^1, Val^5]-ANG II as low as 5 ng.kg bw^{-1} are well within the physiological range and are the lowest yet reported to give a cardiovascular response to ANG I or II, in any species of fish. In mammals, ANG III possess only 40% of the pressor activity of ANG II (Dzau *et al.* 1988, Hall *et al.* 1992). Initially, it had been reported that [Asn^4]-ANG III has virtually no pressor activity in Japanese eels, *Anguilla japonica* (Hirano & Hasegawa 1984). However, Butler and Oudit (1995) have recently shown that 25 ng.kg bw^{-1} of [Asn^4]-ANG III increased CO by 23% by way of increasing stroke volume but not heart rate. Even though 150 ng.kg bw^{-1} increased CO by 47%, there was still no measurable effect on heart rate. This lack of change in heart rate represented a clear difference in the cardiac responses to ANG II and ANG III. Peak P_{DA} increased by 25% in response to 25 ng and by 52% in response to 150 ng.kg bw^{-1} of ANG III which may have been due to a combination of an increased CO and peripheral vasoconstriction. The above observations (Butler & Oudit 1995) were supported by the present study. For example, Figure 2 shows that even though a 20 ng. kg bw^{-1} i.v. injection of ANG III increased significantly CVBF (30%) it had no measurable effect on DABF. At the higher dose of 50 ng.kg bw^{-1} of [Val^4]-ANG III , CVBF increased by 52% and DABF by 24%. Therefore

DABF was less affected. My observations may show that [Val^4]- ANG III differs from [Asn^1, Val^5, Gly^9]- ANG I and [Asn^1, Val^5]-ANG II insofar as the hormone increases CO slightly through its direct effect on stroke volume but not on cardiac frequency. The increase in P_{DA} is achieved by peripheral vasoconstriction.

The effect of [Asn^1, Val^5, Gly^9]-ANG I, [Asn^1, Val^5]-ANG II and [Val^4]-ANG III on blood flow were tested in North American eels. We have shown that i.v. injections of ANG I, ANG II or ANG III, cause dose-dependent increases in arterial (dorsal aorta) and venous (caudal vein) blood flows. These results augment the findings of earlier studies of the effect of these peptides on the arterial blood pressure and blood flow in some fishes.

Evidence for a renin or renin-like enzyme in the CS: flow responses to CS-EXT and hRS

Now that the experimental set-up has been shown to be sensitive to angiotensins and that CVBF and DABF responses to as little as 5-10 ng.kg bw^{-1} of [Asn^1, Val^5]-ANG II, one may proceed with the next step. That is to show that the CS contain renin or a renin-like substance. Figure 3 illustrates three experiments that are related to this hypothesis. In order to exclude the possibility that the posterior kidney contains substantial or measurable amounts of renin, we tested the flow responses in the DA and CV following the i.v. injection of 5.0 mg of a saline extract of the posterior or functional kidney. Since this region contains the majority of the filtering glomeruli, and therefore the bulk of the juxtaglomerular cells containing renin (Capreol and Sutherland 1968) one might expect a pressor or flow response from the extract. However, there

was no measurable response to this extract but there was hormonal activity in the CS extracts.

This is the first study to demonstrate that approximate physiological doses of an extract containing 0.50, 1.25 or 2.50 mg. Kg bw^{-1} of fresh CS can cause immediate and sustained, dose-dependent increases in arterial and venous blood flows. Only the lowest dose (0.50 mg. kg bw^{-1}), equivalent to one-tenth of a corpuscle per kg bw, failed to elicit a significant flow response, whereas a 1.25 or 2.50 mg. kg bw^{-1} dose of CS increased arterial and venous blood flows well beyond baseline levels (Figure 3). The results of my study strongly support earlier findings on the pressor effects of the CS.

Extracts from the CS of four different species of teleosts (*A. anguilla, C. carpio, C. auratus, L. litulon*) were all found to have significant pressor effects in anaesthetized rats (Chester-Jones *et al.* 1966, Sokabe *et al.* 1970). Surgical removal of the CS (stanniectomy) in European eels, *A. anguilla* and North American eels, *A. rostrata* is followed by a rapid decline in arterial blood pressure (Chester-Jones *et al.* 1966, Bailey & Fenwick 1975). In a recent study, as much as a 15% decline in dorsal aortic blood pressure and a 45% decline in dorsal aortic blood flow were reported in the North American eel following stanniectomy (Butler *et al.* 1995). In stanniectomized eels, the blood pressure depression was corrected by intravenous injections of extracts of eel CS (Chester-Jones *et al.* 1966). However, there are no published reports on the effect of CS extracts on dorsal aortic or caudal venous blood flow rates. Remarkably, the duration and the intensity of the response to just 2.50 mg. kg bw^{-1} of CS is equivalent to a 50 ng. kg bw^{-1} injection of [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II. Our

experiments clearly show that the CS contain a vasoactive principle that is a potent stimulator of arterial and venous blood flow, in the intact North American eel.

A saline extract of 0.5 mg .kg bw^{-1} of CS-EXT failed to change DABF or CVBF, but an i.v. injection of 1.25 mg of CS-EXT was followed by a 127% increase in CVBF and a 45% increase in DABF. Both of these increases were statistically significant ($P<0.05$) compared with saline-injected control values (Figure 3). Even greater responses were observed following the injection of an extract from 2.5 mg CS. kg bw^{-1}. CVBF increased by 237% and DABF by 64%, both flows being significantly greater than both the saline injected controls and in the 1.25 mg group. This observation, added to the discovery by Chester Jones *et al.* (1966) that extracts of freshwater European eel, *A. anguilla*, CS contained a renin-like pressor substance, strengthens my hypothesis that the CS contain significant renin-like activity in teleost fishes. Finally, I injected three doses (50, 100 and 150 ng.kg bw^{-1} i.v.) of human renin substrate (hRS) to show if endogenous eel renin from either the posterior kidney JG cells and/or the CS cleave the decapeptide ANG I from this substrate. It would be rapidly converted to ANG II by endogenous ACE and one might expect to observe changes in CVBF and DABF similar to those shown in Figure 2 in response to injected [Asn^1, Val^5, Gly^9]-ANG I and [Asn^1, Val^5]-ANG II. Figure 3 shows that at the intermediate dose there was a statistically significant ($P<0.05$) 38% increase in CVBF but no change in DABF. At the highest dose of 150 mg.kg bw^{-1} i.v., both the CVBF (16%) and DABF (42%) increased significantly. Apparently hRS was an adequate but not quantitatively strong substrate for endogenous eel renin (Figure 3).

The response to human renin substrate [Asp^1, Ile^5, His^9] (hRS) has not been previously tested in freshwater North American eels. It had been reported that kidney extract from the eels can form angiotensin from synthetic hRS as well as fowl renin-substrate (Nishimura 1980). The results of our study suggest that in the eel, the conversion of hRS to angiotensins can also occur *in vivo.* It should be considered that in mammals, the reaction of renin on renin-substrate shows strong species specificity (Schaffenburg *et al.* 1960, Grill *et al.* 1972). In our case, the reaction between eel renin and heterologous substrate may not have proceeded with great efficiency, resulting in the inadequate production of angiotensins and hence the observed weak flow responses.

Inhibition of putative renin in eel CS-EXT and of hRS by Pepstatin A

Flow responses to extracts of CS were inhibited by prior i.v. injections of 1 mg. kg bw^{-1} of pepstatin A. This implied that the CS contained "renin" (Figure 7). Pepstatin A is a bacterially derived pentapetide (Umezawa *et al.* 1970) which is a highly effective, competitive inhibitor of acid proteases such as human pepsin, gastricin, cathespin D (Marciniszyn *et al.* 1976) and human renin (Gross *et al.* 1977). It has been suggested that Pepstatin A is unsatisfactory for use in studies of the RAS because it lacks specificity for renin (Gauten *et al.* 1984, Haber 1985). However, of all the acid proteases, renin is the only enzyme that can form ANG I at a significant rate. Pepstatin A decreases renin activity (PRA) by at least 80% (Hidaka *et al.* 1985). The intravenous injection or constant infusion of Pepstatin A reduced blood pressure and blocked the

pressor effects of exogenous renin in rats (Evin *et al.* 1978, Miyazaki *et al.* 1979, Antonaccio 1982).

This is the first experimental test of the effects of pepstatin A in any species of fish. The effects of pepstatin A in freshwater eels were found to be analogous to its effects in mammals (Figure 6), revealing the highly conserved nature of the RAS. We showed that pepstatin A can inhibit renin-activity *in vivo* since the flow response to hRS, the renin substrate, was also abolished (Figure 6). Furthermore, the inhibitory effect of pepstatin A was shown to be renin-specific, since the flow response to i.v. injections of [Asn^1, Val^5, Gly^9]-ANG I (Figure 4) were not prevented by prior administration of Pepstatin A. This result was expected since the conversion of [Asn^1, Val^5, Gly^9]-ANG I to [Asn^1, Val^5]-ANG II the active peptide, depends only upon ACE which is not blocked by Pepstatin A. Finally, Pepstatin A had no measurable effect on CVBF or DABF responses to ANG II (Figure 5) because no enzymatic conversion of the peptide takes place in the RAS. ANG II is therefore able to act with full force.

The functional connection between the CS and the RAS has been investigated previously using a variety of different experimental approaches. In each case, the investigators concluded that the CS contain a renin-like pressor that is capable of forming ANG I. It is known that extracts made from the CS of freshwater eels contain a powerful pressor that has many of the physical and functional properties of mammalian renin (Chester-Jones 1966). Studies show that the CS from the Japanese eel (Ogawa *et al.* 1982), the chum salmon (Takemoto *et al.* 1983) and the goosefish (Hasegawa *et al.* 1984) can produce ANG I when it is incubated with homologous plasma. In our study, we compared the flow response to CS-EXT in intact animals with the same

response under the condition of renin-inhibition, carried out by a 1 mg. kg bw^{-1} injection of pepstatin A. For instance (Fig. 7), in the unblocked state, blood flows in the dorsal aorta and the caudal vein becomes significantly elevated for 24 to 33 min following CS-EXT injection, compared to blood flow during the preceding (55 min) vehicle injected period (Duncan's Multiple Range Test, $p < 0.05$). However, in the blocked state, CS-EXT failed to elicit any significant increase in flow. Therefore, we clearly demonstrated that renin activity underscores the flow and presumably the pressor response to CS-EXT.

