# VASOACTIVITY OF THE CORONARY ARTERY IN *SALMO GAIRDNERI*

by

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VASOACTIVITY OF THE CORONARY ARTERY IN SALMO GAIRDNERI

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**ABSTRACT**

Coronary blood flow is essential in order to supply oxygen and substrates to the heart muscle. Salmo gairdneri represents a transitional phase in the evolution of cardiac myoarchitectural design. This study is a preliminary survey of vascular smooth muscle cell receptors which may be involved in the control of coronary circulation in Salmo gairdneri. Responses of arterial rings from the coronary artery to various pharmacologically active agents were measured using an isometric force transducer. During the course of the experiment, the endothelium was stripped from the coronary artery.

Adenosine, adenosine diphosphate (ADP) and adenosine triphosphate (ATP) caused vasoconstriction indicating the presence of purinergic receptors. This excitatory response to adenosine is not observed in the mammalian coronary vasculature. Theophylline blocked the action of adenosine, ADP and ATP, although it blocks only the action of adenosine in the mammalian system. I propose a model for Salmo gairdneri in which a single, generalized purinergic receptor is present as opposed to the specialized subtypes of purinergic receptors found in mammals.

Adrenaline and noradrenaline caused vasoconstriction and vasodilatation indicating a mixed population of $\alpha$- and $\beta$-adrenergic receptors. Studies with a $\beta_1$-antagonist suggest the presence of $\beta_1$-adrenergic receptors.

Acetylcholine caused vasoconstriction indicating the presence of muscarinic receptors. Serotonin caused weak vasodilatation.

Nitroglycerine, an endothelium-derived relaxing factor (EDRF) analogue, and sodium nitroprusside, a similar nitrodilator, caused relaxation indicating that the coronary artery can respond to EDRFs.

Steelhead trout, an anadromous population of Salmo gairdneri, have a higher frequency of arteriosclerotic lesions than rainbow trout, a freshwater population of Salmo gairdneri. No difference was seen in the response to acetylcholine and adenosine in these two populations. Responses to ATP and ADP in steelhead trout were significantly greater than in rainbow trout. However, no difference was seen in the responses to acetylcholine, adenosine and isoproterenol in fish with lesions compared to fish without lesions. Therefore, the difference in the ATP and ADP responses probably cannot be attributed to the presence of lesions. Sensitivity to the agents tested was not different between steelhead and rainbow trout except for adenosine which was more potent in rainbow trout.

**Acknowledgements**

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## Dedication

to Mark and my family

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# Introduction

## Coronary vasculature

The coronary circulation supplies oxygen and substrates to the working cardiac muscle. Increased oxygen demand in the myocardium due to increased work can be met either by increasing myocardial oxygen extraction from arterial blood or by increasing the blood flow to the muscle. In mammals, myocardial oxygen extraction at rest is 75% and is already high compared with 25% oxygen extraction in skeletal muscle. Consequently, there is little potential for increasing oxygen extraction from the coronary circulation (Haeusler and Holck, 1982). Preliminary experiments show that this may also be true for rainbow trout (Farrell et al., 1988). Therefore, changes in blood flow with oxygen demand are critical to avoid anaerobic metabolism in the myocardium. In mammals, the ability of the heart muscle to function under anaerobic conditions is limited and only briefly sustainable, therefore, there is a close relationship between myocardial oxygen consumption and coronary blood flow (Shipley and Gregg, 1945; Eckenhoff et al., 1947; Marchetti et al., 1966; Lichtlen et al., 1971; Berne and Rubio, 1979). The anaerobic capacity of the fish heart is also limited (Driedzic et al., 1983) making coronary blood flow to the myocardium important but different from mammals because of the myoarchitectural design of the fish heart.

The presence or absence of coronary vasculature and the structure of the myocardium in teleosts depends upon the activity level of the species. Sluggish benthic species of teleosts such

as plaice, cod and saithe lack a coronary circulation (Grant and Regnier, 1926; Santer and Cobb, 1972). Their myocardium is trabeculated and spongelike in appearance, sandwiched between the continuous external epicardial and inner endocardial layers (Santer, 1976). The ventricle is supplied with oxygen and metabolites from the venous blood bathing the lumen of the heart.

Active pelagic species of teleosts have a more specialized cardiac design. The innermost layer is covered by an outer compact layer which is perfused by a true coronary vasculature (Tota, 1983). The coronary arteries branch from the hypobranchial arteries which arise from the efferent branchial arteries of the second, third and fourth gill arches (Parker and Davis, 1899). Coronary arteries in fish are homologous to mammalian coronary arteries, although the branchial origin of the fish coronary artery is distant compared with the proximal origin of the mammalian coronary artery at the aortic arch (Grant and Regnier, 1926). The coronary circulation delivers oxygenated blood to the compact layer while oxygen is supplied to the inner spongy layer by the venous blood in the ventricle. This 'mixed type' myocardium (Tota, 1983) is present in salmonids, tuna, anchovy (Santer and Greer Walker, 1980), marlin (Davie and Daxboeck, 1984), carp (Bass et al., 1973), powan and scorpionfish (Stewart, 1974). In salmonids and tuna, the compact myocardium constitutes 25 to 76% of the ventricular muscle mass (Santer and Greer Walker, 1980). In Salmo gairdneri, the compact myocardium makes up approximately 40% of the ventricle (Farrell et al., 1988). This arrangement differs from the mammalian heart in

which the myocardium is mostly compact tissue and only the innermost endocardial layer, which is very thin, is spongy (Tota, 1983). Salmo gairdneri is an example of a teleost species which has a well developed coronary circulation and, as such, reresents a transitional phase in the evolution of cardiac design. Salmo gairnderi is the animal model used in this study.

The importance of coronary blood flow for the functioning of the fish heart has also been demonstrated. Ligation of the coronary artery decreases the critical swimming speed ($U_{crit}$) of chinook salmon (Oncorhynchus tshawytscha) by 35.5% (Farrell and Steffensen, 1987). $U_{crit}$ is a measure of the maximum aerobic capacity of a fish (Webb, 1971; Jones and Randall, 1978) which, in part, depends on cardiac performance. While decreased swimming performance does not cause immediate death in fish, coronary blood flow in mammals is not only important but essential to sustain life.

There are two components to the coronary vasculature that need to be considered to understand the regulation of this vascular bed. These components are the large epicardial arteries and arterioles and it is the latter which contribute 70-80% of the resistance to flow in mammals (Grega, 1987). The arterioles are of primary importance in the regulation of coronary blood flow, as in other vascular beds. Nonetheless, there has been recent interest in the contribution of the large arteries to regulation under physiological and pathological conditions. Up until the last decade, the large arteries were thought of as fixed-diameter conduits for blood transport and, therefore, could

not regulate coronary flow. Researchers were aware that atherosclerotic lesions in the large arteries reduced blood flow to the ventricles, but they were not aware that large arteries can contract to close off entire lengths of vessel. The recognition that vasospasm is an important contributor to coronary artery disease has stimulated interest in control of the large coronary artery (Kalsner, 1982). Large vessels are innervated by the autonomic nervous system and appear to have receptor types and subtypes similar to those found in small arteries. However, the relative densities of these receptors can vary between the large and small arteries (Grega, 1987). Consequently, large coronary arteries and resistance vessels can respond differently to the same pharmacological and physiological stimuli. For the purposes of this study, a review of the effects of different pharmacological agents on coronary circulation will be restricted, mainly, to studies of the large arteries in mammals.

The large coronary artery consists of three layers. The intimal layer lining the lumen is made up of a single layer of endothelial cells, a subendothelial basement membrane and the internal elastic lamina made up of elastic fibres. The medial layer contains the smooth muscle fibres which are bound by the internal elastic lamina and the external elastic lamina which separates the media from the outermost adventitial layer. The adventitial layer is composed of collagen and elastin fibres and contains the nerve fibres (Rhodin, 1980). The mammalian coronary artery is innervated by cholinergic and adrenergic nerve fibres.

The regulation of the coronary circulation is a complex process involving physical (eg., perfusion pressure, extravascular compression), neural (eg., adrenergic, cholinergic, purinergic), humoral (eg., catecholamines, serotonin, bradykinin, histamine) and metabolic factors (eg., adenosine, oxygen, pH) which interact to control blood flow (Feigl, 1983). The mechanisms by which these factors cause vasoconstriction and vasodilatation of blood vessels are further complicated by the involvement of the endothelial cell.

Endothelium-mediated responses

Endothelial cells react to mechanical force (Lansman, 1988) as well as neural and humoral factors by releasing vasodilator or vasoconstrictor substances and, as such, contribute to the local control of vascular resistance. Furchgott and Zawadzki (1980) demonstrated that vasodilatation in rabbit aorta, in response to acetylcholine, is endothelium-dependent and is mediated by endothelium-derived relaxing factors (EDRFs). Conflicting results were observed for the effect of acetylcholine on coronary arteries and other blood vessels with *in vitro* and *in vivo* studies. Acetylcholine causes vasoconstriction *in vitro* (Foley et al., 1979; Ito et al., 1979;) but vasodilatation *in vivo* (Blesa and Ross, 1970; Gross et al., 1981; Cox et al., 1983). The techniques used for the *in vitro* studies resulted, inadvertently, in the mechanical stripping of the intimal surface of the blood vessel, showing that the direct action of acetylcholine on the vascular smooth muscle is excitatory. Denudation of the vessel did not occur *in vivo* illustrating that

the endothelium-mediated response to acetylcholine is inhibitory for these blood vessels.

Nitric oxide has been identified as an EDRFs (Palmer et al., 1987). Ammonia, a potent vasodilator, has also been suggested to be an EDRF (Vanhoutte, 1988). Nitric oxide is very labile with an extremely short half-life. Vasoconstriction has also been shown to be dependent on, or enhanced by the endothelium (De Mey and Vanhoutte, 1982; Rubanyi and Vanhoutte, 1985) which produces endothelin, a potent vasoconstrictor peptide (Yanagisawa et al., 1988). Endothelin is long lasting and may contribute to the long-term regulation of vascular tone, whereas the short-lived EDRFs may contribute to rapid local control.

Adrenergic receptors

Adrenergic stimulation by the sympathetic nervous system or circulating catecholamines is a major determinant of vascular tone in both large and small coronary arteries in mammals (Silver et al., 1982; Vatner, 1985). In the mammalian system, the large number of *in vitro* and *in vivo* studies provide conflicting information on adrenergic receptor predominance because of the inherent technical difficulties. First, α and β-adrenergic receptors have opposing actions. Second, large and small coronary arteries respond differently to the same physiological and pharmacological stimuli (Malindzak, 1982). Third, there is adrenergic stimulation of myocardial metabolism which also affects coronary resistance (Berne, 1964; Hillis and Braunwald, 1978). General statements can be made about the large and small coronary arteries, individually, however.

The large coronary arteries are densely innervated with sympathetic adrenergic nerve fibres and the control of these vessels appears to be determined primarily by neural and humoral stimulation (Berne et al., 1965; Feigl, 1967; Malindzak et al., 1978a, 1978b; Gerova et al., 1979a, 1979b). The large coronary artery in mammals is predominantly vasoconstrictory (Zuberbuhler and Bohr, 1965; Mekata and Niu, 1969; Anderson et al., 1972; Trinker, 1973; Ross, 1976; Huzulakova et al, 1978; Kelley and Feigl, 1978; Malindzak et al., 1978a, 1978b; Gerova et al., 1979a; Vatner et al., 1974). The large coronary artery contributes little to the control of vascular resistance in the coronary circulation under normal conditions (Fam and McGregor, 1968; Winbury et al., 1969; Malindzak et al., 1978a, 1978b) but may play a potentially important constrictory role under pathological conditions (Yasue et al., 1974).

The small coronary arteries, or the coronary resistance vessels, are less densely innervated than the large coronary artery and appear to be controlled primarily by the metabolic activity of the myocardium, in addition to neural and humoral factors (Malindzak, 1982). Vasodilatation predominates in the resistance vessels (Bayer et al., 1974)., α-receptor-mediated vasoconstriction can occur at rest (Vatner et al.,1974), during exercise (Aung-Din et al., 1981; Longhurst et al., 1985) and under pathological conditions (Heusch and Deussen, 1983) in the coronary circulation. However, β-adrenergic receptor-mediated metabolic vasodilatation usually overrides α-adrenergic stimulation in the coronary circulation. The precise role of α-

adrenergic stimulation in the regulation of the coronary circulation is still unclear.

Alpha$_1$- and $\alpha_2$-adrenergic receptors are present in canine large coronary arteries (Woodman and Vatner, 1985) but there has been some debate over which type predominates (Holtz et al., 1982; Rimele et al., 1983; Heusch et al., 1984). Endothelium-dependent relaxation in response to noradrenaline in the pig and dog coronary arteries has been attributed to $\alpha_2$-adrenergic receptor activation of endothelial cells (Cocks and Angus, 1983).

Beta$_1$- and $\beta_2$-adrenergic receptors are present in mammals. Beta$_1$- dominate over $\beta_2$-receptor subtypes in the large coronary artery of swine (Drew and Levy, 1972; Johansson, 1973), rabbits (de la Lande et al., 1974), kittens (Cornish and Miller, 1975), cows (Vatner et al., 1986) and humans (Berkenboom et al., 1987). Beta$_2$-adrenergic receptors are the dominant subtype in pigs (Bayer et al., 1974). Activation of $\beta_1$-adrenergic receptors on smooth muscle cells causes endothelium-independent relaxation of dog coronary arteries, that is, the removal of the endothelium does not diminishes the relaxation response to $\beta_1$ agonists. Similarly, $\beta_2$-receptor activation was not affected by the removal of the endothelium (MacDonald et al., 1987).

Cholinergic receptors

Acetylcholine, a muscarinic receptor agonist generally associated with cholinergic nerve fibers of the parasympathetic nervous system, has profound effects on the coronary artery. There are, however, species differences in the response to acetylcholine. In dogs (Furchgott and Zawadzki, 1980; Cox et

al., 1983), monkeys (Toda, 1983) and rabbits (Griffith et al., 1984) acetylcholine induces endothelium-mediated relaxation of the coronary artery. In human coronary arteries, acetylcholine rarely causes endothelium-dependent relaxations in vivo or in vitro (Toda, 1983; Ginsburg et al., 1984; Forstermann et al., 1986). Endothelium-independent vasoconstriction is observed in pigs (Ito et al., 1979), cats (Foley et al., 1979), rabbits (de la Lande et al., 1974) and baboons (Young and Vatner, 1986). Acetylcholine causes endothelium-independent vasoconstriction of the descending aorta of the turtle. Acetylcholine also causes vasoconstriction in the descending aorta of the frog with and without endothelium, but the sensitivity to acetylcholine is enhanced by the absence of the endothelial layer. The descending aorta of the cayman is relaxed by acetylcholine in the presence of the endothelium, but is unresponsive to acetylcholine in the absence of endothelium (Miller and Vanhoutte, 1986).

The effects of parasympathetic nerve stimulation are not clearly understood. Studies have demonstrated that parasympathetic nerve endings are present in the adventitia of large and small coronary arteries (Denn and Stone, 1976; Dolezel et al., 1978). Vagal stimulation of small arteries results in vasodilation in dogs (Reid et al., 1985) but has no effect the large coronary artery in dogs (Gerova et al., 1979b). There is some controversy over the extent to which cholinergic fibres are involved in the regulation of the large coronary arteries (Young and Vatner, 1986). Vanhoutte et al. (1981) suggest that cholinergic nerve terminals may serve to modulate the release of

adrenergic transmitters by presynaptic inhibition.

