OXYGEN-SENSITIVE CHEMORECEPTORS AND CARDIOVASCULAR AND VENTILATORY CONTROL IN RAINBOW TROUT

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Zoology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February, 1991

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Date __May 27 1991__

ABSTRACT

Fish respond to changes in external (water) and internal (blood/tissue) O_2 levels by altering cardiovascular and ventilatory performance. These reflexes are mediated, for the most part, by O_2-sensitive chemoreceptors. Although the reflex responses of intact fishes have been characterized in detail, the receptors mediating these reflexes to hypoxia are poorly understood. To this end, afferent neural activity from O_2-sensitive chemoreceptors was recorded from the glossopharyngeal nerve (cranial nerve IX) in the isolated, perfused first gill arches of rainbow trout (*Oncorhynchus mykiss*).

Branchial O_2-sensitive chemoreceptors were sensitive to changes in external and/or internal O_2 tensions. Some receptors were sensitive to either internal or external O_2 levels and others were sensitive to both. External receptors showed an increase in activity as the P_{O2} was decreased to about 40 torr. Below 40 torr, afferent activity was depressed but recovered as P_{O2} was increased. Reversible depression of O_2 receptor activity at low P_{O2}s has been observed in mammalian O_2 receptors and is believed to illustrate the dependence of receptor activity on oxidative metabolism (Lahiri *et al.*, 1983). Occluding perfusate flow through the gill had little effect on afferent activity indicating that the internal receptors were not very flow sensitive. Sodium cyanide, a potent O_2 receptor stimulant, dramatically increased afferent neural activity. The response characteristics of these receptors are similar to tuna gill and mammalian carotid body O_2 receptors and suggest that these may be the receptors that mediate the cardiovascular and ventilatory reflex responses of fishes to hypoxia.

A number of different neurochemicals are thought be involved in O_2 transduction. Catecholamines are released into the circulation of fishes in response to hypoxia and it was hypothesized that adrenergic stimulation of O_2 receptors might contribute to the observed reflex

responses. Epinephrine and norepinephrine stimulate O_2 receptors and ventilation in mammals (Fidone and Gonzalez, 1986). Although fish respond to large dosages of epinephrine and norepinephrine (100-1000 nmol/kg) with hyperventilation (lower dosages, 5 nmol/kg, have little or no effect in intact fish), the afferent neural activity from the branchial O_2 receptors was not affected by these neurochemicals. Thus, ventilatory responses to increased circulating catecholamines do not appear to be mediated by branchial O_2 receptors. Inhibition of O_2 receptor activity by propranolol was probably due to indirect effects. Dopamine elicited a dose-dependent brief burst of chemoreceptor activity followed by inhibition but had only modest effects on DA blood pressure and inhibited opercular pressure amplitude in intact fish.

Serotonin (5-Hydroxytryptamine) caused a transient increase in chemoreceptor activity. In intact fish, serotonin, stimulated heart rate, decreased DA blood pressure and stimulated ventilation.

Cholinergic agonists (acetylcholine, nicotine and muscarine) significantly stimulated O_2 receptor discharge. Acetylcholine and nicotine increased heart rate, DA blood pressure and ventilation in intact fish, whereas, muscarine decreased heart rate and DA blood pressure and increased ventilation. Atropine inhibited O_2 receptor activity but had little affect on ventilation in intact fish.

Cholinergic mechanisms appear to be more important than adrenergic mechanisms in controlling the cardiovascular and ventilatory reflex responses mediated by branchial O_2-sensitive chemoreceptors. The response characteristics of branchial O_2-sensitive chemoreceptors indicates that they are homologous to the O_2 receptors of the mammalian aortic and carotid bodies.

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ACH	Acetylcholine
ATRO	Atropine
bpm	Beats or breaths per minute
CNS	Central nervous system
CSF	Cerebrospinal fluid
DOP	Dopamine
DA	Dorsal aorta
EDF	Extradural fluid
EPI	Epinephrine
f_G	Gill ventilation rate (bpm)
f_H	Heart rate (bpm)
ISO	Isoproterenol
MUSC	Muscarine
NaCN	Sodium cyanide
NIC	Nicotine
NOREPI	Norepinephrine
P_{DA}	Dorsal aorta blood pressure (cm H_2O)
P_{OP}	Opercular pressure amplitude (cm H_2O)
PROP	Propranolol
PVP	Polyvinylpyrrolidone (MW = 40,000)
5-HT	5-Hydroxytryptamine (serotonin)

ACKNOWLEDGEMENTS

INTRODUCTION

Most living organisms possesses metabolic pathways dependent upon oxygen (O_2), and the ability to respond to environmental hypoxia and increased O_2 demand are basic functions of nearly all animals. Thus, mechanisms which allow organisms to sense and respond to variations in O_2 availability and demand are critically important. This is particularly so in water, where life first evolved, because aquatic oxygen availability is spatially and temporally variable. Paradoxically, very little is known about O_2-sensitive chemoreceptors in aquatic vertebrates although a great deal is known about chemoreceptive control of their cardiovascular and ventilatory reflexes. Lacking detailed information about the receptors which sense and control the responses of fishes to hypoxia leaves a large gap in our understanding of the evolution of respiratory control. To this end, this thesis examines the discharge characteristics and effects of neurochemicals on O_2 receptor afferent activity and cardio-ventilatory reflexes in the rainbow trout (*Oncorhyncus mykiss*).

REFLEX RESPONSES TO HYPOXIA AND HYPOXEMIA

While little is known about the structure and location of O_2-sensitive chemoreceptors, their afferent pathways or factors modulating their neural activity in fishes, the reflex responses of fishes to hypoxia have been studied in great detail. The basic responses of fish to aquatic hypoxia or lowered arterial O_2 content (hypoxemia) are: 1) swimming away from an hypoxic region and 2) altering cardiovascular and ventilatory variables to maintain O_2 uptake. The fact that fishes are able to sense and respond to variations in O_2 is taken as *prima facie* evidence that they possess O_2-sensitive chemoreceptors.

Cardiac Responses

• Most fishes respond to aquatic hypoxia by reflexively decreasing heart rate (bradycardia) (Laurent *et al.* 1983; Farrell, 1984), although some species may show little or no cardiac response to hypoxia (Saunders and Sutterlin, 1971). Hypoxemia, on the other hand has no apparent reflex effect on heart rate (Daxboeck and Holeton, 1978; Smith and Jones, 1978). Hypoxic bradycardia is a vagal, cholinergic reflex and is abolished by section of the cardiac branch of the vagus nerve or pre-treatment with atropine (a cholinergic antagonist) (Laurent *et al.* 1983). Although heart rate is reduced during hypoxia, cardiac output is maintained or increased by an increase in stroke volume (Randall, 1968). Hypoxic bradycardia is thought to improve blood oxygenation by allowing the blood a longer residence time in the gills. This longer residence time may increase gas exchange by allowing the blood to more fully saturate with O_2 in the face of a reduced transbranchial O_2 gradient (see Randall, 1968 for review). The importance of this reflex is illustrated by several studies. If hypoxic bradycardia is abolished in dogfish by atropine or by sectioning the cardiac branches of the vagus nerve, arterial O_2 levels are reduced (Taylor *et al.* 1977; Short *et al.* 1979). Extirpation of the first gill arch in rainbow trout, where O_2 receptors responsible for hypoxic bradycardia are located (Daxboeck and Holeton, 1978; Smith and Jones, 1978), lowers arterial P_{O2} more than ligation of any of the other gill arches (Davis, 1971).

Ventilatory Responses

There is some variability in the response of fishes to hypoxia. Although some species reduce ventilation during hypoxia while others seem not to respond at all, a majority of fishes studied to date respond to aquatic hypoxia and/or hypoxemia by increasing ventilation. The respiratory water flow is usually continuous and is maintained by buccal and opercular pumps

(see Hughes and Shelton, 1962 for review). Exceptions, however, include periodic breathing which is characterized by periods of apnea between episodes of ventilation and ram-ventilating which occurs in some fishes while swimming. In all but the latter case, ventilation volume is increased by increasing the frequency and/or stroke volume of the buccal and opercular pumps. The relative contribution of frequency or stroke volume increases to overall ventilation volume depends upon the species of fish and age (see Shelton *et al.* 1986 for review). In the case of ram ventilation, ventilation volume is increased by increasing mouth gape (Roberts and Rowell, 1988). Increased ventilation serves to increase the convective transport of O_2 over the respiratory surface enabling the fish to maintain O_2 uptake as aquatic O_2 concentrations decline.

Thus the hypoxic reflexes amongst fishes (both cardiac and ventilatory) and the mechanisms of these reflexes appear diverse. Reasons for the differences observed between species cannot be fully understood until we understand the patterns of afferent discharge arising from the O_2-sensitive chemoreceptors which initiate the reflex responses.

Oxygen vs Carbon Dioxide

Although O_2 exerts dominant control over the endogenous respiratory rhythm in fishes, there has been a long standing debate about the extent to which hypercapnia or acidosis also modulates their ventilation (Smatresk, 1990). It has been suggested that hypercapnia or hypoxemia may elicit the release of endogenous catecholamines which stimulate ventilation, although the extent of ventilatory modulation by endogenous catecholamines is currently in question (Perry and Kinkead, 1990). Several recent studies also provide evidence for a significant direct effect of CO_2 or pH on ventilation following exercise, or in hyperoxic water (Heisler *et al.*, 1988; Graham *et al.*, 1990; Shipman, 1989; Wood *et al.*, 1990; Wood, 1990). These ventilatory responses are best correlated to arterial pH rather than arterial P_{CO_2}, *per se*,

although this remains in question. Furthermore, there are a number of studies which indicate that a large portion of the reflex responses to CO_2/pH are secondary responses elicited by changes in blood O_2 carrying capacity via Bohr and Root effects (Randall and Jones, 1973; Truchot *et al.* 1980; Smith and Jones, 1982). Given that systemic arterial chemoreceptors in all other animals examined respond to both O_2 and CO_2 (Smatresk, 1990), it seems probable the peripheral chemoreceptors of fishes may mediate reflex responses to hypercapnic acidosis as well as to hypoxemia. Palatine receptors, sensitive to CO_2, have been identified in carp (Yoshii *et al.* 1979), but whether they contribute to ventilatory reflexes has not been determined.

CHEMORECEPTOR LOCALIZATION

Many of the early experiments designed to delimit O_2 chemosensitive areas in fishes have been inconclusive, although most reports place O_2 receptive loci in the head region. Three general locations were suggested by Hughes and Shelton (1962) as possible chemoreflexive regions: 1. The buccal, pharyngeal, branchial region in contact with the respiratory water flow. 2. The vasculature, either arterial or venous. 3. Within tissues at the site of metabolism or the brain. Regardless of the specific O_2 receptor loci, that there appear to be distinct responses to aquatic hypoxia and hypoxemia indicates that there may be receptors monitoring both the internal and external environments (Smatresk *et al.* 1986; Burleson and Smatresk, 1990b).

Evidence for External and Internal Chemoreceptor Loci

Aquatic hypoxia and small bolus injections into inhalant water of NaCN, an O_2-sensitive chemoreceptor stimulant, elicit cardiac and ventilatory reflex responses (Smatresk, 1986; Smatresk *et al.* 1986; Burleson and Smatresk, 1990; McKenzie, 1990). Several experiments

designed to alter the aquatic P_{O_2} without changing arterial P_{O_2} tensions stimulated ventilation and elicited bradycardia (Saunders and Sutterlin, 1971; Smatresk *et al.* 1986). The receptors that mediate hypoxic bradycardia have been localized to the gills of salmonids (Daxboeck and Holeton, 1978; Smith and Jones, 1978), Atlantic cod (Fritsche and Nilsson, 1989) channel catfish (Burleson and Smatresk, 1990a), *Amia* (Burleson *et al.* 1990; McKenzie, 1990; McKenzie *et al.* 1991) and gar (Smatresk, 1991). In dogfish sharks these O_2 receptors appear to have a more diffuse arrangement, since bilateral section of all innervation to the bucco-pharyngeal cavity (cranial nerves V, VII, IX, and X) is necessary to abolish hypoxic bradycardia in these animals (Butler *et al.* 1977).

If blood O_2 content is experimentally reduced (internal hypoxia/hypoxemia) by carbon monoxide (Holeton, 1971), hemoglobin reduction (Cameron and Wohlschlag, 1969) or anemia (Wood *et al.* 1979; Smith and Jones, 1982) heart rate is either not affected or is slightly increased. Even when blood flow through the gills of trout is completely halted by ligation of the ventral aorta, water flowing over the gills must still be made hypoxic to elicit bradycardia (Randall and Smith, 1967). Experiments using NaCN as a chemical probe to stimulate O_2-sensitive chemoreceptors support these earlier studies. External NaCN elicits a significant bradycardia, but internal NaCN has no effect on heart rate in gar (Smatresk *et al.* 1986), channel catfish (Burleson and Smatresk, 1990a) or *Amia* (McKenzie, 1990; Burleson *et al.* 1990). Thus, hypoxic bradycardia appears to be mediated exclusively by externally oriented O_2 receptors.

Branchial ventilation, on the other hand, appears to be regulated by both externally and internally oriented chemoreceptors. Abrupt step changes in aquatic P_{O_2} stimulate ventilation in trout very rapidly (within about 5 sec) (Eclancher, 1972; Bamford, 1974). External bolus injections of NaCN solutions stimulate ventilation in channel catfish after about a 7 sec delay

(Burleson and Smatresk, 1990b). Sea raven sculpins show ventilatory responses to aquatic hypoxia even when blood is shunted past the gills and oxygenated artificially (Saunders and Sutterlin, 1971). Hypoxic depression of gill ventilation in gar is a ventilatory reflex to low aquatic P_{O_2} and is not affected by blood P_{O_2} (Smatresk et al. 1986). Internally oriented chemoreceptors also appear to contribute to ventilatory control. Ventilation increases in hypoxemic fish, even when the aquatic P_{O_2} is normal (Cameron and Wohlschlag, 1969; Holeton, 1971; Wood et al. 1979; Smith and Jones, 1982). Saunders and Sutterlin (1971) were able to bypass the gills of sea raven sculpins and make the blood hypoxic independently from water P_{O_2}. In their experiments internal hypoxia stimulated ventilatory reflexes even during external normoxia. By ventilating the air breathing organ of gar with various gas mixtures, Smatresk et al. 1986 were able to manipulate blood P_{O_2} separate from aquatic P_{O_2} and differentiate cardio-ventilatory reflex responses to internal and external stimulation. Data from experiments using NaCN injections also support the presence of internal O_2 chemoreceptive loci controlling ventilation in fishes. Intravascular injections of cyanide have been shown to stimulate ventilatory reflexes in lungfish (Lahiri et al. 1970), rainbow trout (Eclancher and Dejours, 1975), gar (Smatresk, 1986; Smatresk et al. 1986), channel catfish (Burleson and Smatresk, 1990b) and Amia (Burleson et al. 1990; McKenzie, 1990; McKenzie et al. 1991).

Although there is abundant physiological evidence which suggests that there are both external and internal O_2 chemosensitive loci, most of this evidence is indirect. The presence of external and internal chemoreceptive loci can only be determined unequivocally by recording from chemoreceptor afferents and subsequently identifying their afferent nerve endings.

Identification of Chemoreceptor Afferent Pathways

As described above, it is generally agreed upon that the receptors mediating hypoxic bradycardia are externally oriented and located in or near the gills. However, there is debate about the location and afferent pathways of the chemoreceptors mediating the ventilatory chemoreflexes. Proposed reflexogenic areas include the gills (Powers and Clark, 1942), pseudobranch (Laurent and Rouzeau, 1972), brain (Jones, 1973; Bamford, 1974), venous vasculature (Barrett and Taylor, 1984), arterial vasculature (Randall, 1982) and afferent branchial vasculature (Smatresk *et al.* 1986). This variety of putative loci may reflect the varied and often indirect methods used to study the chemoreceptive control of cardio-ventilatory reflexes and species/ecological differences between fishes studied.

The homology between the branchial arches and the carotid and aortic arches (where O_2 receptors are located in mammals) (Romer, 1962) suggests that this is the most likely location for O_2 receptors in fishes. The pattern of innervation of these regions in fishes is similar to that seen in mammals. As such, most experimental evidence indicates that the O_2-sensitive chemoreceptors which mediate hypoxic reflexes in fishes are in the gills.

The suggestion that O_2-sensitive chemoreceptors controlling hypoxic reflexes are in the gills is supported by both indirect and direct evidence from a number of fish species. Selective irrigation of various gill arches demonstrated that hypoxic bradycardia is mediated by branchial receptors in the first gill arch in trout (Daxboeck and Holeton, 1978). Histological studies have identified potential chemoreceptive cells. De Kock (1963) identified "end-buds"; nerve endings in the pharyngeal epithelium of salmonids in a position to be in contact with the respiratory water flow. Neuroepithelial cells have been identified in the gills of several species of freshwater teleosts (see Laurent, 1984 for review). These cells resemble mammalian Type I (glomus) cells and are located in the primary gill epithelia between the inhalant water and blood flow pathways (Dunel-Erb *et al.* 1982). These neuroepithelial cells, like mammalian Type I

cells, also contain serotonin (Dunel-Erb *et al.* 1982).

Section of the branchial innervation in several fish species has been used to localize O_2 chemoreceptors and identify afferent pathways. The branchial nerves in fish are cranial nerves VII (facial), IX (glossopharyngeal) and X (vagus) (Nilsson, 1984; Fig. 1). Cranial nerve VII innervates the spiracle in elasmobranchs, as well as the pseudobranch (in some species of teleosts; Laurent and Dunel-Erb, 1984) and ventilatory muscles of the jaw and operculum. The pseudobranch, which is not present in all species of fish (see Hughes, 1984 for review), and first gill arch are innervated by cranial nerve IX. The remaining gill arches, (number varies according to species (Hughes, 1984)), are innervated by branches of cranial nerve X (see Nilsson, 1984, for a more complete review of gill innervation).

Sectioning the branches of cranial nerves IX and X to the first gill arch in salmonids abolishes hypoxic bradycardia, however, ventilatory reflexes to environmental hypoxia remain unaltered (Smith and Jones, 1978; Smith and Davie, 1984). Bilateral section of all cranial innervation to the gills of the sea raven (Saunders and Sutterlin, 1971) and tench (Shelton, 1959; Hughes and Shelton, 1962) failed to abolish all ventilatory responses to aquatic hypoxia. In contrast to previous nerve section experiments, complete bilateral section of cranial nerves IX and X has been shown to abolish both cardiovascular and ventilatory responses to cyanide and hypoxia in channel catfish (Burleson and Smatresk, 1990b) and gar (Smatresk, 1991). Branchial denervation and pseudobranch ablation abolishes ventilatory reflex responses to NaCN in *Amia* (Burleson *et al.* 1990; McKenzie, 1990; McKenzie *et al.* 1991).

Figure 1. Simplified diagram showing the innervation pattern of the gills and pseudobranch of

a typical teleost fish (modified from Nilsson, 1984).

VII   IX   X   sympathetic chain
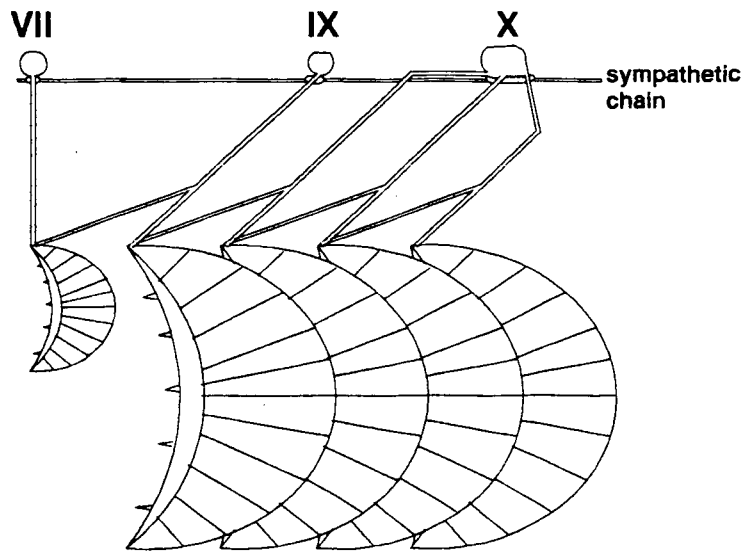
OXYGEN-SENSITIVE CHEMORECEPTORS

To date, O_2 sensitive chemoreceptors have been directly identified by afferent neural recording in decapod crustaceans (Ishii *et al.* 1989), teleost fish (Milsom and Brill, 1986; Burleson and Milsom, 1990a), anuran amphibians (Ishii *et al.* 1985b) chelonian reptiles (Ishii *et al.* 1985a), birds (Bouverot and Leitner, 1972) and mammals (Heymans and Neil, 1958). However, virtually all of the mechanistic and pharmacological studies of O_2 reception have focused on mammalian aortic and carotid bodies. The various hypotheses which have been proposed to explain the mechanisms of O_2 reception in the mammalian carotid body fail to account for all of the available data, and the roles of the numerous neurotransmitters found in these organs remain far from clear.

Mechanisms of O_2 Chemoreception

Mechanistic studies of O_2 chemoreception have only been conducted on mammalian receptors. The presumed mammalian "chemoreceptor unit" consists of two characteristic cell types, Type I (glomus) and Type II (sustentacular) and afferent and efferent nerve endings in close association with blood vessels (Fig. 2). Type I cells are characterized by numerous dense-core vesicles (neurotransmitter substances), ribosomes, mitochondria and other organelles. These cells are also innervated by sensory branches of cranial nerve IX. Type II cells have a glial or supportive function (see McDonald, 1981 for a review of carotid body ultrastructure). Current theories suggest that Type I cells are the primary transducer elements for O_2 chemoreception (López-Barneo *et al.* 1988; López-López *et al.* 1989; Biscoe and Duchen, 1990).

The exact mechanism of O_2 chemoreception has proven to be elusive. The following

Figure 2. Simplified diagram of the presumed O_2 chemoreceptive unit (mammalian) showing

Type I and Type II cells, afferent and efferent nerve endings and nearby blood vessel (modified

from Fidone *et al.* 1988).

Blood

STIMULI
low O2
high CO2
neurochemicals

Efferent Nerve

Afferent Nerve

DOP
NOREPI
EPI
ACH
5-HT

Type I

Type II
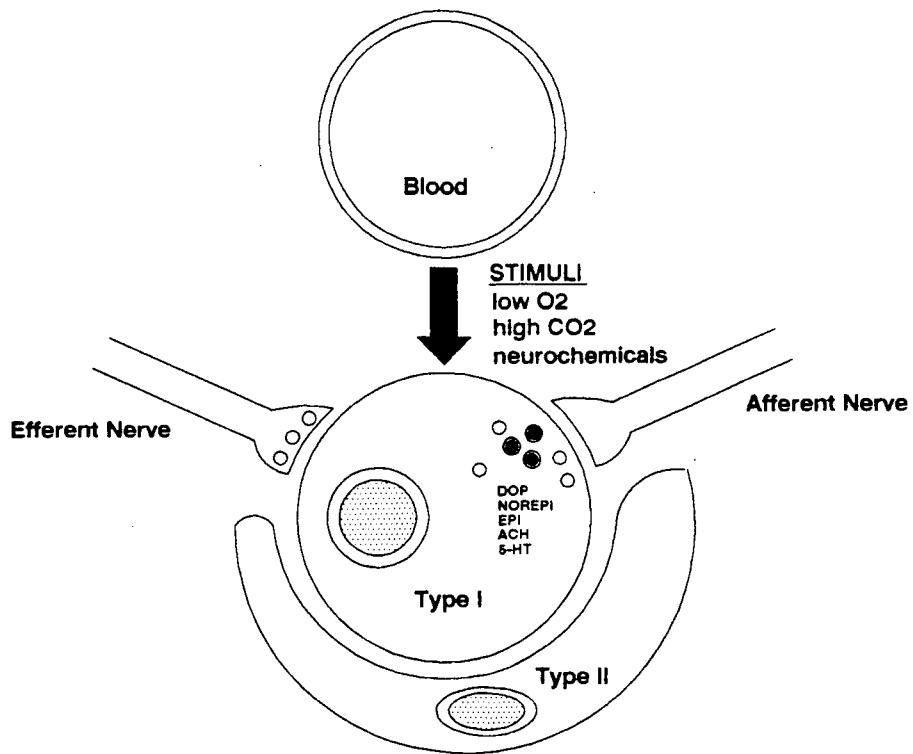
is a summary, based on recent data, of the events which presumably occur when the chemoreceptive unit senses hypoxia:

Low levels of O_2 in the arterial blood supply to the chemoreceptive areas results in lowered tissue P_{O2}. This cellular hypoxia affects either a heme-containing enzyme linked via G proteins to K^+ channels, K^+ channels directly (López-Barneo *et al.* 1988; López-López *et al.* 1989) or mitochondria (Biscoe and Duchen, 1990) causing intracellular Ca^{+2} concentrations to increase. Two hypotheses suggest mechanisms responsible for the rise in intracellular Ca+2: 1) low P_{O2} inhibits activity of K^+ channels in the Type I cells resulting in a depolarization that opens voltage-gated Ca^{+2} channels resulting in an influx of Ca^{+2} into the cell from extracellular stores (López-Barneo *et al.* 1988; López-López *et al.* 1989) or 2) mitochondrial depolarization releases Ca^{+2} into the cytosol (Biscoe and Duchen, 1990). Regardless of the precise mechanism, increased intracellular Ca^{+2} levels increase the rate of exocytotoic secretion, thus the release of neurotransmitter(s) into the synapse between the Type I cell and afferent neuron. The neurotransmitter(s), in turn, depolarizes the post-synaptic afferent nerve fiber and leads to action potentials in the afferent nerve (carotid sinus nerve). This nerve transmits the afferent information from the O_2 receptors to the cardio-respiratory centers of the medulla. The afferent information is integrated in the medulla which coordinates hypoxic reflexes to correct for the low arterial P_{O2}.

NEUROHUMORAL CONTROL OF HYPOXIC REFLEXES

Given the paucity of data on the neurochemical control of hypoxic reflexes in fishes, predictions for the roles of various neurochemicals in O_2 chemotransduction must be based on the mammalian model. The carotid body is a complex organ with efferent as well as afferent

innervation. Chemoreceptor afferent activity may be altered by efferent nerve activity, local vascular effects or by endogenous or exogenous neurochemicals. A number of different neurochemicals have been identified in the mammalian carotid body. Most of these have been localized to the Type I cells and/or afferent or efferent nerve endings (Table I). Hypoxic exposure has been shown to alter the concentration and release of some of these substances (Fidone *et al*. 1983; Hanbauer, 1983). The effects of various neurochemicals on O_2 receptor afferent nerve activity differ between species and within species as a function of anesthetic regime (Fidone *et al*. 1988). Although endogenous neurotransmitters are involved in the process of O_2 chemoreception in the mammalian carotid body, the precise roles that these chemicals play in the control of hypoxic reflexes are controversial. One of the most important questions remaining concerns the nature of the neurotransmitter(s) released from the glomus cell which initiates activity in the afferent nerve. It has proven difficult to answer this question using the mammalian carotid body model because of the plethora of receptor sites, neurochemicals and the potential for indirect effects. If the first gill arch-carotid arch homology holds, then these same neurochemicals should also play a role in the mechanisms of O_2 chemosensory transduction in the gills of fishes. Thus, characterization of the pharmacological responses of piscine O_2 receptors may be an extremely useful step in determining the role of neurotransmission in O_2 chemoreception.

The little neurochemical localization work which has been done on fish gills has concentrated mostly on the vasculature. Adrenergic and cholinergic nerves, mostly associated with the vasculature have been identified (Donald, 1984; Donald, 1987; Bailly and Dunel-Erb, 1986; Dunel-Erb and Bailly, 1986). Thus, these classes of neurochemicals have the potential to affect O_2 receptor afferent activity indirectly.

The neuroepithelial cells identified by Dunel-Erb *et al*. (1982) are monoamine containing

Table I. Neurotransmitters whose presence has been demonstrated in the carotid body (from McQueen, 1983).

Acetylcholine

Dopamine

Norepinephrine

Epinephrine

5-Hydroxytryptamine (Serotonin)

Adenosine

Adenosine triphosphate

Leucine enkephalin

Methionine enkephalin

Substance P

Vasoactive intestinal polypeptide

Taurine

Glutamate

cells. Histoflouresence, immunocytochemistry and the use of parachlorophenylalanine (an inhibitor of 5-HT synthesis) indicate that the major monoamine in these neuroepithelial cells is 5-HT (Laurent, 1984). 5-HT has been localized to mammalian Type I cells, and the fact that aquatic hypoxia causes the release of 5-HT from fish gill neuroepithelial cells suggests a chemoreceptive function (Laurent, 1984). The effects of 5-HT on hypoxic reflexes and afferent O_2-sensitive neural activity, however, have yet to be examined.