Depending on the species examined, the total renin activity of teleost CS is estimated to be only 0.1 to 0.7 % that of the kidney (Sokabe *et al.* 1970). However, I showed that the renin-like activity found in just 1.25 mg. kg bw^{-1} of CS-EXT (equivalent to approximately one quarter of a CS. kg bw^{-1}), could elicit significant increases in arterial and venous blood flow (Figure 3). One may consider the fact that the posterior or functional kidney of eels contains relatively few large nephrons (Audige 1910). Therefore one may hypothesize that the actual number of putative renin-secreting granulated cells may be far greater in the two functioning CS, where their number exceeds 90% of the total cell content. Of particular relevance is the fact that compared to renal renin, isorenins found in both rat brain and hog spleen, exhibit a 1000-fold greater sensitivity to the inhibitory action of Pepstatin A (Hackenthal *et al.* 1978). On the basis of my study, the CS of North American eels may also contain isorenins that are highly sensitive to Pepstatin A, and are capable of forming angiotensins with a higher than expected efficiency.

Blood flow responses to putative renin in eel CS-EXT and to hRS:

(A) Inhibition of the second-step endogenous conversion of ANG I to ANG II with captopril

Captopril is a potent inhibitor of angiotensin-converting enzyme (ACE), and blocks the enzymatic conversion of ANG I to ANG II (Ondetti *et al.* 1977). In my study, Captopril completely blocked the flow responses to i.v. injections of CS-EXT (Figure 11) , hRS (Figure 10) and ANG I (Figure 9) but not to ANG II (Figure 8), as would be predicted.

In mammals, Captopril reduces blood pressure and blocks the pressor response to i.v. injections of ANG I, without affecting the response to ANG II (Ferguson *et al.* 1977, Gavaras *et al.* 1978, Rotmensch *et al.* 1988). In the rainbow trout, *O. Mykiss*, Captopril inhibits ACE-activity which is located primarily in the gills. As a result, there follows a significant reduction in dorsal aortic blood pressure presumably because of decreased circulating levels of ANG II (Lipske *et al.* 1987). Captopril and its natural analogue, teprotide (SQ 20881), block the pressor effects of exogenous goosefish [Asn^1, Val^5, His^9]-ANG I or chum salmon [Asn^1, Val^5, Tyr^9]-ANG I, in unconscious vagotomized rats (Nakajima *et al.* 1971). Similarly, SQ 20881 and presumably Captopril can inhibit the pressor response to ANG I in North American eels but not the pressor response to ANG II (Nishimura *et al.* 1978). Such studies confirm not only the presence of ACE in North American eels, but also the absolute requirement of ACE-activation in the flow and pressor responses to ANG I and its precursor, hRS.

In mammals, ACE catalyzes not only the formation of ANG II, but also the degradation of bradykinins (Erdos 1977). Bradykinins are powerful vasodepressors that act by stimulating the production of arachidonic acid metabolites, nitric oxide and

endothelium-derived hyperpolarized factor (Vanhoutte 1989). Therefore, ACE inhibition should restrict the formation of ANG II, and promote the accumulation of bradykinins. Studies show that bradykinins may also be present in teleosts (Olson *et al.* 1997), although their function is unclear. For instance, kinin-like substances have been identified in the carp, *Cyprinus carpio* (Inouye *et al.* 1961) and the rainbow trout (Dunn & Perks 1975). The occurrence of kinin-like substances in other teleost species, such as the North American eel, cannot be discounted. As stated above, my study shows that the ACE inhibitor Captopril can abolish the blood flow response to CS-EXT, hRS and ANG I. Under these conditions, when ACE is inhibited, kinin-like substances may accumulate in tissues and possibly affect blood flow and pressure. If so, one should interpret the flow responses with caution and consider that the changes in flow in response to Captopril may not be caused only by a reduction in blood levels of ANG II. In my study, one may largely discount any interference from putative kinins because the extent and duration of the flow responses to [Asn^1, Val^5]-ANG II are similar before treatment with Pepstatin A (Figure 5), before treatment with Captopril (Figure 9) and after treatment with Captopril (Figure 9).

(B) Effects of the AT_1 and AT_2 receptor antagonist Sarile

In this study, i.v. injection of 50 ug.kg bw^{-1} of Sarile, a potent ANG II- receptor antagonist (Yamamoto *et al.* 1972), abolished the increased CVBF and DABF which followed the i.v. injection of 2.5 mg. kg bw^{-1} of CS-EXT (Figure 13). This implies that renin-like activity is contained within the CS-EXT's, and had ultimately generated endogenous [Asn^1, Val^5]-ANG II which in turn, increased CVBF and DABF. When i.v.

injection of the same amount of CS-EXT was preceded by mammalian AT_1 and AT_2 receptor blockers, the extract failed to generate the earlier increases in CVBF and DABF (Figure 13). The next experiment (Figure 12) showed that the amplitude and duration of the increased CVBF and DABF responses to ANG II almost matched the responses to CS-EXT (Figure 13). If an i.v. injection of 50 ug.kg bw^{-1} of Sarile was given before the second injection of [Asn^1, Val^5]-ANG II, the flow responses were blocked completely (Figure 12). Sarile was fully effective as an ANG II inhibitor in these freshwater eels, a finding that is in accordance with earlier observations showing that Sarile inhibits the pressor effects of ANG II in rats and canines (Johnson *et al.* 1972, Turker *et al.* 1972). Moreover, Sarile can inhibit the other actions of ANG II, including the stimulation of aldosterone synthesis and the myotrophic effects on vascular smooth muscle (Johnson *et al.* 1973, Hall *et al.* 1974). Through the use of radioimmunoassays, Sarile has been shown to bind indiscriminately to both AT_1 and AT_2 receptors (Timmermans *et al.* 1993).

Sarile also blocks the pressor responses to ANG II in birds, reptiles, amphibians and fishes other than eels. For example, Sarile can block the pressor response to ANG II in Pekin ducks, *Anas platyrhynchos* (Butler *et al.* 1998) and chickens, *Gallus domesticus* (Moore *et al.* 1981, Nakamura *et al.* 1982). Moreover, a clear inhibitory effect of Sarile on the pressor response to ANG II has been observed in Western painted turtles, *Pseudemys scripta elegans* (Zehr *et al.* 1981) and in bullfrogs, *Rana catesbeiana* (Harper *et al.* 1985).

In some cases, ANG II receptors which were unaffected by nonpeptide antagonists such as Losaratan or PD 123319 were fully blocked by peptide antagonists

such as Sarile. For example, ANG II receptors in the adrenal gland of Pekin ducks, *A. platyrhynchos* (Gray *et al.* 1989) and the heart of African clawed toads, *Xenopus laevis* (Bergsma *et al.* 1993) bind Sarile but not other known ANG II antagonists such as Losaratan and PD 123319. On the other hand, my experiments have shown that in *A. rostrata*, ANG II receptors respond to both Sarile (Figures 12 and 13) and nonpeptide antagonists such as the AT_1 receptor blocker Losartan (Figures 14 and 15) and the AT_2 blocker PD123319 (Figures 16 and 17). The latter experiments with nonpeptide ANG II receptor antagonists reveal further that these receptors may represent two distinct subtypes, similar to AT_1 and AT_2 receptors in mammals.

(C) *Effects of the AT_1 receptor antagonist Losartan and the AT_2 receptor antagonist PD 123319*

My experiments are the first to show that i.v. injection of the synthetic nonpeptide ANG II receptor antagonists, Losartan and PD123319 have inhibitory effects on the CVBF and DABF responses to CS-EXT (Figure 15 and 17) and ANG II (Figure 14 and 16) in freshwater eels. Earlier studies on rat and bovine tissues have shown that Losartan and PD123319 bind discriminately to 2 subpopulations of ANG II receptors, designated as AT_1 (Losartan) and AT_2 (PD123319) receptors (Chang *et al.* 1990, Bumpus *et al.* 1991). AT_1 receptors are found in the heart, vascular tissues, adrenal cortex, renal tubules and the circumventricular organs of the brain (Chiu *et al.* 1989, Herblin *et al.* 1991). As such, these receptors mediate all of the classical responses to ANG II including vasoconstriction, blood pressure elevation, aldosterone release, renal tubule sodium reabsorption, thirst, sodium appetite, as well as hyperplesia and hypertrophy of vascular

and cardiac smooth muscle (Timmermans 1993). All of the aforementioned central and peripheral actions of ANG II are inhibited by the AT_1 receptor antagonist Losartan, (see reviews by Griendling *et al.* 1994, Messerli *et al.* 1996). In studies on rats and dogs, i.v. injections of Losartan blocked the pressor effects of ANG II, while the pressor response to other hormones such as norepinephrine or vasopressin were unaffected (Chiu *et al.* 1990, Wong *et al.* 1991).

AT_2 receptors on the other hand, are less prevalent and their function is unknown. In rats, the highest concentrations of AT_2 receptors occur in the adrenal medulla, uterus, thalamus and the developing fetus, though their numbers diminish dramatically post-partum (Smith *et al.* 1992, Viswanathan *et al.* 1991). Inhibition of AT_2 receptors with PD123319, PD123177 or CGP42112 does not affect the responses to ANG II, including pressor responses (Wong *et al.* 1990, Dudley *et al.* 1990, Griendling *et al.* 1994).

The results of our study show that blood flow responses to ANG II in freshwater eels, may be mediated concurrently by AT_1 receptors and AT_2 receptors. The first experiment showed that the increase in CVBF and DABF which followed the i.v. injection of CS-EXT (Figure 15) were only partially blocked by an i.v. injection of 5 mg.kg bw^{-1} of the mammalian AT_1 antagonist losartan (Figure 15). The reduced flow response was not due to a failure of the CS-EXT initiated [Asn^1, Val^5]-ANG II generating system (Figure 15) because in a second experiment using [Asn^1, Val^5]-ANG II, the result was much the same. Figure 14 shows that an i.v. injection of 50 ng.kg bw^{-1} of [Asn^1, Val^5]-ANG II gave a typical and clear increase in both CVBF and DABF which again, as in the CS-EXT experiment was only partially blocked with Losartan.

