OXYGEN SENSING, PLASTICITY AND CATECHOLAMINERGIC FUNCTIONS IN CULTURED CHROMAFFIN CELLS OF RAT CAROTID BODY AND ADRENAL MEDULLA: MODULATION BY CHRONIC HYPOXIA AND ACETYLCHOLINE RECEPTORS

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ABSTRACT

The mammalian carotid body is a chemosensory organ, located at the bifurcation of the common carotid artery. It senses blood levels of oxygen, carbon dioxide, and acidity and maintains homeostasis via the control of breathing. Type I or glomus cells of the carotid body are the putative chemoreceptors which transduce blood-borne chemical stimuli into electrical signals carried by the carotid sinus nerve, which projects to the respiratory control center in the brainstem. It is likely that oxygen chemoreception in the carotid body involves the concerted actions of multiple neurotransmitters or neuromodulators, e.g. dopamine, acetylcholine and substance P, released from glomus cells onto apposed sensory terminals of the carotid sinus nerve. Further, carotid body catecholamines have been implicated in the resetting of chemoreceptor sensitivity after birth, ventilatory acclimatization to chronic hypoxia, and "blunting" of the ventilatory response in high-altitude dwellers. Catecholamine release is also critical for the animal's ability to survive the hypoxic stress associated with delivery and the transition to extrauterine life, though in this case, the origin is from a different source, the adrenomedullary chromaffin cells. The primary goal of this thesis was to elucidate the cellular and molecular mechanisms underlying oxygen chemoreception and the adaptive responses of chemoreceptors to chronic hypoxia. The use of dispersed cell cultures of the rat carotid body and adrenal medulla permitted the *direct* exposure of putative oxygen

chemoreceptors to low oxygen. In addition, high performance liquid chromatography, immunocytochemistry, and pharmacological tools were used to delineate the cellular and molecular mechanisms which underlie oxygen sensing and adaptation of the isolated chemoreceptors to chronic hypoxia *in vitro*.

In normoxic carotid body cultures, acute hypoxia stimulated dopamine release in a dose- and Ca^{2+}-dependent manner, possibly via closure of Ca^{2+}-dependent K^+ channels. Exposure of glomus cells to chronic hypoxia *in vitro* triggered a wide array of adaptive responses with the potential to modify the level of released neurotransmitter. These "plastic" responses include: (1) an apparent down-regulation of functional oxygen-sensitive, Ca^{2+}-dependent K^+ channels; (2) up-regulation of GAP-43 immunoreactivity; and (3) enhanced basal extracellular dopamine, which appears to be set by positive and negative feedback regulation via nicotinic and muscarinic acetylcholine receptors, respectively, and inhibition of dopamine transporters. In chronic hypoxia, acetylcholine appears to be an important autocrine/paracrine modulator of dopaminergic function in carotid body cultures. These cellular adaptations may relate to changes in carotid body chemosensitivity during chronic hypoxia *in vivo*.

Similar to carotid body glomus cells, *neonatal* adrenomedullary chromaffin cells express oxygen-chemoreceptive properties. Exposure of neonatal chromaffin cultures to acute hypoxia or a specific blocker of Ca^{2+}-dependent K^+ channels stimulated catecholamine (predominantly epinephrine) release *in vitro*. These findings in glomus and adrenomedullary chromaffin cells suggest that hypoxia may close Ca^{2+}-dependent K^+ channels, leading to membrane depolarization, entry of extracellular Ca^{2+} and

catecholamine release. However, unlike glomus cells, adrenomedullary chromaffin cells possess a developmentally regulated oxygen-sensing mechanism, since hypoxia had no significant effect on catecholamine release in *juvenile* adrenomedullary chromaffin cells.