It has been suggested that catecholamines, particularly EPI and NOREPI, may play an important role in the cardio-respiratory reflex responses of fishes to hypoxia (Perry and Wood, 1989). Catecholamines are released from sympathetic nerve endings at specific target tissues (i.e. heart or vascular smooth muscle) and into the circulatory system from chromaffin tissue. Catecholamines are liberated in response to "stressing" stimuli including aquatic hypoxia, anemia, hypercapnia, low external Ca^{+2}, acid-infusion, air exposure and exercise (see Perry *et al.* 1989 for review). The role that catecholamines play in the ventilatory responses of fishes to hypoxia is currently debated (see Perry and Kinkead, 1990 and Taylor and Randall, 1990).

Evidence supporting a role for catecholamines include studies which show that aquatic hypoxia stimulates the release of catecholamines, exogenously administered catecholamines stimulate ventilation and ventilatory reflexes are blocked by hyperoxia or the ß-antagonist, PROP (see Aota *et al.* 1990). However, there are other studies which show that although catecholamines may be released during hypoxic exposure, moderate hypoxia stimulates ventilation but not catecholamine release, β-blockade does not abolish ventilatory reflexes and exogenous catecholamines at dosages which approximate physiological concentrations in the blood often do not stimulate ventilation (Perry and Kinkead, 1990; Playle *et al.* 1990).

Uncertainties about effects of catecholamines on ventilatory reflexes makes it difficult to speculate about mechanisms and loci for observed responses. In mammals, the ventilatory

effects of increased blood levels of circulating catecholamines are mediated by carotid body O_2 receptors (Fidone and Gonzalez, 1986). It has been proposed that the ventilatory effects of catecholamines in fishes could also be mediated centrally (Aota and Randall, 1989; Taylor and Randall, 1989; Aota *et al.* 1990). The piscine blood-brain barrier differs from that of mammals in that it allows catecholamines to cross (Peyraud-Waitzenegger *et al.* 1979; Nekvasil and Olson, 1986). Microinjections of catecholamines into respiratory areas of the medulla in dogfish stimulate ventilatory activity (Taylor and Randall, 1989), and perfusing the cranial space of *Amia* with artificial EDF containing EPI stimulates branchial ventilation (Burleson and Hedrick, unpublished observations).

Do catecholamines modulate ventilation via central effects or are catecholamines a component of the peripheral O_2-sensitive chemoreceptor response? The effects of catecholamines on peripheral O_2 receptors need to be assessed in order to help resolve this debate.

GOALS

The first purpose of the experiments presented in this thesis was to examine the discharge characteristics of O_2 sensitive chemoreceptor activity in the glossopharyngeal nerve to the first gill arch of rainbow trout during aquatic and internal hypoxia for comparison with the response characteristics of O_2 receptors in other poikilothermic vertebrates and mammals. We must know about the discharge characteristics of the O_2 receptors in order to understand reflex responses to hypoxia. For example, in mammals hypoxia results in an increase in O_2 receptor afferent discharge which stimulates ventilation. In fishes, the effects of hypoxia on O_2 receptors are known for only one species, the yellowfin tuna (Milsom and Brill, 1986). It is

not known how O_2 receptor activity relates to reflex responses in other fish, especially those which show variable responses to hypoxia. The presence of different internal and external O_2 chemoreceptive loci has been indirectly demonstrated in a variety of fish species but only directly by afferent nerve recordings in yellowfin tuna (Milsom and Brill, 1986).

The chemical link between glomus cell depolarization and afferent nerve activity is not understood. It is presumed that neurochemical(s) present in Type I cells and released during hypoxia initiate neural activity in the afferent neuron, however, the specific neurochemical(s) responsible are unknown. To determine the extent of the homology between fish gill O_2 receptors and mammalian carotid body O_2 receptors and to gain insight into O_2 sensory transduction mechanisms, the effects of various neurochemicals on fish chemoreceptor afferent activity will be examined.

The final purpose of this study was to examine the effects of various neurochemicals on cardiovascular and ventilatory variables in whole animals for comparison with their effects on gill O_2-sensitive chemoreceptors. Although the results will not necessarily reveal anything about the principle site of action, they will indicate the extent to which *in vivo* responses mimic predicted responses to *in vitro* stimulation of O_2 chemoreceptors. The results will also indicate the extent to which *in vivo* effects may be due to gill O_2 receptor stimulation as well as the extent to which these neurochemicals act on cardiovascular and ventilatory control at other sites.

MATERIAL AND METHODS

Animals

Rainbow trout (*Oncorhyncus mykiss*) were obtained from a local supplier and maintained outdoors in fiberglass tanks of circulating, dechlorinated tap water at the University of British Columbia. Fish were fed commercial trout food daily. The fish were generally in good health although occasionally ectoparasitic copepods, commonly called "anchor worms" (*Ergasilus sp.*), were found attached to the gill arches and epithelium of the buccal cavity and pharynx. Any fish that did not appear to be in good health or had parasites attached to the gills was discarded.

NERVE RECORDING STUDIES

The isolated, perfused gill preparation

The cranial anatomy of rainbow trout is such that the nerves to the gill arches are not easily accessible. It was found to be virtually impossible to expose enough of the nerve, without damaging the afferent branchial vasculature, to use traditional fiber picking nerve recording techniques. An *in vivo* preparation would require the use of anesthetics or decerebration which have the potential to alter chemoreceptor activity indirectly through effects on blood flow or directly (i.e. anesthetic effects). However, the isolated, perfused gill preparation allows direct control of perfusion and eliminates the need for anesthetics thus resolving some of these potential problems.

Surgery

On the day of an experiment a fish was netted from the tank, visually examined to confirm that it was in good health and was not parasitized, given an intracardiac injection of 5,000 units of sodium heparin (Allen and Hanburys, Canada). The fish was killed by a sharp blow to the head and pithed with a dissecting needle, thus avoiding the use of anesthetics which could interfere with nerve and chemoreceptor function. The fish was then placed, right side down, in a dissecting pan about half full of crushed ice to lower its temperature and presumably its metabolism. The left operculum was removed exposing the gills. During the procedure to remove the first gill arch the gills were periodically irrigated with ice cold hyperoxic saline to prevent desiccation and hypoxia. The first gill arch on the left side was cut from where it attaches to the floor of the pharynx and the other gill arches. It was then reflected anteriorly, and a scalpel was used to make an incision in the epithelia between the first and second gill arches where they attach to the roof of the pharynx. Using scissors, the incision was extended along the base of the filaments of the second gill arch exposing the underlying muscles, blood vessels and nerves. The pretrematic branch of the vagus innervating the first gill arch was located underneath a thin layer of muscle and sectioned. Fine forceps were used to dissect tissue and expose the glossopharyngeal nerve. The glossopharyngeal nerve was sectioned central to the petrosal ganglion where it leaves the neurocranium and carefully freed from the surrounding tissue. The gill arch was then cut away from it's dorsal attachment and placed in a petri dish containing ice cold hyperoxic gill perfusion solution (Table I). Excess tissue was trimmed away and the efferent branchial artery was cannulated (PE-50, Intramedic) and flushed with 3 ml of perfusate to clear it of blood. Silk suture (000, Ethicon) was attached to the dorsal and ventral cut portions of the gill for suspension in the gill chamber (Fig. 3). The gill was then placed in the water-jacketed chamber (Fig. 3), thermostatically controlled to 10°C, and

Figure 3. Diagram of the experimental apparatus used to perfuse gills and record afferent neural activity from branchial nerves.

electrode

syringe

air valve

air/nitrogen

mineral oil

perfusate

pressure transducer

air/nitrogen

water jacketed chamber
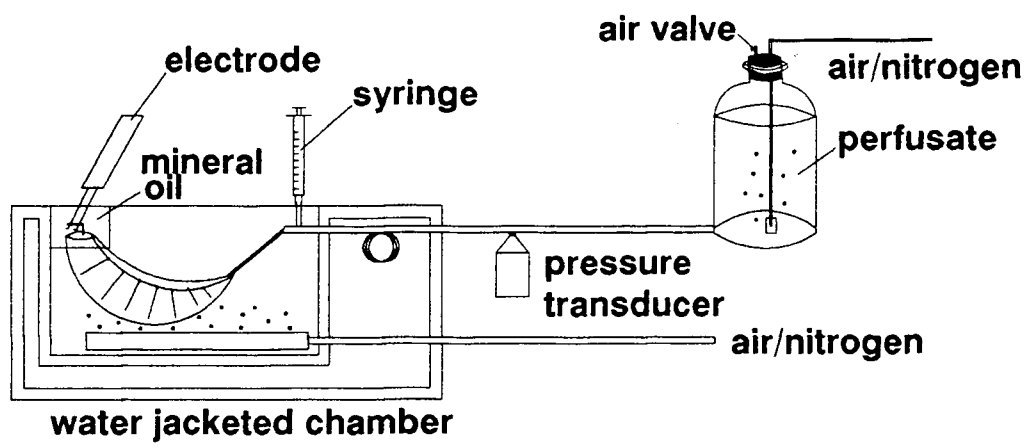
Table II. Perfusion solution for isolated gill arches (from Perry *et al.* 1984).

CHEMICAL	grams/liter
NaCl	6.59
KCl	0.31
Na_2HPO_4	0.14
$MgSO_4$	0.14
$CaCl_2$	0.14
$NaHCO_3$	1.10
$(NH_4)_2SO_4$	0.02
KH_2PO_4	0.05
Glucose	1.00
PVP	40.0
Heparin	5000 units

perfused at constant physiological pressure (30-60 cm H_2O) with filtered (Millipore 45 μm) physiological saline with PVP (MW=40,000) added as a colloid osmotic filler (Perry et al. 1984; Table II). In some experiments ISO (10^{-8} M) was added to keep the gill vasculature open. The ISO may have had a mild inhibitory effect on gill O_2 receptor discharge. If so, however, this would have been constant throughout the study and would not affect the interpretation of the data. In later experiments, high initial perfusion pressure (100-120 cm H_2O) was used to open the gill vasculature, and ISO was not added to the perfusate.

Once suspended in the gill chamber and attached to the perfusion apparatus, the nerve was cleaned of connective tissue and desheathed under a dissecting microscope (Wild Heerbrugg Ltd., Switzerland). The nerve was placed on a stainless steel platform under mineral oil to prevent desiccation during recording. Small bundles of axons were peeled from the main nerve trunk using fine forceps and placed over bipolar platinum wire (1/1000" diameter) electrodes. Extracellular nerve activity was measured using standard techniques.

The nerve signal was amplified using an A.C. pre-amplifier (Grass P5 series model P511K) with associated regulated power supply (Grass model RPS 107E) and high impedance probe (Grass model HIP511). A window discriminator (WPI model 121) was used to distinguish between different units in multiunit preparations. Window discriminator output was averaged over 1 sec time intervals using an integrating amplifier (Gould model 13-4615-70). Nerve activity was displayed on a storage oscilloscope (Tektronix model 5111A) and also monitored with an audio monitor (Grass model AM 8B). Perfusion pressure was measured using a pressure transducer (Statham model P23 Db) with associated pre-amplifier and amplifier (FRAMP model GPA-2). The window discriminator output (electroneurogram, ENG), integrated window discriminator output and perfusion pressure were displayed on a pen recorder (Beckman Type R Dynograph). These same signals along with the amplified nerve activity

were also recorded on audio tape using a reel to reel instrumentation tape recorder (Hewlett-Packard model 3968A). Signal records were played back for analysis on a 4-channel pen recorder (Gould model 2400S) and computer data acquisition system; an Olivetti M24 personal computer (IBM XT compatible) equipped with an analog to digital converter card (Data Translation model DT2801) using a commercial software data acquisition program (Labtech Notebook, Laboratory Technologies Corporation, Wilmington, MA).

The P_{O2} and pH of the perfusate were measured using a Radiometer acid base analyzer (PHM71 Mk 2) and associated electrodes. The O_2 electrode was zeroed with a solution of 0.1 M sodium borate and sodium sulphite and calibrated with air equilibrated water. The pH electrode was calibrated using Radiometer precision buffers. The gill chamber and electrodes were maintained at 10°C using a circulating water chiller (Lauda model RM 6).

Oxygen sensitivity of active units was tested by either bubbling the bath with N_2 to lower the external P_{O2}, applying NaCN externally to the gill filaments, perfusing the gill with hypoxic perfusate or injecting NaCN into the perfusion cannula. Bubbling the bath with N_2 to achieve various levels of external P_{O2} was a simple procedure, but I was not able to alter internal (perfusate) P_{O2} levels with any precision. Thus, stimulus-response curves for changing O_2 tensions were done for external receptors only.

Drugs and Neurochemicals

Drug solutions were made fresh daily using the before mentioned gill perfusate and stored away from light in a refrigerator until used. All injections were given via a side arm in the perfusion cannula in a single rapid 0.1 ml bolus. The following drugs were used: sodium cyanide (NaCN), (-)- norepinephrine hydrochloride (NOREPI), (±)-epinephrine bitartrate (EPI), (±)- isoproterenol (ISO), 3-hydroxytyramine (dopamine, DOP), (±)-propranolol (PROP),

5-hydroxytryptamine (serotonin, 5-HT), acetylcholine chloride (ACH), nicotine (hydrogen

tartrate salt, NIC), (±)-muscarine chloride (MUSC) and atropine sulfate (ATRO) (all drugs

obtained from Sigma Chemical Company).

REFLEX STUDIES IN INTACT FISH

Surgery

Fish were initially anesthetized in a solution of MS-222 (tricaine methanesulphonate)

(1:10,000), buffered with $NaHCO_3$, until ventilatory movements ceased then transferred to a

surgical table where the gills were artificially ventilated with a more dilute solution of MS-222

(1:20,000). The dorsal aorta (DA) was cannulated with polyethylene tubing (PE-50) using the

technique of Soivio *et al.* (1972). The DA cannula was secured to the roof of the mouth with

silk suture and led out a hole in the roof of the mouth in front of the nares via a flared section

of polyethylene tubing (PE-160). Once secured, the cannula was filled with heparinized

Cortland saline and plugged with a 23 gauge needle.

A small hole was drilled into the center of the operculum with a high speed drill

(Dremel moto-tool) for attachment of an opercular cannula. Opercular cannulae were fashioned

from a short length of 18 gauge stainless steel tubing bent at a 90° angle, flared pieces of

PE-160 and PE-200 tubing and silicone tubing (0.03" x 0.065" x 0.0175"; A-M Systems, Inc.

Everett, WA.). This assembly was secured to the fish with 00 silk suture. The silicone tubing

was much more flexible than polyethylene tubing, and this configuration prevented the cannulae

from kinking in the narrow confines of the perspex fish chamber. It was confirmed visually that

the opercular cannula did not interfere with normal ventilatory movements. After surgery the

fish were allowed to recover for at least 24 hours in a darkened perspex box with a flow of

aerated, dechlorinated tap water (Fig. 4).

Drugs and Neurochemicals

The drugs used in this experiment were the same as those used in the nerve recording studies. The drugs were dissolved in Cortland saline and injected via the DA cannula. The drug dosages used in this series of experiments were based on the dosages which elicited neural responses in the O_2 receptor experiments and were comparable to those reported in previous studies on cardiovascular regulation in fish (i.e. Wood and Shelton, 1980). Selective use of agonists and antagonists were used to confirm which neurochemicals are involved in cardio-ventilatory regulation. Control injections of Cortland saline, of the same volume as experimental injections, were also administered. External NaCN (250 µg dissolved in dechlorinated tap water) was given as a 0.5 ml bolus injected into the inflow water port (see Fig. 4). External control was a 0.5 ml bolus of dechlorinated tap water injected into the inflow water port.

Protocol

Heart rate (f_H) and blood pressure (P_{DA}; peak systolic) were measured from the DA cannula using a pressure transducer (Narco RP1500i) connected to one channel of a two-channel ink pen recorder (Gould 2400S model# 2107-4200-00) via a transducer amplifier (Gould model# 13-4615-50). Gill ventilation rate (f_G) and opercular pressure amplitude (P_{OP}) were measured from the opercular cannula using a pressure transducer (Statham P23BB) connected to the other channel on the pen recorder via another transducer amplifier.

After 24 to 48 hours recovery from surgery the DA and opercular cannulae were flushed with saline and water, respectively, and attached to the pressure transducers. The general

Figure 4. Diagram of experimental apparatus used to measure cardiovascular and ventilatory reflex responses of conscious, intact trout to internal and external NaCN and injections of various neurochemical solutions.

water

air

pressure
transducers

injection syringe

dorsal aorta pressure

opercular pressure

fish box

aeration column
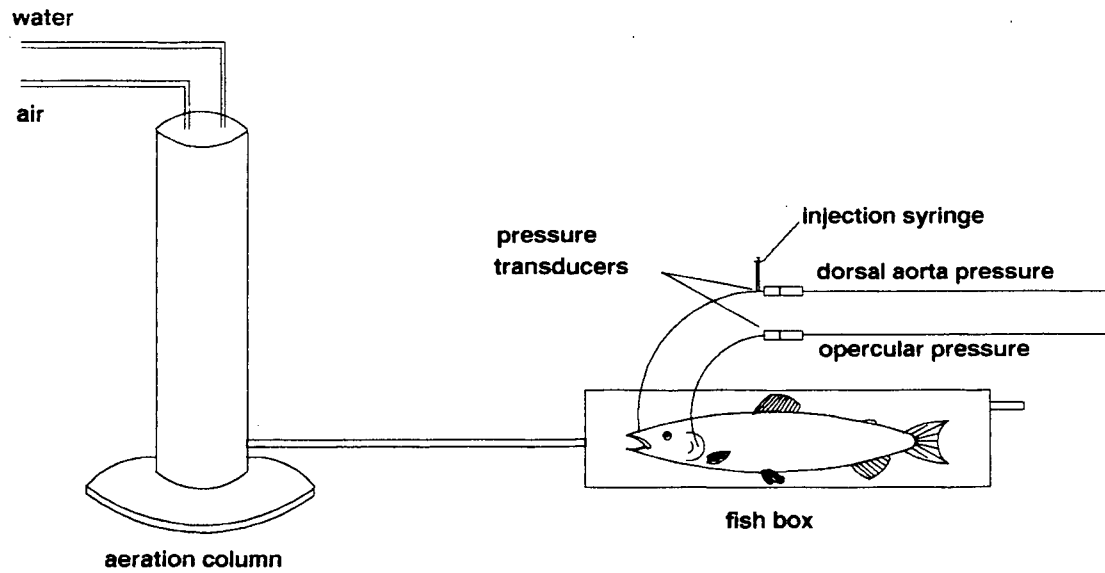
condition of the fish was assessed by observing the DA blood pressure and ventilation traces. If there was excessive struggling or if cardiovascular or ventilatory variables appeared abnormal (i.e. erratic or elevated: $f_H > 50$ bpm, $P_{DA} > 50$ cm H_2O, $f_G > 80$ bpm, $P_{OP} > 1.5$ cm H_2O), the fish was allowed more time for recovery. If levels still did not return to normal, the fish was discarded from the study.

All experiments were performed on resting fish in normoxic water. At least one minute of normal, resting cardiovascular and ventilatory variables was recorded before each injection. Following each injection, cardiovascular and ventilatory variables were recorded for 10 minutes. The order of drugs was randomized except for ATRO and PROP which, when given, were always the last injection to any fish. Fish were allowed at least 2 hours between injections, and only two drugs were injected into any individual fish each day. Not all of the drugs were injected into each fish. At the end of an experiment the fish was killed by injecting 3 ml of saturated KCl into the DA cannula.

Data analysis and statistics

Cardiovascular and ventilatory variables were averaged for 30 sec bins during the pre-injection control period and at each min of the 10 min record. Heart rate and P_{DA} were determined from the pressure trace measured from the DA cannula. Gill ventilation rate and P_{OP} were determined from the pressure trace from the opercular cannula.

The effects of control and experimental injections on cardiovascular and ventilatory variables were analyzed using a two-way analysis of variance (ANOVA) (Sokal and Rohlf, 1981) with time and type of injection (experimental or control) as covariates. Results were judged significant at $P \leq 0.05$. Thus, significance, when referred to in the text, means statistically significant. n values for each injection are reported in the figure legends.

RESULTS

Discharge characteristics of O_2-sensitive chemoreceptors

More than 800 glossopharyngeal nerve fibers were tested for O_2 sensitivity during the course of this study. Approximately 5% of these fibers displayed increased activity in response to hypoxia (internal and/or external); were insensitive to mechanical stimuli and were considered to be specific O_2-sensitive chemoreceptors. Afferent information arising from gill mechanoreceptors, sensitive to filament or raker displacement or changes in perfusion pressure, were by far the most numerous receptors observed but did not respond to either hypoxia or NaCN. On several occasions neural discharge from the branchial branch of cranial nerve X (vagus) to the first gill arch was screened for O_2-sensitive activity. Only raker and filament mechanoreceptor activity which did not respond to hypoxia or NaCN was recorded. Although no O_2-sensitive afferents were identified in cranial nerve X, only a small number of fibers were examined.

Resting branchial O_2-sensitive chemoreceptor afferent discharge was generally erratic. Bursting discharge patterns of afferent activity, as described by Milsom and Brill (1986) in the first gill arch of yellowfin tuna, were observed in only two pilot experiments. The erratic or random discharge pattern of O_2 receptors was not altered as P_{O2} was lowered, the net frequency of action potentials simply increased.

Of nineteen fibers in cranial nerve IX that exhibited O_2 sensitivity and were subjected to both external and internal stimulation; seven were sensitive to external changes in P_{O2} only, seven were sensitive to changes in internal perfusate composition only and five showed sensitivity to changes in both the internal and external milieu. An inability to control internal P_{O2} levels accurately with the experimental apparatus precluded any quantitative comparison of

internal and external receptor chemosensitivity.

External receptors showed an increase in activity as bath P_{O2} was decreased to 40 torr (Figs. 5 and 6). The P_{O2} and the flow rate of the perfusate were maintained during external hypoxia, demonstrating that the activity of these receptors could be altered independently of internal O_2 stimulus levels. Below about 40 torr, nerve activity in most units became depressed (Fig 6). The depressant effect of hypoxia was reversible, and O_2 sensitivity returned as O_2 tensions were increased. Oxygen receptor activity increased dramatically in all O_2-sensitive fibers when NaCN was applied externally onto the gill filaments and promptly returned to resting levels when the NaCN was rinsed from the filaments.

Internal O_2-sensitive chemoreceptors showed increased discharge rates in response to hypoxic perfusate and bolus injections of NaCN (Figs. 7 and 8). Some of the internal receptors also showed sensitivity to changes in the external P_{O2}. Internal receptors, however, did not appear to be very sensitive to O_2 delivery. These receptors showed remarkably low sensitivity to alterations in perfusate flow (Fig. 9). The response of internal chemoreceptors to occlusion of the perfusate flow was slight in comparison to the responses to hypoxia, NaCN and certain neurochemicals. This may indicate that O_2 consumption locally at the receptor level is low under these experimental conditions. Given that the gill perfusate was cell-free and did not contain any O_2 carrying pigments it was impossible to manipulate O_2 delivery independent of P_{O2}.

The average pH of the normoxic bath fluid ($P_{O2} \approx 120$-160 torr) (external) was 7.67 (n=24). The pH of normoxic perfusate ($P_{O2} \approx 120$-160 torr) was 7.64 (n=27) and of the hypoxic perfusate ($P_{O2}=9$-33 torr) was 8.80 (n=11).

Figure 5. Electroneurograms (ENG) and integrated activity (impulses/sec) showing the response of a single chemoreceptor unit to varying levels of external P_{O2}.
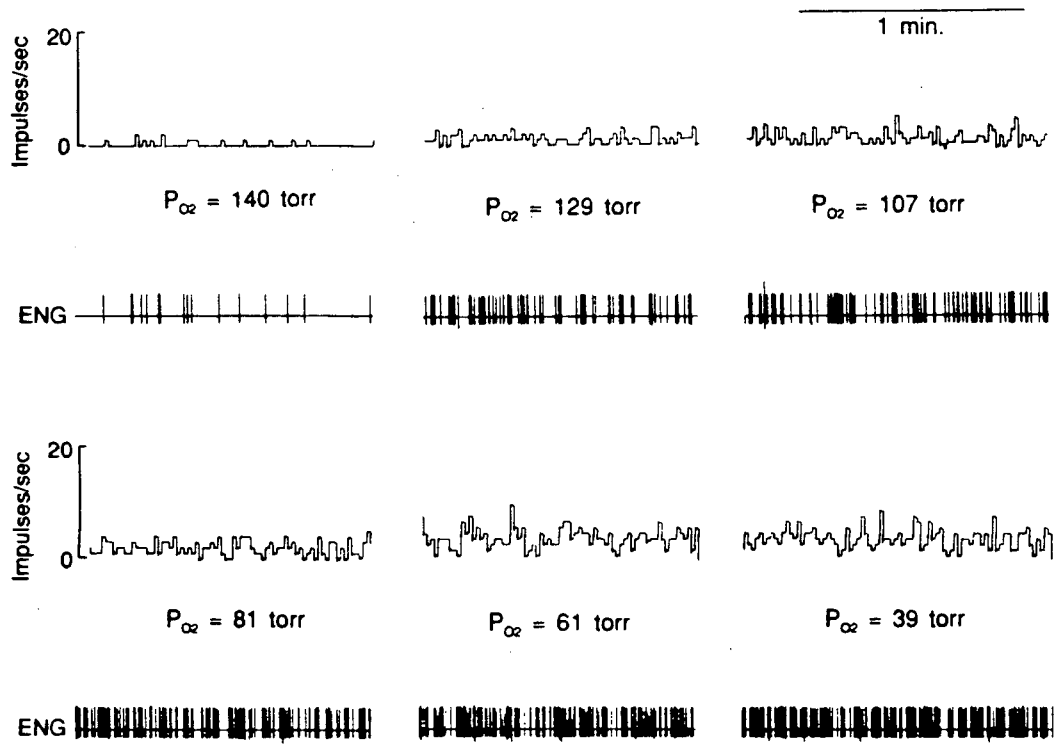
Figure 6. Changes in discharge frequency of eight O_2 sensitive units plotted against external P_{O2}. The solid line is a second order polynomial calculated from all values except those below a P_{O2} of 50 torr. Dashed line (drawn by eye) shows the hypothetical rate of decrease in discharge frequency as P_{O2} continues to decrease. Horizontal and Vertical bars are ± 1 SEM.

CHEMORECEPTOR ACTIVITY
(impulses/sec)

P$_{O2}$ (torr)
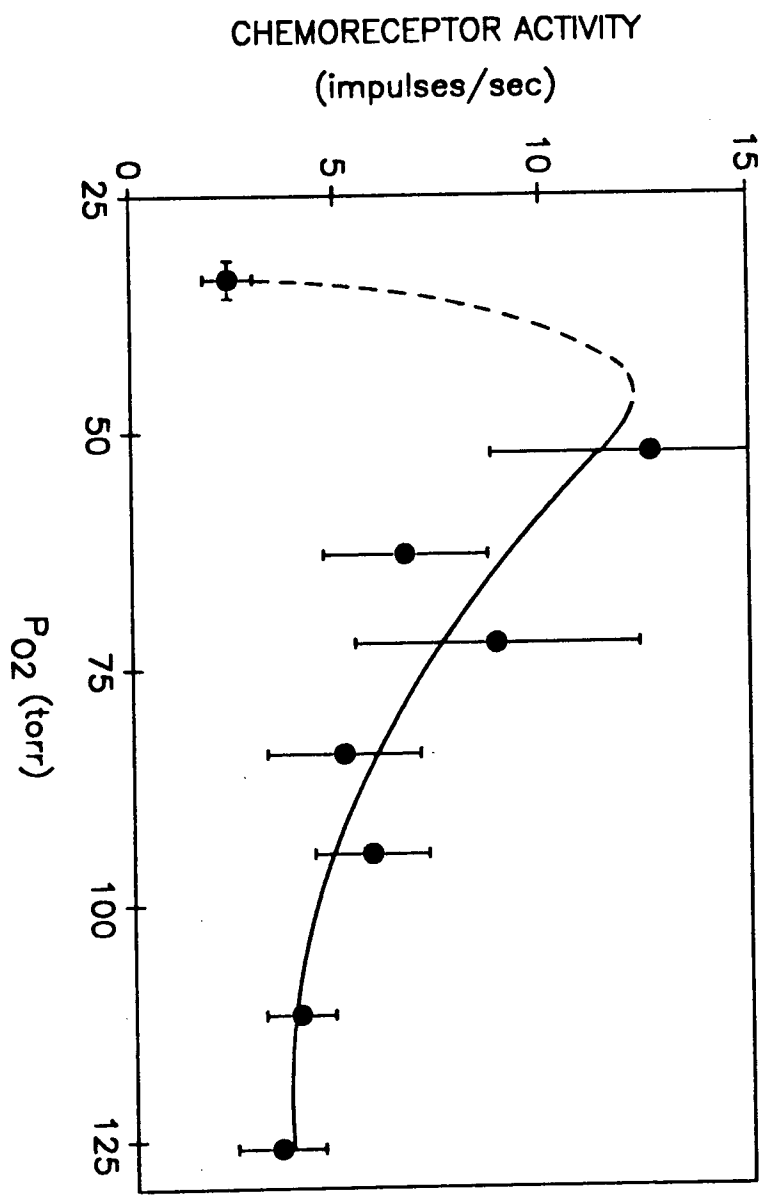
Figure 7. Response of a single unit to 25 µg NaCN injected into the perfusion cannula (internal receptor).