Since only partial blockade of the flow responses to [Asn^1, Val^5]-ANG II was achieved with the mammalian AT_1 antagonist Losartan, it was important to repeat the experiment using the AT_2 antagonist PD 123319. Figure 17 shows that eel CVBF and DABF both increased, as before (Figure 15) following an i.v. injection of 2.5 mg of fresh CS. This increase was diminished partially if the eels were given an i.v. injection of 5 mg.kg bw^{-1} of the AT_2 antagonist PD 123319 before the subsequent injection of CS-EXT (Figure 17). This result implied that the CS extract generated endogenous ANG II. In the second experiment (Figure 17), I observed that, as with Losartan, PD 123319 only partially blocked the flow responses to ANG II (Figure 17). I concluded that both AT_1 and AT_2 receptors may collaborate to directly and/or indirectly regulate CVBF and DABF. Although AT_1 or AT_2 inhibition alone, failed to abolish the DABF and CVBF responses to i.v. injections of ANG II or CS-EXT, their combined inhibitory effect on DABF and CVBF is close to 100%. Therefore, one can argue that AT_1 and AT_2 receptors effectively mediate all of the DABF and CVBF responses to ANG II or CS-EXT.

My studies augment previous findings on the characterization of ANG II receptors in teleost fishes. In the rainbow trout, *O. Mykiss*, ANG II receptors have been found in many tissues including the heart, gills, kidney, brain and the adrenocortical homologue (Cobb *et al.* 1992). Of particular relevance to my investigation is the identification of AT_1-like receptors in the liver, gill, kidney and intestines of freshwater European eels, *A. anguilla*. In addition to the AT_1-like receptors, in these eels, there is evidence for a second receptor subtype in the liver (Marsigliante *et al.* 1994, 1995). Similarly, AT_1-like receptors have been identified in toadfish vascular smooth muscle

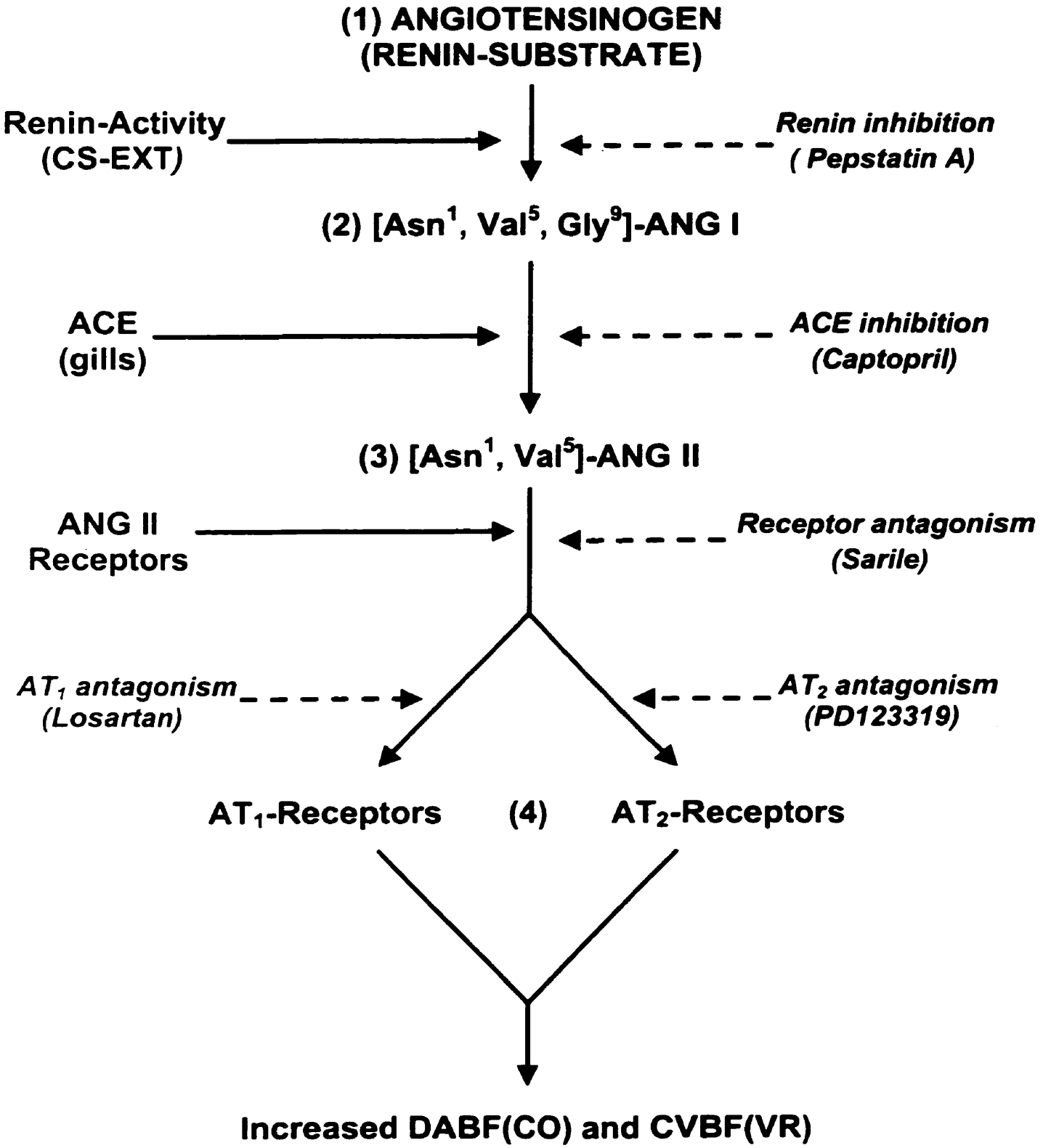
(Nishimura & Qin 1994). The existence of multiple ANG II receptor subtypes in the eel is strongly supported by the results of our study which show that the blood flow response, and presumably the pressor response to [Asn^1, Val^5]-ANG II (Butler *et al.* 1995), is mediated by two subtypes of ANG II receptors, akin to mammalian AT_1 and an AT_2 receptors.

Alternatively, ANG II receptors in North American eels may represent a unique receptor subtype that has characteristics of both AT_1 and AT_2 receptors. Experimental evidence suggests that ANG II receptor heterogeneity is prevalent among lower vertebrates. Receptors for ANG II have been well characterized in birds and amphibians, where they mediate many of the classical responses to ANG II, including pressor effects (see review by Nishimura 1980). Avian ANG II receptors may also differ from mammalian AT_1 and AT_2 receptors, since the predicted effects of nonpeptide ANG II receptor antagonists on the actions of ANG II, are not observed. In Pekin ducks, *A. platyrhynchos*, i.v. injections of both losartan and PD123319 failed to block either the pressor response, or the attenuation of nasal salt gland secretion following i.v. infusion of ANG II (Butler *et al.* 1998). Furthermore, studies on the domestic fowl, *G. domesticus*, showed that neither losartan nor PD123319 inhibit the actions of ANG II on isolated blood vessels and adrenal tissues (Le Noble *et al.* 1991, Hasegawa *et al.* 1993, Gray *et al.* 1989). In clawed toads, *Xenopus laevis* ANG II receptors are found in the heart, kidney, interrenals and ovarian follicles. They have a low affinity for Losartan (Kloas *et al.* 1992, Sandberg *et al.* 1990, Sandberg *et al.* 1991). Thus the presence of novel ANG II receptors that mediate the flow and pressor effects of ANG II in North American eels would not be inconsistent with evolutionary trends.

Figure 19. Stepwise pharmacological inhibition of the blood flow responses to peptide components of the eel RAS, incuding the possible role of the CS.

DABF (Dorsal Aortic Blood Flow), CO (Cardiac Output)

CVBF (Caudal Venous Blood Flow), VR (Venous Return)


(1) ANGIOTENSINOGEN
(RENIN-SUBSTRATE)
Renin-Activity
(CS-EXT)
Renin inhibition
(Pepstatin A)
(2) [Asn1, Val5, Gly9]-ANG I
ACE
(gills)
ACE inhibition
(Captopril)
(3) [Asn1, Val5]-ANG II
ANG II
Receptors
Receptor antagonism
(Sarile)
AT1 antagonism
(Losartan)
AT2 antagonism
(PD123319)
AT1-Receptors
(4)
AT2-Receptors
Increased DABF(CO) and CVBF(VR)

SUMMARY

1) DABF and CVBF increased in freshwater North American eels, *A. rostrata*, in a dose-dependent manner following i.v. injection of 5, 10, 20 and 50 ng. kg bw^{-1} [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II. The effects of [Asn^1, Val^5, Gly^9]-ANG I and [Asn^1, Val^5]-ANG II on DABF and CVBF were identical, the minimum effective dose was 5 ng. kg bw^{-1}.

2) Physiological doses of [Val^4]-ANG III (5, 10, 20 and 50 ng. kg bw^{-1}) increased DABF and CVBF in a dose-dependent manner. The minimum effective dose of [Val^4]-ANG III was 10 ng. kg bw^{-1}.

3) Intravenous injection of 0.5, 1.25 or 2.5 mg. kg bw^{-1} CS-EXT caused immediate and sustained dose-dependent increases in DABF and CVBF. Similar increases in DABF and CVBF was observed following the i.v. injection of 50, 100 or 150 ng. kg bw^{-1} hRS. The DABF and CVBF responses to the 2.50 mg. kg bw^{-1} injection of CS-EXT were comparable to the responses to 50 ng. kg bw^{-1} of [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II.

4) Renin-like activity mediates the DABF and CVBF responses to hRS and CS-EXT. The blood flow responses to 150 ng. kg bw^{-1} hRS or 2.5 mg. kg bw^{-1} CS-EXT were completely blocked by a prior injection of 1 mg. kg bw^{-1} of the mammalian renin

inhibitor, Pepstatin A. Pepstatin A did not affect the flow responses to 50 ng. kg bw^{-1} of [Asn^1, Val^5, Gly^9]-ANG I or [Asn^1, Val^5]-ANG II.