Purinergic receptors

Purinergic receptors which are activated by adenosine and the adenine nucleotides (AMP, ADP and ATP) are probably involved in the local regulation of blood flow (Paddle and Burnstock, 1974). Adenosine is released from the working heart and activates $P_1$-purinergic receptors (Burnstock, 1978) on the vascular smooth muscle cells and, in some cases, the endothelial cells to cause an inhibitory response. Adenosine generally causes endothelium-independent relaxation of mammalian vascular beds (Berne et al., 1983). The inhibitory and excitatory actions of adenosine are blocked by theophylline, a specific $P_1$-purinergic antagonist (Burnstock, 1978). Whether or not adenosine plays an important role in the local regulation of coronary blood flow remains controversial. Adenosine concentration increased in response to cardiac work, ischaemia and hypoxia and was, therefore, hypothesized to be a putative mediator of metabolic vasodilation (Berne and Rubio, 1979; Belloni, 1979; Berne, 1980; Feigl, 1983). Recent studies with adenosine deaminase suggest that adenosine may play only a minor role in regulation under basal (Gewirtz et al., 1986) and hypoxic (Gewirtz et al., 1987) conditions. Infusion of adenosine deaminase did not decrease myocardial blood flow under these conditions indicating some mediator other than adenosine was involved.

AMP, ADP and ATP activate $P_2$-purinergic receptors to cause endothelium-dependent relaxation of blood vessels. Their actions

are not usually blocked by theophylline (Burnstock, 1978). Burnstock and Kennedy (1985a) distinguished between two types of $P_2$ receptors; $P_{2x}$ receptors are excitatory and are present on the vascular smooth muscle while $P_{2y}$ receptors are inhibitory and are present on the endothelium. In the coronary artery of the dog (Cocks and Angus, 1983) and rabbit (Griffith et al., 1984), ATP causes vasodilatation in the presence of the endothelium and this response is reduced or abolished in denuded vessels.

Purines are released from the intracellular stores of vascular cells (Paddle and Burnstock, 1974; Pearson et al., 1983, Born and Kratzer, 1984), endothelial and smooth muscle cells (Pearson and Gordon, 1979; Nees and Gerlach, 1983) and blood cells during intravascular platelet aggregation (Mills et al., 1968; Born and Kratzer, 1984) in concentrations sufficient to activate local purinergic receptors (Born and Kratzer, 1984). ATP which acts via $P_{2x}$-purinergic receptors has been implicated as an excitatory co-transmitter with noradrenaline from sympathetic perivascular nerves causing vasoconstriction in blood vessels. ATP also has prejunctional inhibitory actions in conjunction with adenosine (Burnstock and Kennedy, 1985b).

Serotonergic, histaminergic and bradykinin receptors

Aggregating platelets release serotonin, histamine and thromboxane $A_2$, in addition to ATP and ADP. Serotonin is one of the most potent vasoconstrictors of the large coronary artery *in vivo* and *in situ* (Mena and Virdio, 1976; Bove and Dewey, 1983; Lamping et al.,1985) and can elicit vasospasm in dogs (Perez et al., 1983). Removal of the endothelium enhances the

vasoconstrictory response (Brum et al., 1984; Lamping et al., 1985). In isolated coronary vessels which have been precontracted to achieve resting tone, serotonin causes vasodilatation, but in the absence of endothelium serotonin causes further vasoconstriction (Cocks and Angus, 1983; Cohen et al., 1983). Serotonin-mediated vasodilation is weak and only sufficient to counteract a portion of the potent vasoconstriction (Young and Vatner, 1986). Histamine causes vasoconstriction of human coronary arteries (Toda, 1983) which is enhanced when they are atherosclerotic (Ginsburg et al., 1984). In monkey and dog coronary arteries, histamine causes vasodilatation (Konishi et al., 1981; Toda, 1983,1986). Other substances involved in haemostasis, such as thrombin which is produced in abundance during activation of the coagulation cascade, causes endothelium-dependent relaxation of the dog coronary artery (Ku, 1982).

Bradykinin, a circulating hormone, causes endothelium-dependent vasodilatation of the coronary artery in the dog (Cherry et al., 1982) and vasoconstriction in sheep (Kovalcik, 1962).

Arteriosclerosis

Arteriosclerorsis of the coronary artery in salmonids (Robertson et al., 1961) involves proliferation of smooth muscle cells to produce lesions on the intimal surface of the blood vessel (Van Citters and Watson, 1968; Maneche et al., 1973; Moore et al., 1976a, 1976b; House and Benditt, 1981) which is similar to the early stages of that found in mammalian coronary arteries (Robertson et al., 1961). Endothelium-dependent responses are

impaired by the presence of atherosclerotic lesions in mammals (Freiman et al., 1986; Sreeharan et al., 1986; Bossaller et al., 1987; Jayakody et al., 1987). Anadramous Salmo gairdneri, have a higher incidence of arteriosclerotic lesions than nonanadromous populations (Robertson et al., 1961).

Coronary artery of fish

The structure of the coronary artery in fish is similar to that of mammals (Laurent et al., 1983), however, the pattern of innervation is different. The ventricle in trout is innervated by adrenergic nerve fibres which travel along the coronary artery (Holmgren, 1977). Thus, it is likely that the coronary artery is innervated as well. The vagus innervates the atrium in the pacemaker region (Holmgren, 1977) but the existence of cholinergic nerve nerve fibres on the ventricle or the coronary artery has not been determined. Acetylcholine has been shown to cause endothelium-independent vasoconstriction of the ventral aorta of the trout (Miller and Vanhoutte, 1986). Alpha- and β-adrenergic receptors are also present throughout the vasculature in fish (Wood, 1974, 1975; Wood and Shelton, 1975; Farrell, 1981). In the coronary circulation of Pacific blue marlin (Makaira nigricans), Atlantic salmon (Salmo salar) and rainbow trout (Salmo gairdneri), α-adrenergic receptors dominate over β-adrenergic receptors. This results in coronary artery vasoconstriction in response to adrenaline and weak vasoconstriction in response to noradrenaline or even vasodilatation at lower concentrations of noradrenaline. Isoproterenol, a nonspecific β-agonist, results in vasodilatation

of the coronary circulation (Davie and Daxboeck, 1984; Farrell and Graham, 1986; Farrell, 1987). In the conger eel, β-adrenergic receptors are predominant (Belaud and Peyraud, 1971). There is very little known about the control of the coronary vascular bed in fish and what is known is restricted to the resistance vessels. Nonetheless, observations of dissected coronary rteries in Salmo gairdneri indicate that they can contract to significantly reduce the vessel diameter. This suggests that receptors to control vascular tone in the large coronary artery exist and, therefore, may be important in the regulation of coronary blood flow.

Objectives

The purpose of this study was to document receptor types in the coronary artery of Salmo gairdneri. This is the first step towards understanding the regulation of coronary blood flow in trout. In this study, the term receptor is defined as any cellular macromolecule to which a pharmacologically active agent binds to initiate its effects. The receptors in mammals are well defined but little is known about receptors in other vertebrate classes. Consequently, only synthetic agonists and antagonists specific for mammalian receptors are available. Therefore, in order to document receptor types in Salmo gairdneri, the assumption is made that mammalian-specific agents interact with non-mammalian receptors. The following two criteria were used to establish whether the responses in Salmo gairdneri were receptor-mediated. First, if agents have known receptor-mediated effects in mammals and elicit a response in Salmo gairdneri, it is

assumed that the response is mediated by receptors. Responses which are the same in mammals and Salmo gairdneri are probably mediated by a similar type of receptor, likewise, responses which differ are probably not mediated by the same type of receptor. Second, if agents have known receptor-mediated effects in mammals but elicit no response in Salmo gairdneri, it is assumed that the response is not mediated by receptors.

Vascular rings from the coronary artery of rainbow and steelhead trout were mounted on an isometric force transducer to measure the vasoactivity in response to various pharmacologically active agents. Several objectives were addressed. First, I wanted to test for the presence of purinergic, muscarinic, α- and β-adrenergic, serotonergic, histaminergic and bradykinin receptors. Second, I wanted to determine if the denuded coronary arteries could respond to EDRF analogues. Third, I wanted to compare the responsiveness of arteriosclerotic coronary arteries to normal coronary arteries to a given agent. The comparison of the regulation of the coronary circulation in Salmo gairdneri with its regulation in mammals may provide clues to the evolution of receptor types in the coronary artery of vertebrates.

## Materials and Methods

### Experimental Animals

The rainbow trout (Salmo gairdneri) used in this study were obtained year round from West Creek Farms, Aldergrove, British Columbia. The fish were held indoors in 2000 L tanks supplied with dechlorinated water, exposed to a natural photoperiod regime and fed commercial trout chow ad libitum. The fish were acclimated at 15°C for at least two weeks before they were used for experiments. The body weights ranged from 914 to 1755 g with a mean of 1178 g (SD=203; N=32).

Steelhead trout (Salmo gairdneri) were transported to Simon Fraser University from Robertson Creek Hatchery, Port Alberni, British Columbia and were held in outdoor aquarium facilities. The fish were held in 2000 L tanks supplied with dechlorinated water and were exposed to the natural winter (October to January) temperatures and photoperiod. The temperature range over this time period was 4 to 10°C. The fish did not eat when food was offered during this time period. The body weights ranged from 1260 to 6000 g with a mean body weight of 2506 g (SD=1193; N=20 where N=number of fish and n=number of vascular rings).

Male Wistar rats (150 to 200 g) were used to test the efficacy of the experimental setup. The femoral artery from the rat was used to repeat the study of Kennedy et al., 1985.

### Isometric Force Measurements

Fish were killed by a blow to the head. The whole heart was dissected out of the animal by making an 8 to 10 cm incision on the ventral surface approximately three cm below the isthmus.

The main branch of the coronary artery runs along the ventral side of the bulbus arteriosus and bifurcates at or near the ventricle/bulbus arteriosus junction (Figure 1). The bulbus arteriosus was cut away from the ventricle and refrigerated (4°C) for at least 12 hours in physiological saline (118 mM NaCl, 2.9 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.1 mM $NaH_2PO_4 \cdot H_2O$, 2.5 mM $Na_2HPO_4$ and 1.9 mM $NaHCO_3$) (Farrell, 1987). Arterial rings used for generating the acetylcholine concentration-response curve for rainbow trout were fresh pieces which were allowed to equilibrate in the organ bath for 1 to 2 hrs. Refrigerated vessels gave a higher yield of viable rings, consequently, all other curves were generated with coronary arteries which had been refrigerated for 1 to 4 days. The duration of refrigeration had no effect on the response to 45 mM KCl (Figure 2). The atrium was removed from the ventricle and discarded. The ventricle was blotted dry and weighed; the mass in rainbow trout ranged from 0.71 to 2.52 g with a mean of 1.19 g (SD=0.07; N=32). The ventricular mass in steelhead ranged from 1.15 to 3.90 g with a mean of 2.55 g (SD=0.19; N=18).

The vessels were prepared for isometric force measurements using a modifation of the method described in Hooker et al. (1977). Sections of the vessel, 2 mm in length, were cut from the coronary artery upstream from the point of bifurcation and were mounted onto two 36 gauge platinum wires (Figure 1) with the aid of a Jena dissecting microscope. The vessel was kept in chilled physiological saline while cutting and mounting it in preparation for tension measurements. The lower wire was firmly

Figure 1. Diagrammatic representation of the trout heart and preparation of the coronary artery for isometric force measurements.