↑ Sodium Cyanide (25 ug)
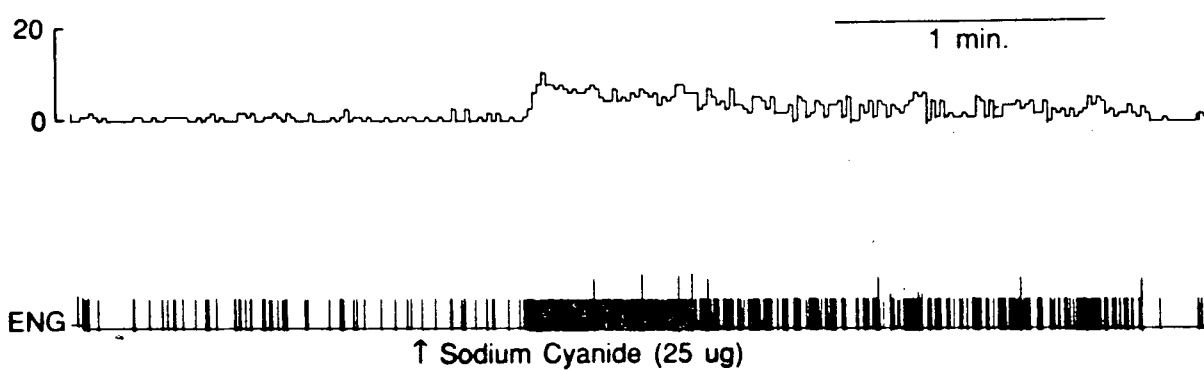
Figure 8. Response of an internal O_2 sensitive unit to hypoxic perfusate ($P_{O_2} \approx 20$ torr).

↑ Begin Hypoxic Perfusion   ↑ End Hypoxic Perfusion

Figure 9. Mean response (n=13) of internal O_2-sensitive chemoreceptors to occlusion (OFF) of perfusate flow.
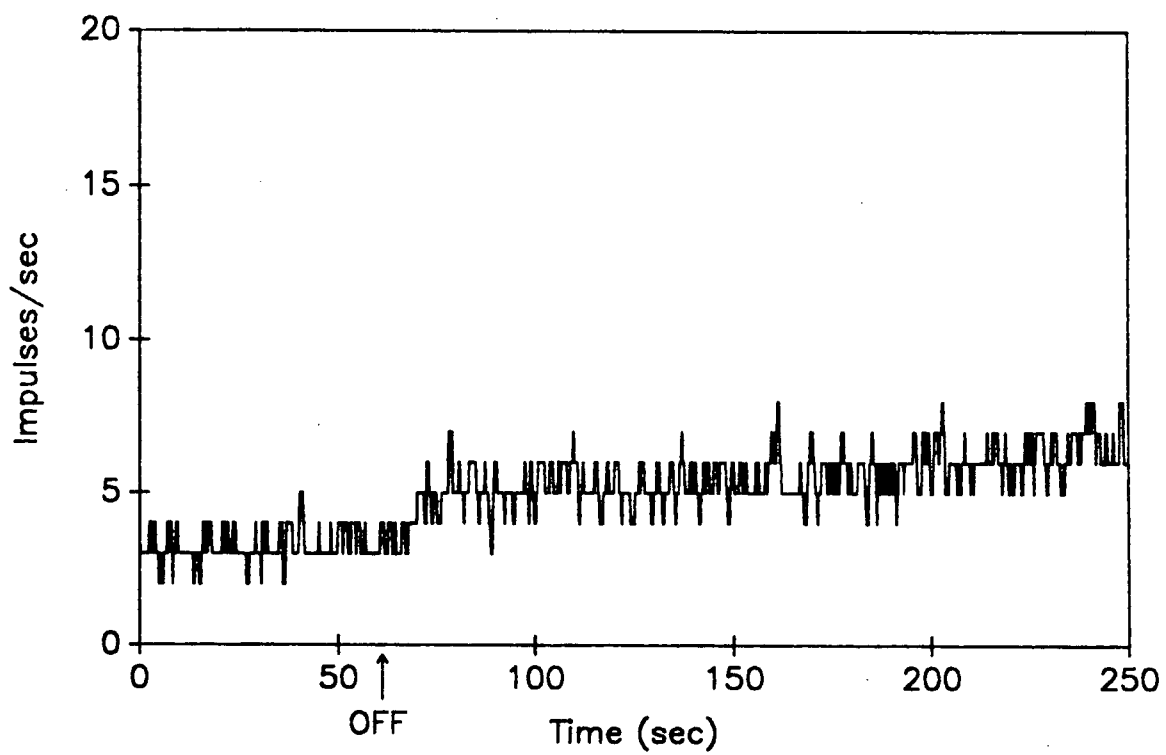
PHARMACOLOGICAL AGENTS

With the exception of NaCN, pharmacological agents were presented to internal receptors only.

Sodium Cyanide (NaCN)

Cyanide was a potent stimulant of both external and internal O_2-sensitive chemoreceptors. Injections of 25 µg NaCN into the perfusate flow promptly stimulated afferent nerve activity in all identified internal O_2-sensitive fibers in the trout gill (Fig. 10). NaCN caused a more prolonged stimulation of afferent nerve activity than any of the other chemicals tested.

Intact fish showed cardiovascular and ventilatory reflex responses to both waterborne (external) and bloodborne (internal) NaCN. The responses to NaCN injections were transient and recovery usually occurred within 15 minutes. The cardiovascular and ventilatory reflex responses of trout to NaCN were similar to the reflex responses to hypoxia. Conscious trout responded to NaCN (250 µg) injected externally into the ventilatory water flow with bradycardia while P_{DA} was unaffected (Fig. 11). Internal NaCN (25 µg), on the other hand, had no effect on f_H but P_{DA} was significantly increased (Fig. 12A and B). Both f_G and P_{OP} were significantly stimulated by internal (Fig. 12C and D) and external NaCN. There were no significant changes in cardiovascular or ventilatory variables over time in response to either external or internal saline control injections.

Norepinephrine

The low dosages (100-500 nmol) of NOREPI did not affect afferent neural discharge from any of the O_2 receptors studied (Fig. 13). Higher dosages (1000 nmol) may have had

Figure 10. Mean response of gill O_2 receptors to NaCN (25 µg; n=12).
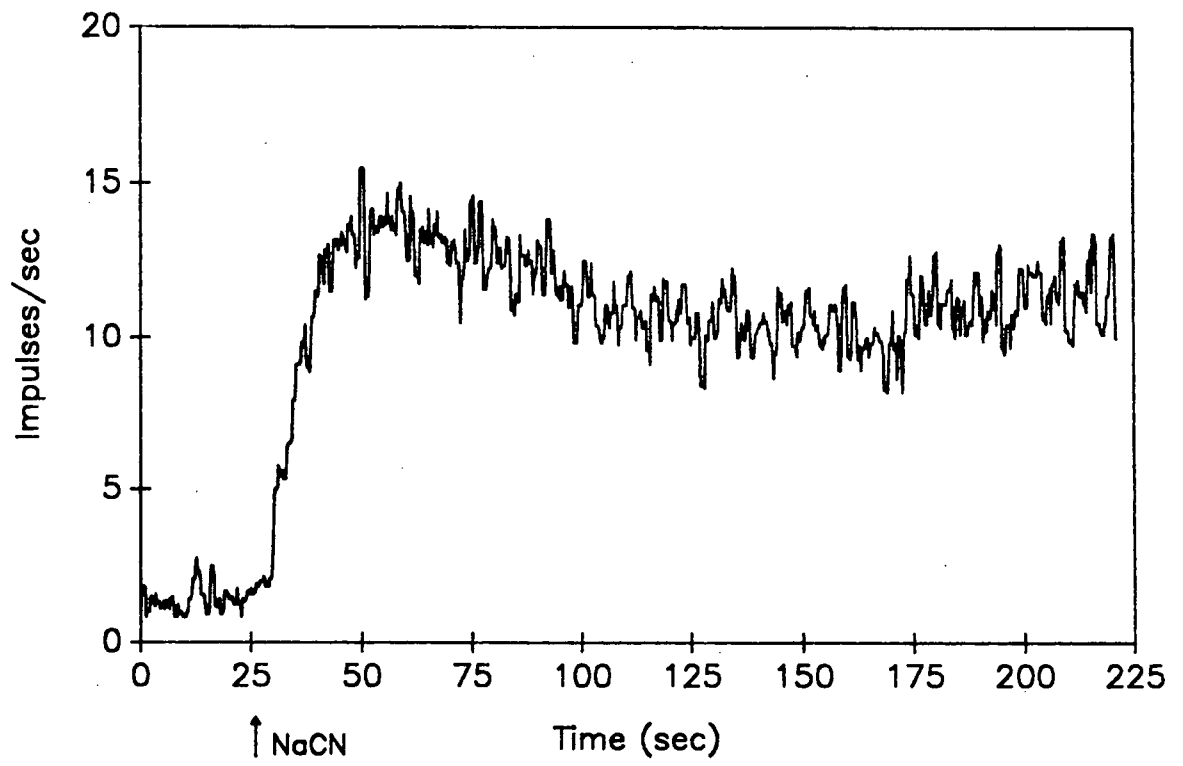
Figure 11. Mean (n=13) cardiovascular and ventilatory responses of fish to external injections of NaCN (250 µg; ●) and water controls (○). Vertical bars are ± 1 SEM.
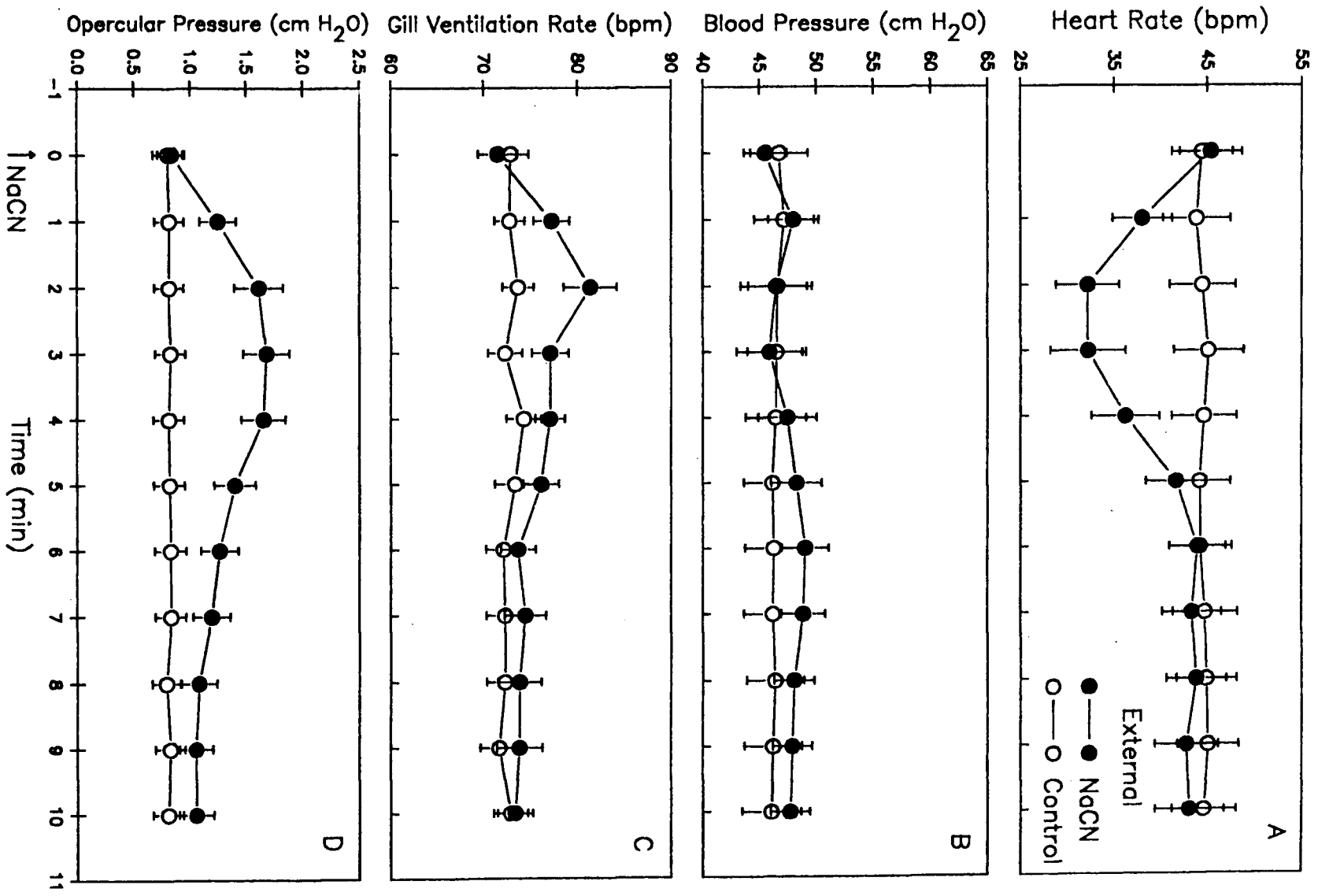
Figure 12. Mean (n=11) cardiovascular and ventilatory responses of fish to internal injections of NaCN (25 µg; ●) and internal saline controls (○). Vertical bars are ± 1 SEM.
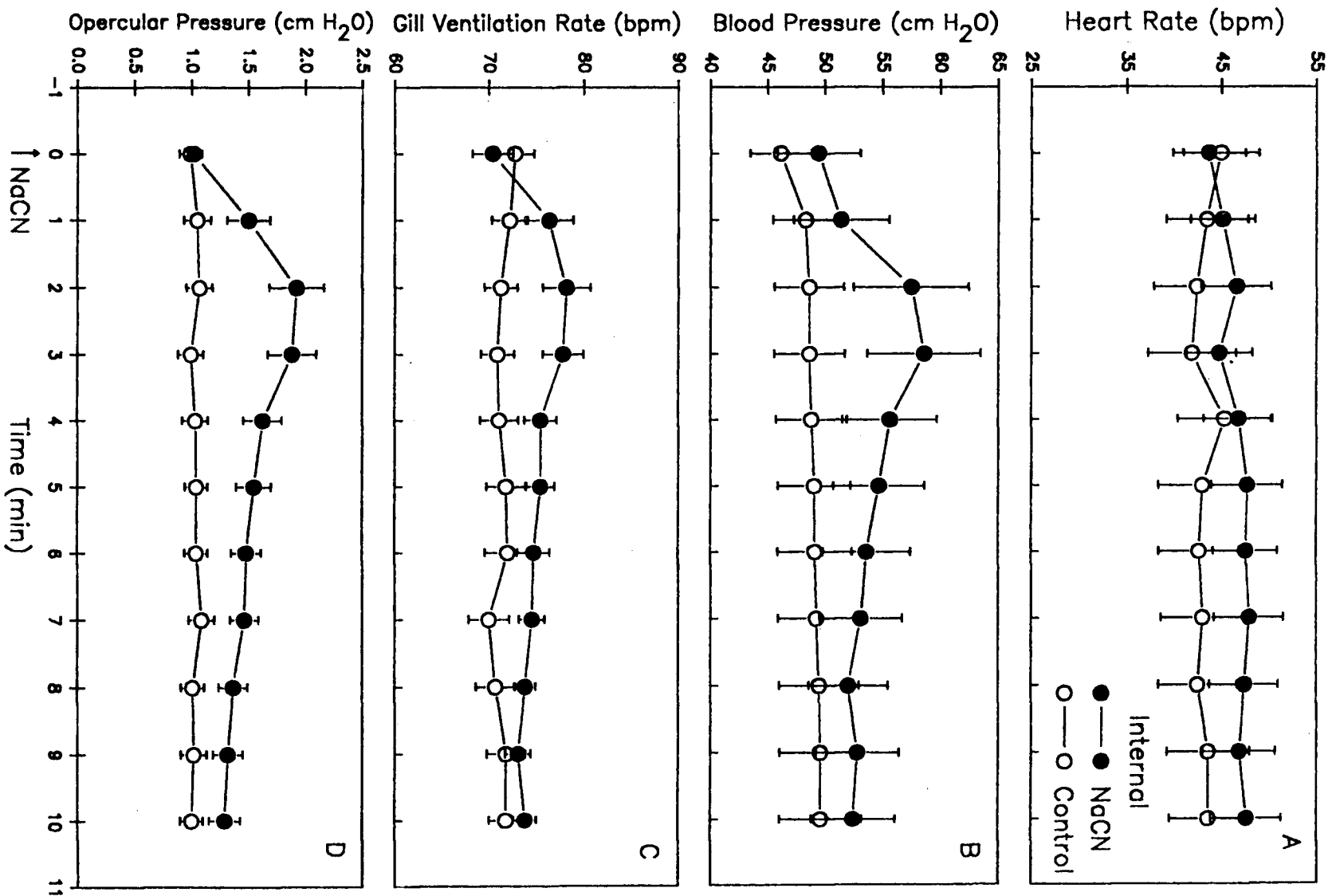
Figure 13. Mean effect of different dosages of NOREPI (100 nmol, n=5; 200 nmol, n=6; 500 nmol, n=8; 1000 nmol, n=5) on O_2-sensitive chemoreceptor afferent activity.

52
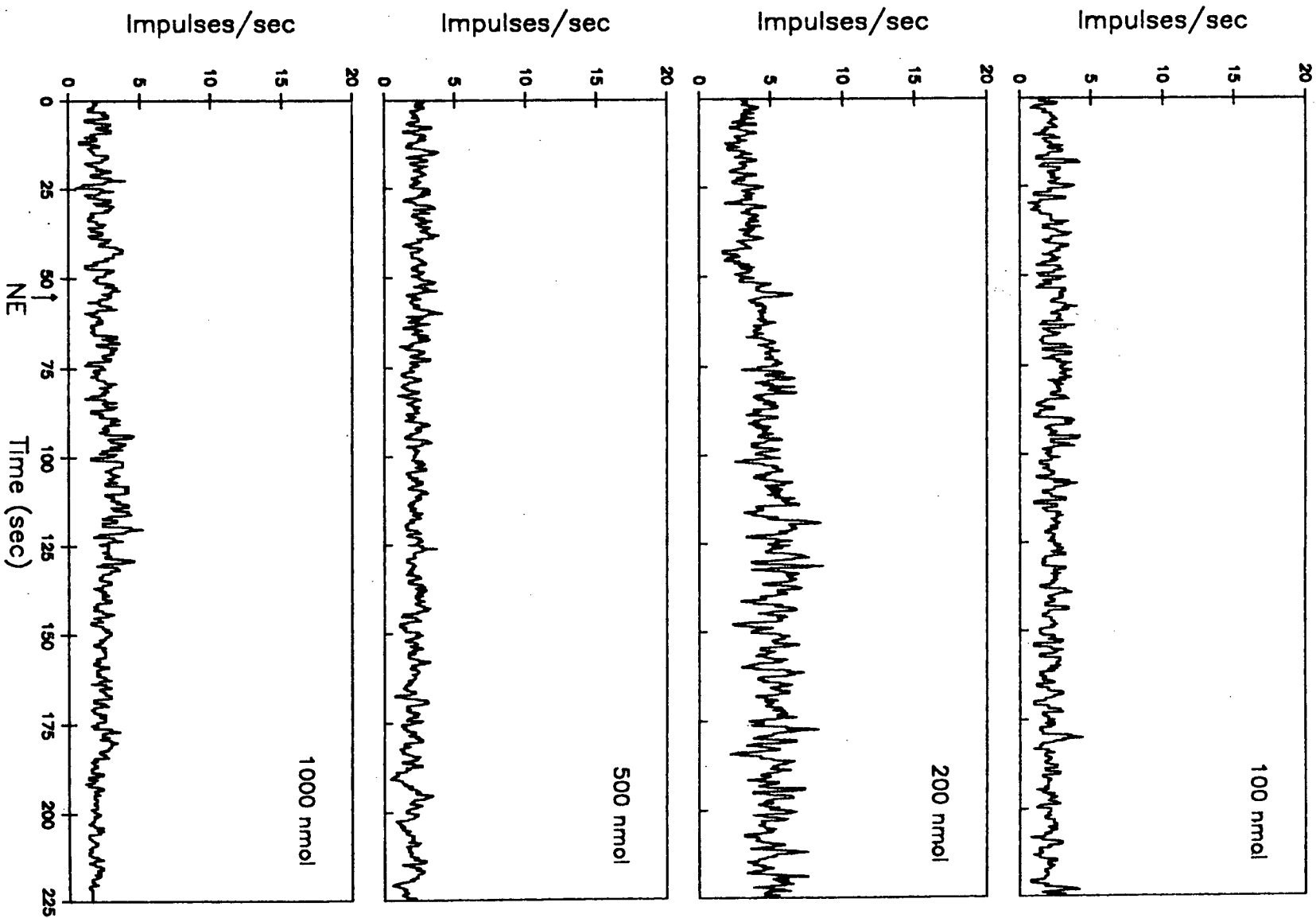
Figure 14. Mean responses of four gill O_2 receptors to 1000 nmol NOREPI. This is the same data presented in the bottom panel of Figure 13 with data from the one receptor that responded with an increase in activity omitted.
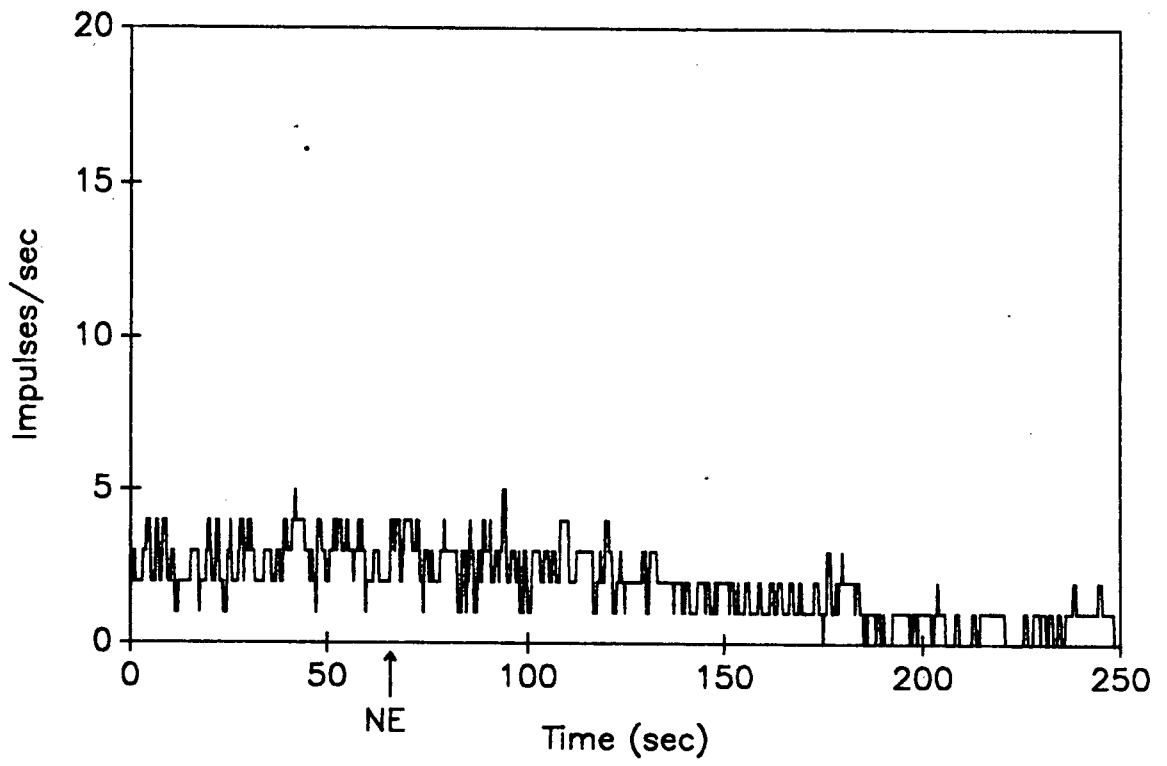
a slight inhibitory effect on chemoreceptor discharge in all but one receptor (Figs. 14 and 15) which did increase activity in response to NOREPI (Fig. 15). The increase in discharge shown by this one receptor was modest and was inhibited by administration of PROP (200 nmol).

Low dosages of NOREPI (5 nmol/kg) in intact fishes stimulated moderate increases in f_H and P_{DA} that returned to pre-injection levels within 10 min (Fig. 16A and B). Higher dosages of NOREPI (100 nmol/kg) caused a large increase in f_H ($\approx$62 bpm). Increasing the dose of NOREPI to 1000 nmol/kg increased f_H only slightly higher than a 100 nmol/kg dose and prolonged the response (Fig. 16A). There was never a bradycardia concurrent with increasing P_{DA}. The low dosage of NOREPI (5 nmol/kg) caused P_{DA} to increase to a peak of about 80 cm H_2O within the first minute. Increasing the dose of NOREPI roughly doubled P_{DA} to approximately 100 cm H_2O and prolonged the time course of the response although the peak response still occurred at one minute (Fig. 16B).

Ventilation was not significantly modified by low doses of NOREPI. 5 nmol/kg NOREPI had no effect on f_G, but higher concentrations significantly increased f_G in a dose-dependent fashion (Fig. 16C). The latency to response was longer than for NaCN injections, and ventilation did not begin to increase until two minutes post-injection. Norepinephrine never elicited changes in P_{OP} regardless of the concentration given (Fig. 16D).

Epinephrine

Epinephrine injections also indicated that there was little adrenergic modulation of O_2 receptor activity in the isolated, perfused trout gill. Responses to EPI injections (500 nmol) were somewhat variable, and there was either no effect (n=5) or a decrease in afferent activity (n=3) (Figs. 17 and 18) in all gill O_2 chemoreceptors tested.