5) The DABF and CVBF responses to [Asn^1, Val^5, Gly^9]-ANG I, hRS or CS-EXT are ACE-dependent. 1 mg. kg bw^{-1} of the mammalian ACE-inhibitor, Captopril completely abolished the flow responses to 50 ng. kg bw^{-1} [Asn^1, Val^5, Gly^9]-ANG I , 150 ng. kg bw^{-1} hRS or 2.5 mg. kg bw^{-1} CS-EXT. Captopril did not affect the flow responses to 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II.

6) The mammalian AT_1 and AT_2 receptor antagonist [Sar^1, Ile^8]-ANG II (Sarile) is a potent inhibitor of the DABF and CVBF responses to [Asn^1, Val^5]-ANG II or CS-EXT. 50 ug. kg bw^{-1} Sarile completely blocked the flow responses to 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II or 2.5 mg. kg bw^{-1} CS-EXT.

7) The CS of freshwater North American eels, *A. rostrata*, contain a potent renin-like pressor substance which participates in the synthesis of ANG II, in conjunction with other endogenous elements of the eel RAS

8) Losartan, the mammalian AT_1 receptor antagonist or PD123319, the mammalian AT_2 receptor antagonist partially blocks the DABF and CVBF responses to 50 ng. kg bw^{-1} of [Asn^1, Val^5]-ANG II or 2.5 mg. kg bw^{-1} CS-EXT.

9) Cardiovascular regulation in *A. rostrata* may be mediated through two subtypes of ANG II receptors; an AT_1-like, Losartan sensitive subtype and an AT_2-like, PD123319 sensitive subtype.

REFERENCES

Akagi H, Hayashi T, Nakayama T, Nakajima T, Watanabe TX & Sokabe H 1992 Comparative studies on angiotensin. *Chemical Pharmaceutical Bulletin* **30** 2498-2502.

Antonaccio MJ 1982 Inhibitors of the renin-angiotensin system as new antihypertensive agents. *Clinical and Experimental Hyperpertensin Part A Theory and Practice* **A4 (1-2)** 27-46.

Aoyagi T, Kunimoto S, Morishima H, Tekeuchi T & Umezawa H 1971 Effect of pepstatin on acid proteases. *Journal of Antibiotics* **24** 687-694.

Arillo A, Uva B & Vallarino M 1981 Renin activity in rainbow trout (*Salmo gairdneri* Rich.) and effects of environmental ammonia. *Comparative Biochemistry and Physiology* **68A** 307-311.

Armour KJ, O'Toole LB & Hazon N 1993 Mechanisms of ACTH- and angiotensin II-stimulated 1- α -hydroxycorticosterone secretion in the dogfish, *Scyliorhinus canicula*. *Journal of Molecular Endocrinology* **10** 235-244.

Arnold-Reed DE & Balment RJ 1994 Peptide hormones influence in vitro interrenal secretion of cortisol in the trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology* **96** 85-91.

Audigé J 1910 Contribution à l'étude des reins des poissons téléostéens. *Archives de Zoologie Expérimentale et Générale* **4** 275-625.

Bailey JR & Fenwick JC 1975 The effect of stanniectomy and plasma clacium levels on blood pressure in the eel, *Anguilla rostrata* LeSueur. *Comparative Biochemistry and Physiology* **51** 693-697.

Bailey JR & Randall DJ 1981 Renal perfusion pressure and renin secretion in the rainbow trout, *Salmo gairdneri*. *Canadian Journal of Zoology* **59** 1220-1226.

Balment RJ & Carrick S 1985 Endogenous renin-angiotensin system and drinking behaviour in flounder. *American Journal of Physiology* **248** R157-R160.

Bauchot R 1953 Anatomie comparée des corpuscles de Stannius chez les Téléostéens. *Archives de Zoologie Expérimentale et Générale* **89** 147-169.

Bean JW 1942 Specificity in the renin-angiotensinogen reaction. *American Journal of Physiology* **136** 732-742.

Beldent V, Michaud A, Wei L, Chauvet M-T & Corvol P 1993 Proteolytic release of human angiotenisin-converting enzyme. *Journal of Biological Chemistry* **268** 26428-26434.

Belsare DK 1973*a* Comparative anatomy and histology of the corpuscles of Stannius in teleosts. *Zeitschrift für Mikroskopisch-Anatomische Forschung (Leipzig)* **87** 445-456.

Belsare DK 1973*b* Histogenesis of the interrenal corpuscles, the chromaffin tissue and the corpuscles of Stannius in *Channa punctatus Bloch*. (Actinopyerygii, Percomorphi). *Cell and Tissue Research* **6** 343-349.

Bergsma DJ, Ellis C, Nuthulaganti PR, Nambi P, Scaife K, Kumar C & Aiyar N 1993 Isolation and expression of a novel angiotensin II receptor from *Xenopus laevis* heart. *Molecular Pharmacology* **44** 277-284.

Bhattacharya TK & Butler DG 1978 Fine structure of the corpuscles of Stannius in the toadfish. *Journal of Morphology* **155** 271-286.

Blair-West JR, Coghlan JP, Denton DA, Gibson AP, Oddie CJ, Sawyer WH & Scoggins BA 1977 Plasma renin activity and blood corticosteroids in the Australian lungfish, *Neoceratodus forsteri*. *Journal of Endocrinology* **74** 137-142.

Borriraja V, Henderson IW & Chester-Jones I 1973 Renal fractions affecting the concentration of plasma cortisol in the eel, *Anguilla anguilla L. Journal of Endocrinology* **57** 13-17.

Boyd GW 1977 An inactive higher-molecular-weight renin in normal subjects and hypertensive patients. *Lancet* **1** 215-218.

Brown AJ, Clark SA & Lever AF 1983 Slow rise and diurnal changes of blood pressure with saralasin and angiotensin II in rats. *American Journal of Physiology* **244** F84-F88.

Buckley JP 1972 Actions of angiotensin on the central nervous system. *Federation Proceedings* **31** 1332-1337.

Bumpus FM, Catt KJ, Chiu AT, DeGasparo M, Goodfriend T, Husain A, Peach MJ, Taylor DG & Timmermans, PBMWM 1991 Nomenclature for angiotensin receptors. *Hypertension* **17** 270-723.

Burton J, Poulsen K & Haber E 1975 Competitive inhibitors of renin: inhibitors effective at physiological pH. *Biochemistry* **14** 3892-3898.

Butler DG & Cadinouche AMZ 1995 Fractional reabsorption of calcium, magnesium and phosphate in the kidneys of freshwater North American eels (*Anguilla rostrata*

LeSueur) following removal of the corpuscles of Stannius. *British Journal of Comparative Physiology* **165** 348-358.

Butler DG & Oudit GY 1995 Corpuscle of Stannius and blood flow regulation in freshwater North American eels, *Anguilla rostrata* LeSueur. *Journal of Endocrinology* **145** 181-194.

Butler DG, Oudit GY & Cadinouche AZM 1995 Angiotensin I-and II-and norepinephrine-mediated pressor responses in an ancient holostean fish, the bowfin (*Amia calva*). *General and Comparative Endocrinology* **98** 289-302.

Butler DG, Zandevakili R & Oudit GY 1998 Effect of ANG II and III and angiotensin receptor blockers on nasal salt gland secretion and arterial blood pressure in conscious Pekin ducks (*Anas platyrhynchos*). *Journal of Comparative Physiology and Biochemistry* **168** 213-224.

Campbell DJ, Bouhnik J, Coexy E, Menad J & Corvol P 1985 Characterization of precursor and secreted forms of human angiotensinogen. *Journal of Clinical Investigation* **75** 1880-1893.

Capelli JP, Wesson LG & Aponte GE 1970 A phylogenetic study of the renin-angiotensin system. *American Journal of Physiology* **218** 1171-1178.

Capreol SV & Sutherland LE 1968 Comparative morphology of juxtaglomerular cells. I. Juxtaglomerular cells in fish. *Canadian Journal of Zoology* **46** 249-256.

Carpenter SJ & Heyl HL 1974 Fine structure of the corpuscles of Stannius of Atlantic salmon during freshwater spawning journey. *General and Comparative Endocrinology* **23** 212-223.

Carrick S & Balment RJ 1983 The renin-angiotensin system and drinking in the euryhaline flounder, *Platichthys flesus*. *General and Comparative Endocrinology* **51** 423-433.

Carroll RG & Opdyke DF 1982 Evolution of angiotenisn II-induced catecholamine release. *American Journal of Physiology* **243** R65-R69.

Chang RSL & Lott VJ 1990 Two distinct angiotensin II receptor binding sites in rat adrenal revealed by new selective nonpeptide ligands. *Molecular Pharmacology* **29** 347-351.

Chester-Jones I, Henderson IW, Chan DKO, Rannklin JC, Mosley W, Brown JJ, Lever AF, Robertson JIS & Tree M 1966 Pressor activity in extracts of the corpuscles of Stannius from the European eel (*Anguilla anguilla* L.). *Journal of Endocrinology* **34** 292-408.

Chiu AT, Herblin WF, Ardecky RJ, McCall DE, Carini DJ, Duncia JV, Pease LJ Wexler RR, Wong PC, Johnson AL & Timmermans PBMWM 1989 Identification of angiotensin II receptor subtypes. *Biochemical and Biophysical Research Communications* **165** 196-203.

Chiu AT, McCall DE, Price WA, Wong PC and Carini DJ 1990 Nonpeptide angiotensin II receptors antagonists VII. Cellular and biochemical pharmacology of DuP 753, an orally active antihypertensive agent. *Journal of Pharmacology and Experimental Therapeutics* **252** 711-718.

Churchill PC, Malvin RL, Churchill MC & McDonald FD 1979 Renal fucntion in *Lophius americanus*: Effects of angiotensin II. *American Journal of Physiology* **236** R297-R301.