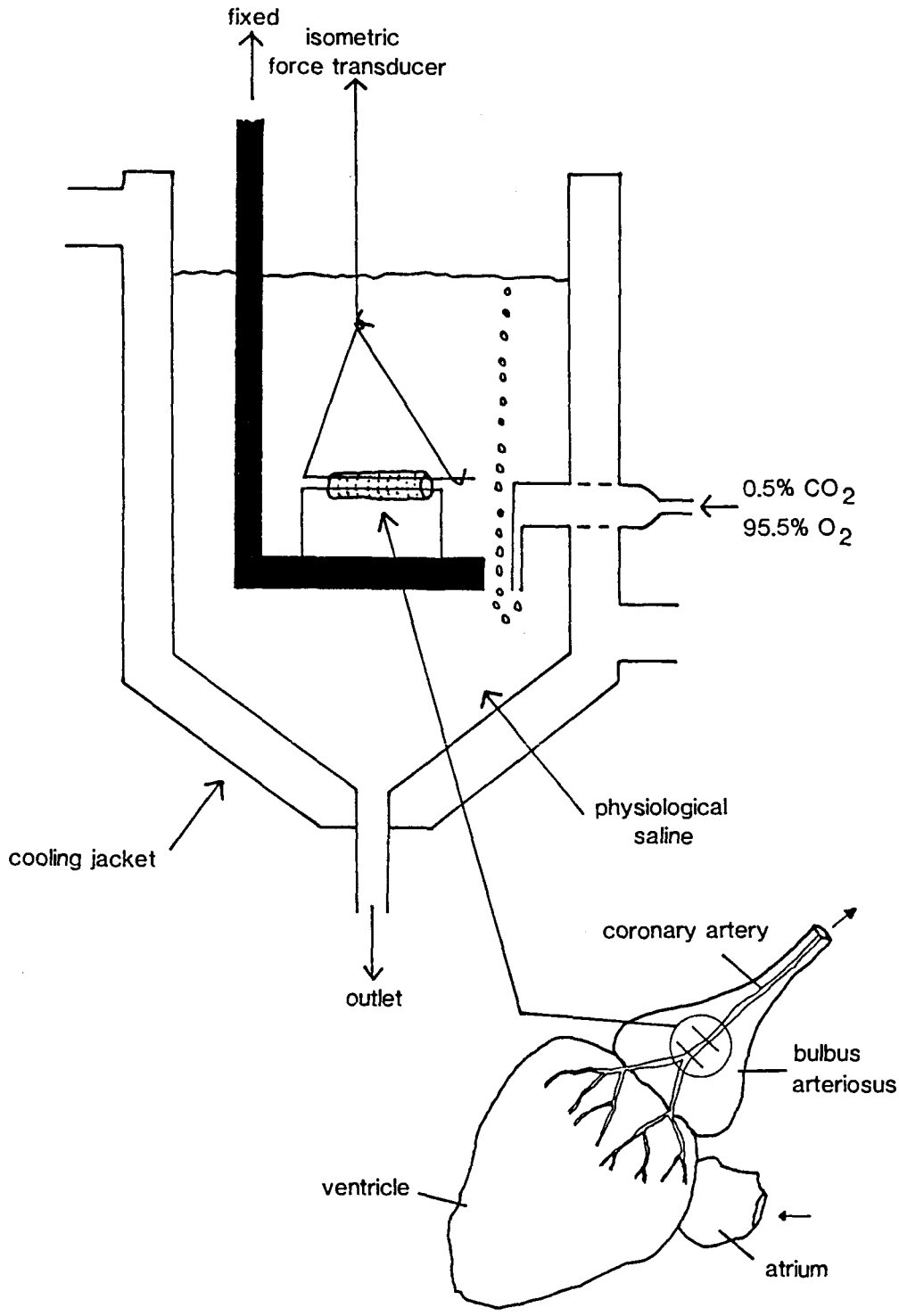
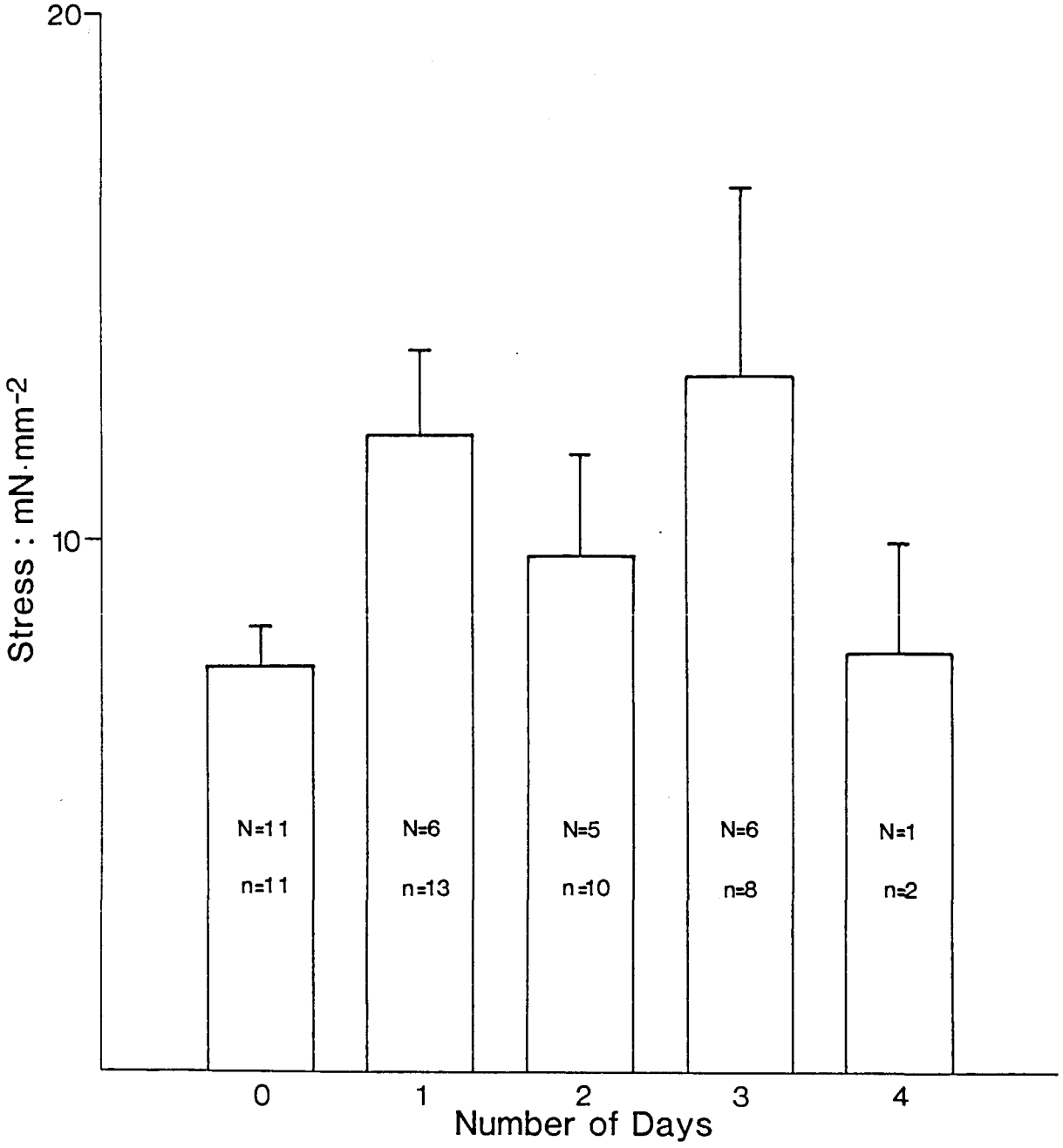
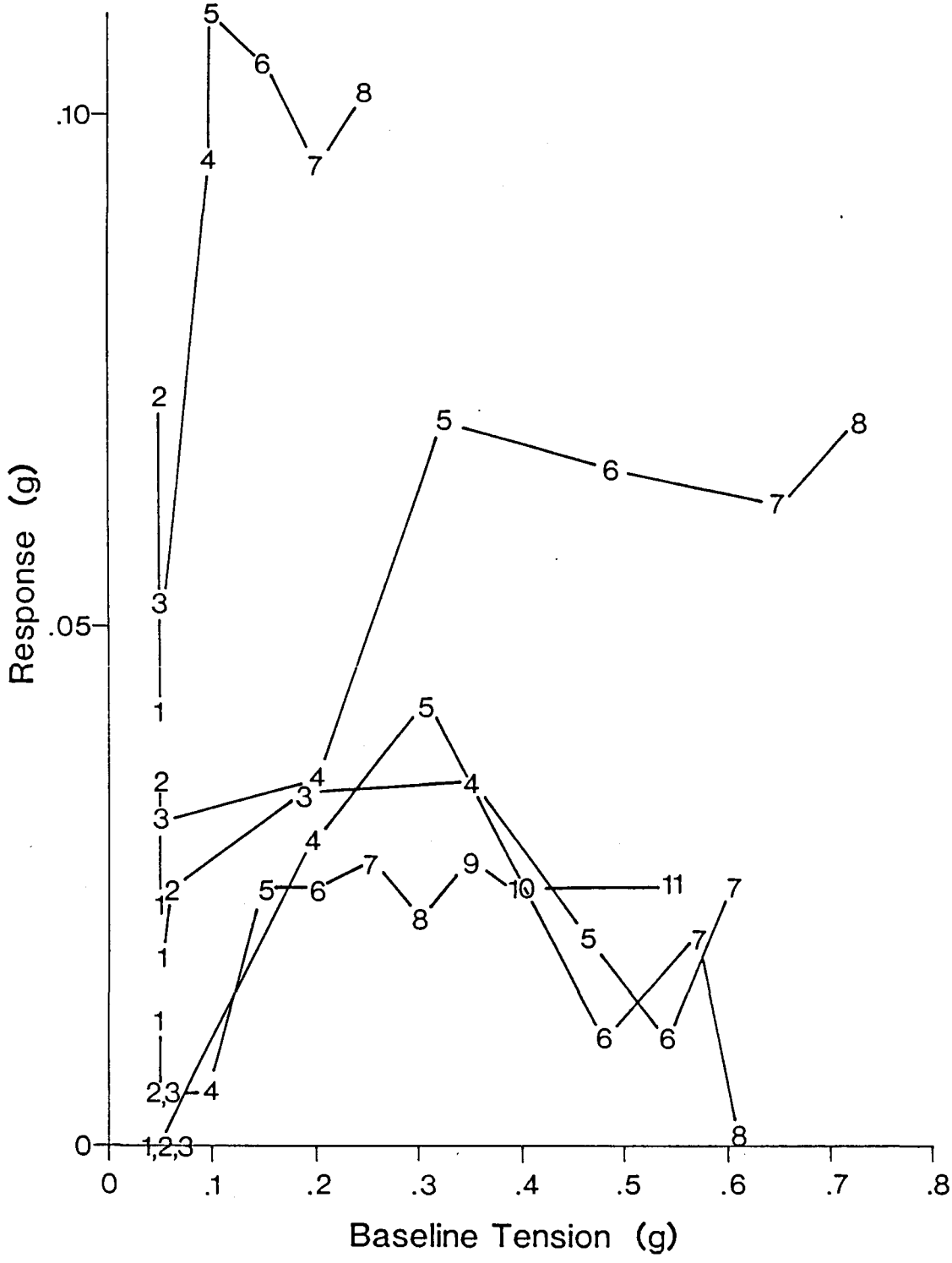

fixed
isometric
force transducer
0.5% $CO_2$
95.5% $O_2$
physiological
saline
cooling jacket
outlet
coronary artery
bulbus
arteriosus
ventricle
atrium

Figure 2. The effect of vascular ring age on the response of the coronary artery in rainbow trout to a single dose of 45 mM KCl. Vascular rings which responded with a stress value of <0.05 $mN/mm^2$ were excluded from analysis. No significant difference was found. N=number of fish; n=number of vascular rings.


20
10
Stress : mN·mm-2
N=11
n=11
N=6
n=13
N=5
n=10
N=6
n=8
N=1
n=2
0
1
2
3
4
Number of Days

secured in a clamp-like device while the upper wire was attached to a Statham UC2 Universal Transducing Cell with a piece of 5-0 suture thread, looped at one end. The mounted vascular ring was placed in a 15 ml bath filled with physiological saline at 15°C containing 5.6 mM D-glucose and bubbled with 0.5% $CO_2$ and 95.5% $O_2$ (pH=7.9). The amount of stretch applied to the arterial ring to obtain a biological zero or baseline tension was optimized for each piece of vessel to adjust for differences in vessel diameter. The integrity of each segment of vessel was tested by adding a single dose of 90 mM KCl to the bath after an initial 0.15 g of tension was applied to the vessel. The optimization of baseline tension was arrived at by repeatedly adding single doses of 90 mM KCl to the bath at increasing tension values until the magnitude of the response to KCl was relatively constant (Figure 3). Vascular rings with optimized KCl responses less than 0.05 g of tension were discarded. Any change in force from the optimal baseline tension constituted active force which was recorded over time on a Graphtec chart recorder (Linercorder F WR3701) and was expressed as grams of tension. Transducers were calibrated and tested for linearity using additions of known masses. The average mechanical drift was insignificant at 0.0053 g/hr. Drugs were added to the bath in volumes of 120 to 300 µl. The maximum volume added ranged from 1.38 to 1.92 ml. The addition of a single 2 ml dose of saline to the bath caused an increase of tension of 0.005 g. Three washes over at least a 15 min time interval followed each single drug addition or cumulative concentration curve. Examples of typical vasoconstrictory and

Figure 3. Optimization of the response of the coronary artery in rainbow trout to a single dose of 45 mM KCl by increasing baseline tension. The responses were expressed as changes in tension. Numbers represent the order in which individual doses were administered. N=2; n=5.


Response (g)
.10
.05
0
0
.1
.2
.3
.4
.5
.6
.7
.8
Baseline Tension (g)

vasodilatory responses are shown in Figure 4. The agents and their suppliers are listed in Table 1.

Rat vascular rings were sections 2 mm in length taken from the femoral artery as described in Kennedy et al. (1985). Force measurements were determined using the technique described above except that experiments were done at 37°C using Kreb's solution (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $K_2HPO_4$, 25 mM $NaHCO_3$ and 11 mM D-glucose) bubbled with 5% $CO_2$ and 95% $O_2$ (Kennedy et al., 1985).

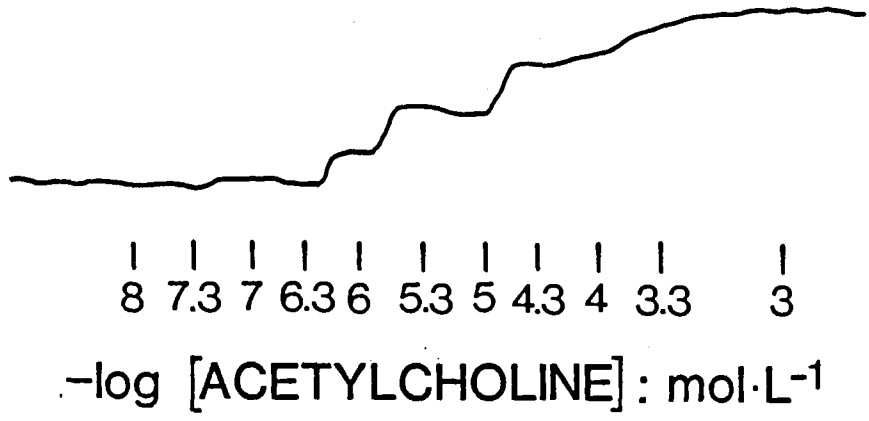
Arteriosclerotic lesion grading and cross-sectional area measurements

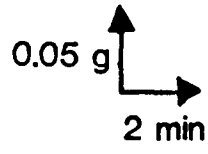
At the end of each experiment, the fish vessels were placed in 10% buffered formalin for at least 48 hours and then stored in 70% ethanol in preparation for histological studies. The vessels were routinely dehydrated, cleared and embedded in paraffin wax for sectioning at 6 to 7 µm. Sections were stained with Verhoeff's elastic stain and counterstained with picro-ponceau (Farrell et al., 1988). Approximately 20 serial sections were taken from two locations along the length of the vessel. Twenty representative sections were graded for each vessel.

The grading system used, assigning atherosclerotic lesion severity on a scale of 0 to 5, was based on systems by Moore et al. (1967a, 1967b), McKenzie et al. (1978) and Farrell et al. (1986). Grade '0' represented a normal artery in which lesions were absent. Grade '1' was assigned to lesions which consisted of between 1 and 6 cells while lesions with greater than 6 cells but fewer than 20 cells were assigned grade '2'. Sections which

Figure 4. Tracings of typical cumulative-concentration response curves. (A) Cumulative-concentration curve for acetylcholine. (B) Cumulative-concentration curve for nitroglycerine. The vascular ring was precontracted with 45 mM KCl.

A


8 7.3 7 6.3 6 5.3 5 4.3 4 3.3 3
-log [ACETYLCHOLINE] : mol·L-1


0.05 g
2 min

B

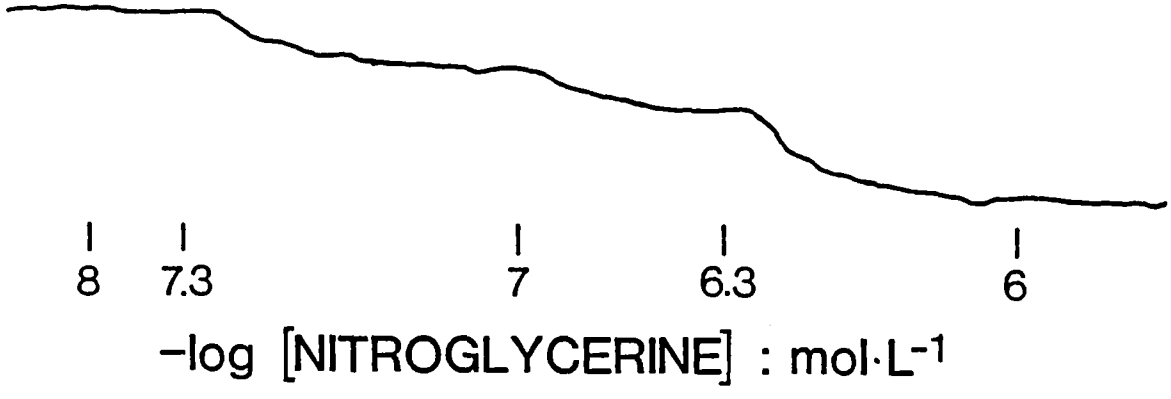

8 7.3 7 6.3 6
-log [NITROGLYCERINE] : mol·L-1

TABLE 1. List of Agents Used

| Agent | Action | Supplier |
|---|---|---|
| Acetylcholine chloride | muscarinic agonist | Sigma |
| Adenosine | $P_1$-purinergic agonist | Sigma |
| Adenosine diphosphate (sodium salt) | $P_2$-purinergic agonist | Pharmacia |
| Adenosine triphosphate (disodium salt) | $P_2$-purinergic agonist | Pharmacia |
| -Adrenaline (+Bitartrate salt) | $\alpha$-, $\beta$-agonist | Sigma |
| Atenolol | $\beta_1$-antagonist | Sigma |
| Bradykinin (triacetate salt) | agonist | Sigma |
| Clonidine | $\alpha_2$-agonist | Sigma |
| Histamine | agonist | Sigma |
| ICI 118,551 hydrochloride | $\beta_2$-antagonist | donated by Imperial Chemical Industries |
| ±Isoproterenol | $\beta$-agonist | Sigma |
| Nitroglycerine (Nitrostat) | EDRF analogue | gift from Dr. Jack Diamond |
| L-phenylephrine | $\alpha$-agonist | Sigma |
| Serotonin | agonist | Sigma |
| Sodium nitroprusside | nitrodilator | Sigma |
| Theophylline | $P_1$-purinergic antagonist | Sigma |

contained multiple sites of grade '1' lesions represented grade '3' severity while grade '4' represented sections which had multiple sites of lesions with grade '2' severity. Grade '5' was assigned to lesions to which consisted of more than 20 cells.

The cross-sectional area of the wall of each vessel was measured for the calculation of stress values by tracing the outside diameter of the histological section onto sheets of paper using a Leitz compound microscope and camera lucida. The tracings were cut out and weighed and the cross-sectional area was calibrated relative to the weight of a piece of paper with a known area.