In intact fish, f_H decreased in response to 5 nmol/kg EPI but was significantly

Figure 15. Response of a single gill O_2 receptor showing increased activity in response to 1000 nmol NOREPI. The response of this neuron to NOREPI was inhibited by PROP (not shown).
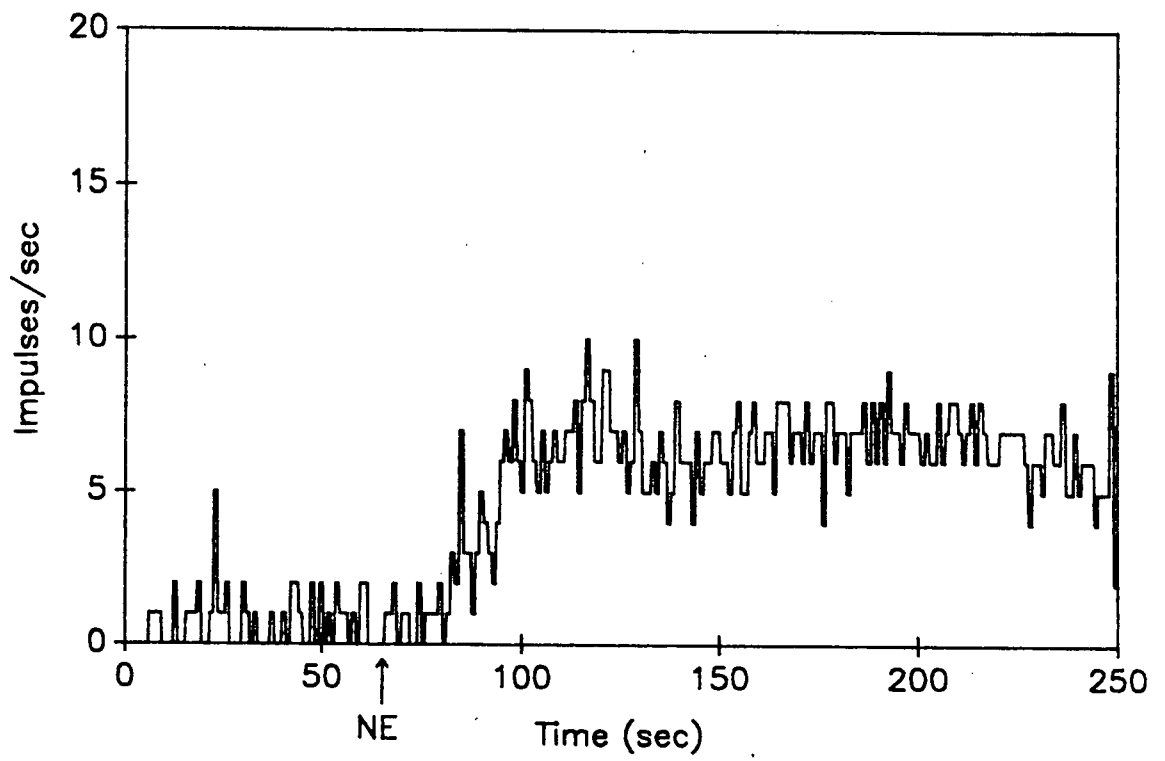
Figure 16. Mean (n=7) cardiovascular and ventilatory responses of fish to injections of 5 nmol/kg (●), 100 nmol/kg (■) and 1000 nmol/kg (▲) NOREPI and saline (○). Vertical bars are ± 1 SEM.
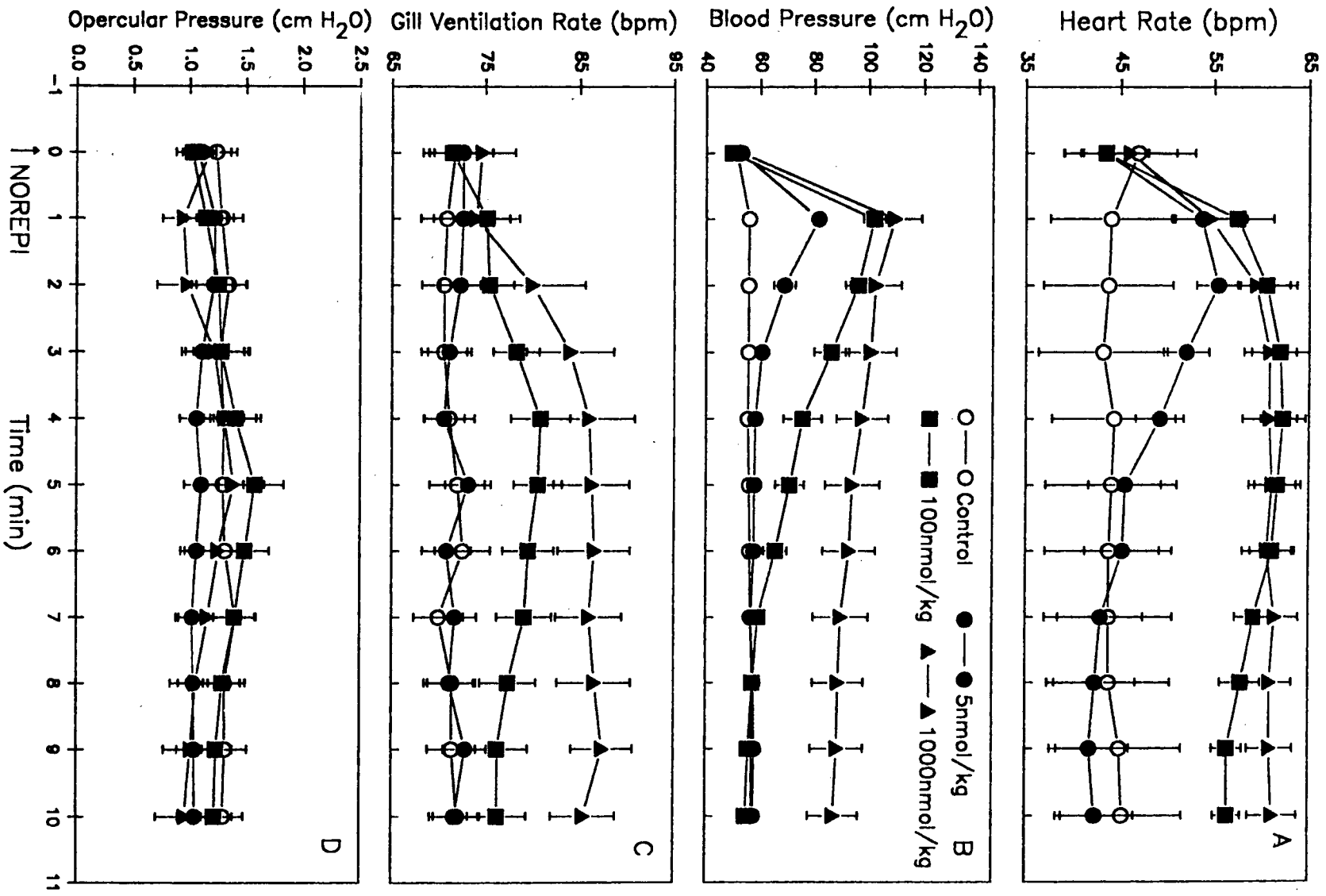
Figure 17. Mean responses of gill O_2 receptors to 500 nmol EPI (n=8).
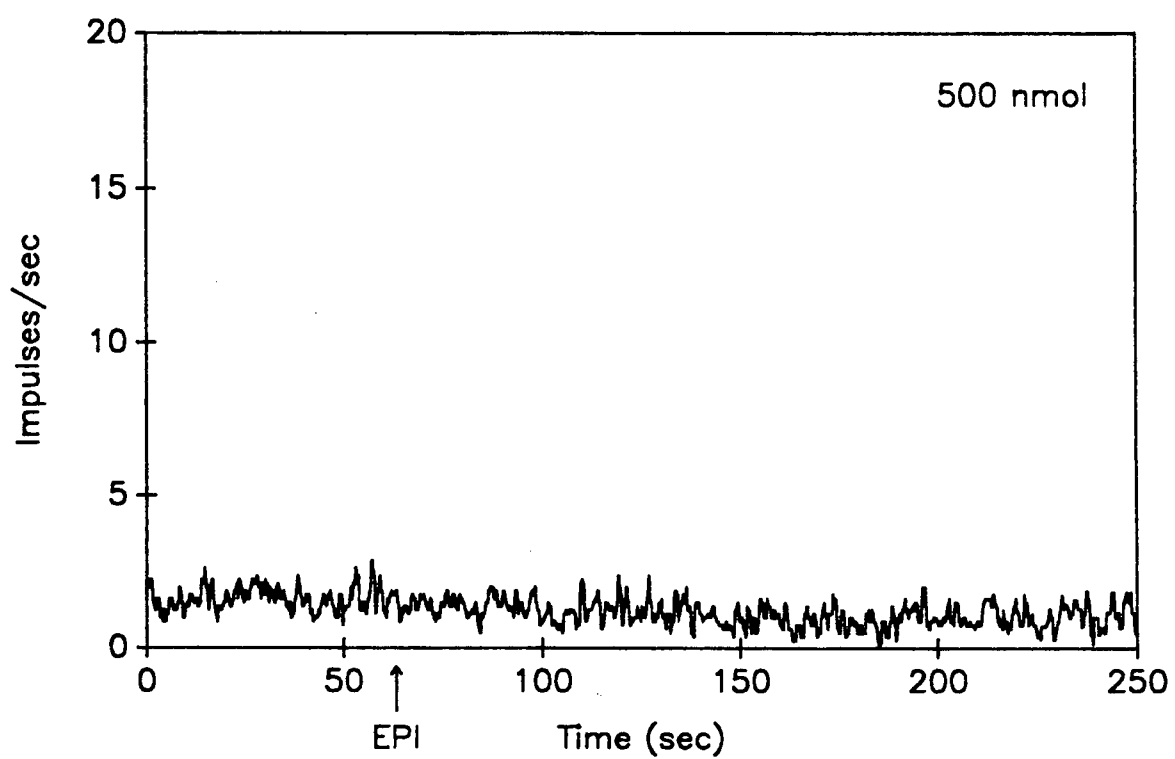
Figure 18. Response of a single chemoreceptive unit to 500 nmol of EPI showing a decrease in afferent neural activity.
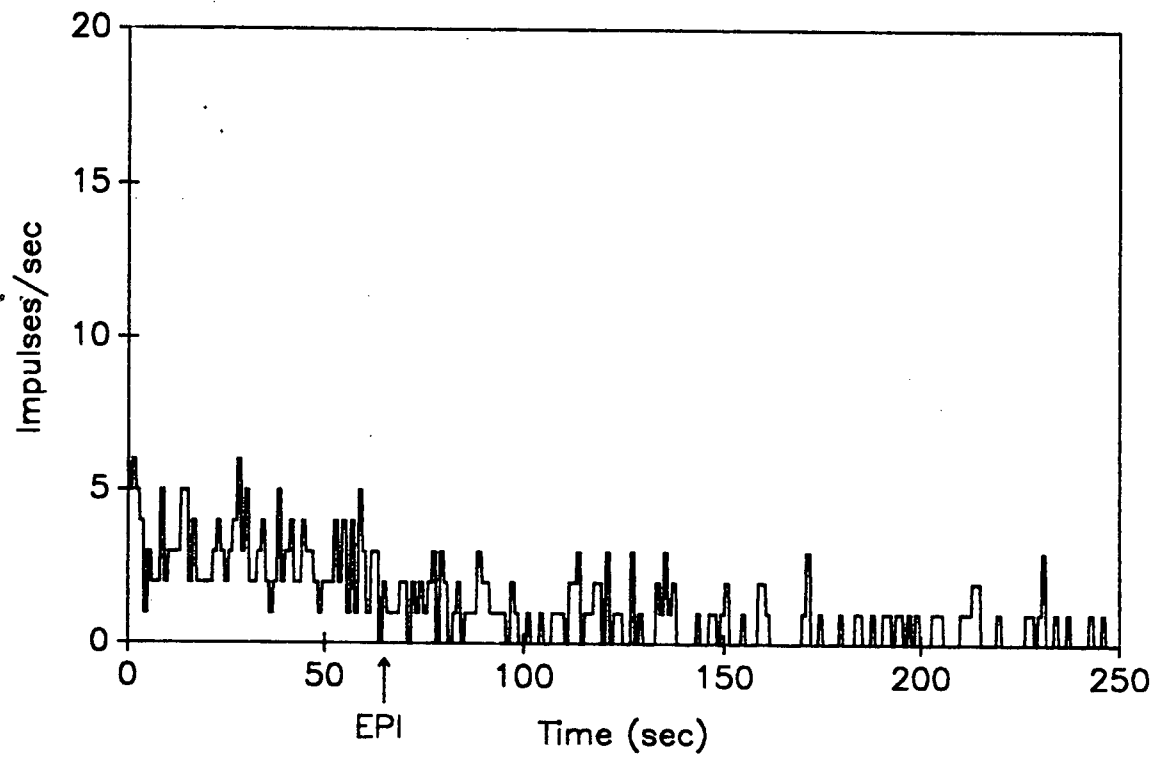
stimulated by higher dosages (Fig. 19A). Dorsal aortic blood pressure was stimulated by EPI in a dose-dependent manner (Fig. 19B) which was very similar to the effects of NOREPI injections (Fig. 19B). The peak response also occurred at one minute with injections of EPI. The 5 nmol/kg EPI injection had no effect on f_G, but higher dosages resulted in a significant stimulation (Fig. 19C). EPI had no significant effect on P_{OP} (Fig. 19D).

Isoproterenol

There appeared to be little or no ß-adrenergic control of O_2-sensitive afferent neural activity. Injections of the model β-agonist, ISO (100-1000 nmol), had a slight inhibitory effect, if any, on O_2 chemoreceptor activity (Fig. 20).

In contrast to its affects on O_2 receptor afferent discharge, ISO (100 nmol/kg) significantly stimulated some cardiovascular and ventilatory variables. Heart rate (f_H) was significantly stimulated, but the effect on P_{DA} was negligible (Fig. 21A and B). Gill ventilation rate (f_G) was significantly stimulated by ISO after about one minute but, like the other adrenergic agonists, ISO had no effect on P_{OP} (Fig. 21C and D).

Propranolol

Paradoxically, PROP injections (100-200 nmol) significantly decreased O_2 receptor activity. Figure 22 illustrates the response of an internal O_2 chemoreceptor to changing perfusate P_{O2} both before and after PROP injection. The P_{O2} of the bathing medium remained constant at 157 torr. The P_{O2} of each perfusate is given in the figure. The mean responses for all receptors studied are given in Fig. 23. The excitation of chemoreceptors by NaCN injection was also greatly reduced after propranolol was administered (Figs. 24 and 25). The inhibitory effect of PROP was reversible, and receptors regained their full sensitivity to NaCN and

Figure 19. Mean (n=5) cardiovascular and ventilatory responses of fish to injections of 5 nmol/kg (●), 100 nmol/kg (■) and 1000 nmol/kg (▲) EPI and saline (O). Vertical bars are ± 1 SEM.
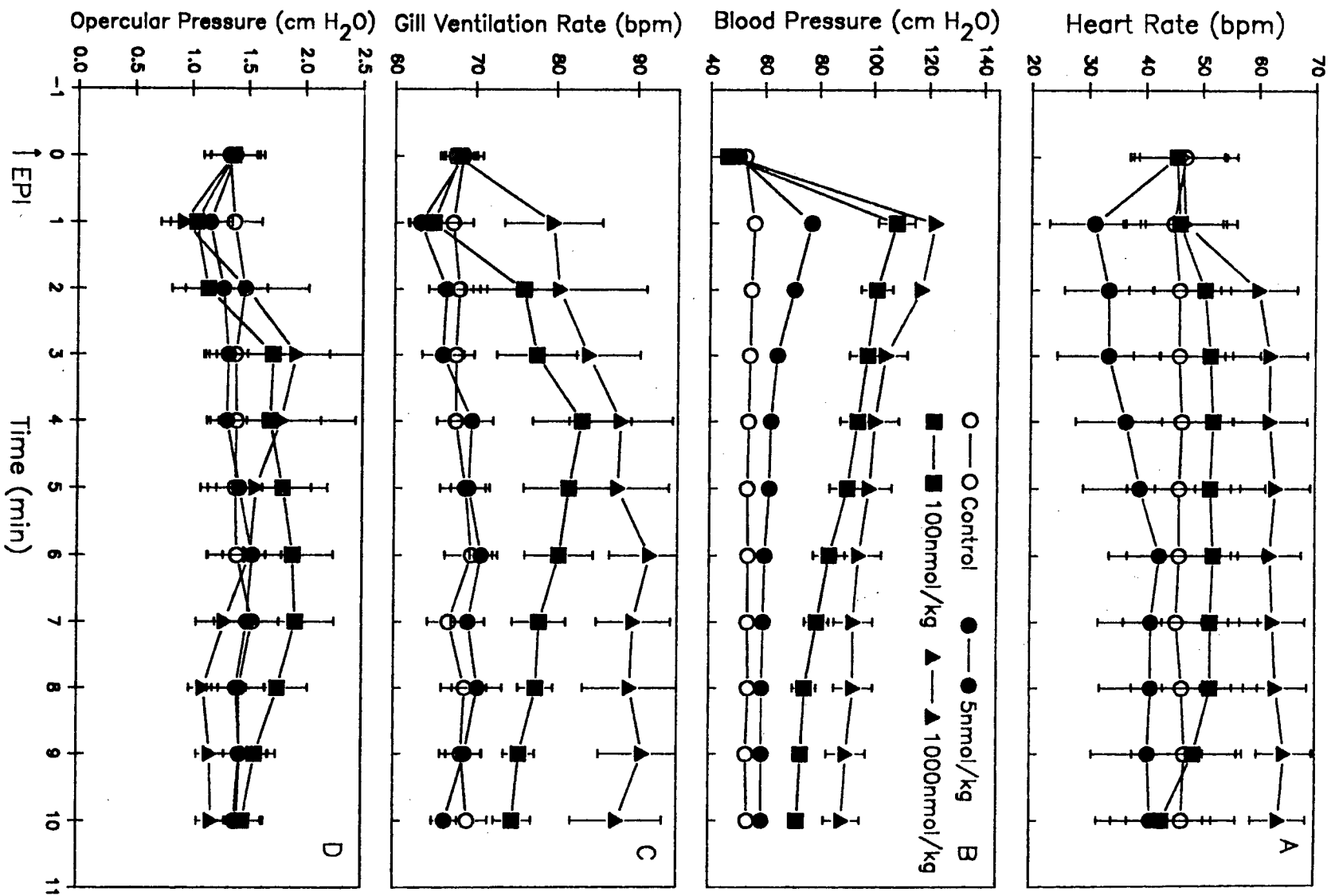
Figure 20. Mean response of gill O_2 receptors to ISO (500 nmol; n=10).

Figure 21. Mean (n=4) cardiovascular and ventilatory responses of fish to injections of 100 nmol/kg ISO (●) and saline (○). Vertical bars are ± 1 SEM.
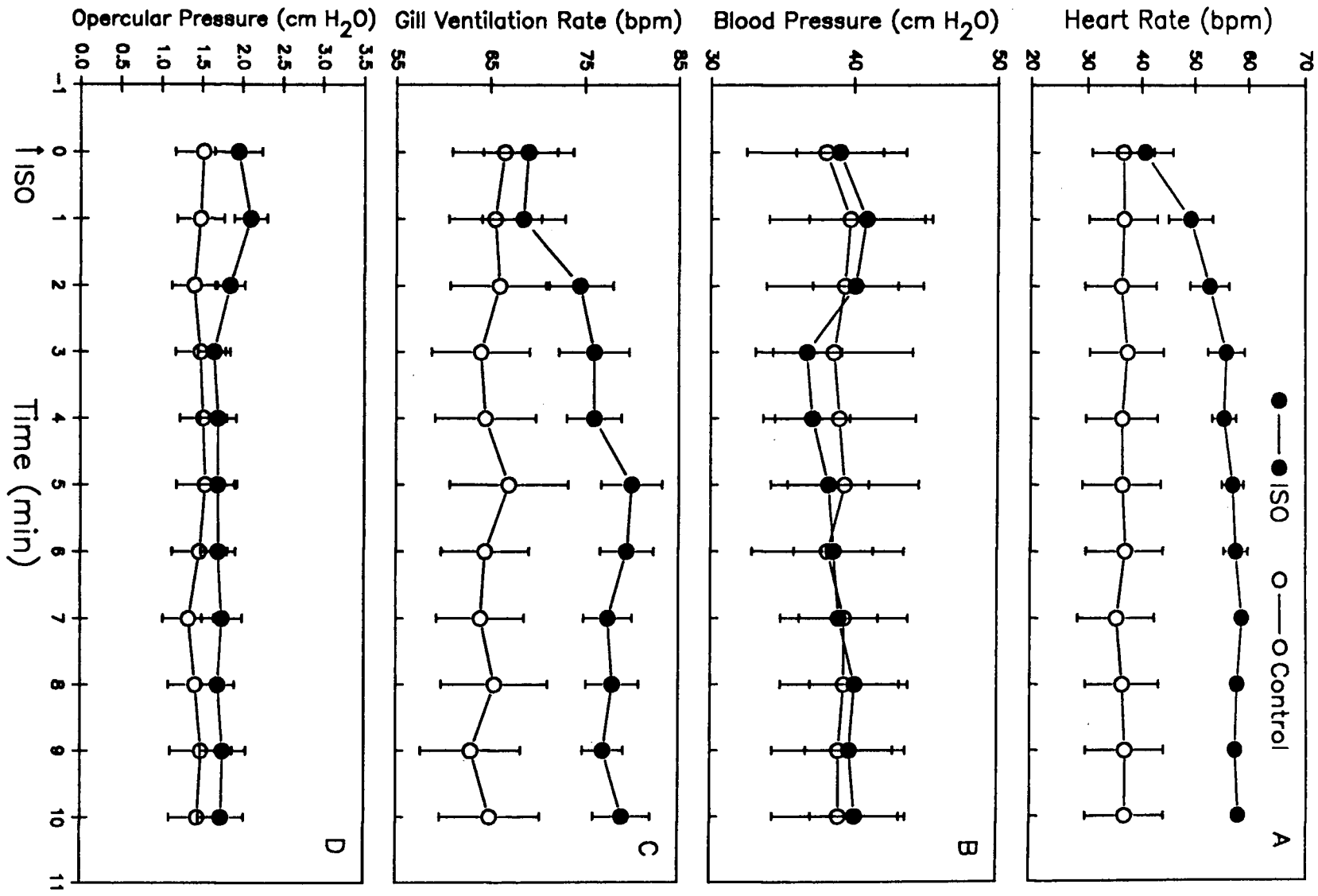
Figure 22. Activity of a single O_2 sensitive chemoreceptor during perfusion with A. normoxic,

B. hypoxic and C. hypoxic perfusate after 100 nanomoles of propranolol. Upper trace in each

pair is the integrated discharge rate. Lower trace is the window discriminator output. The time

marker applies to all three panels.

A

Impulses/sec

10 ⌐
0 ⌐

ENG

Normoxia (P_{O_2} = 146)

B

Impulses/sec

10 ⌐
0 ⌐

ENG

Hypoxia (P_{O_2} = 19)

1 min

C

Impulses/sec

10 ⌐
0 ⌐

ENG

Hypoxia After Propranolol (P_{O_2} = 19)

72
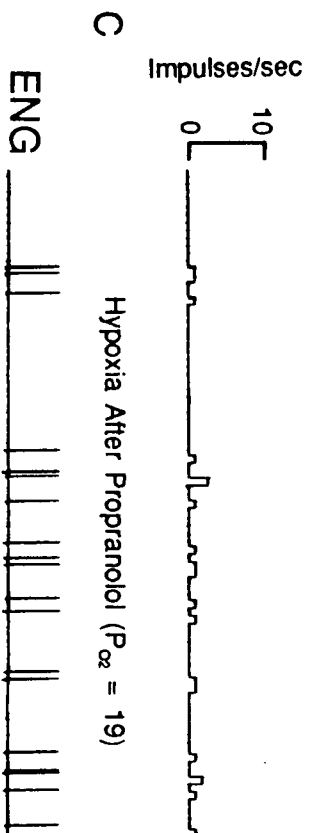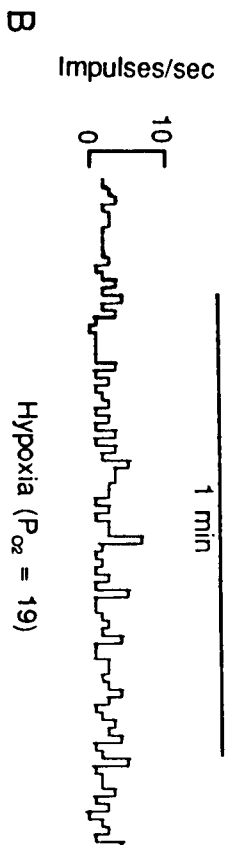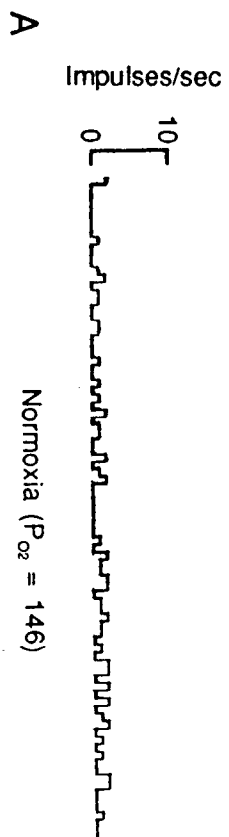
Figure 23. Effect of propranolol on chemoreceptor discharge during normoxia (P_{O_2}=150-160 torr) and hypoxia (P_{O_2}=10-30 torr). Data are means ± SEM. n = number of chemoreceptor units.
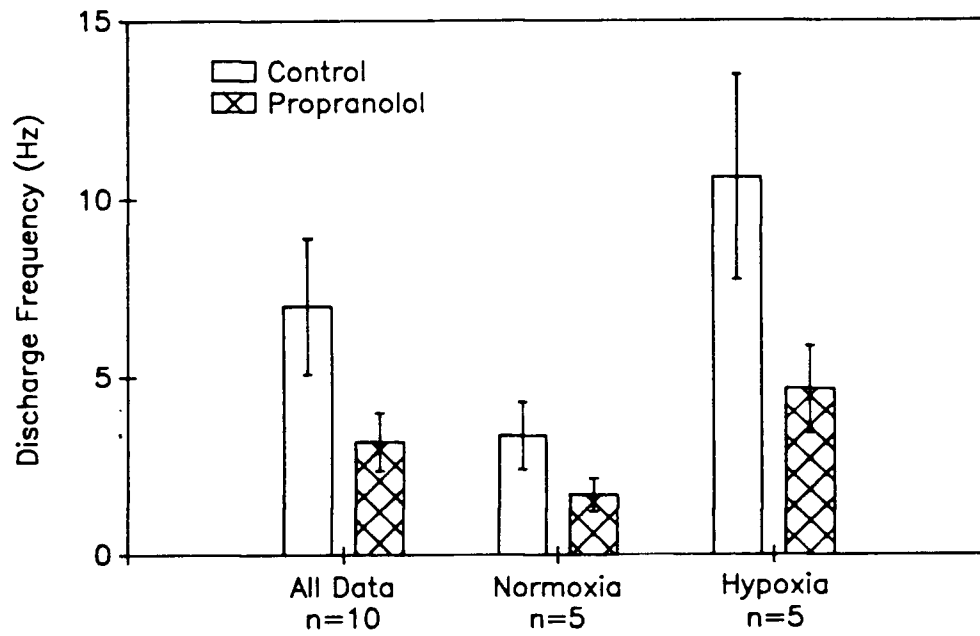
Figure 24. Effect of NaCN (25 µg) on chemoreceptor activity before and after propranolol (200 nanomoles). Upper trace in each pair is the integrated discharge rate. Lower trace is the window discriminator output. Bottom pair of traces are a continuation of the upper pair in this figure.
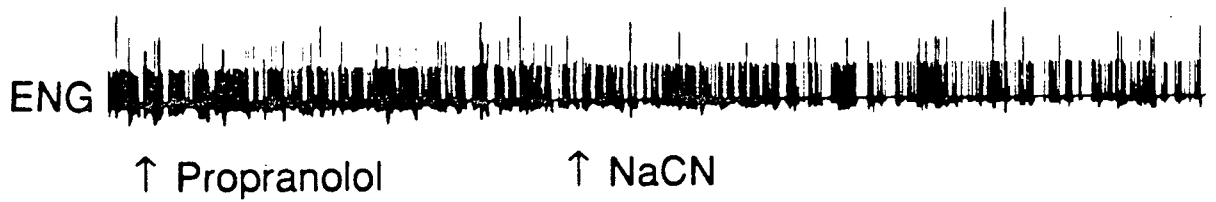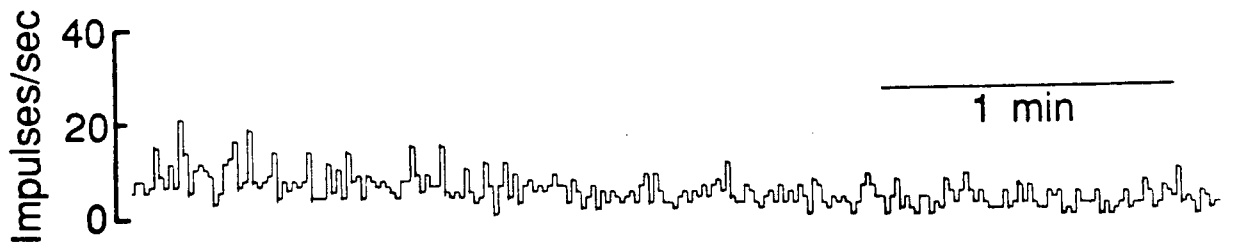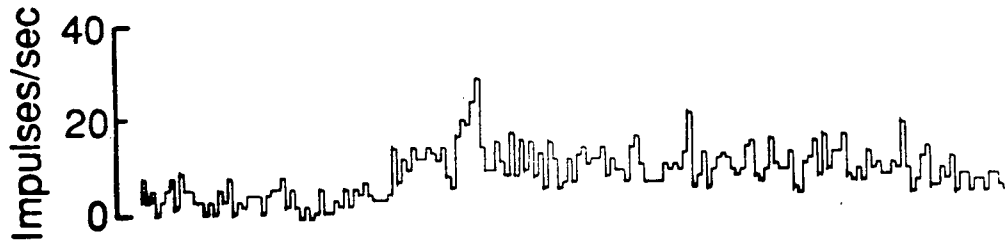
Figure 25. Mean responses of O_2 chemoreceptors to internal 25 µg NaCN before (top trace) and after 200 nmol propranolol (lower, filled in trace) (n=5).
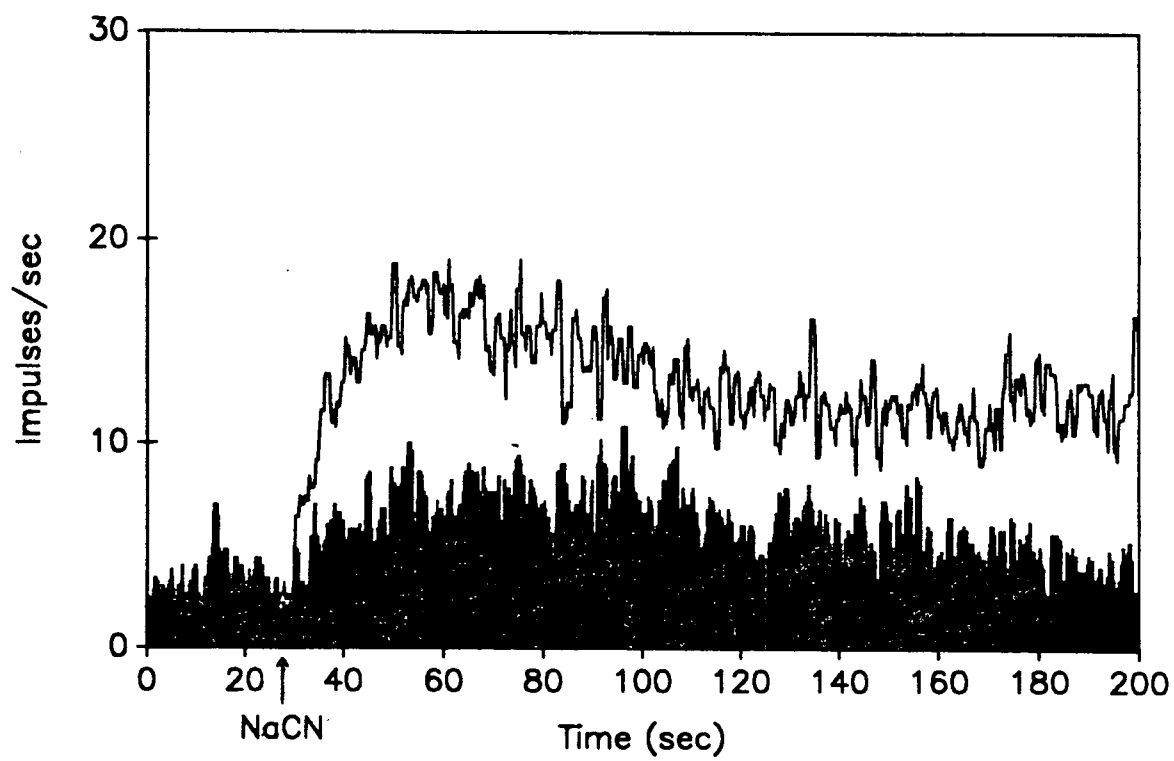
hypoxia after approximately one hour.