Cobb CS & Brown JA 1992 Angiotensin II binding to tissues of the rainbow trout, *Salmo gairdneri*, studies by autoradiography. *Journal of Comparative Physiology* **162** 197-202.

Cohen RS, Pang PKT & Clark NB 1975 Ultrastructure of the Stannius corpuscles of the killifish, *Fundulus heteroclitus*, and its relation to calcium regulation. *General and Comparative Endocrinology* **27** 413-423.

Davis JO, Freeman RH, Johnson JA & Spielman WS 1974 Agents which block the actions of the renin-angiotensin system. *Circulation Research* **34** 279-285.

De Gasparo M, Levens NR, Kamber B, Furet P, Whitebread S, Brechler V & Bottari SP 1993 The Angiotenisn II AT_2 Receptor Subtype. In *Angiotensin receptor*, pp 95-118. Eds JM Saavedra & Timmermans PBMWM. New York: Plenum Press.

De Smet D 1962 Considerations on the Stannius corpuscles and interrrenal tissue of bony fishes, especially based on researches into *Amia calva*. *Acta Zoologica (Stockholm)* **43** 201-219.

Dudley DT, Panek RL, Major TC, Lu GH, Bruns RF, Klinkefus BA, Hodges JC & Weishaar RE 1990 Subclasses of angiotensin II binding sites and their functional significance. *Molecular Pharmacology* **38** 370-377.

Dunn RS & Perks AM 1975 Comparative studies of plasma kinins: The kallikrein-kinin system in poikilotherm and other vertebrates. *General and Comparative Endocrinology* **26** 165-178.

Dzau VJ, But DW & Pratt RE 1988 Molecular biology of the renin-angiotensin system. *American Journal of Physiology* **255** F563-F573.

Edwards JG 1940 The vascular pole of the glomerulus in the kidney of vertebrates. *The Anatomical Record* **76** 381-389.

Ehlers MRW & Riordan JF 1989 Angiotensin-converting enzyme: New concepts concerning its biological role. *Biochemistry* **28** 8311-5318.

Ehlers MRW & Riordan JF 1990 Angiotensin-converting enzyme. Biochemistry and molecular biology. In *Hypertension: Pathophysiology, Diagnosis, and Management*, pp.1217-1231. Eds LH Laragh & BM Brenner. New York: Raven Press.

Ehlers MRW & Riordan JF 1991 Angiotensin-converting enzyme. Zinc- and inhibitor-binding stoichiometries of the somatic and testis isozymes. *Biochemistry* **30** 7118-7126.

Erdos EG 1976 Conversion of angiotensin I to angiotensin II. *American Journal of Medine* **60** 749-759.

Erdos EG 1977 The angiotensin I converting enzyme. *Federation Proceedings* **36** 1760-1765.

Erdos EG 1990 Angiotensin I converting enzyme and the changes in our concepts through the years. *Hypertension* **16** 363-367.

Erdos EG & Skidgel RA 1987 The angiotensin I-converting enzyme. *Laboratory Investigation* **56** 345-348.

Evin G, Gardes J, Kreft C, Castro B, Corvol P & Menard J 1978 Soluble pepstatins: a new approach to blockade *in vivo* of the renin-angiotensin system. *Clinical Science and Molecular Medicine* **55** 1673-1695

Fernley RT, John M, Niall HD & Coghlan JP 1986 Purification and characterization of ovine angiotensinogen. *European Journal of Biochemistry* **154** 597-601.

Ferguson RK, Brunner HR & Turini GA 1977 A specific orally active inhibitor of angiotensin converting enzyme in humans. *Lancet* **1** 75-779.

Ford P 1959. Some observations on the corpuscles of Stannius. In *Comparative Endocrinology*, pp. 728-734. Ed A Gorbman. New York: Wiley.

Fujita H & Honma Y 1967 On the fine structure of corpuscles of Stannius of the eel, *Anguilla japonica*. *Zeitschrift für Zellforschung und Mikroskopische Anatomie* **77** 175-187.

Galli-Phillips SM 1991 Evidence for the presence of an active angiotensin system in the nurse shark, *Gynchlymostoma cirratum*. In *Book of Abstracts, 5th International Symposium on Fish Physiology* pp. 25.

Garrett FD 1942 The development and phylogeny of the corpuscles of Stannius in ganoid and teleostean fishes. *Journal of Morphology* **70** 41-67.

Gauten D, Paul M, Deboden A, Unger TH & Lang RE 1984 The brain renin angiotensin system in central cardiovascular control. *Contributions to Nephrology* **43** 114-128.

Gavaras H, Brunner HR & Turini GA 1978 Antihypertensive effect of the oral converting enzyme inhibitor SQ 14225 in humans. *New England Journal of Medicine* **298** 991-995.

Goodfriend TL, Furhquist F, Gutmann F, Knych E, Hollemans H, Allmann D, Kent K & Cooper T 1972 Clinical and conceptual use of angiotensin receptors, pp. 549-563. In *Hypertension '72*. Eds J Genest & E Kiow. New York: Springer Verlag.

Gray DA, Gerstberger R & Simon E 1989 Role of angiotensin II in aldosterone regulation in the Pekin duck. *Journal of Endocrinology* **123** 445-452.

Griendling KK, Lassique B, Murphy TJ & Alexander RW 1994 Angiotensin II receptor pharmacology. *Advances in Pharmacology* **28** 269-306.

Grill G, Granger P & Thourau K 1972 The renin angiotensin system of amphibians. I. Determination of the renin content of amphibian kidneys. *Pflugers Archiv; European Journal of Physiology* **331** 1-12.

Gross F, Lazar J & Orth H 1972 Inhibition of the renin-angiotensin reaction by pepstatin. *Science* **175** 656.

Haber E 1985 Will renin inhibitors influence decision-making in antihypertensive therapy. *Journal of Hypertension* **3 (Suppl. 2)** S71-80.

Hackenthal E, Hackenthal R & Hilgenfeld U 1978 Isorenin, pseudorenin, cathepsin D and renin. A comparative enzymatic study of angotensin-forming enzymes. *Biochimica et Biophysica Acta* **522** 574-588.

Hall JE & Brands MW 1992 The renin-angiotensin-alsosterone system: Renal mechanisms and circulatory homeostasis. In *The Kidney: Physiology and Pathophysiology*, pp. 1455-1504. Eds DW Seldin & G Giebisch. New York: Raven Press.

Hall MM, Khosla MC, Khairallah PA & Bumpus FM 1974 Angiotensin analogs: the influence of sarcosine substituted in position 1. *Journal of Pharmacology and Experimental Therapeutics* **158** 222-228.

Harper RA & Stephens GA 1985 Blockade of the pressor response to angiotensin I and II in the bullfrog, *Rana catesbeiana*. *General and Comparative Endocrinology* **60** 227-235.

Hasegawa Y, Cipolle M, Watanabe TX, Nakajima T, Sokabe H & Zehr JE 1984*a* Chemical structure of angiotensin in the turtle, *Pseudemys scripta*. *General and Comparative Endocrinology* **53** 159-162.

Hasegawa K, Nishimura H & Khosla MC 1993 Angiotensin-induced endothelium-dependent relaxation of fowl aorta. *American Journal of Physiology* **264** R903-911.

Hasegawa Y, Nakajima T & Sokabe H 1983*a* Chemical structure of angiotensin formed wih kidney renin in the Japanese eel, *Anguilla japonica*. *Biomedical Research* **4** 417-420.

Hasegawa Y, Watanabe TX, Nakajima T & Sokabe H 1984*b* Chemical structure of angiotensin formed by incubating plasma with corpuscle of Stannius in the

Japanese goosefish, *Lophius litulon*. *General and Comparative Endocrinology* **54** 264-269.

Hasegawa Y, Watanabe TX, Sokabe H & Nakajima T 1983*b* Chemical structure of angiotensin in the bullfrog, *Rana catesbeiana*. *General and Comparative Endocrinology* **50** 75-80.

Hayashi T, Nakayama T, Nakajima T & Sokabe H 1978 Comparative studies on angiotensins. V. Structure of angiotensin formed by the kidney of Japanese goosefish and its identification by dansyl method. *Chemical and Pharmaceutical Bulletin* **26** 215-219.

Hazon N, Balment RJ, Perrott M & O'Toole LB 1989 The renin-angiotensin system and vascular and dipsogenic regulation in elasmobranches. *General and Comparative Endocrinology* **74** 230-236.

Hazon N & Henderson IW 1985 Factors affecting secretory dynamics of 1-alpha-hydroxycorticosterone in the dogfish, *Scyliorhinus canicula*. *General and Comparative Endocrinology* **59** 50-55.

Henderson IW, Jotisankasa V, Mosely W & Oguri M 1976 Endocrine and environmental influences upon plasma cortisol concentrations and plasma renin activity of the eel, *anguilla anguilla* L. *Journal of Endocrinology* **70** 81-95.

Henderson IW, Oliver JA, McKeever A & Hazon N 1981 Phylogenetic aspects of the renin-angiotensin system. In *Advances in Animal and Comparative Physiology*, pp. 355-363. Eds G Pethes & VL Freny. Oxford: Pergamon Press.

Herblin WF, Chiu AT, McCall DE, Ardecky RJ & Carini DJ 1991 Angiotensin II receptor heterogeneity. *American Journal of Hypertension* **4** 299S-302S.

Heyl HL 1970 Changes in the corpuscles of Stannius during the spawning journey of atlantic salmon (*Salmo salar*). *General and Comparative Endocrinology* **14** 43-52.

Hidaka H, Sawada S, Sato R & Oka H 1985 An improved method for measuring angiotensin I converting enzyme activity using a highly sensitive angiotensin II radioimmunoassay. *Endocrinologia Japonica* **32** 803-809.

Hirano T 1989 The corpuscle of Stannius. In *Vertebrate Endocrinology: Fundamentals and Biomedical Implications* Vol 3, pp. 139-162. Eds. PKT Pang & MP Schreibman. San Diego: Academic Press Inc.

Hirano T & Hasegawa S 1984 Effects of angiotensins and other vasoactive substances on drinking in the eel, *Anguilla japonica*. *Zoological Science* **1** 106-113.