Endothelium Detection

The presence or absence of endothelium on the intimal surface of the coronary arterial rings was assessed using a silver staining procedure (Abrol et al., 1984). Fresh vascular rings were pinned flat on a paraffin block using 0.25 mm insect pins. The samples were kept in 5% D-glucose for 2 min and then stained in 0.25% silver nitrate for 20 sec. Excess silver nitrate was removed by rapidly immersing the samples six times in 5% D-glucose. The samples were then placed in 3% cobalt(II) bromide for three min to improve contrast. The samples were then washed six times in 5% D-glucose to remove excess cobalt(II) bromide. The samples were placed at a distance of 15 cm from a germicidal ultraviolet tube for 30 min to develop the stain. The samples were fixed in 10% buffered formalin overnight. The samples were removed from the paraffin blocks and mounted on slides using Kaiser's glycerin-jelly, an aqueous mounting medium

(10 g gelatine, 70 ml distilled water, 60 ml pure glycerin and 0.25 g phenol crystals) (Drury and Wallington, 1967). The margins of the coverslips were ringed with clear nail polish (Revlon) to prevent evaporation or seepage of the mountant.

Data analyses

The responses of the coronary artery to the agents tested were analyzed to yield $EC_{50}$ values and the maximum or minimum stress produced. An $EC_{50}$ value is the concentration that produces a 50% maximal response and is a measure of agonist potency (Leslie, 1987). Potency is the ability of a drug to produce a biological response and is not a measure of agonist affinity. The $EC_{50}$ values were interpolated from curves in which individual vessel responses were expressed as a percentage of their own maximum.

Normalization of the tension values to account for differences in vascular smooth muscle mass and thickness between vessels was done by expressing responses in terms of force produced/cross-sectional area (stress). Stress ($mN/mm^2$) was calculated as a change in tension (g) x 9.81 $m/s^2$ (mN)/cross-sectional area ($mm^2$) (Kenakin, 1984). This transformation is necessary when comparing responses of blood vessels from animals which vary greatly in body size.

Statistical analyses

Paired and unpaired t-tests, one way analysis of variance and randomized block analysis of variance were used to determine statistically significant differences (set at $p<0.05$) in $EC_{50}$ values and maximum and minimum stress values.

## Results

Responsiveness of the test system

ATP ($5x10^{-4}$ M) (N=1) caused vasoconstriction of the rat femoral artery with and without being precontracted with noradrenaline ($1x10^{-6}$ M). Adenosine ($5x10^{-4}$ M) (N=4) caused vasodilation in vessels precontracted with noradrenaline, but elicited no response in vessels at resting tension. Kennedy et al. (1985) found that adenosine dilated the rat femoral artery independent of the presence of the endothelium. ATP relaxation was endothelium-dependent in the femoral artery; vasoconstriction resulted when the endothelium was absent.

Purinergic receptors

Adenosine, ADP and ATP, over the range $5x10^{-6}$ to $1x10^{-3}$ M, caused concentration-dependent contractions of the coronary artery from eight rainbow and eight steelhead trout (Figures 5, 6 and 7). One of the eight steelhead trout did not respond to adenosine. The potencies of adenosine, ADP and ATP were equal ($p>0.05$) within the steelhead group and rainbow group. Similarly, the maximum stress values for ATP and ADP were the same ($p>0.05$) but greater than adenosine ($p<0.05$). Between rainbow and steelhead trout, the $EC_{50}$ values (Table 2) for ADP and ATP were not significantly different ($p<0.05$) but ADP and ATP produced approximately 4.2 and 3.9 times more stress, respectively, on the coronary artery from steelhead trout than vessels from rainbow trout. The $EC_{50}$ value (Table 2) for adenosine was significantly greater (1.2 times) in steelhead than in rainbow trout, yet the maximum stress produced by adenosine

Figure 5. Cumulative-concentration curve for the coronary artery of rainbow and steelhead trout in response to adenosine. The percent maximum contraction of rainbow trout (A) and steelhead trout vascular rings (B) as well as the maximium stress of rainbow trout (C) and steelhead trout vascular rings (D) are shown. The values are expressed as means ± SEM. Statistical analysis of $EC_{50}$ and maximum stress values are summarized in Tables 2 and 3, respectively. Rainbow trout (N=5; n=8); steelhead trout (N=7; n=7).

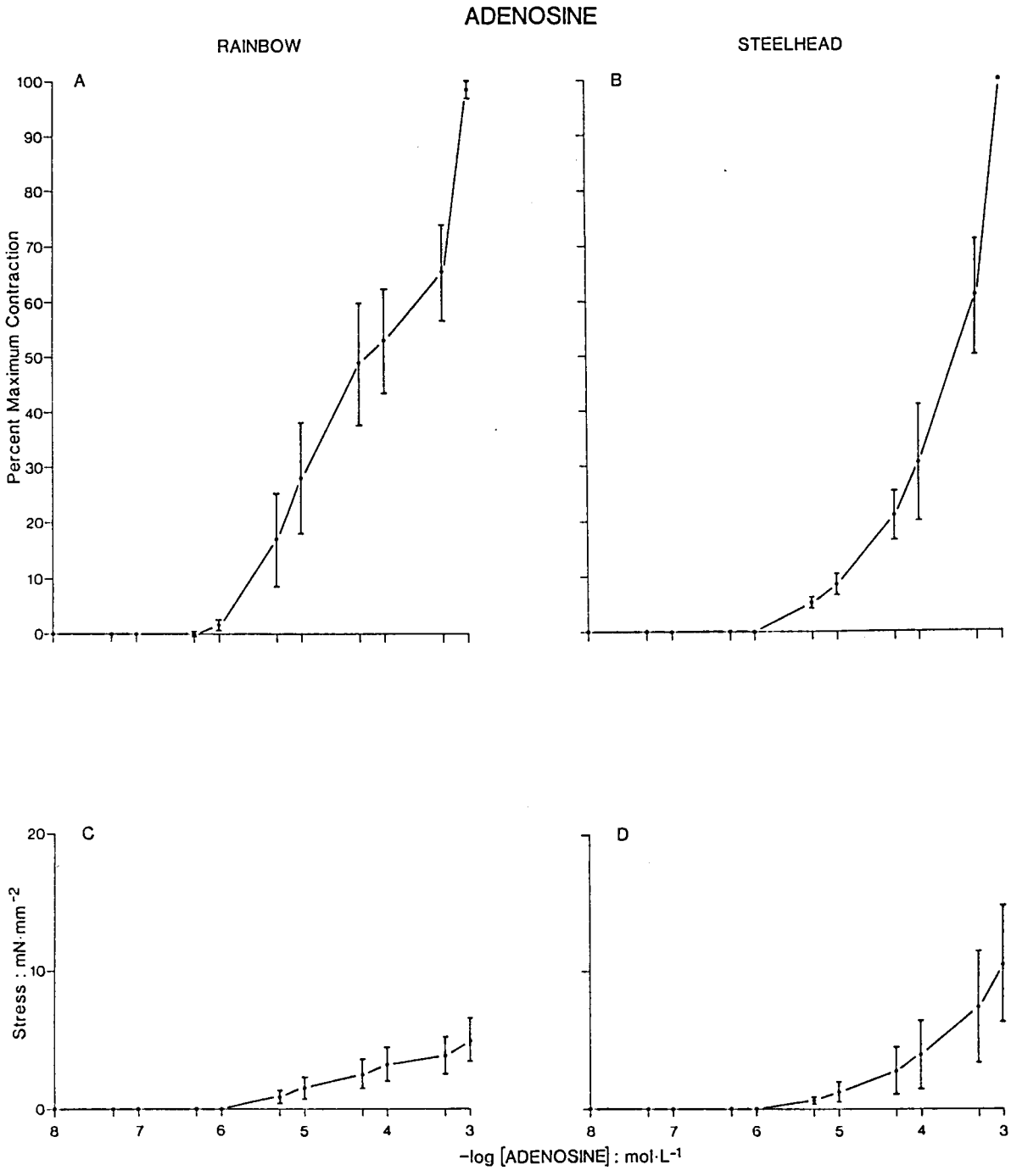

ADENOSINE
RAINBOW
STEELHEAD
A
B
C
D
Percent Maximum Contraction
Stress : mN·mm⁻²
−log [ADENOSINE] : mol·L⁻¹
100
90
80
70
60
50
40
30
20
10
0
20
10
0
8
7
6
5
4
3

Figure 6. Cumulative-concentration curve for the coronary artery of rainbow and steelhead trout in response to ADP. The percent maximum contraction of rainbow trout (A) and steelhead trout vascular rings (B) as well as the maximium stress of rainbow trout (C) and steelhead trout vascular rings (D) are shown. The values are expressed as means ± SEM. Statistical analysis of $EC_{50}$ and maximum stress values are summarized in Tables 2 and 3, respectively. Rainbow trout (N=5; n=8); steelhead (N=8; n=8).

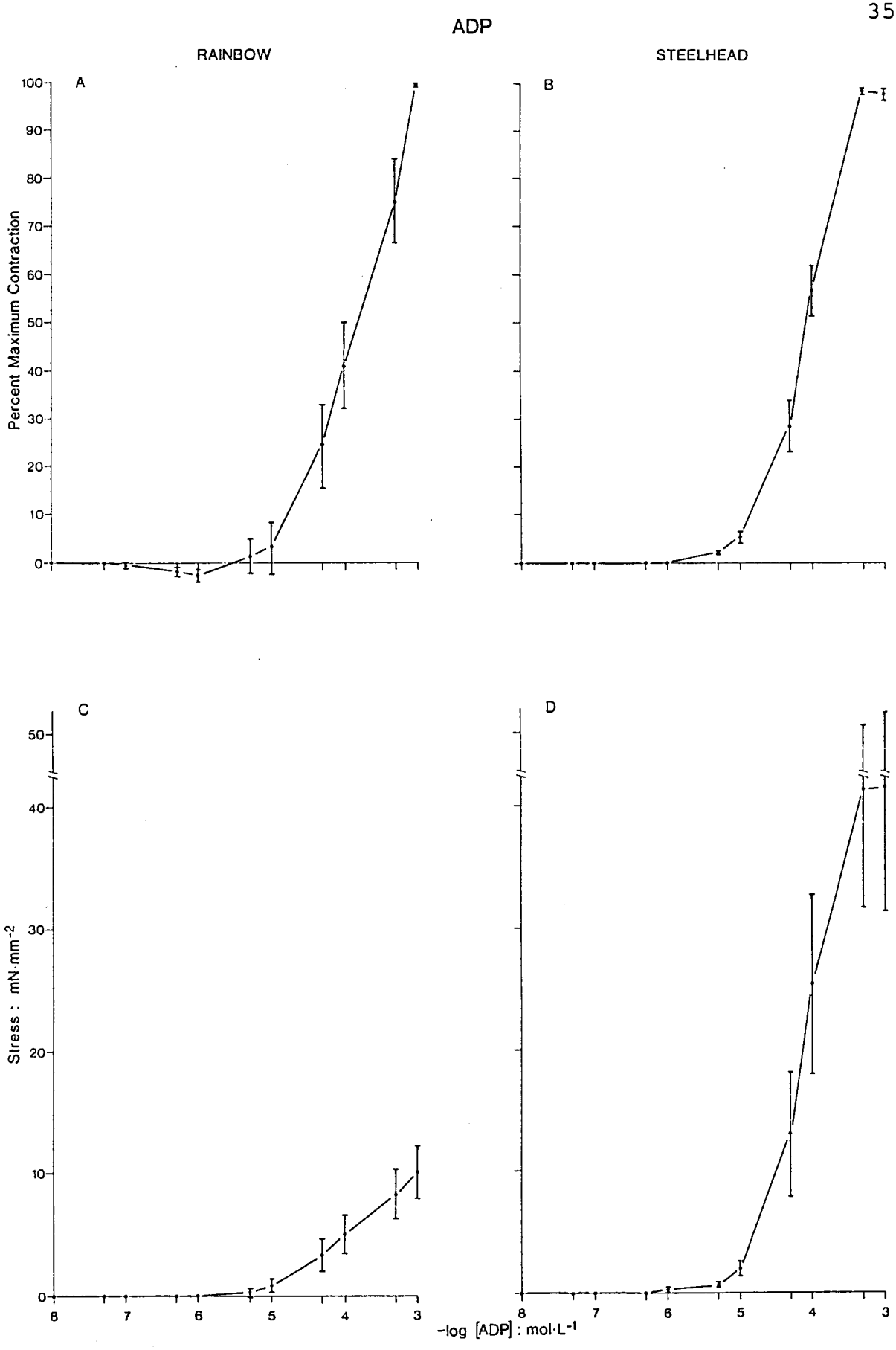

ADP
RAINBOW
STEELHEAD
A
B
C
D
Percent Maximum Contraction
Stress : mN·mm⁻²
-log [ADP] : mol·L⁻¹
100
90
80
70
60
50
40
30
20
10
0
8
7
6
5
4
3

Figure 7. Cumulative-concentration curve for the coronary artery of rainbow and steelhead trout in response to ATP. The percent maximum contraction of rainbow trout (A) and steelhead trout vascular rings (B) as well as the maximium stress of rainbow trout (C) and steelhead trout vascular rings (D) are shown. The values are expressed as means ± SEM. Statistical analysis of $EC_{50}$ and maximum stress values are summarized in Tables 2 and 3, respectively. Rainbow trout (N=5; n=8); steelhead (N=8; n=8).