In intact fish, bolus injections of the β-adrenergic antagonist PROP (500 nmol/kg) modified resting, normoxic P_{DA} (Fig. 26). Heart rate was significantly higher to begin with in experimental animals but did not change significantly over time in response to PROP (Fig. 26A). Blood pressure increased very slowly (Fig. 26B). Opercular pressure was also unaltered by PROP but f_G was slightly stimulated (Fig. 26C and D).

Dopamine

Injections of DOP (100-1000 nmol) elicited a dose-dependent transient increase in chemoreceptor discharge followed by a slight decrease in discharge (Fig. 27).

In intact fish, bolus injections of DOP (100 nmol/kg) did not alter heart rate (Fig. 28A) although blood pressure was significantly elevated by this drug (Fig. 28B). The ventilatory responses of trout to DOP injections were distinctly different from the responses to the previous catecholamines. Gill ventilation rate was unaffected (Fig. 28C) but P_{OP} showed a significant, transient depression in response to DOP (Fig. 28D).

Serotonin (5-HT)

The indolamine 5-HT (100 nmol) elicited a transient burst of activity followed by an inhibition of activity when injected into the trout gill (Fig. 29). This pattern of response was similar to that of DOP (Fig. 27).

Serotonin (5-HT; 100 nmol/kg) also had significant effects on all cardiovascular and ventilatory variables in intact fish (Fig. 30). 5-HT injections caused a dramatic decrease in P_{DA} and stimulated a significant increase in f_H (Fig. 30A and B). Ventilatory variables began to increase after about one minute. There was a very brief apneic period during the first 30 sec

Figure 26. Mean (n=7) cardiovascular and ventilatory responses of fish to injections of 500

nmol/kg PROP (●) and saline (○). Vertical bars are ± 1 SEM.
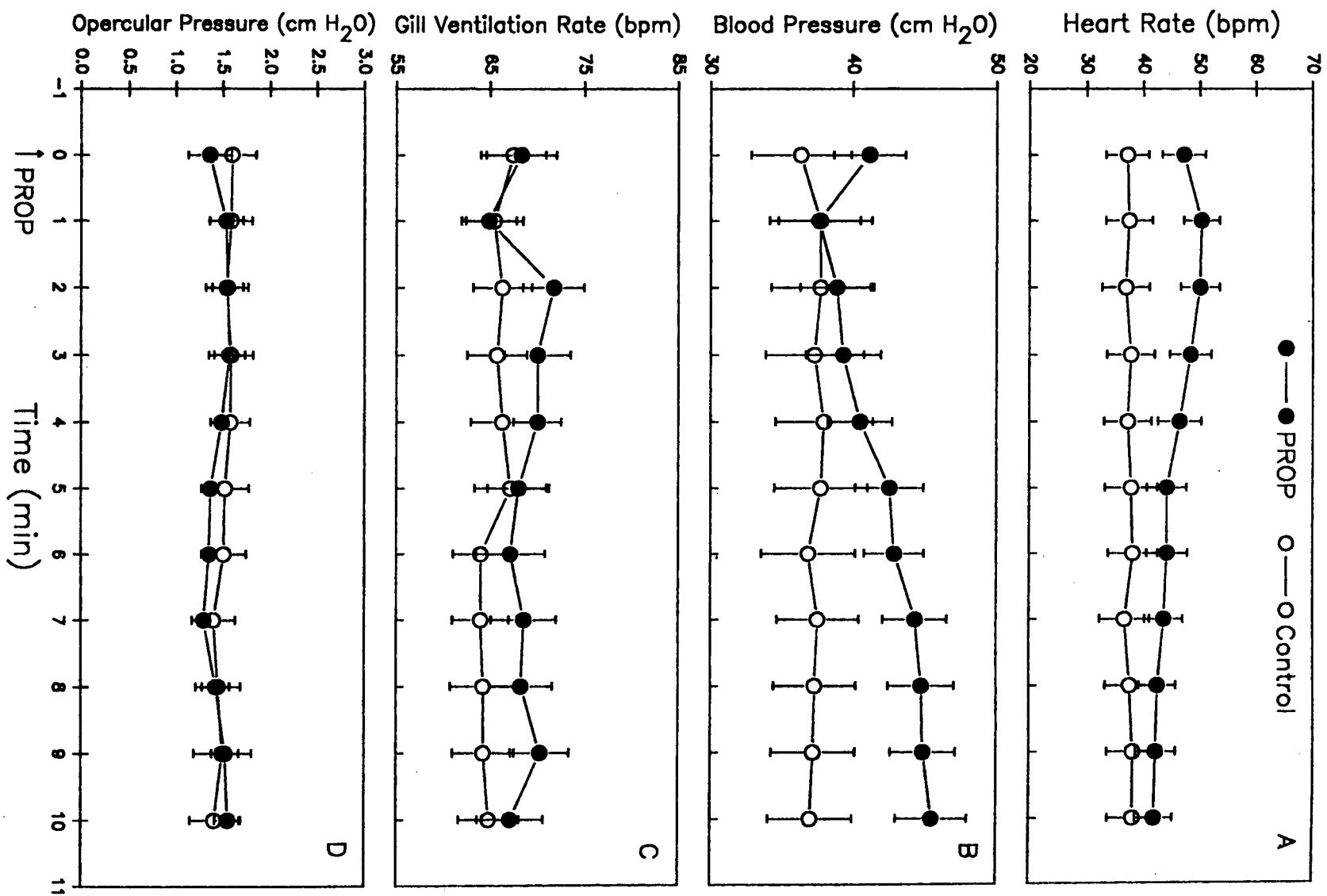
Figure 27. Effects of different doses of dopamine (100 nmol, n=4; 200 nmol, n=3; 1000 nmol,

n=4) on O_2-sensitive chemoreceptor afferent activity.
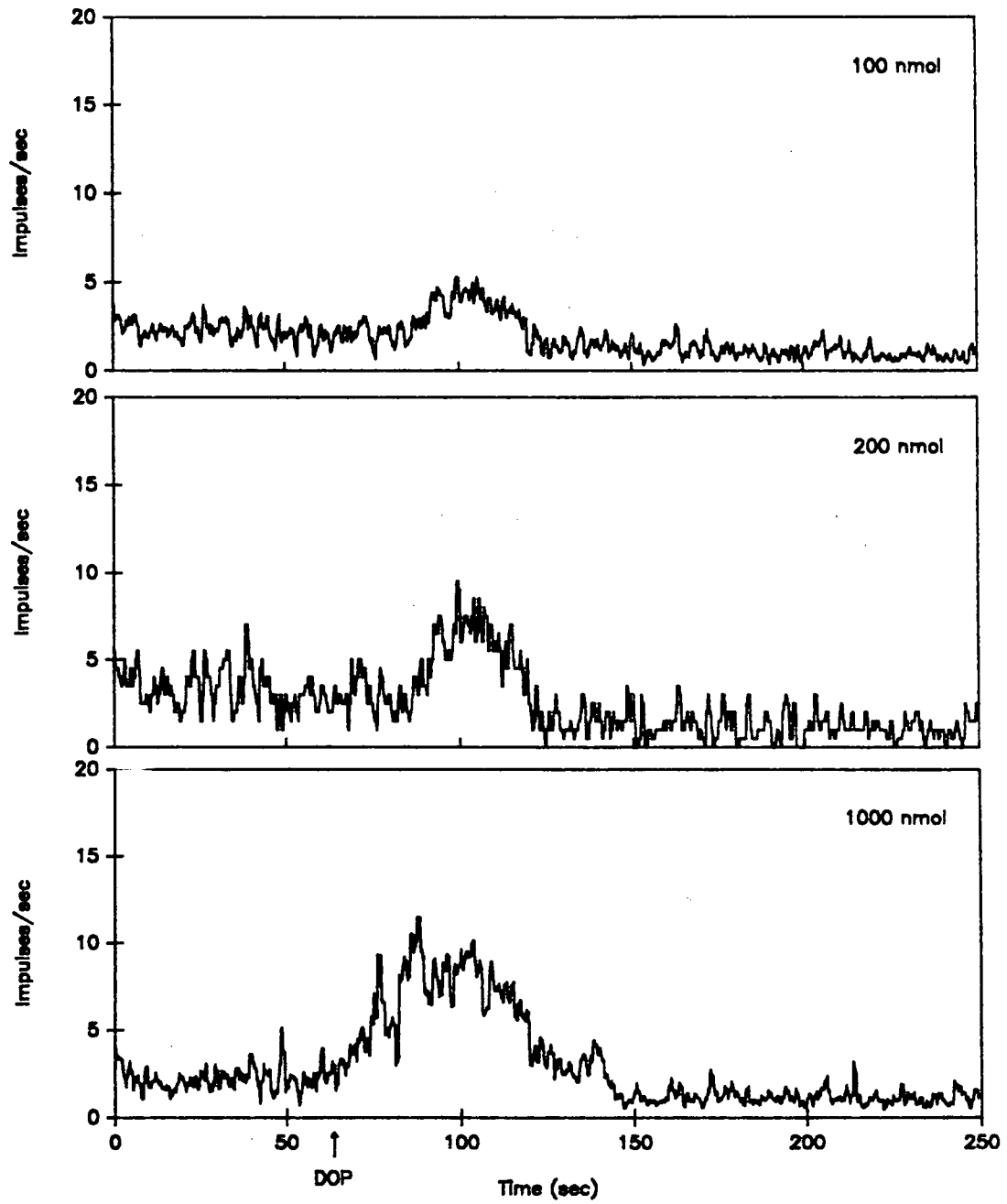
Figure 28. Mean (n=6) cardiovascular and ventilatory responses of fish to injections of 100 nmol/kg DOP (●) and saline (O). Vertical bars are ± 1 SEM.
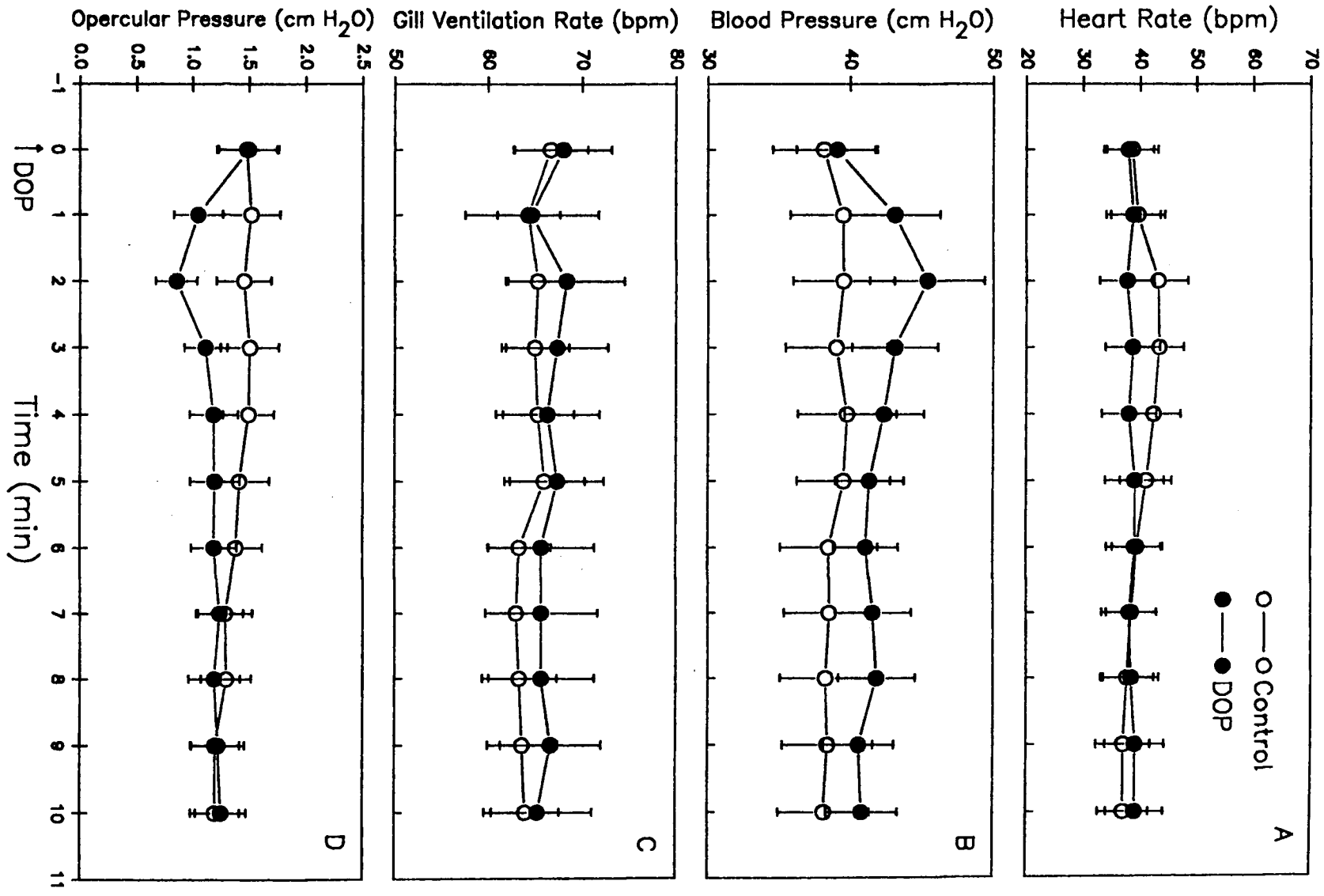
Figure 29. Mean response of gill O_2-sensitive chemoreceptors to 5-HT (100 nmol, n=8).
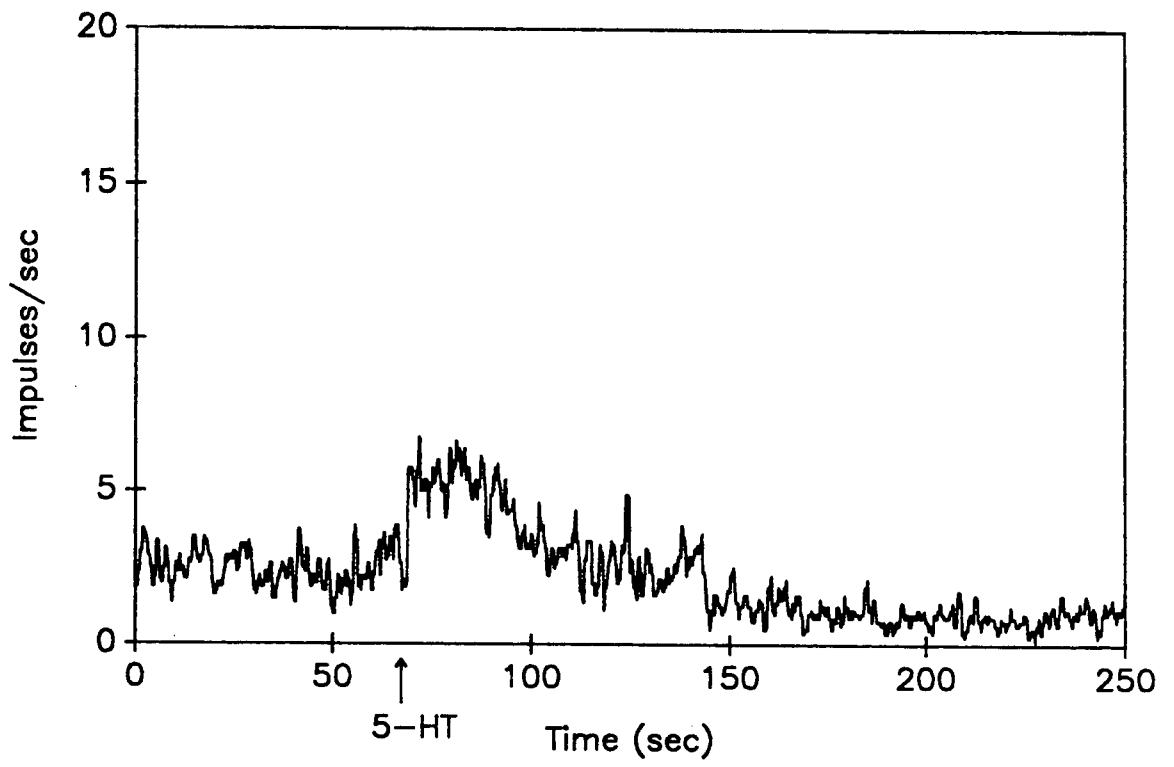
Figure 30. Mean (n=6) cardiovascular and ventilatory responses of fish to injections of 100 nmol/kg 5-HT (●) and saline (○). Vertical bars are ± 1 SEM.
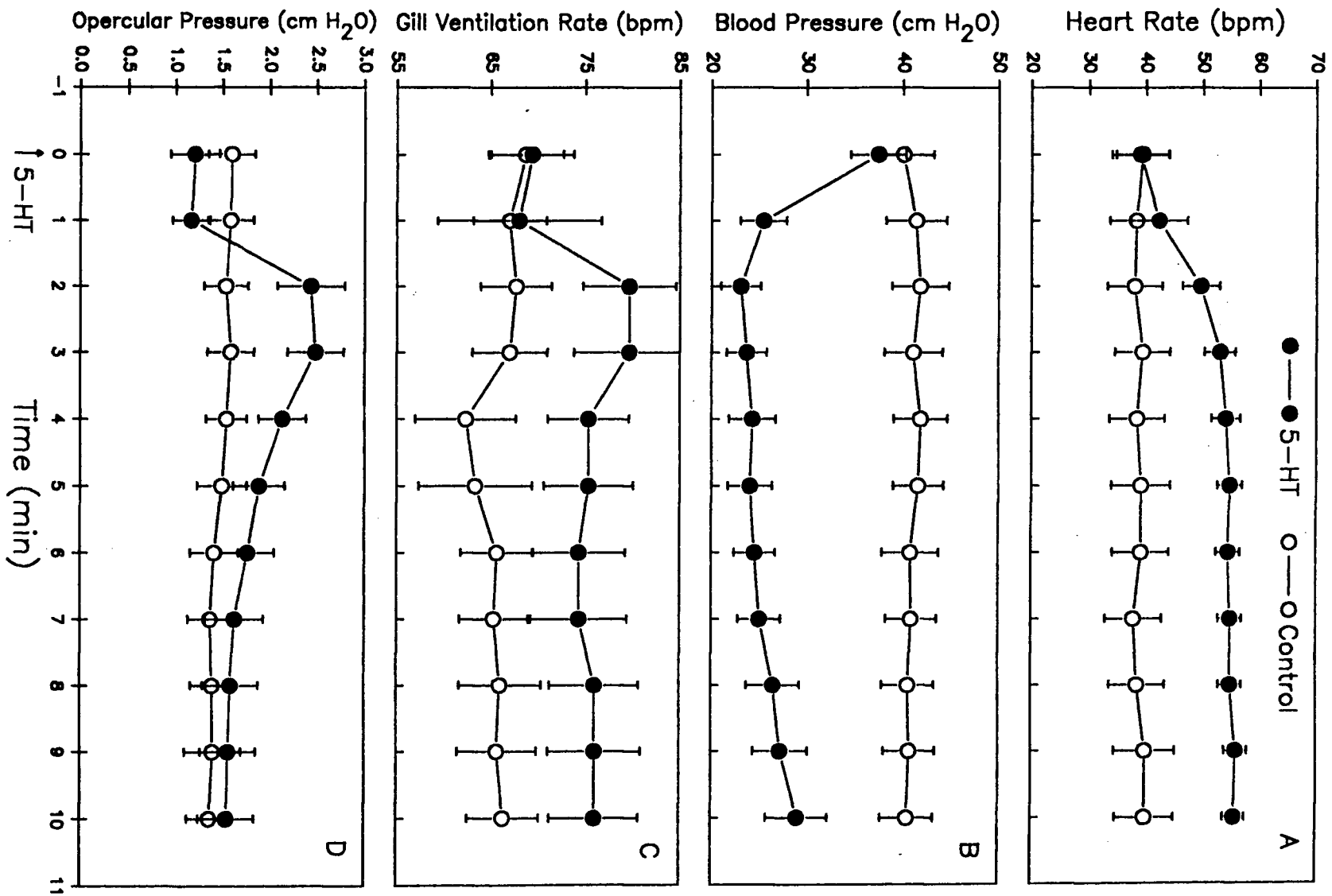
following 5-HT injection observed in some fish. Gill ventilation rate increased significantly after a delay of one minute and remained elevated for the duration of the 10 minute recording period (Fig. 30C). Opercular pressure was also significantly stimulated after a one minute delay but returned to pre-injection levels within 10 minutes (Fig. 30D).

Acetylcholine

In contrast to adrenergic chemicals, cholinergic agents had profound effects on chemoreceptor afferent nerve activity. Acetylcholine (100 nmol) was a potent stimulant of O_2 receptors (Fig. 31).

In intact fish, all cardiovascular and ventilatory variables increased significantly in response to 100 nmol/kg ACH injected via the DA cannula. Curiously, both f_H and P_{DA} were stimulated by ACH injections (Fig. 32A and B). There was a long latency to response for f_G (about 4 min post-injection) but P_{OP} increased almost immediately (Fig. 32C and D).

Nicotine

Nicotine was a potent stimulant of O_2 receptor afferent neural activity. The magnitude of the response to NIC injections (100 nmol; Fig 33) was similar to the response to ACH (Fig. 31).

In intact fish, NIC (100 nmol/kg), like ACH, elicited significant increases in all measured cardiovascular and ventilatory variables (Fig. 34). The increase in f_H was moderate but P_{DA} showed a large and prolonged elevation (Fig. 34A and B). The ventilatory responses to NIC were larger and had a shorter latent period (Fig. 34C and D) than the responses to ACH.