Hirano T, Takei Y & Kobayashi H 1978 Angiotensin and drinking in the eel and the frog. In *Osmotic and volume regulation, Alfred Benzon Symposium XI*, pp. 123-128. Eds C Barker-Jorgensen & E Skadhauge Copenhagen: Munksgaard.

Hirose S & Murakami K 1992 Molecular biology of renin. *Blood Vessels* **2** 65-69.

Hooper NM 1990 Angiotensin converting enzyme: implications from molecular biology for its physiological functions. *International Journal of Biochemistry* **23** 641-647.

Hulthen I & Hokfelt T 1978 The effect of the converting enzyme inhibitor SQ20 881 on kinins, renin-angiotensin-aldosterone and catecholamines in relatons to blood pressure in hypertensive patients. *Acta Medica Scadinavica* **204** 497-502.

Ikemoto F, Takaori K, Iwao H & Yamaoto K 1982 Intrarenal localization of renin binding substances in rats. *Life Sciences* **31** 1011-1016.

Inagami T & Murakami K 1977 Purification of high molecular weight forms of renin from hog kidney. *Circulation Research* **41 (Suppl. 2)** 11-16.

Inouye A, Kataoka K & Tsujioka T 1961 On a kinin-like substance in the nervous tissue extracts treated with trypsin. *Japanese Journal of Physiology* **11** 319-324.

Itskowitz HD & McGiff JC 1974 Hormonal regulation of the renal circulation. *Circulation Research* **34 (Suppl. 1)** 65-73.

Jackson BA, Brown JA, Oliver JA & Henderson IW 1977 Actions of angiotensin on single nephron rate of trout, *Salmo gairdneri*, adapted to fresh- and sea-water environments. *Journal of Endocrinology* **75** 32P.

Johnson CI 1990 Biochemistry and pharmacology of the renin-angiotensin system. *Drugs* **39 (Suppl. 1)** 21-31.

Johnson CI, McGrath BP, Millar JA & Matthews PGF 1979 Long-term effects of captopril (SQ14225) on blood pressure and hormone levels in essential hypertension. *Lancet* **2** 193-196.

Johnson DW 1972 Variations in the interrenal and corpuscles of Stannius of *Mugil cephalus* from the Colorado river and its estuary. *General and Comparative Endocrinology* **19** 7-25.

Johnson JA & Davis JO 1972 Effects of an angiotensin II analog on blood pressure and adrenal steroid secretion in dogs with thoracic caval constriction (abstract). *Physiology* **15** 184.

Johnson JA & Davis JO 1973 Effect of a specific competitive antagonist of angiotensin II on arterial pressure and adrenal steroid secretion in dogs. *Circulation Research* **32 (Suppl. I)** 159-168.

Kaneko T, Hasegawa S & Hirano T 1992 Embryonic origin and development of the corpuscles of Stannius in chum salmon (*Oncorhynchus keta*). *Cell and Tissue Research* **268** 65-70.

Khosla MC, Nishimura H, Hasegawa Y & Bumpus FM 1985 Identification and synthesis of [1-asparagine, 5-valine, 9-glycine] angiotensin I produced from plasma of American eel, *Anguilla rostrata*. *General and Comparative Endocrinology* **57** 223-233.

Khosla MC, Smeby RR & Bumpus FM 1974 Structure-activity relationship in angiotensin II analogs. In *Angiotensin*, pp. 126-161. Eds IH Page & FM Bumpus. Berlin: Springer-Verlag.

Kloas W & Hanke W 1992 Angiotensin II binding sites in frog kidney and adrenal. *Peptides* **13** 349-354.

Kobayashi H & Takei Y 1996a In *The Renin-Angiotensin System: Comparative aspects. Zoophysiology, Vol. 35*, pp. 1-9. Berlin: Springer International.

Kobayashi H & Takei Y 1996*b* In *The Renin-Angiotensin System: Comparative aspects. Zoophysiology, Vol. 35*, pp. 77-88. Berlin: Springer International.

Kohler G & Milstein C 1975 Continuous cultures of fused cells producing antibody of predefined specificity. *Nature* **256** 495-497.

Krishnamurthy VG 1967 Development of the corpuscles of Stannius in the anabantid teleost, *Colisa lalia*. *Journal of morphology* **123** 109-120.

Krishnamurthy VG 1976 Cytophysiology of corpuscles of Stannius. *International Review of Cytology* **46** 177-246.

Krishnamurthy VG & Bern HA 1971 Innervation of the corpuscles of Stannius. *General and Comparative Endocrinology* **16** 162-165.

Krishnamurthy VG & Bern HA 1969 Correlative histological study of the corpuscles of Stannius and the juxtaglomerular cells of teleost fishes. *General and Comparative Endocrinology* **13** 313-335.

Krishnamurthy VG & Bern HA 1973 Juxtaglomerular cell changes in the euryhaline freshwater fish, *Tilapia mossambica*, during adaptation to sea water. *Acta Zoologica* **54** 9-14.

Lacy ER & Reale E 1990 The presence of a juxtaglomerular apparatus in elasmobranch fish. *Anatomy and Embryology* **182** 249-262.

Lafeber FPJG, Flik G, Wendelaar-Bonga SE & Perry SF 1988a Hypocalcin from Stannius corpuscles inhibits gill calcium uptake in trout. *American Journal of Physiology* **254** R891-R896.

Lafeber FPJG, Hanssen RGJM, Choy YM, Flik G, Hermann-Erlee MPM, Pang PKT & Wendelaar-Bonga SE 1988b identification of hypocalcin (teleocalcin) isolated from trout corpuscles of Stannius. *General and Comparative Endocrinology* **69** 19-30.

Lagois MD 1974 Granular epitheloid (juxtaglomerular) cell and renovascular morphology of the coelacanth *Latimeria chalumnae* Smith (Crossopterygii) compared with that of other fishes. *General and Comparative Endocrinology* **22** 296-307.

Le Noble FAC, Hekking JWM, Van Straaten HWM., Slaaf DW & Struyker-Bourdier HAJ 1991 Angiotensin II stimulates angiogenesis in chorio-allantoic membrane of the chicken embryo. *European Journal of Pharmacology* **195** 305-306.

Lipke DW, Thomas RL & Olson KR 1987 Characterization of angiotensin-converting enzyme in the gills of rainbow trout, *Salmo gairdneri* (Richardson). *Fish Physiology and Biochemistry* **3** 91-97.

Lopez E, Tisserand-Jochem E-M, Vidal B, Milet C, Lallier F & MacIntyre I 1984 Are corpuscles of Stannius the parathyroid glands in fish? Immunocytochemical and ultrastructural arguments. In *Endocrine control of Bone and Calcium Metabolism, Vol. 8B*, pp. 283-286. Eds DV Cohn, JT Potts & T Fujita. Amsterdam: Elsevier/North-Holland Biomedical Press.

Lumbers ER 1971 Activation of renin in human amniotic fluid by low pH. *Enzymologia* **40** 329-336.

Lund-Johansen P & Omvik P 1984 Long-term haemodynamic effects of enalapril (alone and in combination with hydrochlorothiazide) at rest and during exercise in essential hypertension. *Journal Hypertension* **2 (Suppl. 20)** 49-56.

Ma SWY & Copp DH 1978 Purification, properties and action of a glycopeptide form the corpuscle of Stannius which affects calcium metabolism in the teleost. In *Comparative Endocrinology*, pp 283-286. Eds PJ Gaillard *et al.* Amsterdam: Elsevier.

Mandich A & Massari A 1994 Presence of angiotensin II immunoreactivity in the ovary of the rainbow trout, *Salmo gaidneri*. *General and Comparative Endocrinology* **96** 1-5.

Marciniszyn J, Hartsuck JA & Tang J 1976 Mode of inhibition of acid proteases by pepstatin. *Journal of Biological Chemistry* **251** 7088-7094.

Marra LE, Butler DG, Zhang DH, Oudit GY & Youson JH 1998 Corpuscles of Stannius and stanniocalcin-like immunoreactivity in the white sucker (*Catostomus commersoni*): evidence for a new cell-type. *Cell and Tissue Research* **294** 155-164.

Marsigliante S, Muscella A, Vilella S, Nicolardi G, Ingrosso L, Ciardo V, Zonno V, Vinson GP, Ho MM & Storelli C 1995 A monoclonal antibody to mammalian angiotensin II AT_1 receptor recognizes one of the angiotensin II receptor isoform expressed by the eel (*Anguilla anguilla*). *Journal of Molecular Endocrinology* **16** 45-56.

Marsigliante S, Verri T, Barker S, Jimenez E, Vinson GP & Storelli C 1994 Angiotensin II receptor subtypes in eel (*Anguilla anguilla*). *Journal of Molecular Endocrinology* **12** 61-69.

Messerli FH, Weber MA & Brunner HR 1996 Angiotensin II receptor inhibition: A new therapeutic principle. *Archives of Internal Medicine* **156** 1957-1966.

Milet C, Buscaglia M, Chartier MM, Martelly E & Lopez E 1984. Comparative effect of Stannius corpuscles extracts and 1-34 hPTH in the Anura, *Xenopus laevis*. *General and Comparative Endocrinology* **53** 497.

Miyazaki M, Komori T, Okunishi H & Toda N 1979 Inhibition of dog renin by pepstatin A. *Japanese Circulation Journal* **43** 818-823.

Moore AF, Strong JH & Buckley JP 1981 Cardiovascular actions of angiotensin in the fowl (*Gallus domesticus*) II. Angiotensin analog agonists and antagonists. *Research Communications in Chemical Pathology and Pharmacology* **32** 447-457.

Murakami E, Eggena P, Barret JD & Sambhi MP 1984 Heterogeneity of renin substrate released from hapatocytes and in brain extracts. *Life Sciences* **34** 385-392.

Nakajima T, Nakayama T & Sokabe H 1971 Examination of angiotensin-like substances from renal and extrarenal sources in mammalian and nonmammalian species. *General and Comparative Endocrinology* **17** 458-466.