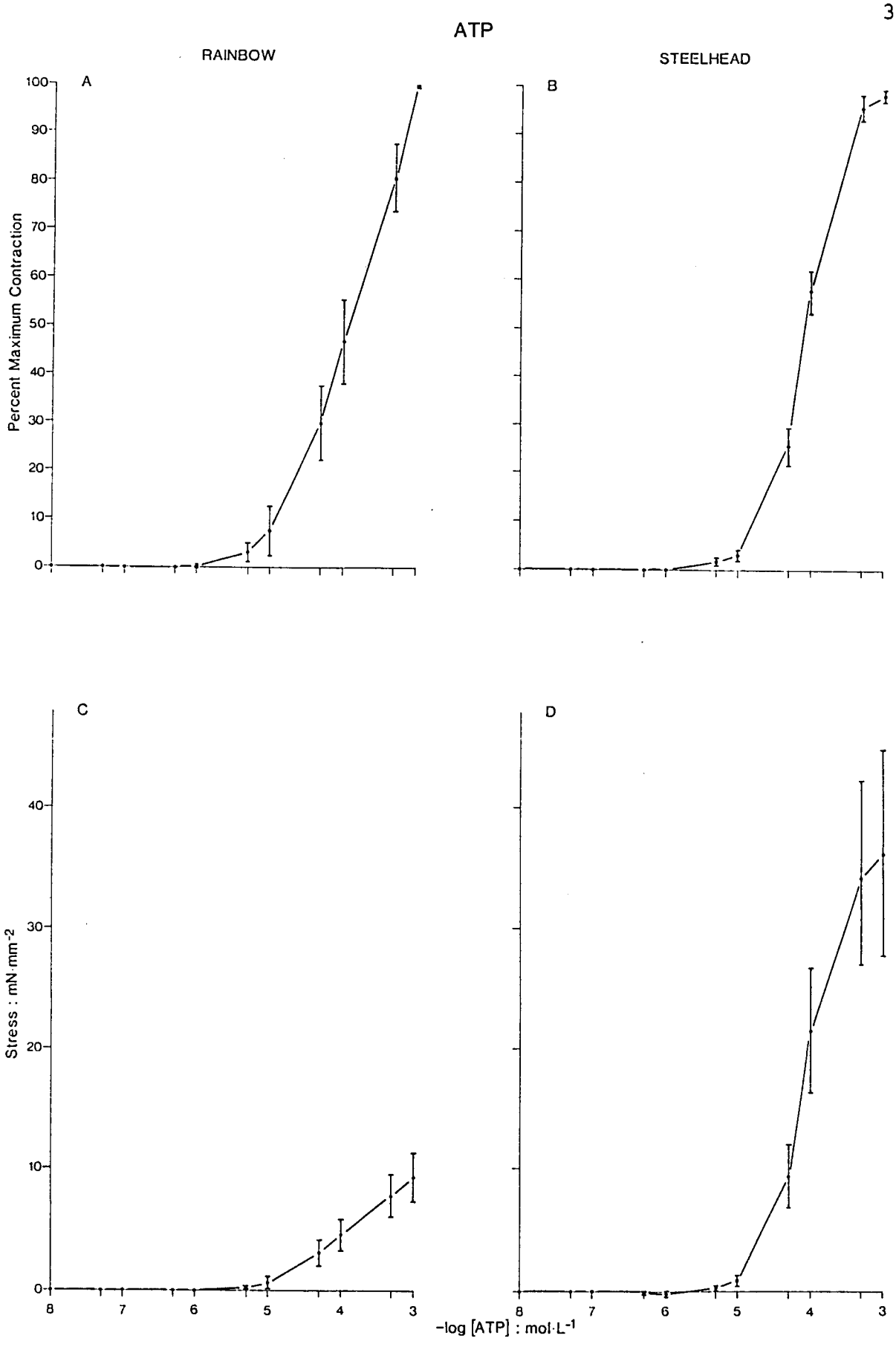

ATP
RAINBOW
STEELHEAD
A
B
C
D
Percent Maximum Contraction
Stress : mN·mm-2
-log [ATP] : mol·L-1
100
90
80
70
60
50
40
30
20
10
0
8
7
6
5
4
3

TABLE 2. $EC_{50}$ values for different agents in coronary vascular rings from Salmo gairdneri

| Agent | -log $EC_{50}$ $\bar{x}$ (SEM) | | p value |
|---|---|---|---|
| | Rainbow trout | Steelhead trout | |
| Adenosine | 4.33 (0.27) N=8 | 3.60 (0.15) N=7 | p=0.04* |
| ADP | 3.87 (0.18) N=8 | 4.07 (0.06) N=8 | p=0.35 |
| ATP | 3.97 (0.17) N=8 | 3.98 (0.11) N=8 | p=0.93 |
| Acetylcholine | 5.57 (0.18) N=8 | 5.91 (0.10) N=9 | p=0.13 |
| Nitroglycerine | 5.85 (0.41) N=5 | 6.51 (0.50) N=5 | p=0.34 |
| Sodium nitroprusside | 5.47 (0.52) N=6 | 4.39 (0.33) N=5 | p=0.12 |

* statistically significant difference ($p<0.05$)

TABLE 3. Maximum or minimum stress values for coronary vascular rings of Salmo gairdneri in response to different pharmacological agents

| Agent | maximum or minimum stress $\bar{x}$ (SEM) ($mN/mm^2$) | | p value |
|---|---|---|---|
| | Rainbow trout | Steelhead trout | |
| Adenosine | 0.497 (0.16) N=8 | 1.060 (0.43) N=7 | p=0.25 |
| ADP | 1.014 (0.22) N=8 | 4.210 (1.0) N=8 | p=0.018* |
| ATP | 0.934 (0.20) N=8 | 3.660 (0.85) N=8 | p=0.016* |
| Acetylcholine | 0.573 (0.11) N=8 | 0.691 (0.12) N=9 | p=0.48 |
| Isoproterenol | -0.600 (0.09) N=4 | - | - |
| Atenolol | -0.175 (0.11) N=4 | - | - |
| ICI 118,551 | -0.607 (0.12) N=4 | - | - |
| Serotonin | -0.175 (0.04) N=5 | - | - |
| Nitroglycerine | -0.422 (0.06) N=5 | -0.400 (0.12) N=5 | p=0.88 |
| Sodium nitroprusside | -0.617 (0.18) N=6 | -0.425 (0.07) N=5 | p=0.35 |

* statistically significant difference ($p<0.05$)

(Table 3) was the same in both groups.

Theophylline ($1x10^{-3}$ M), which blocks adenosine action via $P_1$-purinergic receptors in mammals, inhibited the excitatory response of the vessels to single doses of $1x10^{-4}$ M adenosine (N=6), $1x10^{-4}$ M ADP (N=2) and $1x10^{-4}$ M ATP (N=2) by 99%, 91% and 82%, respectively.

Cholinergic receptors

Acetylcholine, over the range $1x10^{-7}$ to $1x10^{-3}$ M, caused concentration-dependent contractions in the coronary artery from eight rainbow and seven steelhead trout (Figure 8). A typical response to acetylcholine is shown in Figure 4A. The $EC_{50}$ (Table 2) value for acetylcholine and the maximum stress (Table 3) developed in response to this drug were the same for rainbow and steelhead trout. Three vascular rings from two rainbow trout and one steelhead trout gave no response to acetylcholine.

Adrenergic receptors

Preliminary experiments were performed with adrenaline and noradrenaline to determine the relative dominance of α- and β-adrenergic receptors in the coronary artery of rainbow trout (Figure 9). Vasodilatation in response to adrenaline or noradrenaline indicates a predominance of β-adrenergic receptors, while vasoconstriction indicates a predominance of α-adrenergic receptors. In general, adrenaline causes relaxation at low concentrations and constriction at higher concentrations. Adrenaline caused vasodilatation in three fish at concentrations $<1x10^{-5}$ M. At concentrations $\geq 1x10^{-5}$ M, vasoconstriction occurred in two fish while one continued to undergo

Figure 8. Cumulative-concentration curve for the coronary artery of rainbow and steelhead trout in response to acetylcholine. The percent maximum contraction of rainbow trout (A) and steelhead trout vascular rings (B) as well as the maximium stress of rainbow trout (C) and steelhead trout vascular rings (D) are shown. The values are expressed as means ± SEM. Statistical analysis of $EC_{50}$ and maximum stress values are summarized in Tables 2 and 3, respectively. Rainbow trout (N=8; n=8); steelhead (N=7; n=9).

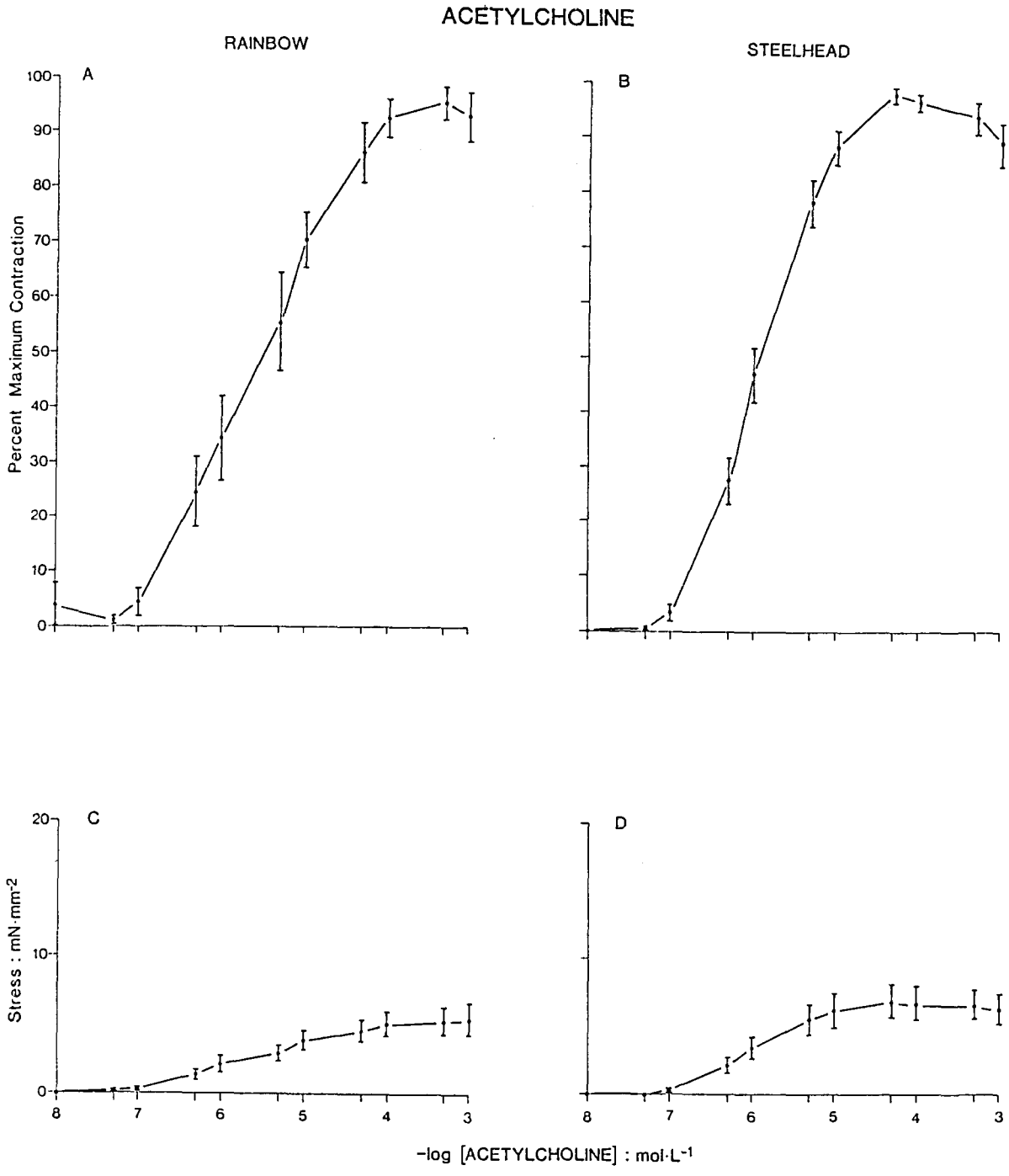
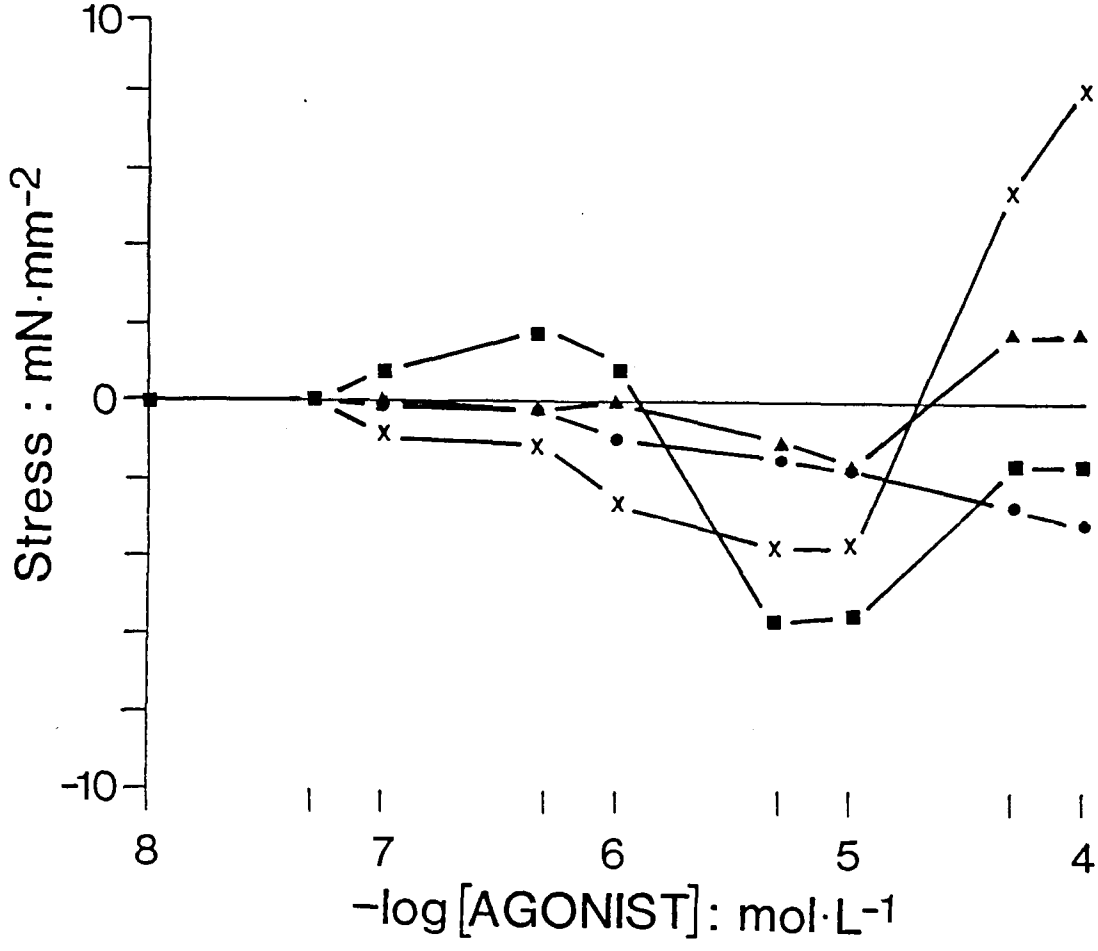

ACETYLCHOLINE
RAINBOW
STEELHEAD
A
B
C
D
Percent Maximum Contraction
Stress : mN·mm-2
-log [ACETYLCHOLINE] : mol·L-1
100
90
80
70
60
50
40
30
20
10
0
8
7
6
5
4
3

Figure 9. Cumulative-concentration curves for the coronary artery of rainbow trout in response to adrenaline and noradrenaline. The response to adrenaline was tested on individual vascular rings precontracted with $5x10^{-5}$ M acetylcholine (x), $1x10^{-5}$ M acetylcholine (▲) and 50 mM KCl (•). The response to noradrenaline was tested on a vascular ring precontracted with $5x10^{-5}$ acetylcholine (▪). Values are expressed as maximium stress.


10
0
-10
Stress : mN·mm-2
8
7
6
5
4
-log [AGONIST]: mol·L-1

vasodilatation. Noradrenaline, in one fish, caused vasodilatation from $5x10^{-7}$ to $5x10^{-6}$ M then caused vasoconstriction at concentrations $\geq 5x10^{-6}$ M. In another rainbow trout, a single dose of noradrenaline ($5x10^{-4}$ M) (n=2) produced complete relaxation after precontraction with $5x10^{-5}$ M acetylcholine.

Because adrenergic receptor stimulation could have either an excitatory or inhibitory response, each vessel was tested at the beginning of the experiment with a single high dose of adrenaline ($1x10^{-5}$ M) to determine whether α-or β-adrenergic receptors appeared to dominate. The response of the coronary arteries, precontracted with $5x10^{-4}$ M ATP, to this single dose of adrenaline varied within and between rainbow and steelhead trout, within the same animal and occasionally within the same arterial ring when tested repeatedly. In rainbow trout (N=6), 8 out of 12 rings relaxed in response to $1x10^{-5}$ M adrenaline, while the other four contracted. In steelhead trout (N=5), 2 out of 12 rings relaxed while 10 out of 12 rings contracted. Based on this response, further characterization of the adrenoceptor subtypes was performed with selective adrenoceptor agonists and antagonists. Characterization of β-adrenergic subtypes was done on those that relaxed, while characterization of α-adrenergic receptor subtypes was done on those that contracted.