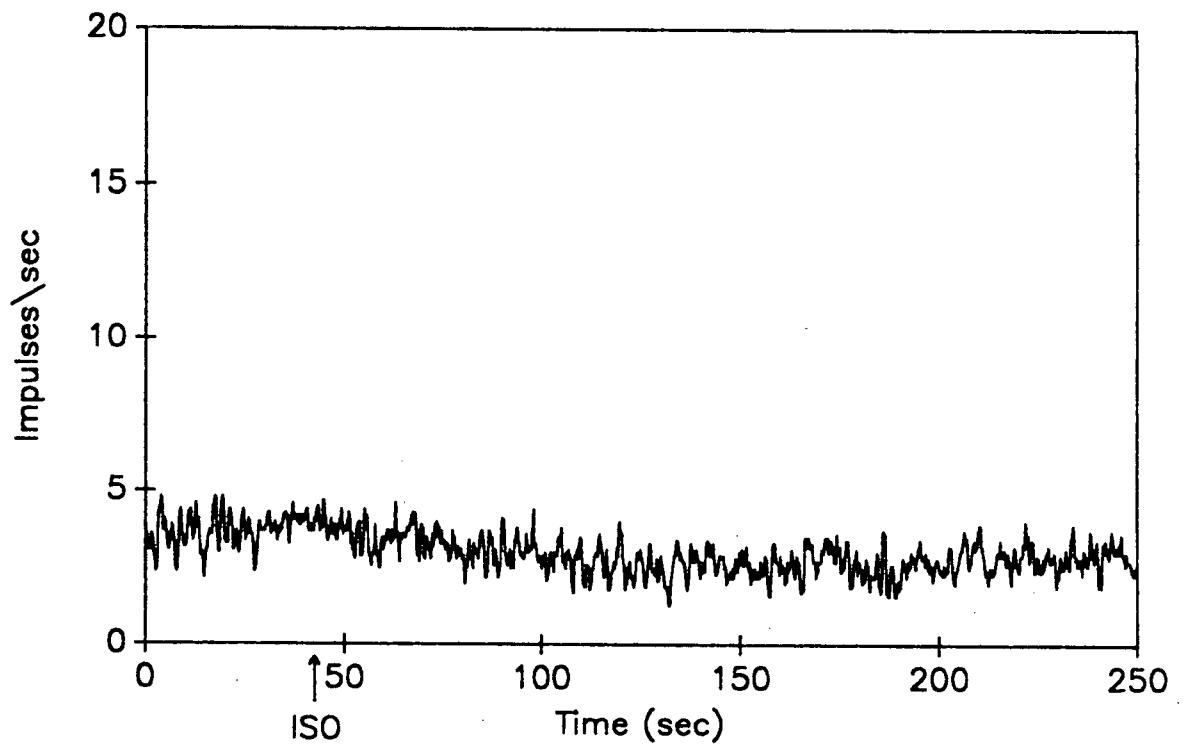Figure 31. Mean response of gill O_2-sensitive chemoreceptors to ACH (100 nmol; n=8).
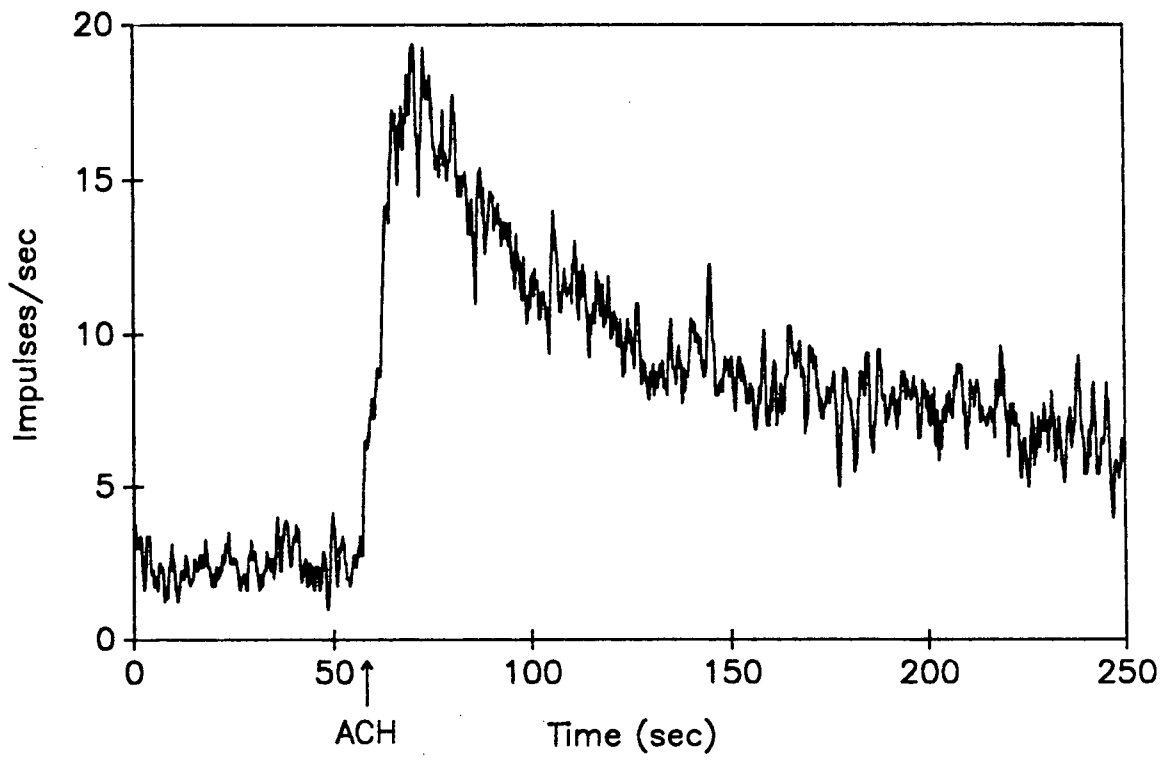
Figure 32. Mean (n=6) cardiovascular and ventilatory responses of fish to injections of 100 nmol/kg ACH (●) and saline (O). Vertical bars are ± 1 SEM.
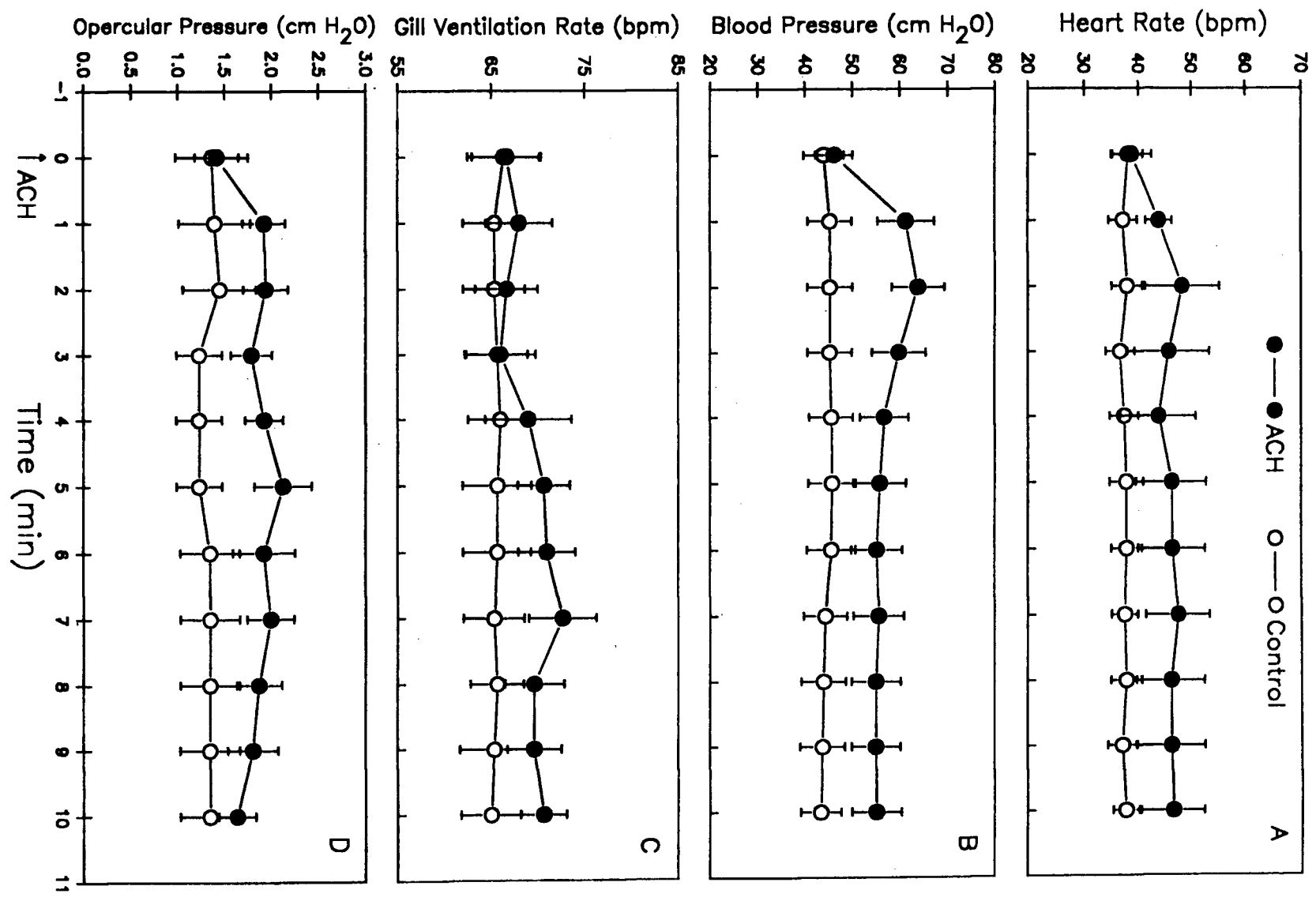
Figure 33. Mean response of gill O_2-sensitive chemoreceptors to NIC (100 nmol, n=12).
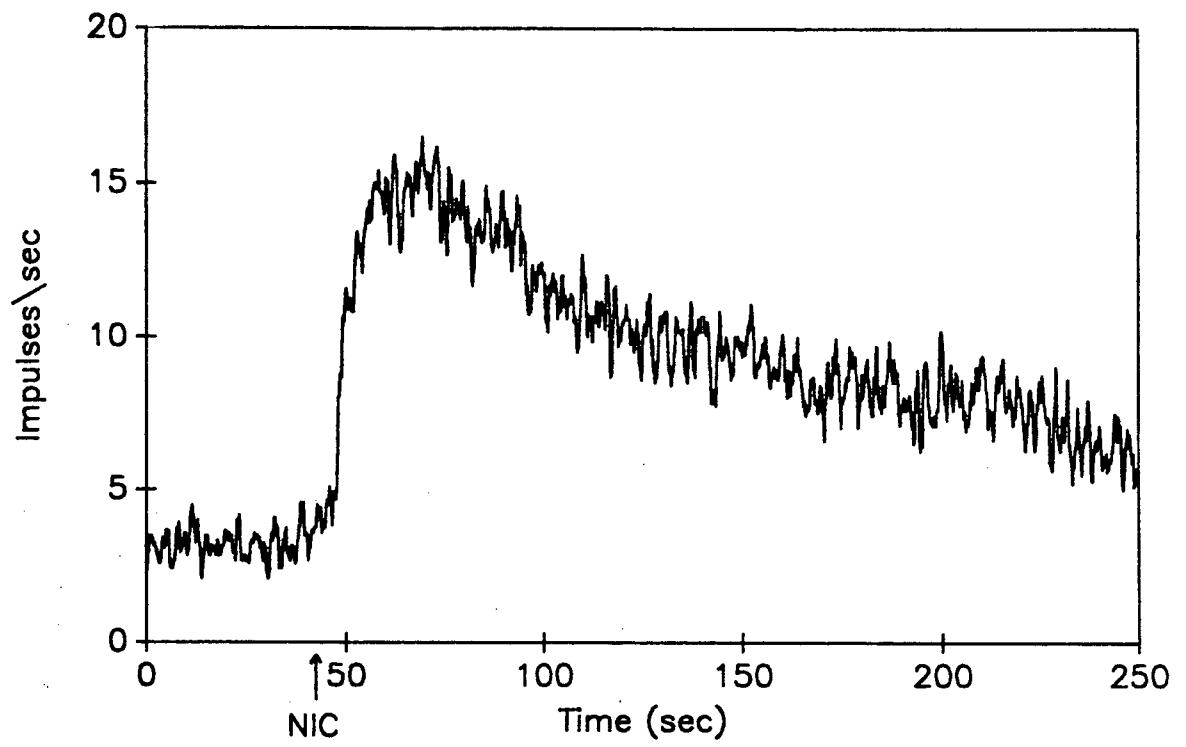
Figure 34. Mean (n=6) cardiovascular and ventilatory responses of fish to injections of 100 nmol/kg NIC (●) and saline (O). Vertical bars are ± 1 SEM.
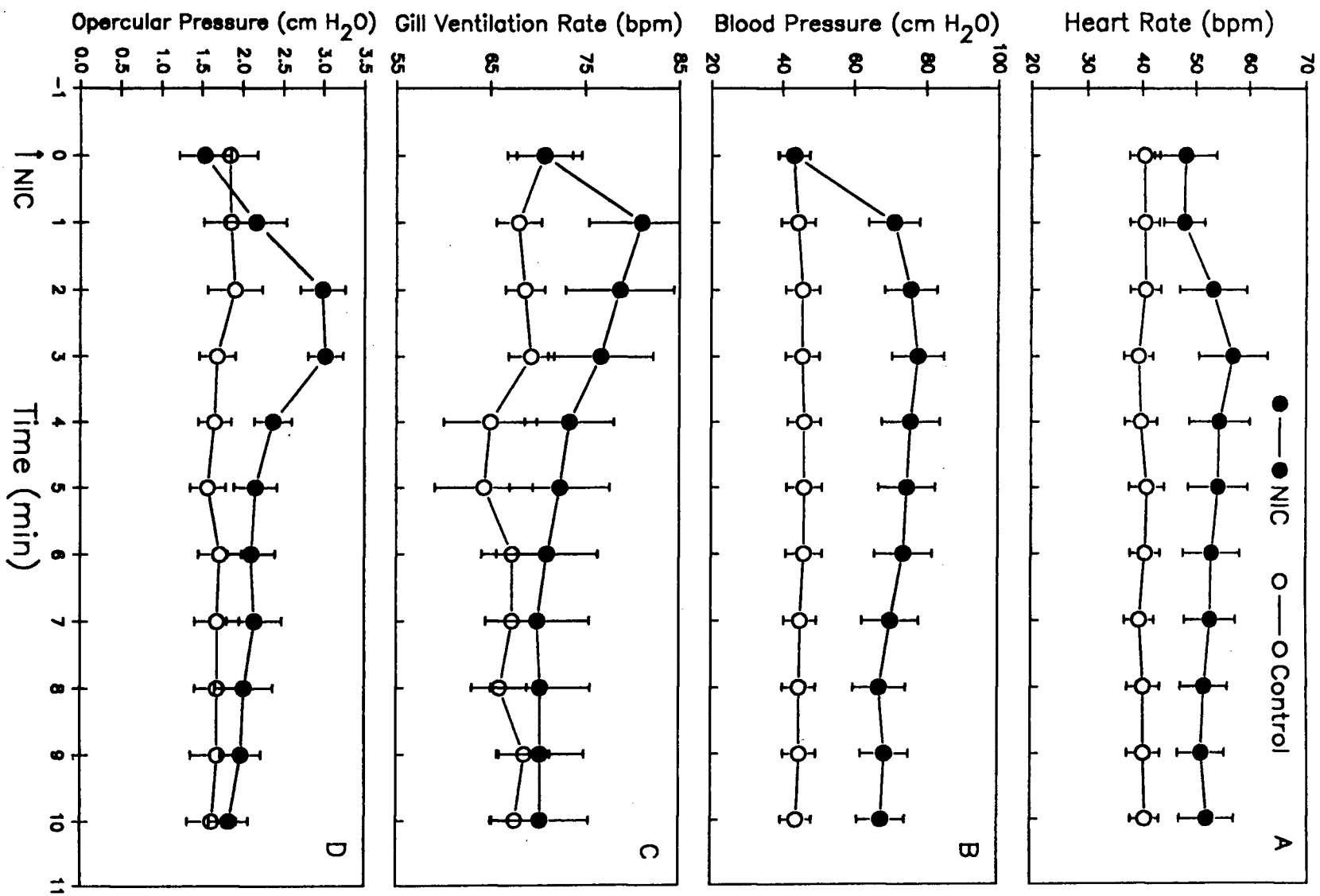
Muscarine

Muscarine, like the other cholinergic agonists, had an excitatory effect on O_2 receptor afferent nerve activity. The response to MUSC injections (100 nmol; Fig. 35), however, was moderate and not as immediate in comparison to the responses to ACH and NIC.

Cardiovascular and ventilatory variables in whole fish were altered significantly in response to MUSC (100 nmol/kg) but in a markedly different fashion than in response to the other cholinergic agonists. Heart rate was dramatically decreased (Fig. 36A), and a reduction in P_{DA} (Fig. 36B) followed the decrease in f_H. Gill ventilation rate showed a triphasic response consisting of an initial increase in rate then a decrease at 2 minutes followed by a gradual increase (Fig. 36C). Opercular pressure showed a very large increase and did not return to pre-injection levels within the 10 min recording period (Fig. 36D).

Atropine

Pre-treatment with ATRO (100-200 nmol) decreased resting neural discharge in some experiments and inhibited the responses to ACH (Fig. 37), NaCN (Fig. 38), NIC and MUSC.

In intact fish, the cholinergic antagonist ATRO (100 nmol/kg) significantly stimulated f_H and P_{DA} but had no significant effects on ventilation (Fig. 39A-D).

Summary

Many of the neurochemicals known to alter mammalian O_2 receptor afferent neural activity also affect branchial O_2 receptors in trout. Table III is a summary of pharmacological effects on O_2 chemoreceptor activity and Table IV is a summary of the cardiovascular and ventilatory responses of intact trout to the various neurochemicals.

Figure 35. Mean response of gill O_2-sensitive chemoreceptors to MUSC (100 nmol, n=7).
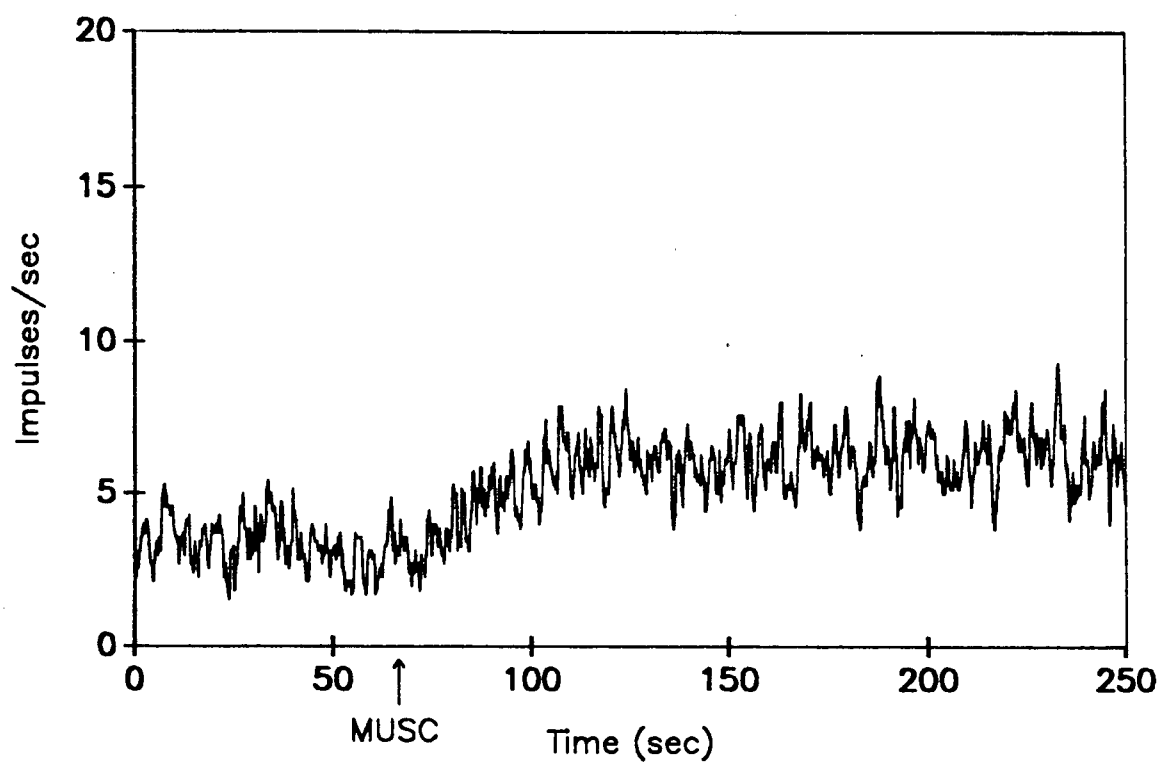
Figure 36. Mean (n=6) cardiovascular and ventilatory responses of fish to injections of 100

nmol/kg MUSC (●) and saline (○). Vertical bars are ± 1 SEM.
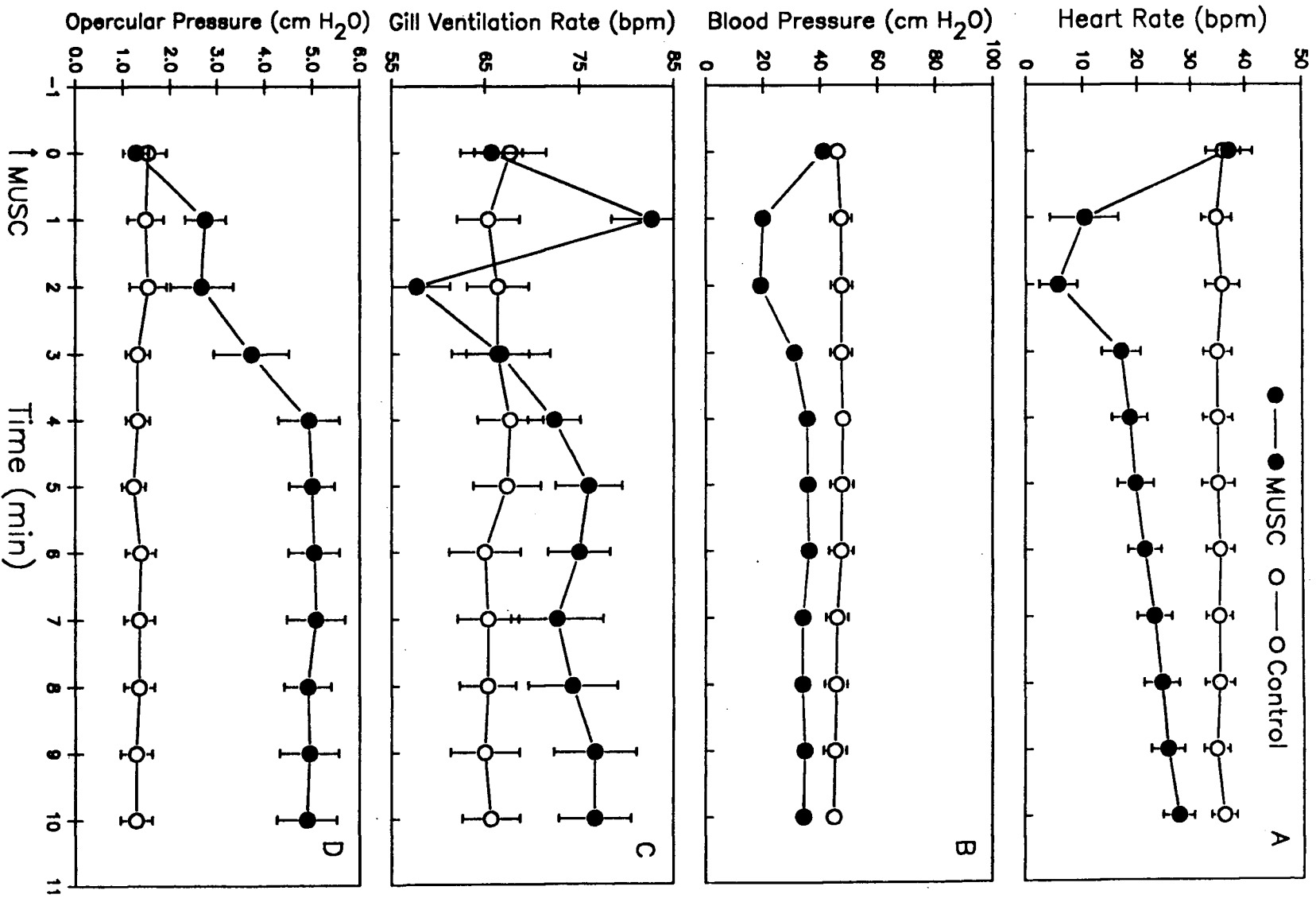
Figure 37. Responses of a single O_2-sensitive chemoreceptor to ACH (100 nmol) before (top trace) and after ATRO (200 nmol; filled in trace).
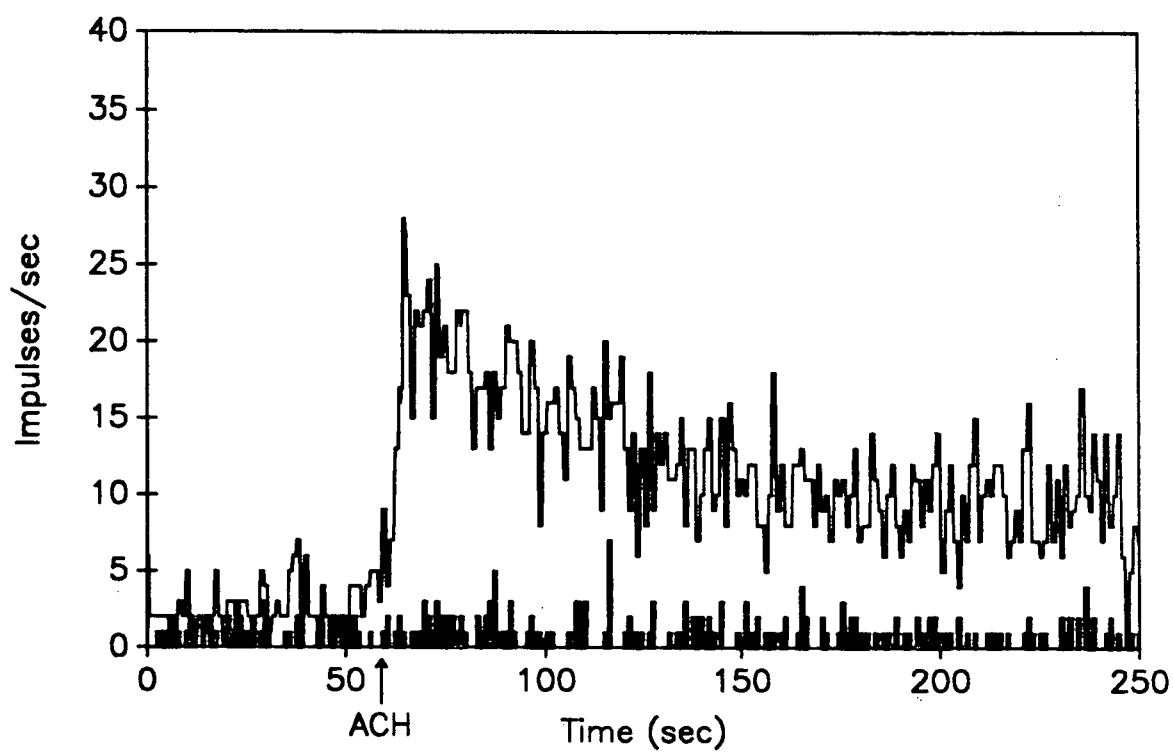
Figure 38. Mean response of gill O_2-sensitive chemoreceptors to NaCN injections before (top trace) and after ATRO (filled in trace) (n=4).
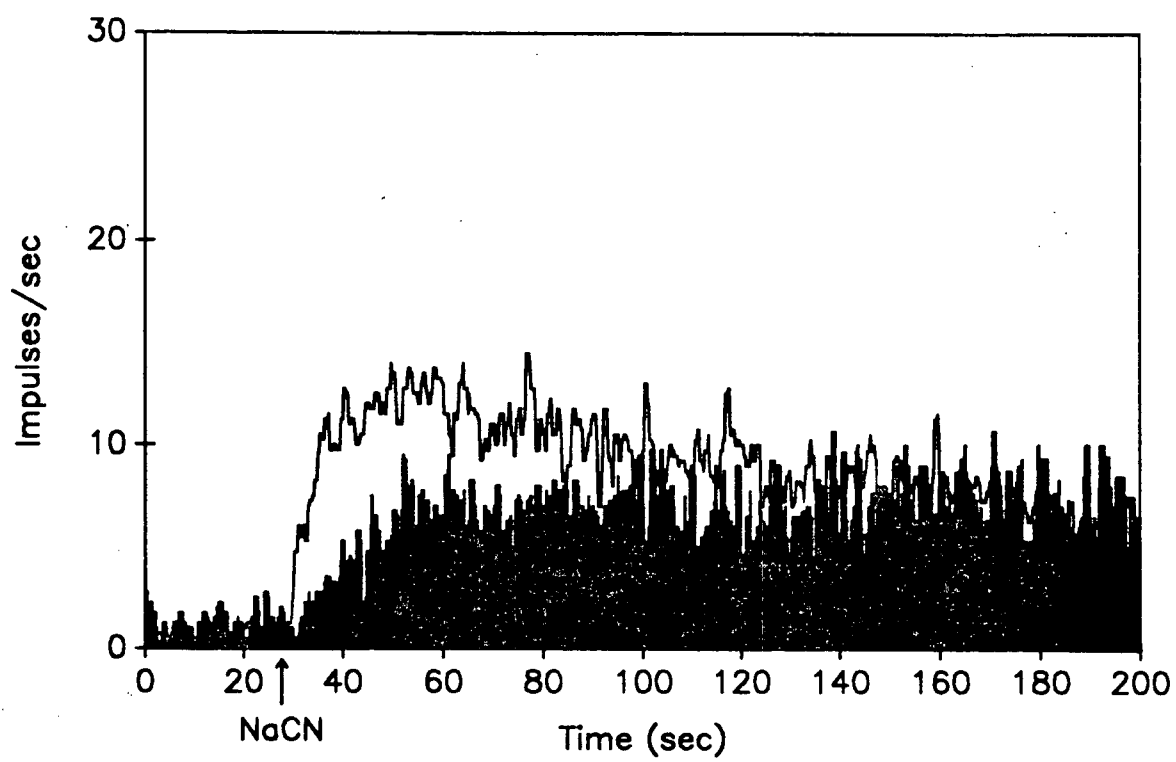
Figure 39. Mean (n=7) cardiovascular and ventilatory responses of fish to injections of 100 nmol/kg ATRO (●) and saline (○). Vertical bars are ± 1 SEM.