Nakamura Y, Hishimura H & Khosla MC 1982 Vasodepressor action of angiotensin in conscious chickens. *American Journal of Physiology* **243** H456-H462.

Nakayama T, Nakajima T & Sokabe H 1973 Comparative studies on angiotensin. III. Structure of fowl angiotensin and its identification by DNS-method. *Chemical and Pharmaceutical Bulletin* **21** 2085-2087.

Nakayama T, Nakajima T & Sokabe H 1977 Comparative studies on angiotensin. IV. Structure of snake (*Elaphe climocophora*) angiotensin. *Chemical and Pharmaceutical Bulletin* **25**, 3255-3260.

Nishimura H 1980a Comparative endocrinology of renin and angiotensin. In *The Renin-Angiotensin System*, pp. 29-77. Eds JA Johnson & Anderson RR. New York: Plenum Press.

Nishimura H 1980b Evolution of the renin-angiotensin system. In Evolution of vertebrate endocrine systems. Eds PKT Pang & A Epple. *Graduate Studies, Texas Tech University* **21**, 373-404.

Nishimura H 1985 Evolution of the renin-angiotensin system and its role in control of cardiovascular function in fishes. In *Evolutionary Biology of Primitive Fishes*, pp

275-293. Eds RE Foreman, A Gorbman, JM Dodd, R Olsson. New York: Plenum Press.

Nishimura H & Qin Z 1994 Control of renin release in teleosts. (abstract) *Physiology* **37** A34.

Nishimura H & Sawyer WH 1976 Vasopressor, diuretic, and natriuretic responses to angiotensins by the American eel, *Anguilla rostrata*. *General and Comparative Endocrinology* **29** 337-348.

Nishimura H, Norton VM & Bumpus FM 1978 Lack of specific inhibition of angiotensin II in eels by angiotensin antagonsts. *American Journal of Physiology* **235** H95-103.

Nishimura H & Ogawa M 1973 The renin-angiotensin system in fishes. *American Zoologist* **13** 823-838.

Nishimura H, Ogawa M & Sawyer WH 1973 Renin-angiotensin system in primitive bony fishes and a holocephalian. *American Journal of Physiology* **224** 950-956.

Nishimura H, Oguri M, Ogawa M, Sokabe H & Imai M 1970 Absence of renin in kidneys of elasmobranches and cyclostomes. *American Journal of Physiology* **218** 911-915.

O'Toole LB, Armour KJ, Decourt C, Hazon N, Lahlou B & Henderson IW 1990 Secretory patterns of 1-alpha-hydroxycorticosterone in the isolated perfused interrenal gland of dogfish, *Scyrhiolinus canicula*. *Journal of Molecular Endocrinology* **5** 55-60.

Ogawa M 1968 Osmotic and ionic regulation in goldfish following removal of the corpuscles of Stannius or the pitutary gland. *Canadian Journal of Zoology* **46** 669-676.

Ogawa M 1977 Evolution of juxtaglomerular apparatus. *Gunma Symposium on Endocrinology* **14** 75-81.

Ogawa M & Sokabe H 1982 Hypocalcemic effect of homologous angiotensin-like substances produced by the renin-like enzyme in the corpuscle of Stannius in the Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology* **47** 36-41.

Ogawa M & Oguri M 1978 Occurrence of the renin-angiotensin system in the vertebrates. *Japanese Heart Journal* **19** 791-798.

Ogawa M, Oguri M, Sokabe H & Nishimura H 1972 Juxtaglomerular apparatus in the vertebrates. *General and Comparative Endocrinology* **Suppl. 3**, 374-381.

Oguri M 1966 Electron-microscopic observtions on the corpuscles of Stannius in goldfish. *Bulletin of the Japanese Society of Scientific Fisheries* **32** 903-908.

Oguri M 1978 Presence of juxtaglomerular cells in the holocephalian kidney. *General and Comparative Endocrinology* **26** 170-173.

Oguri M, Ogawa M & Sokabe H 1970 Absence of juxtaglomerular cells in the kidneys of chrondrichthyes and Cyclstomi. *Bulletin of the Japanese Society of Scientific Fisheries* **36** 881-884.

Oguri M & Sokabe H 1968 Juxtaglomerular cells in the teleost kidneys. *Bulletin of the Japanese Society of Scientific Fisheries* **34**, 882-888.

Olson KR 1992 Blood and extracellular fluid volume regulation. In *Fish Physiology, The Cardiovascular system Vol. XIIB* , pp. 135-254. Eds WS Hoar, DJ Randal & AP Farrell. New York: Academic Press.

Olson KR, Conklin DJ, Weaver L Jr, Duff DW, Herman CA, Wang X & Conlon JR 1997 Cardiovascular effects of homologous bradykinin in rainbow trout. *American Journal of Physiology* **272** R1112-11120.

Olson KR, Lipke DW, Kullman D, Evan AP & Ryan JW 1989 Localization of angiotensin-converting enzyme in the trout gill. *Journal of Experimental Zoology* **250** 109-115.

Ondetti MA & Cushman DW 1982 Enzymes of the renin-angiotensin system and their inhibitors. *Annual Review of Biochemistry* **51** 283-308.

Ondetti MA, Rubin B & Cushman DW 1977 Design of specific inhibitors of angiotensin converting enzyme: New class of orally active antihypertensive agents. *Science* **196** 441-443.

Opdyke DF & Holcombe R 1976 Response to angiotensin I and II and to the AI-converting enzyme inhibitor in a shark. *American Journal of Physiology* **231** 1750-1753.

Oudit GY & Butler DG 1995 Angiotensin II and cardiovascular regulation in a freshwater teleost, *Anguilla rostrata* LeSueur. *American Journal of Physiology* **269** R726-735.

Panthier JJ, Foot S, Chambreaud B, Strosberg AD, Corvol P & Rougeon F 1982 Complete amino acid sequence and maturation of mouse submaxillary gland renin precursor. *Nature* **298** 90-92.

Parsons JA, Gray D, Rafferty B & Zanelly JM 1978 Evidence for a hypercalcemic factor in the fish pituitary, immunologically related to mammalian parathyroid hormone.

In *Endocrinology of Calcium Metabolism*, pp. 111-114. Eds DH Copp & RV Talmage. Amstedam: Excerpta Medica.

Peach MJ 1977 Renin-angiotensin system: biochemistry and mechanism of action. *Physiological Reviews* **57** 313-370.

Peach MJ, Bumpus FM & Khairallah PA 1971 Release of adrenal catecholamines by angiotensin I. *Journal of Pharmacology and Experimental Therapeutics* **176** 366-376.

Perrott MN & Balment RJ 1990 The renin-angiotensin system and the regulation of plasma cortisol in the flounder, *Platichtyys flesus*. *General and Comparative Endocrinology* **78** 414-420.

Perrott MN & Balment RJ 1985 Drinking behavior and the renin angiotensin system in euryhaline and stenohaline fish (abstract). *Journal of Endocrinology* **107** 193.

Perrott MN, Grierson CE, Hazon N & Balment RJ 1992 Drinking behavior in sea water and fresh water teleosts, the role of the renin-angiotensin system. *Fish Physiology and Biochemistry* **10** 161-168.

Polanco MJ, Mata MI, Agapito MT & Recio JM 1990 Angiotensin-converting enzyme distribution and hypoxia response in mammals, bird, and fish. *General and Comparative Endocrinology* **79** 240-245.

Poulsen K, Burton J & Haber E 1973 Competitive inhibitors of renin. *Biochemistry* **12** 3877-3882.

Rasquin P 1956 Cytoogical evidence for a role of the corpuscles of Stannius in the osmoregulation of teleosts. *The Biological Bulletin* **111** 399-409.

Rotmensch HH, Vlasses PH and Ferguson RK 1988 Angiotensin-converting enzyme inhibitors. *Medical Clinics of North America* **72** 399-425.

Rowe BP, Grove KL, Sayler DL & Speth RC 1991 Discrimination of angiotensin II receptor subtypes in the rat brain using nonpeptidic receptor antagonists. *Regulatory Peptides* **33** 45-53.

Ryan GB, Alcorn D, Coghlan JP & Scoggins BA 1979 The granulated peripolar epithelial cell: a potential secretory component of the renal juxtaglomerular complex. *Nature* **277** 655-656.

Salzet M, Verger-Bocquet M, Wattez C & Malecha J 1992 Evidence for angiotensin-like molecules in the central nervous system of the leech *Theromyzon tessulatum*

(O.F.M.). A possible diuretic effect. *Comparative Biochemistry and Physiology* **101A** 83-90.

Salzet M, Wattez C, Baert J-L & Malecha J 1993 Biochemical evidence of angiotensin II-like peptide and proteins in the brain of the rhynchobdellid leech *Theromyzon tessulatum*. *Brain Research* **631** 247-255.

Sandberg K, Bar M, Ji H, Markorck A, Millan MA & Catt KI 1990 Angiotensin II-induced calcium mobilization in oocytes by signal transfer through gap junctions. *Science* **249** 298-301.

Sandberg K, Ji H & Millan MA 1992 Amphibian myocardial angiotensin II receptors are distinct from mammalina AT_1 and AT_2 receptor subtypes. *FEBS Letters* **284** 281-283.

Sandor T, Vinso GP, Chester-Jones I, Henderson IW & Whitehouse BJ 1966 Biogenesis of corticosteroids in the European eel (*anguilla anguilla* L.). *Journal of Endocrinology* **34** 105-115.

Saruta T, Cook R & Kaplan NM 1972 Adrencortical steroidogenesis: studies on the mechanism of action of angiotensin and electrolytes. *Journal of Clinical Investigation* **51** 2239-2245.

Schaffenburg CA, Haas E & Goldblatt H 1960 Concentration of renin in kidneys and angiotensinogen in serum of various species. *American Journal of Physiology* **199** 788-792.

Schiffrin EL & Genest J 1983 Tonin-angiotensin II system. In *Hypertension*, pp. 309-319. Eds J Genest, O Kuchel, P Hamet & M Cantin. New York: McGraw-Hill.

Sen S, Smerby RR & Bumpus FM 1969a Antihypertensive effect of an isolated phospholipid. *American Journal of Physiology* **214** 337-341.