Cumulative concentration-response curves for the nonspecific β-agonist, isoproterenol ($1x10^{-9}$ to $1x10^{-3}$ M) (Figure 10A), were generated for rainbow trout coronary arteries, precontracted with $5x10^{-4}$ M ATP. Isoproterenol caused concentration-dependent

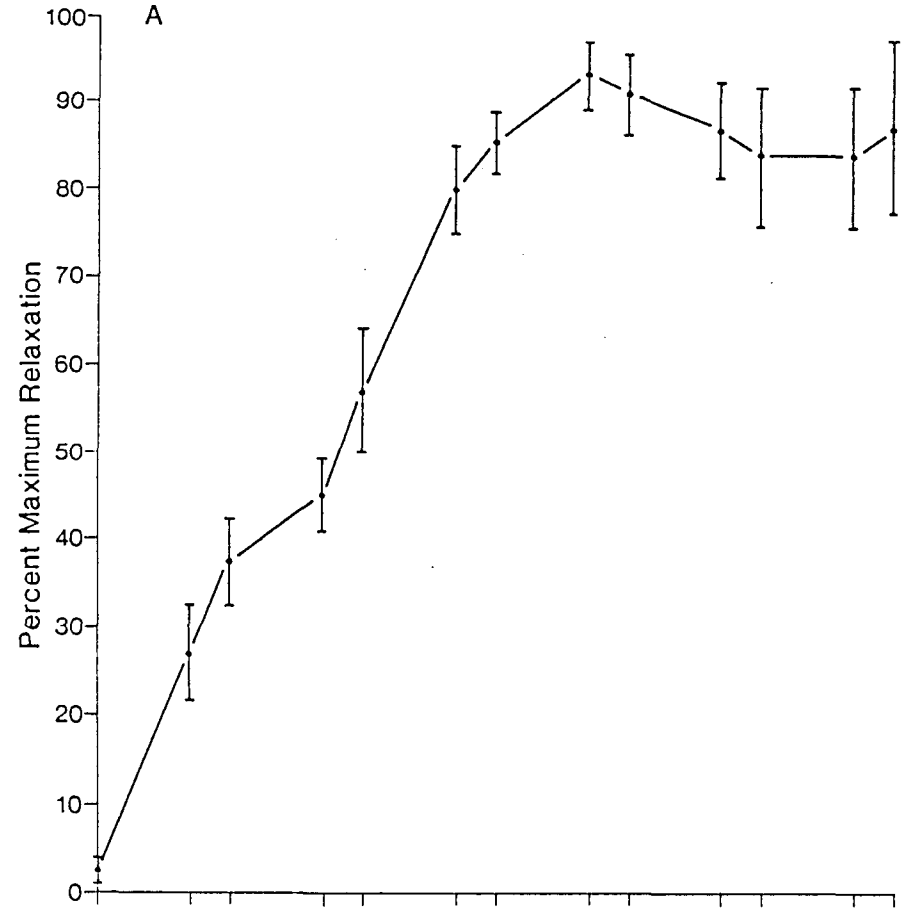
vasodilatation of the coronary artery. The maximum decrease in stress on the coronary artery in response to isoproterenol was -0.600 (SEM=0.087) g/mm$^2$ (Table 3). Identification of β-adrenergic receptor subtypes requires specific antagonists. Pretreatment of the vessel with the specific $\beta_1$-adrenergic antagonist, atenolol ($5x10^{-4}$ M), diminished the effect of isoproterenol by approximately 71% causing a maximum decrease in stress of only -0.175 (SEM=0.106) g/mm$^2$ (Figure 10B and Table 3). ICI 118,551, a specific $\beta_2$-antagonist had no significant inhibitory effect on the maximum response of the vessels to isoproterenol (Figure 10B and Table 3). Therefore, vasodilatation, when present, is through $\beta_1$-adrenergic receptors. Isoproterenol was not tested in steelhead trout because so few vascular rings relaxed with adrenaline.

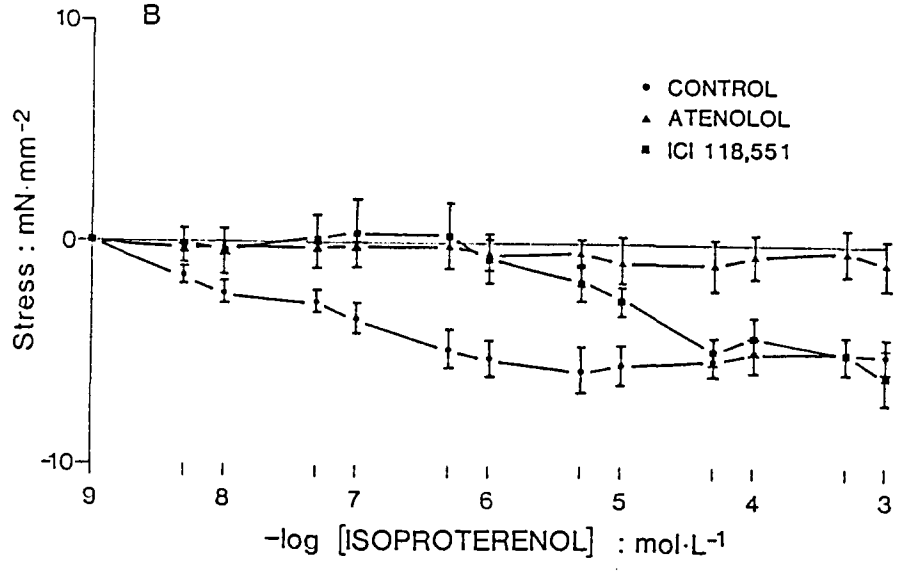
The α-adrenergic receptors appeared to be desensitized by repeated doses of adrenaline since the magnitude of the vasoconstrictory response in rainbow and steelhead trout decreased with repeated application of adrenaline. The desensitization and the greater frequency of spontaneous activity observed in steelhead compared with rainbow trout made it difficult to interpret α-adrenergic cumulative-concentration responses. The α-adrenergic agonist, phenylephrine ($5x10^{-4}$ M) elicited no response when added to the coronary artery at resting tension (N=4) or precontracted with ATP ($5x10^{-4}$ M) (N=3). The $\alpha_2$-adrenergic agonist, clonidine ($5x10^{-4}$ M), had no effect on the rainbow trout coronary artery at resting tension (N=2) or precontracted with ATP ($5x10^{-4}$ M) (N=2).

Figure 10. Cumulative-concentration curves for the coronary artery in rainbow trout in response to isoproterenol and β-antagonists. Vascular rings were precontracted with $5x10^{-4}$ M ATP. (A) The percent maximum relaxation of rainbow trout vascular rings is expressed as mean ± SEM. (N=2; n=4). (B) The minimum stress of rainbow trout control vascular rings (•), vascular rings incubated with $5x10^{-4}$ M atenolol (▲) and vascular rings incubated with $5x10^{-4}$ M ICI 118,551 (▪) are shown. The values are expressed as means ± SEM. Statistical analysis of the minimum stress values are summarized in Table 3. Rainbow trout (N=2; n=4).

# ISOPROTERENOL

RAINBOW



Serotonergic, histaminergic and bradykinin receptors

Serotonin caused concentration-dependent vasodilatation of the coronary artery in five rainbow trout over the range $5x10^{-8}$ to $1x10^{-6}$ M (Figure 11) when precontracted with 45 mM KCl. Over the range $5x10^{-5}$ to $1x10^{-4}$ M, serotonin caused vasoconstriction to the baseline level. Vessels at resting tension showed similar, but smaller responses to serotonin. The maximum stress value in response to serotonin is listed in Table 3. In one rainbow trout, serotonin elicited no response.

Histamine ($1x10^{-8}$ to $1x10^{-4}$ M) (N=6, n=6) elicited no response in the coronary artery of the rainbow trout at resting tension or precontracted with 45 mM KCl. Bradykinin ($1x10^{-8}$ to $1x10^{-4}$ M)(N=4, n=5) had no effect on the coronary artery of rainbow trout under similar conditions.

Non-receptor mediated responses

Nitroglycerine ($1x10^{-8}$ to $1x10^{-4}$ M) (Figure 12) and sodium nitroprusside ($1x10^{-8}$ to $1x10^{-3}$ M) (Figure 13) caused concentration-dependent relaxation of the coronary artery from the rainbow and steelhead trout when precontracted with 45 mM KCl. A typical response to nitroglycerine is shown in Figure 4B. Six out of seven vessels tested in rainbow trout responded to sodium nitroprusside; all five vessels in steelhead trout responded. The $EC_{50}$ and minimum stress values (Tables 2 and 3) were not significantly different for the two groups of fishes.

Endothelium detection

Experimental vessels had an insignificant number of endothelial cells left intact compared with control rings.

Figure 11. Cumulative-concentration curve for the coronary artery of rainbow trout in response to serotonin. Vascular rings were precontracted with 45 mM KCl. The response of rainbow trout vascular rings is expressed as minimum stress. The values are expressed as means ± SEM. (N=5; n=5).

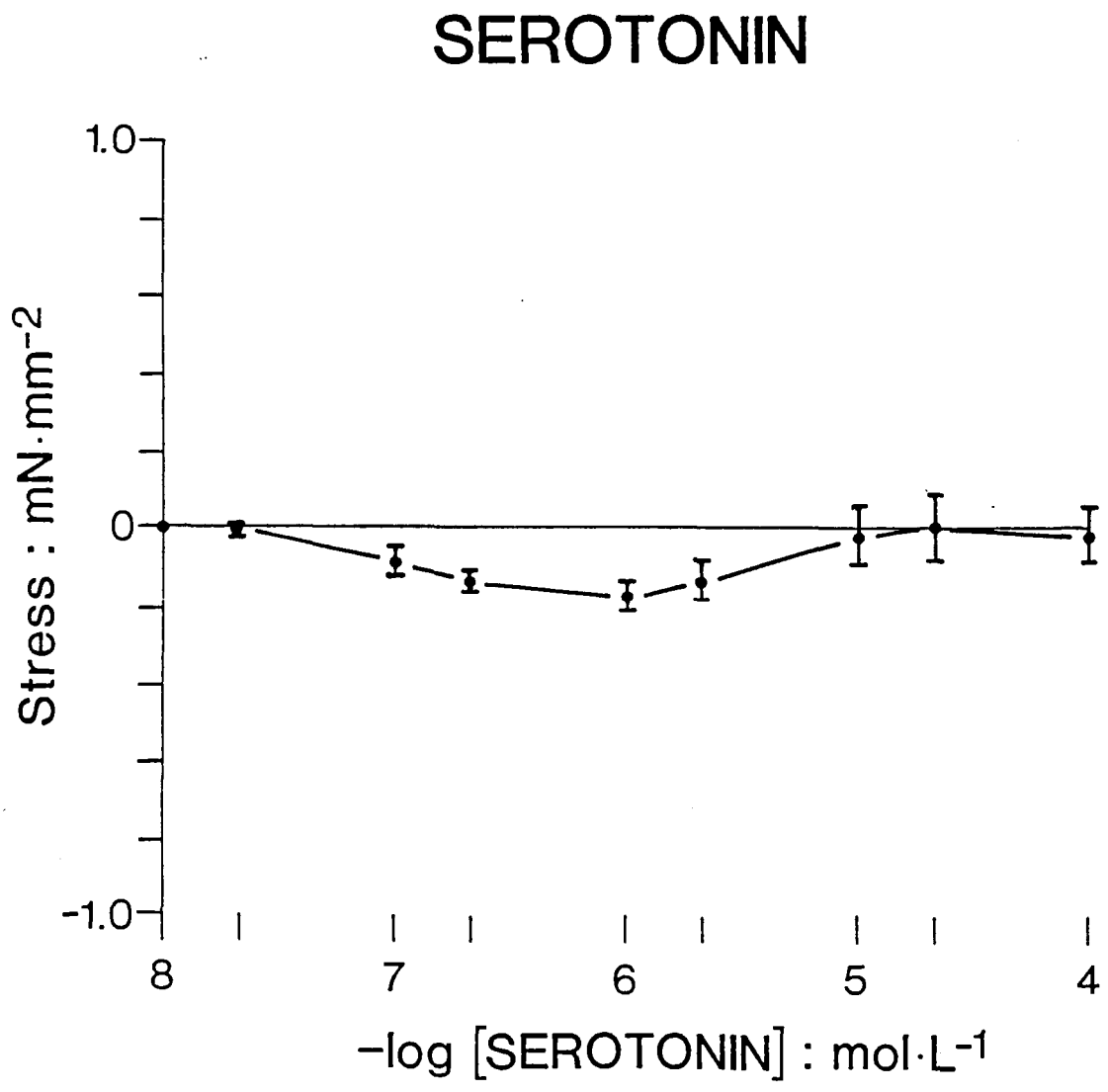

SEROTONIN
1.0
0
-1.0
Stress : mN·mm-2
8
7
6
5
4
-log [SEROTONIN] : mol·L-1

Figure 12. Cumulative-concentration curve for the coronary artery of rainbow and steelhead trout in response to nitroglycerine. Vascular rings were precontracted with 45 mM KCl. The percent maximum relaxation of rainbow trout (A) and steelhead trout vascular rings (B) as well as the minimum stress of rainbow trout (C) and steelhead trout vascular rings (D) are shown. The values are expressed as means ± SEM. Statistical analysis of $EC_{50}$ and maximum stress values are summarized in Tables 2 and 3, respectively. Rainbow trout (N=4; n=5); steelhead (N=4; n=5).