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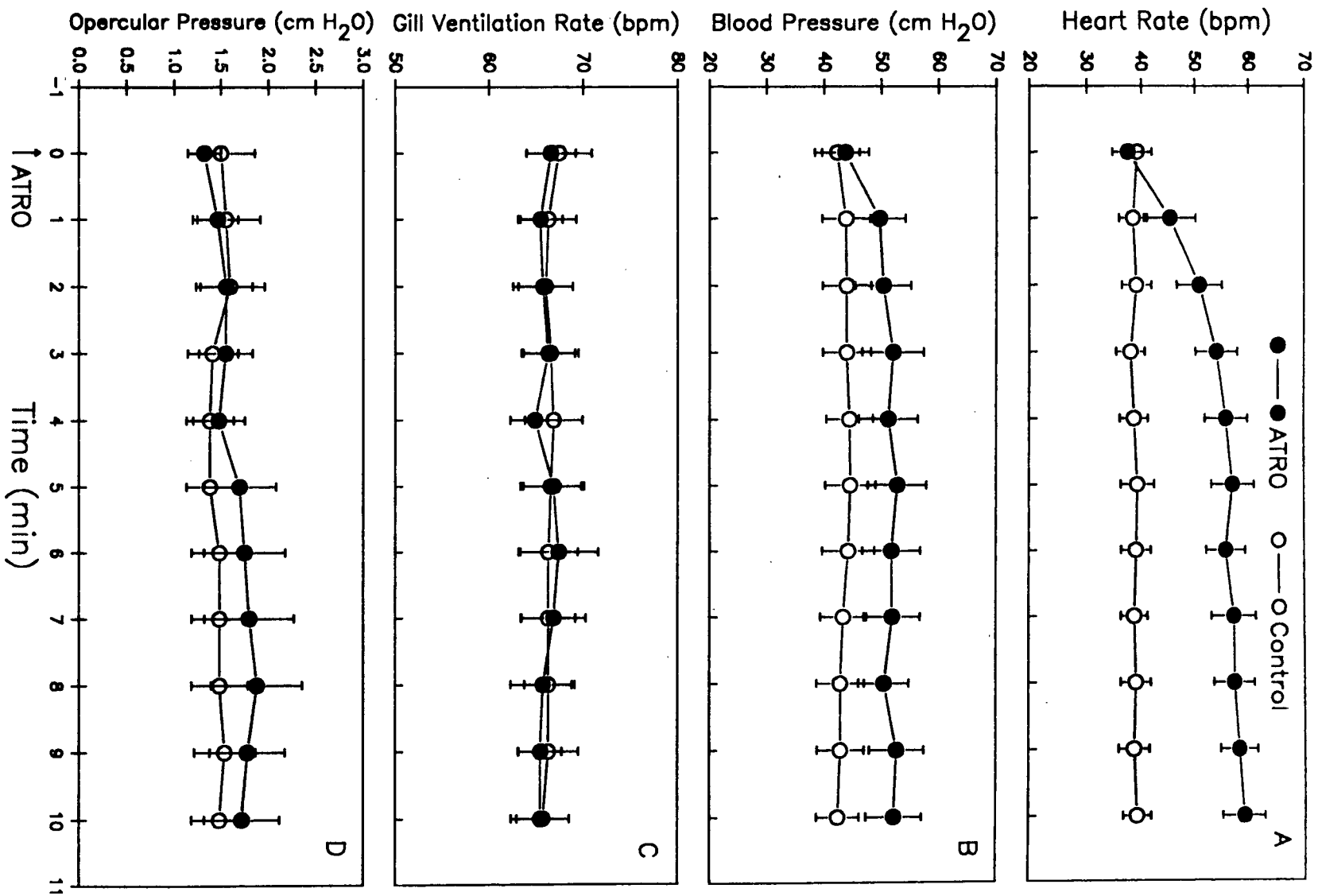
Table III. Summary of pharmacological effects on afferent neural activity.

Drug	Dose	effect
NaCN	External 1000 µg/ml	+
NaCN	Internal 25 µg	+
NOREPI	5-1000 nmol	ns (n=17) + (n=1)
EPI	500 nmol	ns
ISO	100 nmol	ns
DOP	100 nmol	+/-
PROP	500 nmol	-
5-HT	100 nmol	+/-
ACH	100 nmol	+
NIC	100 nmol	+
MUSC	100 nmol	+
ATRO	100 nmol	-

+ = increase; - = decrease; ns = no significant change;

+/- = increase followed by decrease

Table IV. Summary of pharmacological effects in intact fish.

Drug	Dose	f_H	P_{DA}	f_G	P_{OP}
NaCN	External 250 µg	-	ns	+	+
NaCN	Internal 25 µg	ns	+	+	+
NOREPI	5 nmol/kg	+	+	ns	ns
	100 nmol/kg	+	+	+	ns
	1000 nmol/kg	+	+	+	ns
EPI	5 nmol/kg	-	+	ns	ns
	100 nmol/kg	+	+	+	ns
	1000 nmol/kg	+	+	+	ns
ISO	100 nmol/kg	+	ns	+	ns
DOP	100 nmol/kg	ns	+	ns	-
PROP	500 nmol/kg	ns	+	+	ns
5-HT	100 nmol/kg	+	-	+	+
ACH	100 nmol/kg	+	+	+	+
NIC	100 nmol/kg	+	+	+	+
MUSC	100 nmol/kg	-	-	+/-	+
ATRO	100 nmol/kg	+	+	ns	ns

+ = increase; - = decrease; ns = no significant change; +/- = increase followed by decrease

DISCUSSION

Given the widespread occurrence of O_2 dependent metabolic pathways demonstrated in all animal phyla one must assume that the mechanisms of O_2 chemosensitivity are evolutionarily very old and perhaps phylogenetically diverse. Regardless of how many different organisms are able to detect O_2, most of what we know about O_2 reception is based on data from studies performed on mammals. Given that ventilation in aquatic organisms is far more dependent on changes in P_{O2} (arterial and environmental) than ventilation in terrestrial organisms, which responds primarily to changes in arterial P_{CO2}, O_2-sensitive chemoreceptors are believed to play a far more important role in the former group. As a consequence, fish should provide an excellent model to study the evolution and mechanisms of O_2-sensitive chemoreception and hypoxic reflexes.

ARE HYPOXIC REFLEXES IN FISHES DUE SOLELY TO STIMULATION OF BRANCHIAL O_2 RECEPTORS?

O_2 is demonstrably the most important respiratory variable with respect to cardiovascular and ventilatory control in fish, however, the receptor(s) mediating cardio-respiratory reflex responses to changes in O_2 in fish have proven difficult to localize. It has been proposed that, in fish, O_2-sensitive chemoreceptors are located primarily in the CNS, based on experiments which, by default, concluded that O_2 receptors must be present at extrabranchial sites. It was suggested that the latencies to response ($\approx$5 sec) when trout (anesthetized) were exposed to hypoxic water were too long to be mediated by external receptors (Bamford, 1974). In addition, ventilatory responses to aquatic hypoxia were not completely abolished in tench and sea raven

after branchial nerve sections (Hughes and Shelton, 1962; Saunders and Sutterlin, 1971).

Subsequent nerve section experiments, however, on channel catfish (Burleson and Smatresk, 1990b), *Amia* (McKenzie, 1990) and longnose gar (Smatresk, 1991) have demonstrated that it is possible to abolish hypoxic reflexes by total branchial denervation. Furthermore, experiments using isolated, spontaneously breathing carp heads, reveal that respiratory movements are depressed, and eventually cease, when vascular perfusion is stopped (Kawasaki, 1980). This suggests that central hypoxia depresses, rather than stimulates, ventilation in isolated preparations and indirectly supports the suggestion that fish do not possess central O_2 sensitive chemoreceptors.

The results of experiments in which the cranium was superfused with different solutions in *Amia* (Burleson *et al*. 1988; Hedrick *et al*. 1990) lend further and more direct support to the suggestion that there are no central chemoreceptors in fish. If there are central chemoreceptors sensitive to O_2 and/or CO_2/pH present in *Amia*, then they are insensitive to EDF and CSF composition. In fact, the insensitivity of these animals to cranial superfusion with NaCN and extremes in gas tensions (i.e. CO_2 levels 10X greater and O_2 levels 7X greater than physiological levels) is remarkable. Although it is possible that putative central chemoreceptors in fish are sensitive to changes in the vascular compartment of the brain and completely insensitive to the composition of extracellular fluids, this seems unlikely. The piscine blood-brain barrier is quite permeable (Peyraud-Waitzenegger *et al*. 1979; Nekvasil and Olson, 1986) and thus EPI, which does not stimulate peripheral O_2 chemoreceptors, does stimulate breathing, presumably by directly affecting brainstem respiratory neurons, when injected systemically in trout (present study), introduced via EDF in *Amia* (Burleson and Hedrick, unpublished data) or microinjected into the brainstem of dogfish (Taylor and Randall, 1989).

The different results of nerve section experiments have two possible explanations. First, the ventilatory responses of branchially denervated tench and sea ravens may be attributed, in part, to the presence of O_2-sensitive chemoreceptors in the pseudobranch innervated by nerves other than cranial nerves IX and X. This organ is a reduced gill arch usually located bilaterally on the inside surface of each operculum. Although pseudobranchs are absent in some fish, they have variable patterns of blood supply and innervation in the different species which possess them (see Hughes, 1984; Laurent and Dunel-Erb, 1984 and Nilsson, 1984 for reviews). In many species they are innervated by branches of cranial nerves VII and IX (Nilsson, 1984; Laurent and Dunel-Erb, 1984). Furthermore, afferent nerve activity from the pseudobranch has been shown to increase in response to hypoxia (Laurent and Rouzeau, 1972). Both the sea raven and tench posses pseudobranchs, whereas channel catfish lack a pseudobranch completely. It is possible that O_2 sensitive receptors in the pseudobranch could still stimulate ventilation via afferent fibers in cranial nerve VII during hypoxia even when all other gills were denervated. Furthermore, De Kock (1963) suggested that the ventilatory reflexes observed by Shelton (1959) in tench "might conceivably be due to the residual activity of end buds innervated by branches of the V cranial nerve". It is true that surgical removal or denervation of the pseudobranch does not abolish hypoxic reflexes (Hughes and Shelton, 1962; Randall and Jones, 1973; Bamford, 1974), but this does not necessarily argue against the pseudobranch being able to elicit these same reflexes. In *Amia*, bilateral section of the branchial branches of cranial nerves IX and X did not completely abolish ventilatory reflexes to internal NaCN unless the pseudobranchs were also ablated (McKenzie, 1990). Hypoxic reflexes were preserved in channel catfish when only one branchial nerve was left intact (Burleson and Smatresk, 1990a). These observations support the suggestion that the O_2 chemoreceptive areas in fish are not localized to a specific region, as they are in mammals, but are distributed in all gill arches and, when present, the

pseudobranch and that the little afferent input which remains following section of cranial nerves IX and X may be sufficient to elicit a response in fish possessing pseudobranchs.

The second possible explanation for hypoxic ventilatory reflexes following branchial denervation in sea raven and tench involves circulating catecholamines. Blood titers of catecholamines increase during hypoxia and may contribute to ventilatory reflexes (see Perry and Wood, 1989 for review). It has recently been suggested that the ventilatory response to increased circulating catecholamines may be mediated centrally (Taylor and Randall, 1988; Aota and Randall, 1989; Aota et al. 1990). Catecholamines stimulate gill ventilation in branchially denervated Amia which show no responses to either external or internal NaCN (Burleson et al. 1990; McKenzie, 1990; McKenzie et al. 1991). For this to explain the different results of branchial denervation on hypoxic reflexes in sea raven and tench on one hand and Amia, gar and catfish on the other, however, would require species differences in the catecholamine release in response to hypoxia.

Although presently a subject of debate, the two previous suppositions could possibly explain hypoxic ventilatory reflexes in some experimental preparations after complete branchial denervation. They imply that O_2 chemoreception arises from a diffuse network of receptors located throughout all gill arches and the pseudobranchs of species which possess them, innervated by cranial nerves VII, IX and X.

BRANCHIAL O_2-SENSITIVE CHEMORECEPTORS

Recording from the nerves of aquatic animals is technically difficult. In addition, the branchial nerves of the trout gill are not very accessible *in vivo* and, as noted by Milsom and

Brill (1986) in tuna, are very delicate and more easily damaged than some other vertebrate nerves. Despite careful dissections and handling, there were some preparations in which I was unable to record neural activity of any kind. Furthermore, only a very low percentage of the active afferent fibers showed O_2 sensitivity. Over 800 fibers were tested and approximately 5% showed O_2 chemosensitivity. Proprioceptor afferents, sensitive to filament or raker displacement, were the most numerous active units present in the glossopharyngeal nerve.

The resting firing pattern of trout gill O_2 receptors was, like other vertebrate O_2 receptors, random. Afferent neural activity showing regular bursting discharge patterns, as reported by Milsom and Brill (1986) for some tuna O_2 receptors, was only seen in a few fibers during preliminary experiments. As the experiments progressed, the bursting discharge pattern was no longer observed. Milsom and Brill (1986) suggested that intrinsic vasomotion, as reported in fish gills (Satchell, 1962), may have been the cause of the bursting discharge pattern they observed in the tuna O_2 receptors. The use of either ISO in the perfusate or high initial perfusion, in later experiments may have abolished such intrinsic vasomotion in the present study. Furthermore, given that trout O_2 receptors were much less sensitive to occlusion of the perfusate flow (see Fig. 9) than tuna receptors, any small amount of vasomotion which remained would probably have had little effect on afferent neural activity.

The stimulus-response characteristics of trout O_2 chemoreceptors were similar to those described by Milsom and Brill (1986). Progressive external hypoxia stimulated a gradual increase in neural discharge. The discharge rates of trout and tuna O_2 receptors are similar over the same P_{O_2} range although the temperature difference is about 15° C. Therefore, at equal temperatures trout O_2 receptors would presumably be more sensitive although at physiological temperatures the sensitivities of trout and tuna receptors are about equal. The chemoreceptor responses to cyanide were similar to the responses of mammalian carotid body chemoreceptors

(Mulligan *et al.* 1981; Iturriaga *et al.* 1991) as well as turtle (Ishii *et al.* 1985a), and toad (Ishii *et al.* 1985b) O_2-sensitive receptors. Sodium cyanide has been used previously to localize O_2 chemoreceptors in terrestrial vertebrates, and also has been shown to stimulate cardiovascular and ventilatory reflexes in trout (Eclancher and Dejours, 1975), gar (Smatresk *et al.* 1986), catfish (Burleson and Smatresk, 1990a) and *Amia* (Burleson *et al.* 1990; McKenzie, 1990; McKenzie *et al.* 1991).

In the present study, O_2 chemoreceptor activity was depressed during severe hypoxia. Reversible depression of chemoreceptor activity during severe hypoxia has been demonstrated previously in mammalian carotid and aortic receptors and is believed to reflect a dependence of chemoreceptor activity on oxidative metabolism (Lahiri *et al.* 1983). Milsom and Brill (1986) did not report hypoxic depression in tuna gill O_2 receptors possibly because none of their measurements were made below a P_{O_2} of 60 torr. The physiological significance of the values of P_{O_2} at which depression of chemoreceptor discharge occurred in the present study (30-40 torr) is not clear. It has been shown previously that this species is able to maintain increased ventilation at aquatic O_2 tensions between 30 and 40 torr (Holeton and Randall, 1967; Thomas and Hughes, 1982). One possible explanation for this discrepancy is that the P_{O_2} at the receptor site was much lower than the P_{O_2} of the bathing solution in the present study. Although, bubbling the chamber maintained convection over the gill surface, a boundary layer around the gill might have been present. This should not be the case *in vivo* where high rates of water flow over the gill would reduce boundary layers and bring the P_{O_2} of externally oriented receptors into closer equilibrium with the external medium. Also, the time it took to reach a P_{O_2} of 40 torr in the experimental apparatus could require as long as 3 hours. Depletion of ATP stores due to increased nerve activity over several hours in the *in vitro* preparation, could have

led to a depression of neural activity at the higher P_{O2} levels than would be found *in vivo*. This would not occur in an intact preparation where substrate delivery and ATP synthesis would be maintained.

The internal O_2 receptors recorded from with this preparation were not very sensitive to changes in perfusate flow. In conscious fish, decreasing blood O_2 delivery by inducing hypoxemia, injecting hypoxic blood or reducing blood flow to the gills stimulates ventilation (Randall and Smith, 1967; Cameron and Wohlschlag, 1969; Holeton, 1971; Wood *et al.* 1979; Smith and Jones, 1982). When using a cell-free perfusate, as in this study, the O_2 content cannot be manipulated independently of P_{O2}, thus the only way to decrease delivery is to either decrease perfusate P_{O2} or occlude perfusate flow. Occluding the perfusion flow did not elicit the dramatic change in discharge frequency observed in tuna gills (Milsom and Brill, 1986), vascularly isolated, perfused *Bufo* aorta (Ishii *et al.* 1985b) and the superfused-perfused cat carotid body (Iturriaga *et al.* 1991). In fact, the response to occlusion of perfusate was moderate in comparison to the responses to hypoxia and NaCN. Possible explanations include low sensitivity to O_2 delivery or, given the low temperature (10° C), low O_2 consumption rates at the receptor site.

External O_2 Receptors

A vast majority of fish species are restricted to aquatic ventilation in an environment which is unstable with respect to respiratory gases. O_2 depletion in natural waters is a common phenomenon and can occur as a result of a number of factors. Temperature and salinity are physical factors known to affect the solubility of O_2 in water and many waters experience large fluctuations in both. Where there is an abundance of plant life the production of O_2 by photosynthesis during the day may lead to hyperoxic conditions, however, at night when only

the O_2 consuming process of respiration is occurring, hypoxic conditions can develop. The decomposition of organic matter (i.e. sewage) can also deplete aquatic O_2. Many lakes are seasonally covered by ice which obstructs gas exchange with the atmosphere for a significant portion of the year. Snow cover on the ice may block sunlight preventing the photosynthetic production of O_2 by phytoplankton. The resultant death of fish by anoxia in these situations is commonly referred to as "winter kill" (Lagler *et al.* 1977). In order for fish to survive in a rapidly changing environment they must be able to respond to fluctuations in environmental O_2 availability in order to maintain internal homeostasis.

The hypothesis that bradycardia in water breathing fish is exclusively mediated by externally oriented O_2 receptors sensitive to water O_2 levels is supported by the data which shows that external NaCN elicits a bradycardia in trout (this study), longnose gar (Smatresk, 1986; Smatresk *et al.* 1986), channel catfish (Burleson and Smatresk, 1990a) and *Amia* (Burleson *et al.* 1990; McKenzie, 1990; McKenzie *et al.* 1991) whereas internal NaCN has no effect on f_H. These data contrast with the results of Eclancher and Dejours (1975) which show that internal injections of NaCN via the ventral aorta in trout cause a transient bradycardia and increased ventilation whereas external NaCN had no effect. Ventral aortic injections of NaCN in gar (Smatresk *et al.* 1986) and channel catfish (Burleson and Smatresk, 1990a) occasionally elicit bradycardia, but this was believed due to CN⁻ diffusing through the thin respiratory epithelium and stimulating the exteroreceptors. The external concentrations of NaCN (10-35 µg) used by Eclancher and Dejours (1975) may have been too low to stimulate exteroreceptors, being quickly diluted by the external water flow. Internal hypoxia induced by chemical or mechanical means stimulated ventilation but failed to elicit bradycardia during external normoxia in rainbow trout (Randall and Smith, 1967; Cameron and Davis, 1970; Holeton, 1971; Smith and Jones, 1982), pinfish (Cameron and Wohlschlag, 1969) and starry flounder (Wood

et al. 1979a). In the sea raven, the water flowing over the gills must be made hypoxic in order to stimulate bradycardia when the gill vasculature is bypassed and the blood is oxygenated with an artificial gill (Saunders and Sutterlin, 1971). Thus, it has been demonstrated in a number of different fish species using a variety of indirect techniques that hypoxic bradycardia in fish is mediated exclusively by externally oriented receptors, in the gills, sensitive to O_2 levels in the water.

Previous studies have demonstrated that ventilation in fish is also stimulated in response to external stimuli. A step-wise decrease in water P_{O2} stimulates ventilation in trout after a short (about 5 sec) latency (Bamford, 1974). In gar, aquatic hypoxia inhibited gill ventilation and stimulated air-breathing even when arterial O_2 tensions were maintained by artificially ventilating the air-breathing organ (Smatresk *et al.* 1986). External NaCN stimulates ventilation in trout (Sawyer and Heath, 1988; this study), catfish (Sawyer and Heath, 1988; Burleson and Smatresk, 1990a), gar (Smatresk, 1986; Smatresk *et al.* 1986) and *Amia* (Burleson *et al.* 1990; McKenzie, 1990; McKenzie *et al.* 1991).

Direct evidence from nerve recording data supports earlier, indirect reflex studies showing the existence of internal and external O_2 chemoreceptive loci. Exteroreceptors in the gills of tuna (Milsom and Brill, 1986) and trout are responsive to external O_2 stimulus levels. These trout O_2 receptors are also sensitive to external NaCN. The stimulus-response curves of these piscine receptors are similar to those of mammalian carotid body and tuna gill O_2 receptors (see Milsom and Brill, 1986). The response characteristics of gill O_2 receptors suggest that these are the receptors which mediate hypoxic bradycardia and hypoxic ventilatory reflexes in fish.

Internal O_2 Receptors

The factors responsible for internal hypoxia in the absence of external hypoxia have received little attention. Situations in nature where internal hypoxia (hypoxemia) could occur in the absence of external hypoxia are varied. Although exercise increases O_2 demand and may cause internal (tissue) hypoxia, the cardiovascular and ventilatory adjustments to exercise are thought to be mediated primarily by proprioceptive feed-back (see Shelton *et al.* 1985 for review). Anemia, however, induced by chemical or surgical means, has been used previously to study the effects of hypoxemia on the physiology of fish (Cameron and Wohlschlag, 1969; Cameron and Davis, 1970; Holeton, 1971; Wood *et al.* 1979a; Wood *et al.* 1979b; Perry *et al.* 1989). Hypoxemia due to anemia as a result of blood loss via injury or parasitism may be a common occurrence in wild fish populations (Wood *et al.* 1979a). Diet is another factor which has been shown to have a significant effect on hematocrit in channel catfish (Klar *et al.* 1986). Cameron and Wohlschlag (1969) suggested that chemical pollutants with hemolytic effects, particularly phenol compounds, have the potential to cause anemia in fish populations. A situation not uncommon in nature and aquaculture ponds occurs when excess nitrite (NO_2) and nitrate (NO_3), formed through natural processes, cause a condition in fish known commonly as "brown blood disease". High levels of nitrite in water are able to enter the fish and combine with hemoglobin to form methemoglobin effectively blocking the ability of hemoglobin to bind O_2. Nitrite poisoning has been shown to significantly reduce the blood O_2 content in carp and cause increased ventilation (Jensen *et al.* 1987).

Cardiovascular and ventilatory responses of fish to hypercapnia are somewhat variable (see Shelton *et al.* 1986 for review). Environmental CO_2 can reach very high levels in warm waters where there is a preponderance of plant life and may also be increased by acidification (i.e. acid rain). The effects of CO_2 on cardiovascular and ventilatory parameters in fish may

largely result from decreased blood O_2 carrying capacity due to Bohr and Root effects (Randall and Jones, 1973; Truchot *et al.* 1980; Smith and Jones, 1982). Recent experiments, however, indicate that CO_2 has direct effects on ventilation that are independent of O_2 content (Heisler *et al.* 1988; Wood *et al.* 1990).

Hypoxemia, during external normoxia, stimulates ventilation in fish but f_H does not change or increases slightly, again indicating that bradycardia is mediated by exteroreceptors and ventilation by intero- and exteroreceptors. In the event of hypoxemia the cardio-ventilatory reflexes work to compensate for the reduced blood O_2 carrying capacity and help to maintain oxidative metabolism. The cardiovascular and ventilatory reflex responses to reduced blood O_2 carrying capacity during external normoxia raise plasma O_2 content by increasing arterial P_{O2} and, thus, the physically dissolved O_2 in the plasma (Cameron and Davis, 1970; Wood *et al.* 1979a). These observations suggest that internal O_2 receptors in fish are sensitive to O_2 delivery or flow.

The presence of O_2 receptors sensitive to internal O_2 stimulus levels has been directly demonstrated previously in tuna gills (Milsom and Brill, 1986) and now in trout (this study). Tuna O_2 receptors seem to be much more sensitive to perfusate flow than trout receptors. Interrupting perfusion results in an immediate stimulation of the tuna receptors (Milsom and Brill, 1986). In the isolated perfused trout gill, on the other hand, occluding perfusate flow had only a modest effect on afferent nerve activity. It may be that these differences are due to temperature-related metabolic differences in the two studies. Temperature effects on O_2 receptors in poikilothermic animals is an area of research which warrants further study. The response characteristics of internal gill O_2 receptors indicate that these may be the receptors responsible for ventilatory responses to hypoxemia.

ARE TROUT BRANCHIAL O_2-SENSITIVE CHEMORECEPTORS HOMOLOGOUS TO MAMMALIAN O_2 RECEPTORS?

Before using fish as an experimental model to help explain some of the unanswered questions plaguing studies of O_2 chemoreception in mammals, the extent of homology of fish and mammalian O_2 receptors should be examined. Anatomical evidence indicates that the first gill arch of fish is homologous to the carotid arch of mammals where the carotid body O_2 chemoreceptors are located. The innervation of these two areas is the same; cranial nerve IX (glossopharyngeal) innervates both the first gill arch in fish (Fig. 1) and the carotid bodies in mammals. Boyd (1936) suggested that the baroreceptive regions of mammals (*i.e.* the carotid sinus) were derived from branchial arch arteries of non-mammalian vertebrates such as dogfish. Thus, the first gill arch seem an obvious location for O_2-sensitive chemoreceptors in fish, a fact which has not gone unnoticed. Yapp (1965), for example, suggested that in mammals "The gill capillaries of the first gill remain, as a tangle of capillaries called the carotid body,..." in his text on vertebrate zoology.

Similarities

The discharge characteristics of trout O_2 receptor afferents were similar to mammalian O_2 receptor activity. Resting, normoxic discharge rates in trout and mammals are comparable and average about 2-5 impulses/sec (Fidone and Gonzalez, 1986; Fig. 6). Trout O_2 receptors typically show a random or aperiodic discharge pattern that is similar to mammalian receptor activity recorded *in situ* and *in vitro* (Fidone and Gonzalez, 1986).

The response curve of P_{O2} vs discharge rate for mammalian and trout receptors are alike (approximately hyperbolic; Lahiri *et al.* 1983), indicating that the sensitivities of O_2 receptors

in both groups are similar at physiological temperatures. Trout receptors like mammalian receptors (Lahiri *et al.* 1983) also showed a depression of activity at very low O_2 levels. NaCN is a powerful stimulant of O_2 receptor activity in both trout and mammals.

Although a precise localization of the specific O_2 chemoreceptor cells in fishes has yet to be accomplished, branchial neuroepithelial cells which resemble mammalian Type I cells have been identified (Dunel-Erb *et al.* 1982). Like Type I cells, branchial neuroepithelial cells synapse with nerves, contain vesicles of monoamine neurochemicals and show degranulation during hypoxia (Laurent, 1984).

Differences

Fish are responsive to both external (aquatic) and internal (blood/tissue) O_2 stimulus levels. Ventilation has been shown to increase in response to either internal (lungfish, Lahiri *et al.* 1970; trout, Eclancher and Dejours, 1975) or external cyanide (trout, present study). Nicotine, another potent O_2 receptor stimulant, stimulates both pulmonary and branchial ventilation in lungfish when injected either intravenously or administered externally over the gills (Johansen and Lenfant, 1968). The nerve recordings in the present study indicate there are at least two distinct populations of O_2 sensitive chemoreceptors; one population is sensitive to external stimuli and the other population is sensitive to internal stimuli. This observation fits well with data from reflex studies which suggest that external receptors control gill ventilation and internal receptors set the level of hypoxic drive in longnose gar (Smatresk, 1986; Smatresk *et al.* 1986) and that in channel catfish, exteroreceptors mediate the hypoxic bradycardia while the increase in ventilation is mediated by both extero- and interoreceptors (Burleson and Smatresk, 1990a). Mammalian O_2 receptors, however, are only sensitive to the internal environment.

GILL O_2 CHEMORECEPTOR vs WHOLE ANIMAL RESPONSES TO VARIOUS NEUROPHARMACOLOGICAL AGENTS

Most theories suggest that the generation of afferent nerve activity signalling a change in O_2 availability is linked to the release of one or several neurochemicals present in the chemoreceptive tissue. It has been demonstrated that neurochemicals are also involved in the modulation of afferent nerve activity from O_2 receptors. Virtually all studies examining the roles of neurochemicals in O_2 chemoreception have focused on mammalian carotid and aortic body chemoreceptors. This study represents a first step in understanding the roles of neurochemicals in O_2 transduction in non-mammalian vertebrates and the evolution of O_2 reception.

In short, the results of this study demonstrate that a number of different neurochemicals have the potential to alter cardio-ventilatory performance in rainbow trout through their effects on O_2-sensitive chemoreceptors in the first gill arch. The stimulus-response characteristics of trout O_2 receptors to hypoxia and effects of the various neurochemicals on their afferent discharge indicate that O_2 transduction mechanisms in fish O_2 receptors are similar to other vertebrate O_2 receptors. Finally, some of these neurochemicals appear to affect ventilation through mechanisms other than modulation of O_2 receptor discharge.

Much of the data regarding the effects of the various putative neurotransmitters on mammalian carotid body receptors is equivocal. Exogenous substances may stimulate receptors or nerve fibers directly or the effects may be secondary due to their vascular effects and the resulting changes in O_2 flow (see McQueen, 1983 for review). In the *in vitro* trout gill preparation, however, changes in total perfusate flow had little effect on O_2 receptor discharge. It is assumed, therefore, that relative changes in regional flow caused by any of the drugs had

no effect on receptor discharge and that all effects were direct effects of the chemicals on the receptors.