Sen S, Smerby RR & Bumpus FM 1969b Plasma renin activity in hypertensive rats after treatment with renin preinhibitor. *American Journal of Physiology* **216** 499-503.

Shoumura S, Iwasaki Y, Ishicaki N, Emura S, Hayashi K, Yamahira T, Shoumura K & Isono H 1983 Origin of autonomic fibres innervating the parathyroid gland in the rabbit. *Acta Anatomica* **115** 289-295.

Smith RD, Chiu AT, Wong PC, Herblin WF & Timmermans PBMWM 1992 Pharmacology of nonpeptide angiotensin II receptor antagonists. *Annual Review of Pharmacology and Toxicology* **32** 135-165.

So YP & Fenwick JC 1977 Relationship between net ^{45}calcium influx across a perfused isolated eel gill and the development of post-stanniectomy hypercalcemia. *Journal of Experimental Zoology* **200** 259-264.

So YP & Fenwick JC 1979 The *in vivo* and *in vitro* effects of Stannius corpuscles extract on the branchial uptake of ^{45}Ca in stanniectomized North American eel (*anguilla rostrata*). *General and Comparative Endocrinology* **37** 143-149.

Sokabe H, Nishimura H, Ogawa M & Oguri M 1970 Determination of renin in the corpuscles of Stannius of the teleost. *General and Comparative Endocrinology* **14** 510-516.

Sokabe H & Ogawa M 1974 Comparative studies of the juxtaglomerular apparatus. *International Review of Cytology* **37** 270-327.

Sokabe H, Ogawa M, Oguri M & Nishimura H 1969 Evolution of the juxtaglomerular apparatus in the vertebrate kidneys. *Texas Reports of Biology and Medicine* **27** 867-885.

Sokabe H, Oide H, Ogawa M & Utida S 1973 Plasma renin activity in Japanese eels (*anguilla japonica*) adapted to seawater or in dehydration. *General and Comparative Endocrinology* **21** 160-167.

Swanson LW, Marshall GR, Needleman P & Sharpe LG 1973 Characterization of central angiotensin II receptors involved in elicitation of drinking in the rat. *Brain Research* **49** 441-446.

Swarup K & Ahmad N 1978 Studies of corpuscles of Stannius of Notopterus notopterus in relation to calcium and sodium rich environments. *National Academy of Science Letters USA* **1**, 239-240.

Szelke M, Leckie BJ, Hallett A, Jones OM, Sueiras J, Atrash B & Lever AF 1982 Potent new inhibitors of human renin. *Nature* **299** 555-557.

Szelke M, Leckie BJ, Tree M, Brown A, Grant J, Hallett A, Hughes M, Jones DM & Lever AF 1982 H-77: a potent new renin inhibitor. *In vitro* and *in vivo* studies. *Hypertension* **4 (Suppl. 2)** 59-69.

Takei Y, Hasegawa Y, Watanabe TX, Nakajima K & Hazon N 1993a New angiotensin I isolated from a reptile, *Alligator mississippiensis. General and Comparative Endocrinology* **90** 214-219.

Takei Y, Hasegawa Y, Watanabe TX, Nakajima K & Hazon N 1993b A novel angiotensin I isolated from an elasmobranch fish. *Journal of Endocrinology* **139** 281-285.

Takei Y, Hirano T & Kobayashi H 1979 Angiotensin and water intake in the Japanese eel, *Anguilla japonica*. *General and Comparative Endocrinology* **38** 466-475.

Takei Y, Itahara Y, Butler DG, Watanabe TX & Oudit GY 1998 Tetrapod-type [Asp^1] angiotensin Is present in a holostean fish, *Amia calva*.*General and Comparative Endocrinology* **110** 140-146.

Takei Y, Okubo J & Yamaguchi K 1988 Effects of cellular dehydration on drinking and plasma angiotensin II level in the eel, *Anguilla japonica*. *Zoological Science* **5** 43-51.

Takemoto Y, Nakajima T, Hasegawa Y, Watanabe TX, Kumagae H & Sakakibara S 198. Chemical structure of angiotensin formed by incubating plasma with the kidney and the corpuscles of Stannius in the chum salmon, *Oncorhynchus keta*. *General and Comparative Endocrinology* **51** 219-227.

Tewksbury DA, Dart RA & Travis J 1981 The amino terminal amino acid sequence of human angiotensinogen. *Biochemical and Biophysical Research Communications* **99** 1311-1315.

Timmermans PBMWM, Benfield P, Chiu AT, Herblin WF, Wong PC & Smith RD 1992 Angiotensin II receptors and functional correlates. *American Journal of Hypertension* **5** 2215-2355.

Timmermans PBMWM, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye J-AM & Smith RD 1993 Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacological Review* **45** 205-251.

Turker RK, Hall MM, Yamamoto M, Sweet CS & Bumpus FM 1972 A new, long-lasting competitive inhibitor of angiotensin. *Science* **177** 1203-1204.

Uemura H, Tezuka Y, Hasegawa C & Kobayashi H 1994 Immunohistochemical investigation of neuropeptides in the central nervous system of the amphioxus, *Branchiostoma belcheri*. *Cell and Tissue Research* **277** 279-287.

Umezawa H, Aoyagi T, Morishima H, Matsuzaki M, Hamada M & Tekeuchi T 1970 Pepstatin, a new pepsin inhibitor produced by actinomycetes. *Journal of Antibiotics* **23** 259-262.

Unsicker K, Plonius T, Lindmar R, Lffeleholz K & Wolf U 1977 Catecholamines and 5-hydroxytryptamine in corpuscles of Stannius of the salmonid, *Salmo irideus* L. A study correlating electron microscopical, histochemical and chemical findings. *General and Comparative Endocrinology* **31** 121-132.

Urata H, Kinoshinto A, Misono KS, Bumpus FM & Husain A 1990 Identification of a highly specific chymase as the major angotensin-II forming enzyme in the heart. *Journal of Biological Chemistry* **265** 22348-22357.

Vanhoutte PM 1989 Endothelium and control of vascular function: State of the art lecture. *Hypertension* **13** 658-667.

Verger-Bocquet M, Wattez C, Salzet M & Malecha J 1992 Immunocytochemical identification of peptidergic Neurons in compartment 4 of the supraesophageal ganglion of the leech *Theromyzon tessulatum* (O.F.M.). *Canadian Journal of Zoology* **70** 856-865.

Viswanathan M, Tsutsumi K, Correa FM & Saavedra JM 1991 Changes in expression of angiotensin receptor subtypes in the rat aorta during development. *Biochemical and Biophysical Research Communications* **179** 1361-1367.

Wagner GF, Fargher RC, Milliken C, McKeown BA & Copp DH 1993 The gill calcium transport cycle in rainbow trout is correlated with plasma levels of bioactive, not immunoreactive, stanniocalcin. *Molecular and Cellular Endocrinology* **93** 185-191.

Wagner GF, Fenwick JC, Milliken C, Park CM, Copp DH & Friesen HG 1988 Comparative biochemistry and physiology of teleocalcin from sockeye and coho salmon. *General and Comparative Endocrinology* **72** 237-246.

Wendelaar-Bonga SE & Greven JAA 1975 A second cell type in Stannius bodies of two euryhaline teleost species. *Cell and Tissue Research* **159** 287-290.

Wendelaar-Bonga SE, Greven JAA & Veenhuis M 1977 Vascularization, innervation, and ultrastructure of the endocrine cell types of Stannius corpuscles in the teleost, *Gasterosteus aculeatus*. *Journal of Morphology* **153** 225-244.

West NH 1989 Methods for measuring blood flow and distribution in intermittently ventilating and diving vertebrates. In *Techniques in comparative Respiratory Physiolgy-An experimental approach*, pp. 171-193. Eds CR Bridges & PJ Butler. Cambridge: Cambridge Press.

Whitebread S, Mele M, Kamber B & DeGasparo M 1989 Preliminary biochemical chracterization of two angiotensin II receptor subtypes. *Biochemical and Biophysical Research Communications* **163** 284-291.

Wilson JX 1984 Coevolution of the renin-angiotensin system and the nervous control of blood circulation. *Canadian Journal of Zoology* **62** 137-147.

Wong PC, Hart SD, Duncia JV & Timmermans PBMWM 1991 Nonpeptide angiotensin II receptor antagonists. XIII. Studies with DuP 753 and EXP3174 in dogs. *European Journal of Pharmacology* **202** 323-330.

Wong PC, Hart SD, Zaspel AM, Ciu AT, Ardecky RJ, Smith RD & Timmermans PBMWM 1990 Functional studies of nonpeptide angiotensin II receptor subtype-specific ligands: DuP 732 (AII-1) and PD123177 (AII-2). *Journal of Pharmacology and Experimental Therapeutics* **255** 584-592.

Wyllie AH, Kerr, J FR & Currie AR 1980 Cell death: The significance of apoptosis. *International Review of Cytology* **68** 251-306.

Yamada C & Kobayashi H (1987). Immunoreactive angiotensin II in the corpuscle of Stannius of the rainbow trout, *Salmo gairdneri. Zoological Science* **4** 387-390.

Yamamoto M, Turker RK, Khairallah PA & Bumpus FM 1972 Potent competitive antagonist of angiotensin II. *European Journal of Pharmacology* **18** 316-322.

Youson JH & Butler DG 1976 Fine structure of the adrenocortical homolog and the corpuscles of Stannius of *Amia calva L. Acta Zoologica (Stockholm)* **57**, 217-230.

Youson JH, Butler DG & Chan ATC 1976 Identification and distribution of the adrenocortical homolog, chromaffin tissue and corpuscles of Stannius in *amia calva* L. *General and Comparative Endocrinology* **29** 198-211.

Zehr JE, Standen DJ & Cipolle MD 1981 Characterization of angiotensin pressor responses in the turtle, *Pseudemys scripta*. *American Journal Physiology* **240** R276-R281.

Zuker A & Nishimura H 1981 Renal responses to vasoactive hormones in the aglomerular toadfish, *Opsanus tau*. *General and Comparative Endocrinology* **43** 1-9.