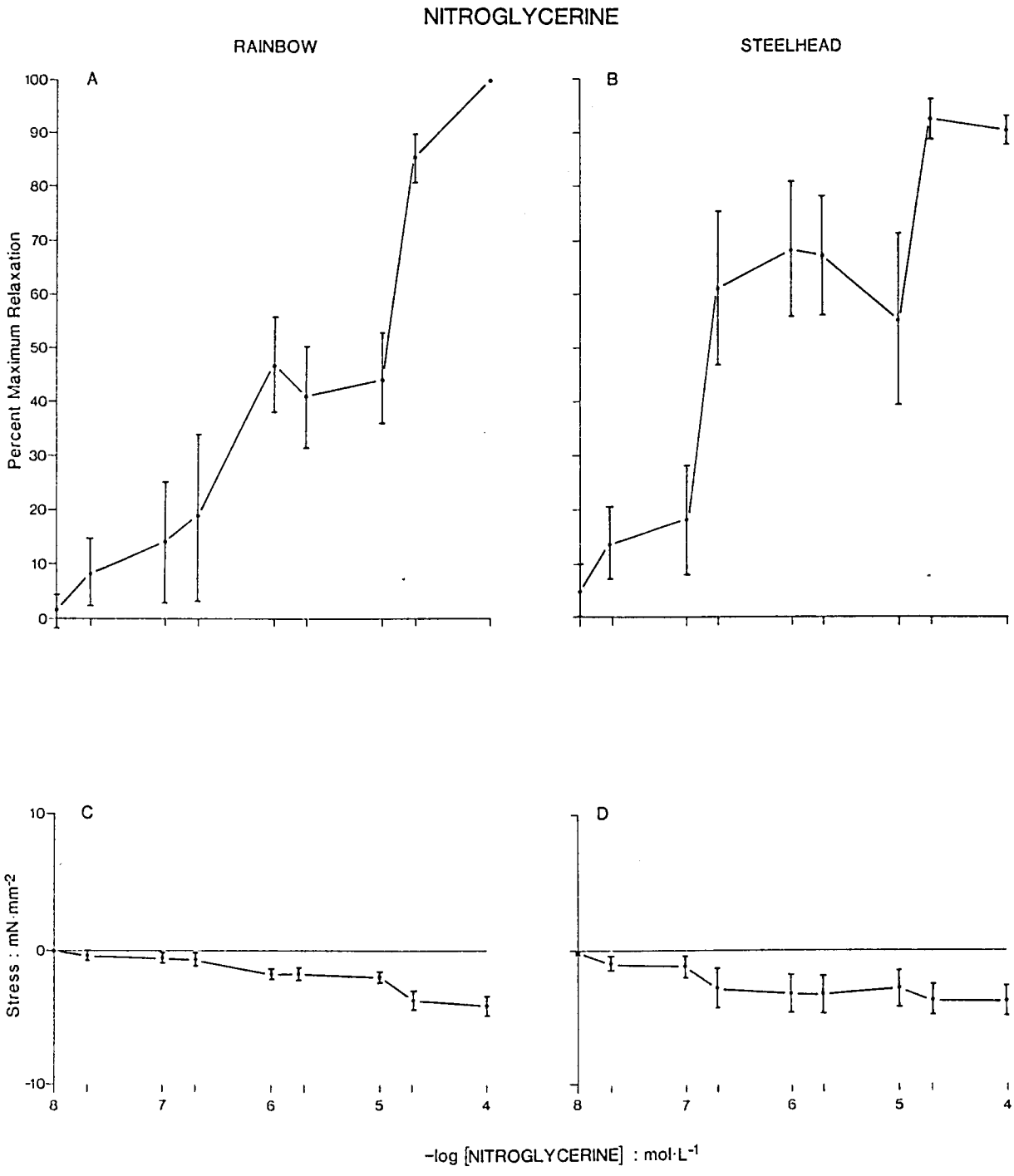
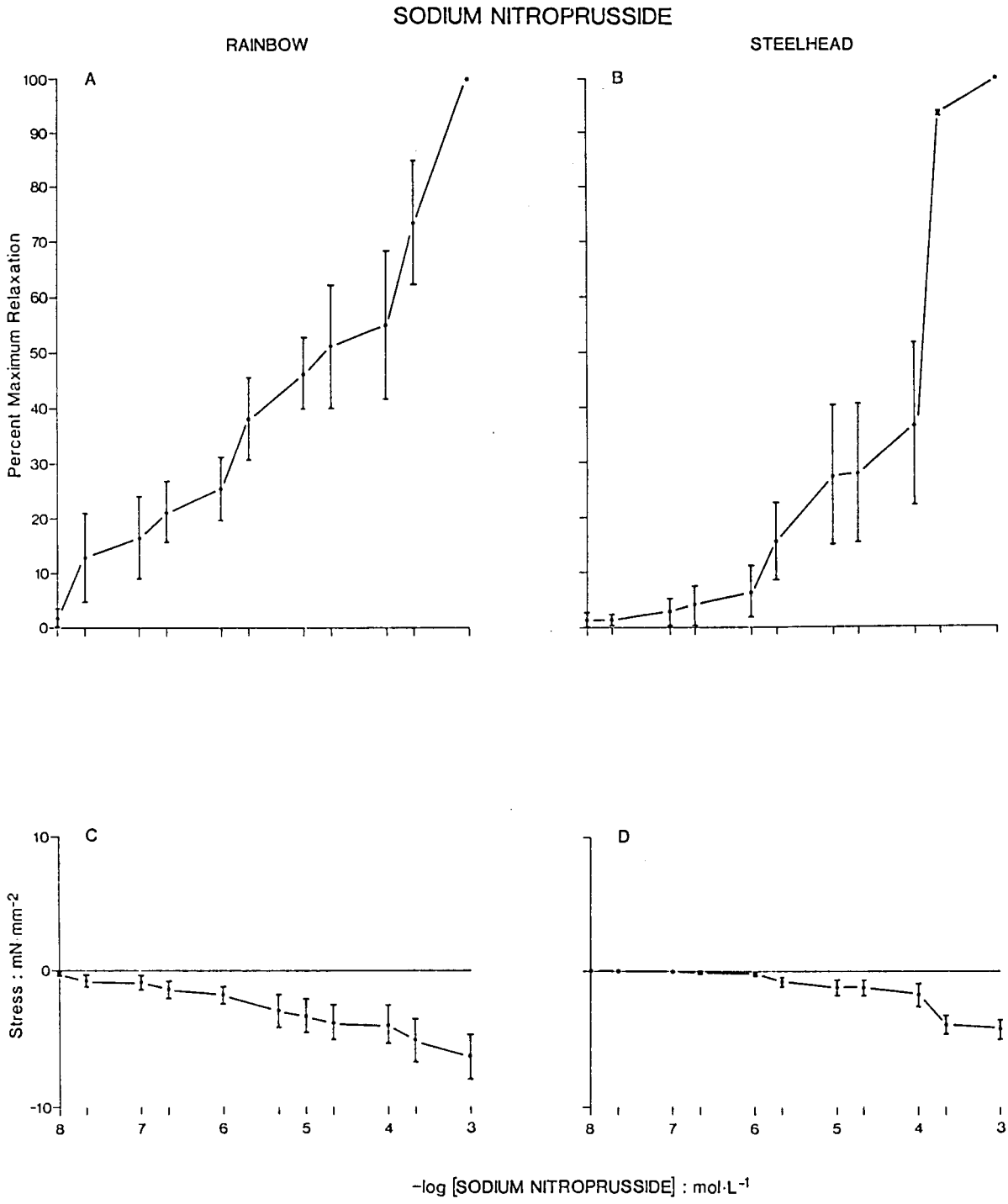

NITROGLYCERINE
RAINBOW
STEELHEAD
A
B
C
D
Percent Maximum Relaxation
Stress : mN·mm⁻²
100
90
80
70
60
50
40
30
20
10
0
-10
8
7
6
5
4
-log [NITROGLYCERINE] : mol·L⁻¹

Figure 13. Cumulative-concentration curve for the coronary artery of rainbow and steelhead trout in response to sodium nitroprusside. Vascular rings were precontracted with 45 mM KCl. The percent maximum relaxation of rainbow trout (A) and steelhead trout vascular rings (B) as well as the minimum stress of rainbow trout (C) and steelhead trout vascular rings (D) are shown. The values are expressed as means ± SEM. Statistical analysis of $EC_{50}$ and minimum stress values are summarized in Tables 2 and 3, respectively. Rainbow trout (N=5; n=6); steelhead (N=4; n=5).


SODIUM NITROPRUSSIDE
RAINBOW
STEELHEAD
A
B
C
D
Percent Maximum Relaxation
Stress : mN·mm⁻²
100
90
80
70
60
50
40
30
20
10
0
-10
8
7
6
5
4
3
-log [SODIUM NITROPRUSSIDE] : mol·L⁻¹

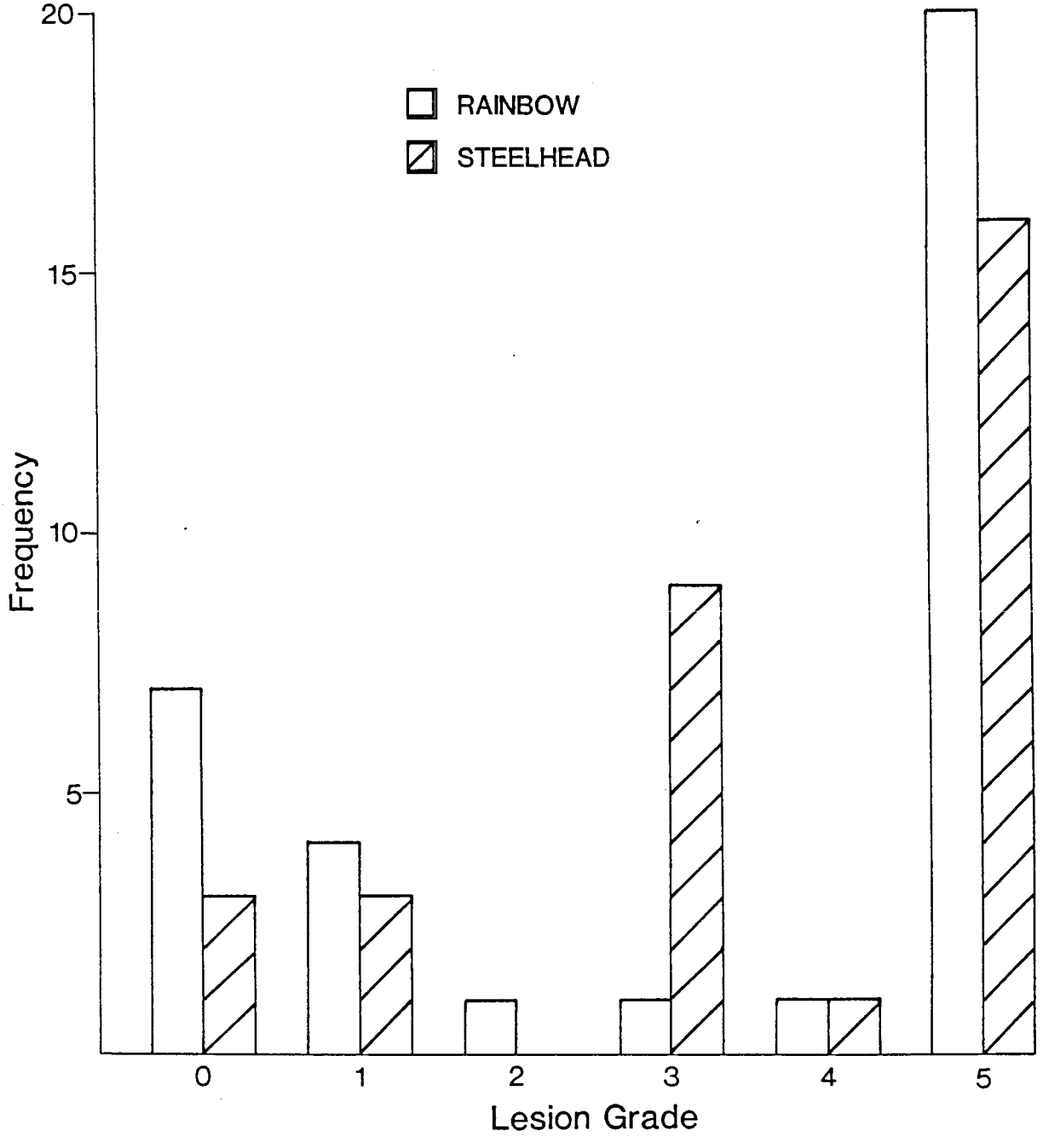
Control rings, which had not been wired, had approximately 90% of the endothelial layer intact. I suspect that inserting the wire through the lumen of the vessel stripped off the endothelium because of the small diameter of the vessel.

Arteriosclerotic lesion grading

The frequency of fish without arteriosclerotic lesions (grade '0') was greater in rainbow trout than steelhead trout (23% vs 9%). The frequency of severe arteriosclerotic lesions (grade '5') in rainbow and steelhead trout were not different (Figure 14). Approximately 50% of the vascular rings from rainbow and steelhead trout had grade '5' lesions.

The response to a given agent in rings graded '0' were compared to rings graded '5' when $n \geq 2$ in both groups. The purpose of this comparison was to examine whether or not lesions had an effect on vasoactivity. The potency ($p=0.15$) of adenosine and the maximum stress ($p=0.14$) produced were not significantly different in rainbow trout with and without severe lesions. Similar results were obtained for the potency ($p=0.5$) of isoproterenol and the minimum stress ($p=0.71$) produced. In steelhead trout, the potency ($p=0.65$) of acetylcholine and the maximum stress ($p=0.18$) produced were not significantly different in coronary arteries with and without severe lesions.

Figure 14. Frequency distribution for the severity of coronary arteriosclerotic lesions in rainbow and steelhead trout. For each of these two groups a subset of 20 arterial cross-sections observed with a given arterial grade is presented. Rainbow trout (n=34); steelhead trout (n=31).


20
15
10
5
Frequency
RAINBOW
STEELHEAD
0
1
2
3
4
5
Lesion Grade

## Discussion

Receptors in the large coronary artery of a lower vertebrate have been investigated for the first time in this study. The technique to study the vasoactivity of the coronary artery in Salmo gairdneri is an adaptation of a vascular ring preparation used to study similar responses of arteries in the mammalian system. The efficacy of this adapted technique was demonstrated by the ability to record dilatory and constrictory responses for the coronary artery in Salmo gairdneri and to reproduce previously reported responses to ATP for the rat femoral artery (Kennedy et al., 1985). Adenosine dilates the rat femoral artery with or without endothelium; ATP constricts the femoral artery in the absence of endothelium and dilates the vessel in the presence of endothelium. In this study, adenosine resulted in vasodilatation of the femoral artery while ATP resulted in vasoconstriction. These results are consistent with earlier studies provided the endothelium was lost during preparation of the vascular ring. Silver staining of the intimal surface of the trout coronary arteries confirmed that the endothelium was stripped during the mounting procedure. Since the femoral artery is comparable in size to the coronary artery of the rainbow and steelhead trout, it was assumed that the endothelium was absent in the femoral arteries.

### Purinergic receptors

Adenosine, ADP and ATP caused vasoconstriction of the coronary artery in Salmo gairdneri. The excitatory response to adenosine is a novel finding for a vertebrate coronary vascular

bed. In mammalian species, adenosine causes endothelium-independent relaxation of the coronary artery (Schnaar and Sparks, 1972; Herlihy et al., 1976; Foley et al., 1979; Detar, 1980; Cocks and Angus, 1983; Hintze and Vatner, 1983, 1984) and endothelium-dependent relaxation of the coronary artery (Rubanyi and Vanhoutte, 1985). Adenosine also causes endothelium-dependent relaxation in other vascular beds (Berne et al., 1983) with the exception of the renal artery of the dog (Osswald, 1975) and the pulmonary artery of the guinea pig (Wiklund et al., 1987) in which adenosine causes endothelium-independent vasoconstriction.

The mammalian purinergic receptor system is divided into $P_1$- and $P_2$-purinergic receptor subtypes. $P_1$-purinergic receptors are activated by adenosine and are blocked by theophylline (Osswald, 1975; Burnstock, 1978; Wiklund et al., 1987 ). $P_2$-purinergic receptors are activated by ADP and ATP and are not blocked by theophylline (Burnstock, 1978; De Mey and Vanhoutte, 1982). Adenosine activates the $P_1$-receptors on the vascular smooth muscle with an inhibitory effect and less commonly activates $P_1$-receptors on the endothelium with the same effect. Adenosine, in most blood vessels, causes endothelium-independent relaxation. Vasodilatation in response to ADP and ATP is endothelium-dependent. Activation of $P_2$-receptors on the endothelium causes relaxation of the blood vessel. When the endothelium is absent, $P_2$-receptors on the vascular smooth muscle are activated and have an excitatory effect. The inhibitory $P_2$-receptors are referred to as $P_{2y}$-receptors while excitatory $P_2$-

receptors are referred to as $P_{2x}$-receptors. Theophylline competitively inhibits $P_1$-receptors activated by adenosine, but does not block $P_2$-receptors activated by ADP and ATP.

The purinergic receptor system in the coronary artery of trout appears to be different to that in the coronary artery of mammals. In the coronary artery of the rainbow and steelhead trout, theophylline blocks the excitatory response to adenosine, ADP and ATP. Thus, adenosine, ADP and ATP probably activate the same purinergic receptor type. Adenosine, AMP, ADP and ATP also cause vasoconstriction in the gill vasculature (Colin and Leray, 1979; Colin et al., 1979; Leray et al., 1979) and in systemic blood vessels (Wood, 1975). Similarly, theophylline blocks the effects of adenosine, AMP, ADP and ATP in the gill vasculature of the trout (Colin and Leray, 1979; Colin et al., 1979). Whether or not the responses to adenosine, ADP and ATP are also endothelium-dependent cannot be determined in the present study. The vasoconstrictory response in the gill vasculature may also be mediated by purinergic receptors on the endothelium because the study was done using an isolated perfused head in which the vascular system was intact. Denudation of the endothelium under these conditions is unlikely. However, this does not rule out the possibility that purinergic receptors may be present only on the vascular smooth muscle.

The purinergic receptor type is similar to a $P_1$-receptor because it is inhibited by theophylline but is more like a $P_{2x}$-receptor because vasoconstriction resulted from purine activation. I am suggesting a model for the trout in which there

is one nonspecific purinergic receptor, $P_0$, located on the vascular smoooth muscle which is activated by adenosine, ADP and ATP and blocked by theophylline. Fish may have what can be termed the 'ancestral' purinergic receptor from which $P_1$- and $P_2$-subtypes have become specialized.