This study also examined the effects of a number of different neurochemicals on cardiovascular and ventilatory variables in intact, conscious trout for comparison with their effects on gill O_2-sensitive chemoreceptors. The agonists used in the study are all putative neuromodulators of mammalian carotid body O_2 receptor afferent activity and consequently may be involved in O_2-chemosensory transduction. Although the results do not necessarily reveal anything about the principle site of action, they do indicate the extent to which *in vivo* responses mimic predicted responses to *in vitro* stimulation of O_2 chemoreceptors. Thus, the results also demonstrate the extent to which *in vivo* effects may be due to gill O_2 receptor stimulation as well as the extent to which these neurochemicals may act on cardiovascular and ventilatory control at other sites.

Sodium Cyanide

Although not a neurochemical *per se*, cyanide has been used for many years to identify O_2-sensitive afferents and test for carotid body denervation. The mechanism of action of cyanide is to block electron transport at cytochrome a_3 in the respiratory chain. Virtually any chemical which interferes with electron transport or oxidative phosphorylation (*i.e.* rotenone, antimycin, oligomycin and 2,4-dinitrophenol) stimulates O_2 receptors in mammals and provides the strongest evidence for the "metabolic hypothesis" of O_2 chemoreception (see Mulligan *et al.* 1981 or Fidone and Gonzalez, 1986 for reviews). Additional studies examining the effects of the various blockers and uncouplers of oxidative phosphorylation in species other than mammals would provide a critical test of the "metabolic hypothesis".

Cyanide was a potent stimulant of trout gill O_2 chemoreceptor activity. Cyanide has previously been demonstrated to stimulate O_2 afferent discharge in all vertebrate, as well as invertebrate, O_2 receptors studied to date (mammals: Heymans and Neil, 1958; chelonian reptiles: Ishii *et al.* 1985a; anuran amphibians: Ishii *et al.* 1985b; salmonid teleosts: Burleson and Milsom, 1990a; decapod crustaceans: Ishii *et al.* 1989). The results of the NaCN experiments on intact trout agree with previous experiments on reflex responses of longnose gar (Smatresk *et al.* 1986), channel catfish (Burleson and Smatresk, 1990a) and *Amia* (Burleson *et al.* 1990; McKenzie, 1990; McKenzie *et al.* 1991). Trout, and presumably most teleost fish, are sensitive to both internal and external O_2 stimulus levels (Smatresk *et al.* 1986; Burleson and Smatresk, 1990a). There was no apnea or coughing in response to NaCN injections, indicating that the reflexes observed were not due to any possible irritating properties of NaCN which could stimulate nociceptors or olfactory receptors. Branchial denervation in channel catfish (Burleson and Smatresk, 1990b), *Amia* (McKenzie, 1990) and gar (Smatresk, 1991) abolishes all cardio-ventilatory responses to both internal and external NaCN, indicating that all receptors sensitive to NaCN, regardless of their physiological stimulus modality, are in the gills. Thus, the present results can be explained by stimulation of gill receptors only.

Catecholamines (Epinephrine and Norepinephrine)

The two major catecholamines most studied in vertebrates are EPI and NOREPI. These substances function both as neurotransmitters and neurohormones. During "stressful" situations catecholamines are released into the circulation from chromaffin tissue. The effects that these neurohormones have on cardio-ventilatory reflexes, blood acid-base balance and O_2 carrying capacity in fish have stimulated a significant amount of research over the past 10 years (see

Perry and Wood, 1989 for review).

Hypoxemia has been demonstrated to be the proximal stimulus for release of EPI and NOREPI into the circulatory system of fish (Iwama et $al.$ 1987; Perry et $al.$ 1989). The release of catecholamines in response to hypoxemia may be due in part to a neural reflex with O_2 receptors on the afferent limb and sympathetic nerves to the chromaffin tissue of the head kidney on the efferent limb. Certainly, release of catecholamines from the head kidney of Atlantic cod is mediated, in part, by spinal autonomic nerves which stimulate cholinergic, nicotinic receptors in the chromaffin tissue which then releases catecholamines into the bloodstream (Nilsson, 1983). This neural mechanism for the release of catecholamines from the adrenal glands in mammals is identical, indicating that this response to hypoxia may be phylogenetically ancient. Recent data shows that hypoxemia induced with carbon monoxide may directly stimulate the release of catecholamines from the mammalian adrenal gland even though arterial P_{O2} does not decrease (Nishijima et $al.$ 1989). If homology holds then, hypoxemia may also directly stimulated the release of catecholamines from chromaffin tissue in fish. A third mechanism for catecholamine release from chromaffin tissue in fish is a positive feedback system in which circulating catecholamines stimulate the release of additional catecholamines, as demonstrated in eels (Epple and Nibbio, 1985). This mechanism is a direct effect of catecholamines on the chromaffin tissue and is not a reflex effect because it occurs even in the absence of the brain and anterior spinal cord (Hathaway et $al.$ 1989). Further studies looking into the control of catecholamine release from chromaffin tissue will be of great interest.

In mammals, circulating catecholamines strongly modulate carotid body O_2 sensitive afferent nerve activity (Dempsey et $al.$ 1986). Thus, it was surprising that high levels of catecholamines had little effect on O_2 chemoreceptor activity in the trout gill. Mammalian

carotid body O_2 receptors are very sensitive to exogenous catecholamines, and it has been suggested that NOREPI may be the primary excitatory neurotransmitter released from Type I cells in response to hypoxia (Dempsey *et al.* 1986). The mechanisms of catecholamine actions on O_2 receptor afferent discharge are not well understood.

The results of the adrenergic agonists on cardio-ventilatory variables demonstrate both α- and β-adrenergic effects in trout and confirm previous studies (i.e. Peyraud-Waitzenegger *et al.* 1980). Many of the cardiovascular responses to exogenous catecholamines are the effects of these neurochemicals on the heart and vasculature, not neural reflexes. Tachycardia is mediated primarily by β-adrenoceptors or a decrease in cholinergic vagal tone (see Farrell, 1984 for review). Bradycardia may be mediated directly by α-adrenoceptors (Peyraud-Waitzenegger *et al.* 1980; Tirri and Rapatti, 1982) or indirectly as a result of a baroreflex in response to the pressor effects of catecholamines (Randall and Stevens, 1967; Helgason and Nilsson, 1973; Chan and Chow, 1976; Wood *et al.* 1979b; Wood and Shelton, 1980; Farrell, 1984). The suggestion that bradycardia in response to EPI is a baroreflex is based on experiments which only interfered with the efferent limb of the reflex (i.e. atropine (Wood and Shelton, 1980) and cardiac vagus nerve section (Helgason and Nilsson, 1973)). Since the baroreceptive regions themselves were never deafferented in any of these studies, it is not certain that this response is, in fact, a baroreflex. ACH, NOREPI and internal NaCN caused similar increases in blood pressure, but f_H was not inhibited. Clearly, the numerous direct and secondary actions of drugs on neural networks and organ systems in the whole animal precludes simple interpretation of the data.

Catecholamines have been demonstrated to stimulate ventilation in mammals (Dempsey *et al.* 1986) and cardiovascular and/or ventilatory reflexes in eel (Peyraud-Waitzenegger *et al.*

1980; Chan and Chow, 1976) *Amia* (McKenzie, 1990; McKenzie *et al*. 1991), Atlantic cod (Helgason and Nilsson, 1973), lingcod (Stevens and Randall, 1972) and rainbow trout (Aota *et al*. 1990). However, the mechanism(s) and receptor(s) mediating the ventilatory reflex responses to catecholamines are controversial. In mammals the possible ventilatory effects of catecholamines include the following: direct effects on O_2 receptors, indirect effects due to alterations in blood flow, direct effects on central respiratory neurons and effects on afferent activity from pulmonary receptors (Dempsey *et al*. 1986). Low dosages (5 nmol/kg) of NOREPI and EPI had no effect on ventilation in trout. Large dosages of NOREPI and EPI, however, did stimulate ventilation in trout, but their effects were only on ventilatory rate; there was no significant effect on amplitude (see Figs. 16 and 19). It seems unlikely that the ventilatory effects of catecholamines in fish are mediated through their effects on peripheral O_2-sensitive chemoreceptors as they are reported to be in mammals (Dempsey *et al*. 1986) since high concentrations of catecholamines had little effect on most O_2 receptors in the isolated, perfused first gill arch of rainbow trout. One gill O_2 receptor, however, did show an excitation in response NOREPI which was inhibited by PROP. This raises the possibility that there is a small sub-population of branchial receptors which are sensitive to NOREPI which could mediate ventilatory reflexes. However, further experiments recording from branchial receptors *in vivo* as well as *in vitro* are needed to resolve this question.

It is interesting to note that EPI, when added to the cranial perfusate (Burleson and Hedrick, unpublished observations), stimulated gill ventilation but had no effect on air-breathing in bimodal-breathing *Amia*. The cardio-ventilatory responses of *Amia* to cranial EPI were similar to the responses elicited by EPI when injected into the DA. Injections of NOREPI and EPI into the DA of unanesthetized, intact *Amia* stimulate branchial ventilation but have no effect

on air-breathing (Burleson *et al.* 1990; McKenzie, 1990; McKenzie *et al.* 1991). However, the low sensitivity of trout gill O_2 receptors to these compounds and the observation that bilateral branchial denervation and pseudobranch ablation attenuate but do not abolish gill ventilatory responses to catecholamine injections in *Amia* indicates that the contribution of branchial O_2 receptors to ventilatory responses to catecholamines is insignificant. These observations argue for a central effect of catecholamines and also indicate that the neural networks controlling aquatic and aerial ventilation in these bimodal-breathing fish are pharmacologically separate. The significance of this in terms of the neural organization of respiratory control systems warrants further research.

Isoproterenol

ISO is a synthetic, β-agonist and has little or no effect on branchial O_2 receptors. The effects of ISO on f_H and f_G were similar to the effects of NOREPI and EPI at the same dosage. In cod, ISO (0.5-20 μg/kg) has no effect or causes a slight increase in f_H (Helgason and Nilsson, 1973). Peyraud-Waitzenegger *et al.* (1980) observed that β-adrenergic stimulation of ventilation was a seasonal phenomenon in eels. ISO had no effect during the winter months but stimulated f_H and ventilation in summer fish. All of the present experiments reported here were performed between April and July at water temperatures between 8 and 15° C, thus would represent "summer fish". Seasonal differences in response to adrenergic stimulation have been reported for carp, eel and trout (see Peyraud-Waitzenegger *et al.* 1980). Thus, the ß-adrenergic stimulation of hypoxic reflexes is not due to its effects on branchial O_2-sensitive chemoreceptor afferent nerve activity.

Propranolol

Propranolol is a potent β-adrenergic antagonist which also has some local anaesthetic effects (see Kruk and Pycock, 1983 for review). The inhibitory effect of PROP on hypoxic reflexes, in some studies, was taken as evidence that circulating catecholamines were an integral component of hypoxic reflexes in fishes (Aota *et al.* 1990). Recently, however, this theory has become the subject of debate (Perry and Kinkead, 1990). The inhibitory effects of PROP on trout O_2 receptor discharge would fit this hypothesis and would not be puzzling if catecholamines had stimulated afferent activity. Since they did not, it is tempting to speculate that the effect of PROP on O_2 receptor discharge was due to its anaesthetic properties. However, I could find no evidence of the reported anaesthetic effects of PROP (100-500 nmol) on neural activity of non-chemosensory neurons (i.e. non-specific activity and filament proprioceptors) at the dosages used in this preparation.

Propranolol had no effect on resting, normoxic ventilatory variables, suggesting that there was no β-adrenergic tonus on ventilation in these trout and that there were no serious anesthetic side effects of PROP at the concentrations used. Since PROP inhibited O_2 receptor activity in trout, if there were no other receptor groups stimulating ventilation, then one would have expected ventilation to be depressed in the resting trout, as occurs during hyperoxia (see Perry and Wood, 1989 for review). Since this was not the case, it suggests that other receptor groups are stimulated (or uninhibited) by PROP. Propranolol does inhibit ventilatory reflexes to adrenergic stimulation and acidosis in fish (Peyraud-Waitzenegger *et al.* 1980; Aota and Randall, 1989; Aota *et al.* 1990). The non-specific actions of PROP on the cardiovascular system of fish complicates interpretation of its effects on whole trout (Stevens *et al.* 1972). The role of adrenoceptors in reflex control of ventilation in fish remains perplexing and appears to be confounded additionally by species, seasonal and experimental conditions.

Dopamine

Another important catecholamine is DOP, and although its effects on ventilatory control in mammals have received much attention, very little is known about its distribution in the nervous systems and effects on cardio-ventilatory reflexes in other vertebrates. DOP is the major catecholamine found in the carotid body of mammals (Hanbauer, 1983; McQueen, 1983). Even within mammals there are species differences in the effects of DOP on O_2 receptor afferent activity. DOP inhibits chemoreceptor activity in cats but is stimulatory in rats, rabbits and dogs (Black *et al.* 1972; Bisgard *et al.* 1979; Dempsey *et al.* 1986). Few studies have investigated the effects of DOP on cardio-ventilatory parameters in lower vertebrates. In trout, DOP elicited a brief burst of activity before inhibition of O_2 chemoreceptor afferent discharge. This pattern of response has also been observed in cats and is dependent on the dose and interval between injections (Okajima and Nishi, 1981). Given the diverse results found in mammals, comparisons are difficult and the significance of the response see in trout is difficult to interpret.

The modest effect that DOP had on P_{DA} in trout is similar to its reported effects on cardiovascular parameters in dogfish (Peirce *et al.* 1970) and eel (Chan and Chow, 1976). Dopamine causes bradycardia via a vagal reflex, which is abolished by atropine, in eels (Chan and Chow, 1976). In the eel, DOP is released from chromaffin tissue and may further stimulate the release of EPI and NOREPI (Hathaway *et al.* 1989). The effects of DOP on ventilation are distinctly different from the effects of the other catecholamines. Dopamine inhibited P_{OP} and had no effect on f_G, whereas, NOREPI, EPI and ISO all had no effect on P_{OP} but stimulated f_G. In the isolated, perfused gill, DOP causes a brief burst of afferent activity, from O_2 receptors, followed by inhibition. In the intact fish there was no brief burst of ventilation corresponding to the O_2 receptor response. There is not enough data available at this time to speculate on the

mechanisms responsible for the differential effects that DOP has on ventilation in comparison to the other catecholamines in fishes, but the data suggest that the *in vivo* effects are not due to gill O_2 receptor stimulation. Thus, the results of this study indicate that DOP is not a primary neurotransmitter involved in the mechanism of O_2 transduction in fish, although, it may have a modulatory role.

5-Hydroxytryptamine

5-HT has been localized in the nervous tissues of animals from all major phyla and in every class of vertebrates (Goodman and Gilman, 1970). Despite this, our knowledge about the distribution and function of 5-HT in the nervous system of fish, as well as other vertebrates, and the effects it has on cardio-ventilatory reflexes is fragmentary. Fluorescence histochemical evidence has demonstrated the presence of 5-HT in the CNS (Parent, 1983), enteric nervous system (see Nilsson, 1983) and gill neuroepithelial cells (Dunel-Erb et al. 1982) of fishes. 5-HT is thought to play a role in the regulation of hypoxic reflexes in mammals for it has been identified in the carotid body and affects ventilation and afferent nerve activity (McDonald, 1981; McQueen, 1983).

Cardio-ventilatory reflex effects of 5-HT injections may be due to nonspecific stimulation of neurons, direct stimulation of serotonergic receptors in the vasculature or gill nociceptors (see Nilsson, 1984) and direct stimulation of gill O_2 receptors. 5-HT may also trigger the release of endogenous catecholamines (Holmgren and Nilsson, 1982).

5-HT is known to directly stimulate a variety of sensory nerve endings in animals (Goodman and Gilman, 1970; Fidone and Gonzalez, 1986). This coupled with potential indirect and secondary effects on cardio-ventilatory reflexes makes interpretation of the results of whole animal reflex studies difficult. 5-HT injections cause a short, transient apnea and bradycardia

followed by elevated ventilation and heart rate. The initial reflex effects of fishes to 5-HT are believed to be homologous to the J-reflex of mammals and are probably mediated by nociceptors in the gills sensitive to irritating substances and edema (Satchell, 1978; Poole and Satchell, 1979). The potent vasodilatory actions of 5-HT have been demonstrated previously in fish (Reite, 1969; Peirce et $al.$ 1970; Chan and Chow, 1976). Reite (1969) suggested that the decrease in P_{DA} after 5-HT injections in fish is due to increased branchial resistance coupled with either direct or indirect actions on the heart. The effects of 5-HT on O_2-sensitive afferent nerve activity in trout were similar to its effects on mammalian carotid body O_2 receptors indicating that 5-HT may play a role in the regulation of hypoxic reflexes in fish. Further research is necessary to ascertain the role of 5-HT in the regulation of hypoxic reflexes in fish as either a neurohormone or neurotransmitter in O_2 receptors.

Acetylcholine

ACH is one of the most widely distributed neurotransmitters in the vertebrate nervous system. This substance is the primary neurotransmitter in all autonomic ganglia, parasympathetic post-ganglionic synapses and skeletal neuromuscular junctions. ACH was the first neurotransmitter shown to have a vigorous stimulatory effect on O_2 receptors. The stimulatory effects of ACH on O_2 receptors have been demonstrated in mammals (von Euler et $al.$ 1941), anuran amphibians (Ishii et $al.$ 1985b) and now trout. However, after nearly 50 years its role as a key neurotransmitter in O_2 reception is still debated (see Fidone and Gonzalez, 1986 for review).

Although ACH is present in the mammalian carotid body (see McDonald, 1981; Fidone and Gonzalez, 1986 for reviews), the cholinoceptors are located primarily on Type I cells, not on the afferent nerve endings (Fidone et $al.$ 1988). Blocking these cholinoceptors with

mecamylamine (a cholinergic antagonist) does not abolish the responses of cat O_2 receptors to hypoxia (Fidone *et al.* 1988). Species specific differences within mammals in the response to ACH also raise questions about its role in O_2 transduction. ACH is excitatory (nicotinic stimulation) in cats, but inhibitory (muscarinic stimulation) in rabbits (McQueen, 1983; Fidone *et al.* 1988). Trout branchial O_2-sensitive chemoreceptors were similar to carotid body receptors in that cholinergic stimulation resulted in an increase in afferent neural activity which was mediated primarily by nicotinic cholinoceptors. The potent stimulatory action of ACH and the inhibitory effects of ATRO on trout O_2 receptors indicates a role for ACH in the control of branchial O_2-sensitive chemoreceptor afferent activity.

Given the potent stimulatory effect ACH has on O_2 receptors in the mammalian carotid body (Fidone and Gonzalez, 1986) and the isolated trout gill one might predict larger ventilatory increases in the intact fish than were observed. In mammals the ventilatory response to ACH is due to its nicotinic action on O_2 receptors and perhaps muscarinic effects on pulmonary receptors (see Dempsey *et al.* 1986 for review). Cardio-respiratory reflex responses of trout to cholinergic drugs were variable, however, which probably reflects the multiple sites of action and effects mediated by cholinoceptors. The stimulatory effect of ACH on f_H was surprising because ACH is the neurotransmitter, released from the vagus nerve, which mediates reflex bradycardia. ACH also stimulates the release of catecholamines from the adrenal medulla in mammals (Kruk and Pycock, 1983). If ACH also causes the release of catecholamines from chromaffin tissue in fish, the increase in f_H in the present study may have been a secondary effect. The modest f_H and ventilatory responses to ACH injections via the DA may also indicate that much of the neurotransmitter is being broken down by cholinesterase in the blood before it reaches reflexogenic areas.

The vigorous stimulation of branchial O_2 receptor afferent discharge by ACH and its

effects on ventilatory reflexes suggests that cholinergic mechanisms may play an important role in the chemoreflexive responses of trout to hypoxia.

Nicotine

NIC, a cholinergic agonist, has both peripheral and central effects. Although NIC is a potent O_2 receptor stimulant, it also stimulates a wide variety of other sensory receptors including mechanoreceptors, thermal receptors and pain receptors (Goodman and Gilman, 1970). Nicotine can also affect cardio-ventilatory variables in mammals via direct effects on the central nervous system and autonomic ganglia or secondarily by stimulating the adrenal medulla to release catecholamines (Goodman and Gilman, 1970). O_2 receptor afferent activity is increased, in cats, in response to nicotinic stimulation.

NIC has been shown to stimulate aquatic and aerial ventilation when injected into the blood or the ventilatory water flow in the African lungfish (Johansen and Lenfant, 1968). The effects of NIC on branchial O_2 receptor discharge and cardio-ventilatory reflexes in intact trout indicate that activation of nicotinic cholinoceptors, in contrast to muscarinic cholinoceptors (see below), is an important mechanism in the control the responses to hypoxia.

Muscarine

MUSC is another important cholinergic agonist. In mammals, stimulation of muscarinic cholinoceptors typically inhibits O_2 receptor afferent neural discharge (Fidone *et al.* 1988). MUSC stimulated O_2 receptor activity in trout, but the time course was slow and the magnitude of the response was not very large. The effect that MUSC had on nerve activity (a gradual increase in discharge rate) was similar to the effect of occluding perfusion. Blood flow through the efferent filament artery in the gill is controlled by sphincters which constrict in response to

muscarinic stimulation (Bailly and Dunel-Erb, 1986). Thus, the response of O_2 receptors in the isolated gill to MUSC may be due to vascular effects.

The effects of MUSC on the cardiovascular system were the opposite of the other cholinergic agonists. The cholinoceptors mediating bradycardia are muscarinic as are the receptors in the vasculature that mediate vasodilation in mammals (Kruk and Pycock, 1983). The muscarinic effect on gill ventilation is difficult to interpret. Cholinoceptors mediating catecholamine release from the mammalian adrenal medulla are primarily nicotinic; thus if a similar mechanism exists in piscine chromaffin tissue, then elevated catecholamine levels would not accompany MUSC injections. The overall response of the intact trout to cholinergic agonists ultimately depends upon the balance of excitatory and inhibitory effects of the drug at that particular dosage. Thus, muscarinic effects do not appear to play a significant role in the control of the cardio-ventilatory reflex responses of trout to hypoxia.

Atropine

ATRO is primarily a muscarinic antagonist, but this drug inhibited the responses to ACH, NIC and NaCN as well as MUSC, indicating that it was also affecting nicotinic cholinoceptors as it has been described to do at high concentrations (Goodman and Gilman, 1970).

The effects of ATRO on cardiovascular variables in intact fish compare favorably with previous studies (Chan and Chow, 1976; Wood *et al.* 1979a). Atropine releases tonic vagal cholinergic tonus on the heart resulting in an increase in f_H (Wood *et al.* 1979). Although ATRO inhibits resting O_2 receptor discharge and the responses to NaCN in the isolated perfused gill, it has no effect on ventilation in the intact fish. This discrepancy may be explained in part by different concentrations of ATRO coming into contact with O_2 receptors in the two different

preparations. In the perfused gill a concentrated bolus of ATRO was injected directly into the afferent branchial artery, whereas in the intact fish the drug was diluted somewhat by the time it had transversed the systemic circulation and entered the gills. These results suggest that there is no cholinergic tonus on ventilation in the resting trout. The increased f_H may have contributed to the increased P_{DA}. Although ATRO blocks hypoxic bradycardia and the cardiac responses to external NaCN in channel catfish, it does not abolish the ventilatory responses (Burleson and Smatresk, 1990a).

Thus, although ATRO is primarily a muscarinic antagonist, its inhibitory effects on branchial O_2 receptor discharge may be due to concurrent nicotinic inhibition at the dosage used. In intact trout, the same dosage was probably diluted and nicotinic cholinoceptors were not affected. Given that there is little or no muscarinic modulation of O_2 receptor activity it is probable that muscarinic inhibition would have little effect on ventilation in intact trout. The effects of ATRO on branchial O_2 receptor activity and ventilatory reflexes in intact trout further suggest that nicotinic cholinoceptors play a more important role in the responses of trout to hypoxia than muscarinic cholinoceptors.

CONCLUSIONS

The results of this thesis suggest that the branchial O_2-sensitive chemoreceptors in fish are homologous to mammalian carotid body O_2 receptors. This study focused on O_2 chemoreceptors located in the first gill arch whose afferent pathway is cranial nerve IX (glossopharyngeal nerve). Based on the nerve recording data there appear to be three functional groups of O_2-sensitive chemoreceptors in the first gill arch: 1) external receptors exclusively sensitive to water O_2 levels, 2) internal receptors exclusively sensitive to blood (perfusate) O_2 levels, and 3) receptors that sense both internal and external O_2 levels. These results support

conclusions of earlier, less direct experiments (see Smatresk *et al.* 1986; Burleson and Smatresk, 1990a). The stimulus response characteristics and hypoxic sensitivity of these O_2 receptors indicate that these are probably the receptors that mediate cardiovascular and ventilatory reflex responses to hypoxia in teleost fishes. In conjunction with results from other studies, it is suggested that external receptors are sensitive to the external milieu and mediate bradycardia and increase ventilation in response to aquatic stimuli. Internal receptors are sensitive to blood/tissue O_2 levels and stimulate ventilation only.

There are two possibilities regarding the way hypoxic reflex responses could be mediated by these receptor groups: 1) Bradycardia could be mediated exclusively by the externally oriented receptors and ventilatory responses could be due to stimulation of the other two receptor groups. This would imply that the receptors mediating cardiac reflexes (group 1) and the receptors mediating ventilatory reflexes (groups 2 and 3) have different central connections. 2) Bradycardia is mediated by group 1 and ventilation is mediated by stimulation of any receptor group (1-3), implying that all receptors synapse with respiratory centers but only group 1 synapses with interneurons to cardiovascular centers. At present it is impossible to distinguish between these two possibilities.

A number of the putative neurotransmitters, which are known to be present in the mammalian carotid body and affect O_2 receptor discharge, also had effects on trout gill O_2 receptors. Trout receptors were sensitive to ACH (primarily nicotinic), DOP and 5-HT, suggesting the presence of multiple receptor sites on/in the chemoreceptive unit.

The results of this study indicate that ventilatory responses to large catecholamine injections may be mediated by both α- and β-adrenoceptors. Stimulation of these adrenoceptors affects ventilatory rate, but there were no adrenergic effects on P_{OP}. At present it is not clear how or where adrenergic mechanisms modulate ventilation in fish, but they do not appear to

involve gill O_2 receptors. A central action is suggested. The effect of PROP, which inhibited O_2 receptor discharge despite the fact ß-agonists failed to stimulate it, may be due to non-specific effects.

Branchial O_2 chemoreceptors were stimulated by 5-HT, ACH, NIC and MUSC and inhibited by ATRO and PROP. The data indicate that ACH is the most likely candidate for primary neurotransmitter in O_2 chemoreception in fishes. The other neuropharmacological agents had modest effects and suggest that adrenergic, dopaminergic and serotonergic receptors play a neuromodulatory role, at best, in the control of O_2 receptor activity. The results of injections of ACH, NIC and MUSC in intact fish indicate that f_H and P_{DA} are stimulated by nicotinic mechanisms and inhibited by muscarinic cholinoceptors. All cholinergic agonists stimulated ventilation, but the mechanisms of this stimulation are difficult to interpret. The ventilatory responses to ACH and NIC are similar to NaCN responses, and a large portion of the response is probably due to direct O_2 receptor stimulation. The responses to MUSC, on the other hand, are unusual and are probably the result of both direct and secondary effects of MUSC on ventilation. Significant effects of cholinergic stimulation on cardio-ventilatory parameters, secondary to O_2 receptor stimulation, may include: 1) Nicotinic stimulation of autonomic ganglia and the release of catecholamines from chromaffin tissue and 2) Muscarinic cardioinhibition and vasodilation.

Thus, branchial O_2-sensitive chemoreceptors are homologous to mammalian carotid body O_2 receptors. However, there appear to be some species differences with respect to the effects of some neurochemicals, particularly catecholamines. These data indicate that catecholamines have only a minimal role, if any, in the modulation of resting O_2 receptor neural discharge but may mediate ventilation in the intact trout by different mechanisms. Cholinergic-nicotinic stimulation, because of its vigorous effect on O_2 receptor discharge appears to be more

important in O_2 transduction than any of the other neurochemicals. The lack of a vigorous response of O_2 receptors to occlusion of perfusate flow indicates that pharmacological effects on afferent activity were not due to vascular effects. Finally, this thesis demonstrates that non-mammalian animal models, especially aquatic organisms, may be very useful for studies of the O_2 chemoreceptive control of respiratory reflexes and may provide insight into the evolution and pharmacological mechanisms of O_2 transduction.

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