Cholinergic receptors

The acetylcholine-induced vasoconstriction of the coronary artery in rainbow and steelhead trout suggests that there is potential for cholinergic regulation of the coronary artery via a muscarinic-type receptor, however, direct innervation of the coronary artery in fish has not been investigated. In the trout aorta, acetylcholine causes endothelium-independent vasoconstriction (Miller and Vanhoutte, 1986) which may also be the case in the coronary artery. In mammalian species, the coronary artery responds to acetylcholine with either endothelium-dependent vasodilatation (Furchgott and Zawadzki, 1980; Cox et al., 1983; Toda, 1983; Griffith et al., 1984) or endothelium-independent vasoconstriction (de la Lande et al., 1974; Foley et al., 1979; Ito et al., 1979; Toda, 1983; Ginsburg et al., 1984; Forstermann et al., 1986; Young and Vatner, 1986). Whether or not the response in the trout coronary artery to acetylcholine is also modulated by EDRFs released from the endothelium cannot be concluded from this study. The absence of an acetylcholine response in some of the rainbow and steelhead trout and the high variability to acetylcholine has at least two explanations. First, a few preparations had sufficient intact endothelium to release EDRFs in adequate amounts to counteract or

completely offset the direct acetylcholine. The second and more likely explanation is that the density of cholinergic receptors varies with the location of the piece of vessel and between animals.

Non-receptor-mediated responses

Nitroglycerine and sodium nitroprusside cause vasodilatation of the coronary artery in the rainbow and steelhead trout. Nitroglycerine is broken down into nitric oxide which has been identified as an EDRF (Palmer et al., 1987). The relaxation of the coronary artery in response to nitroglycerine suggests that this vessel has the potential to respond to at least one EDRF. Sodium nitroprusside acts on the vascular smooth muscle by a mechanism proposed to be similar to that of nitroglycerine (Rapaport and Murad, 1983a, 1983b; Ignarro and Kadowitz, 1985; Rapaport et al., 1985). EDRFs were probably not released in response to endogenous drugs in this study because of the limitations of the isolated vascular ring preparation when using small diameter vessels.

Adrenergic receptors

The net response of a blood vessel which contains α- and β-adrenergic receptors depends upon the relative magnitude of the two receptor densities and is dependent upon the level of vascular tone (Zuberbuhler and Bohr, 1965; Bevan, 1979). Thus, there is either vasoconstriction, vasodilatation or no response to an adrenergic agonist. A mixed population of α-excitatory and β-inhibitory adrenergic receptors was demonstrated for the coronary artery of the rainbow and steelhead trout. This is also

the case in mammals (Feigl, 1967; Pitt et al., 1967; McRaven et al., 1971; Malindzak et al., 1972, 1978a,1978b; Vatner et al., 1974; Gerova, 1982). Although, cumulative concentration curves generated for adrenaline (n=3) and noradrenaline were variable and limited, β-adrenergic receptors appear to be the predominant receptor type in rainbow trout based on the greater number of vasodilatory than vasoconstrictory responses in precontracted vessels to high doses of adrenaline ($5x10^{-4}$ M). In contrast, steelhead trout responded to a single high dose of adrenaline with predominantly vasoconstriction.

An in vitro perfused heart study in the rainbow trout showed that vasoconstriction of the coronary circulation resulted in response to adrenaline at concentrations of $1x10^{-7}$ and $1x10^{-6}$ M given as a bolus at 15°C (Farrell, 1987). Similarly, adrenaline resulted in vasoconstriction of the coronary circulation in Atlantic salmon when administered cumulatively over the range $5x10^{-9}$ to $1x10^{-6}$ M at 9°C using a similar heart preparation (Farrell and Graham, 1986). My results show that adrenaline causes vasodilatation of the coronary artery in rainbow trout at cumulative concentrations less than $1x10^{-5}$ M and in response to single doses of $5x10^{-4}$ M. This observation combined with the vasoconstrictory response of the whole coronary circulation in rainbow trout (Farrell, 1987) suggests that the control of the coronary circulation is primarily in the arterioles. This is consistent with what is known for mammals. In the mammals, the primary source of control of the coronary circulation is also the arterioles, which account for approximately 70-80% of the total

coronary vascular resistance (Grega, 1987).

The predominant response to adrenergic stimulation is vasodilatory for the whole mammalian coronary circulation and vasoconstrictory for the large coronary artery (see introduction for references). The rainbow trout shows the opposite response with vasoconstriction for the whole coronary circulation and vasodilatation for the large coronary artery. There is variability in response to adrenergic stimulation between populations and different teleost species. In steelhead trout, vasoconstriction predominates in the large coronary artery. In Atlantic salmon, vasoconstriction predominates in the whole coronary circulation (Farrell and Graham, 1986) while in marlin vasodilatation predominates at low concentrations but then becomes vasoconstrictory at high concentrations (Davie and Daxboeck, 1984).

The variability in the responses to adrenaline within trout and among teleosts might be explained by the relationship between the magnitude of the $\alpha$ and $\beta$ responses to tone (Zuberbuhler and Bohr, 1965; Bevan, 1979). The level of resting tension for the coronary arteries used in this study was not constant. The resting tension for each piece of artery was optimized to account for the large variation in vessel diameter, thereby introducing a source of error which could potentially affect the net qualitative response of adrenaline. Differences in relative receptor densities could also result in large variations in responses to adrenaline. The non-uniformity of adrenergic receptor distribution has been demonstrated in mammals, for

example, the density of α-receptors in dog coronary arteries decreases distal to the aortic bifurcation (Zuberbuhler and Bohr, 1965; Bohr, 1967).

Preliminary experiments suggest that $\beta_1$-adrenergic receptors are present in the coronary artery of Salmo gairdneri. Atenolol, a specific mammalian $\beta_1$-adrenergic antagonist (Barrat, 1977), is a potent inhibitor of the non-specific β-adrenergic agonist, isoproterenol, in the coronary artery of rainbow and steelhead trout. Vasodilatation in response to noradrenaline which is specifically mediated by $\beta_1$-adrenergic receptors also suggests that $\beta_1$-adrenergic receptors are present. ICI 118,551, a specific mammalian $\beta_2$-adrenergic antagonist (Bilski et al., 1983) had no effect on the maximum response of the coronary artery in rainbow or steelhead trout, suggesting that $\beta_2$-adrenergic receptors may not be present in the coronary artery of Salmo gairdneri. The apparent presence and possible dominance of $\beta_1$-adrenergic receptor subtype in the coronary artery of Salmo gairdneri is consistent with the mammalian literature. In most mammalian species, $\beta_1$- and $\beta_2$-adrenergic receptors are present in the coronary circulation; $\beta_1$-adrenergic receptors are the predominant subtype in the large coronary artery (Zuberbuhler and Bohr, 1965; de la Lande et al., 1974; Brine et al., 1979; Vatner and Macho, 1981; Vatner et al., 1982, 1986; Schwartz and Velly, 1983; O'Donnell and Wanstall, 1984,1985; Nyborg and Mikkelson, 1985; Berkenboom et al., 1987), while $\beta_2$-adrenergic receptors are the predominant subtype in the coronary resistance vessels (Ross and Jorgensen, 1970; McRaven et al., 1971; Mark et al., 1972;

Gross and Feigl, 1975; Hamilton and Feigl, 1976; Ross, 1976).

Vasoconstriction in response to adrenaline and noradrenaline confirmed the presence of α-adrenergic receptors in the coronary artery of rainbow and steelhead trout. However, no vasoconstriction was demonstrated in response to phenylephrine, a nonspecific mammalian α-adrenergic agonist (Bayer et al., 1974). Phenylephrine also has no effect on the circulation in Atlantic salmon (Farrell and Graham, 1986). The characterization of α-adrenergic subtypes was not possible for a number of reasons, including desensitization of the α-adrenergic receptors in response to repeated doses of adrenaline, the lack of effect of specific α-adrenergic agonists and the greater occurrence of spontaneous activity in steelhead trout making it difficult to distinguish small changes in tension. Desensitization of α-adrenergic receptors also occurs in mammals. $Alpha_2$-adrenergic receptors in the saphenous vein in the rat are very susceptible to desensitization, whereas, $\alpha_1$-adrenergic receptors in the rat tail artery are insensitive to desensitization (Cheung, 1985a, 1985b, 1986).

The synthetic agonists and antagonists used in this study are specific for mammalian receptors, consequently, the presence or absence of a response to a given agonist or antagonist may be due to one of three possible explanations. First, there may be no receptors of that type present. Second, the receptor is present, but the drug is so highly specific for mammalian receptors that it has no effect on a non-mammalian receptor in Salmo gairdneri. Third, rainbow and steelhead trout may have

more generalized receptors which are not broken down into the subtypes described for mammals. An example is the turkey erythrocyte β-adrenergic receptor (Minneman et al., 1980). The turkey β-adrenergic receptor has pharmacological properties which are different from those of either the mammalian $\beta_1$- or $\beta_2$-adrenergic receptor. However, the agonist binding properties of the turkey β-adrenergic receptor are more similar to mammalian $\beta_1$-adrenergic receptors. Either one of the latter two possibilities may explain the lack of effect of phenylephrine on the coronary artery in Salmo gairdneri.

Serotonergic, histaminergic and bradykinin receptors

Serotonergic receptors are present in the coronary artery of rainbow trout and cause a modest vasodilatation at low concentrations which is reversed to give a modest vasoconstrictory response at higher concentrations. In rainbow trout, it appears that serotonin causes endothelium-independent relaxation of the coronary artery, suggesting that an inhibitory serotonergic receptor is present on the vascular smooth muscle of the coronary artery. In mammals, serotonin is a potent vasoconstrictor in both intact (Bove and Dewey, 1983; Brum et al., 1984; Kawachi et al., 1984) and isolated coronary arteries (Van Neuten et al., 1980; Brazenor and Angus, 1982; Cohen et al., 1983) with endothelium. However, in precontracted coronary arteries, a weak endothelium-dependent vasodilatation results in response to serotonin; removal of the endothelium enhances the vasoconstrictory effect of serotonin (Cocks and Angus, 1983; Cohen et al., 1983). The location and involvement of

serotonergic subtypes in the vascular action of the mammalian system awaits clarification (Young and Vatner, 1986).

Bradykinin and histamine elicited no response in the coronary artery, therefore it appears that the receptors which are activated by these drugs are not present in this vessel. This suggests that these receptors may not be important for the regulation of the large coronary artery in rainbow trout.

Arteriosclerosis

The basis of the comparison of the responsiveness of the coronary artery in rainbow trout to steelhead trout was the higher frequency of arteriosclerotic lesions in anadromous steelhead relative to freshwater rainbow trout (Robertson et al., 1961). Arteriosclerotic lesions affect the responsiveness of blood vessels to certain drugs by impairing the endothelium-mediated mechanism (Freiman et al., 1986; Sreeharan et al., 1986; Bossaller et al., 1987; Jayakody et al., 1987). There is a higher frequency of lesion-free rainbow trout than steelhead trout, however, the rainbow trout do have lesions and they can be as severe as those in steelhead trout. The presence of severe arteriosclerotic lesions appears to have no effect on the responsiveness of the coronary artery in rainbow or steelhead trout. Adenosine and isoproterenol were equally potent and produced the same amount of stress on the coronary arteries which had no lesions compared with those that had severe lesions in rainbow trout. Similar results were obtained for acetylcholine in steelhead trout coronary arteries. These data are not conclusive due to the limited number of samples available for

comparison of responses to drugs in animals with and without atherosclerotic lesions. The lack of an effect of arteriosclerotic lesions on the responsiveness of the coronary arteries is consistent with the mammalian literature which indicates that the endothelium-mediated responses are impaired. The endothelium was absent in this study, therefore, impairment of its functioning would not be demonstrated. These data do, however, indicate that the variability observed or any differences between rainbow and steelhead trout responses to a given agent probably cannot be explained by the presence or absence of lesions.

Evolutionary implications

Salmo gairdneri is an example of a lower vertebrate species with a coronary circulation, and as such, is representative of a transitional phase in the evolution of cardiac design. The general trends in the evolution of receptor systems in the coronary circulation are highlighted when the vasoactivity of large coronary artery of Salmo gairdneri is compared to that in the large coronary artery in mammals (Table 4). Comparison of the purinergic receptor system between Salmo gairdneri and mammals shows a move away from a generalized purinergic receptor in fish to a group of specialized purinergic receptor subtypes in mammals. A qualitative shift in the effect of purines on the coronary is also evident. Adenosine is excitatory in the fish coronary artery, while in mammalian large coronary arteries, adenosine is always inhibitory. The shift towards a vasodilatory response in mammals is not suprising given that coronary blood

TABLE 4. Summary of the responses of the large coronary artery in Salmo gairdneri and mammals to different receptor agonists.

| Agonist | Salmo gairdneri no endothelium | Mammals endothelium | Mammals no endothelium |
|---|---|---|---|
| Purinergic: | | | |
| adenosine | + | - | - |
| ADP | + | - | 0/+ |
| ATP | + | - | 0/+ |
| Cholinergic: | | | |
| acetylcholine | + | -/+ | + |
| Adrenergic: | | | |
| adrenaline or noradrenaline | -/+ | +/- | +/- |
| Serotonergic: | - | +/- | + |
| Histaminergic: | 0 | -/+ | 0/+ |
| Bradykinin: | 0 | -/+ | + |

+ excitatory
- inhibitory
0 response not detectable

flow to the myocardium is essential to sustain life in mammals which is not the case in fish. The minimal effect of serotonin and the lack of effect of histamine and bradykinin illustrates that the regulation of the coronary circulation in fish may be less complex than regulation in mammals because fewer receptor systems may be involved. The ability to respond to EDRFs is shared by the large coronary arteries of Salmo gairdneri and mammals, although the release of EDRFs in the fish coronary artery remains to be demonstrated. The general trend in the evolution of the receptor systems in the coronary circulation is a move away from generalized, predominantly excitatory receptors in fish to greater numbers of more specialized receptor systems which are predominantly vasodilatory in mammals.

Future studies

The role that the endothelium plays in the control the large coronary in Salmo gairdneri remains unanswered because of the limitations of the experimental set up. Two different approaches could be used to circumvent the problem of the stripping of the endothelium when mounting the vascular rings. One approach is to use much larger fish to deal with the mechanical problem. Another approach is to use the same size coronary arteries mounted in the same way (recipient) but to include a donor coronary artery. The donor artery would consist of a long segment of the coronary left attached to the bulbus arteriosus and cannulated so that the effluent from this segment of artery could be superfused onto the mounted recipient vessel. In this way, pharmacologically active agents could be injected into the

donor coronary artery and EDRFs, if any are released in response to an agent, could be tested directly on the denuded recipient.

## CONCLUSIONS

1. a) Purinergic receptors are present in the coronary artery of Salmo gairdneri and are excitatory in response to adenosine, ATP and ADP in denuded vessels. Adenosine, ATP and ADP are blocked by theophylline and may activate an ancestral-type purinergic receptor.

b) Muscarinic receptors are present and are excitatory in response to acetylcholine in denuded vessels.

c) A mixed population of α-excitatory and β-inhibitory adrenergic receptors are present in the denuded vessel. The β-adrenergic receptors are probably $\beta_1$-adrenergic receptors.

d) Serotonergic receptors are present and are inhibitory in denuded vessels.

e) Histamine and bradykinin receptors are not present in the denuded vessel.

2. Denuded coronary arteries of Salmo gairdneri respond to EDRF analogues.

3. The presence of arteriosclerotic lesions does not appear to affect the responsiveness of the denuded coronary artery to acetylcholine, adenosine and isoproterenol.

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