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TABLE OF ABBREVIATIONS

α-BGT	α-bungarotoxin
AMC	adrenomedullary chromaffin cell
4-AP	4-amino pyridine
ACh	acetylcholine
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BBSS	bicarbonate-buffered salt solution
BK_{Ca}	charybdotoxin-sensitive, calcium-activated potassium channel
BSA	bovine serum albumin
CA	catecholamine
CaM	calcium calmodulin
cAMP	cyclic adenosine monophosphate
CB	carotid body
cGMP	cyclic guanosine monophosphate
CHox	chronically hypoxic
CNic	chronic nicotine-treated
CNS	central nervous system
CO	carbon monoxide
CO_2	carbon dioxide
D	dopaminergic
DA	dopamine
DAT	dopamine transporter
dbcAMP	dibutyryl cyclic adenosine monophosphate
dbcGMP	dibutyryl cyclic guanosine monophosphate

DBH	dopamine-β-hydroxylase
DHBA	di-3,4-hydroxylbenzylamine hydrobromide
DMEM	Dulbecco's Modified Eagle's Medium
EPI	epinephrine
FBS	fetal bovine serum
GAP-43	growth-associated protein 43
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HO	heme oxygenase
HPLC	high performance liquid chromatography
IbTx	iberiotoxin
K_{Ca} channels	calcium-dependent potassium channels
Ko_2	voltage-gated, calcium-independent potassium channel
mAChR	muscarinic acetylcholine receptor
N_2	nitrogen
nAChR	nicotinic acetylcholine receptor
NE	norepinephrine
NF	neurofilament
NGF	nerve growth factor
Nox	normoxic
NTS	nucleus of the tractus solitarius
O_2	oxygen
$O_2^{\cdot}$	superoxide
P	arterial pressure
PBS	phosphate-buffered saline
PGP 9.5	protein gene product 9.5

RA	retinoic acid
SA	sympathoadrenal
SCG	superior cervical ganglion
SIDS	sudden infant death syndrome
SIF	small intensely fluorescent
TEA	tetraethyl ammonium
TH	tyrosine hydroxylase
TTX	tetrodotoxin
VAH	ventilatory acclimatization to hypoxia

GENERAL INTRODUCTION

Oxygen is crucial for the survival of all higher life forms due to its biological role as the final acceptor of electrons in the mitochondrial respiratory chain, and as a result enables the synthesis of chemical energy in the form of adenosine triphosphate (ATP) by oxidative phosphorylation. In mammals, oxygen is delivered to the tissues by respiratory and circulatory systems. O_2-rich air is inhaled from the atmosphere into the lungs and CO_2-rich gas is exhaled from the lungs to the atmosphere. Oxygen then diffuses into the blood at the lungs, binds to hemoglobin, and is transported throughout the body via the circulatory system. Despite the widely differing demands for O_2 uptake and CO_2 output, e.g., during exercise and ascent to or residence at high altitude where Po_2 is reduced, the partial pressure of oxygen (Po_2) and carbon dioxide (Pco_2) in arterial blood are normally kept within close limits. This strict regulation of gas exchange is possible because all elements of the respiratory control system finely control the level of ventilation. The sensors first detect changes in Po_2 , Pco_2, or $[H^-]$ and relay this information to the respiratory center in the brainstem which coordinates the sensory input and, in turn, sends impulses to the respiratory muscles which control ventilation. Hence, mammals have evolved a number of physiological processes geared to protect the individual from harmful levels of hypoxia (low Po_2), hypercapnia (high Pco_2) or acidosis (low pH).

In mammals, peripheral sensors or chemoreceptors are located in the carotid and aortic bodies. However, during the perinatal period, neuroepithelial cells of the lung and adrenomedullary chromaffin cells (see Chapter 4) also appear to function as oxygen sensors. Central chemoreceptors located in the brainstem are most important for the minute-to-minute control of ventilation and sense blood Pco_2 and pH apparently via changes in pH of the cerebrospinal fluid (Dawes et al., 1984). Of the peripheral sensors, the carotid body is the most important for Po_2 sensing, though the carotid body can also sense Pco_2 and pH as well (Gonzalez et al., 1994). Removal of this organ in animals and humans greatly attenuates (or even depresses) the ventilatory and cardiovascular responses to hypoxia (Nakayama, 1961; Bureau et al., 1985; Winter, 1991). Briefly, the carotid body is an arterial chemoreceptor that is sensitive to changes in blood Po_2, Pco_2, and pH and responsible for ~90% of the hyperventilation observed during acute or chronic exposure to low Po_2 and for 30-50% of that seen during acidosis (Fitzgerald and Lahiri, 1986; Weil, 1986). Type I or glomus cells of the carotid body are the putative chemoreceptors which transduce blood-borne chemical stimuli into electrical signals carried by the carotid sinus nerve (CSN), which projects to the respiratory control center. The mechanisms by which these cells sense oxygen and adapt to various situations where they are chronically stimulated, e.g. by low oxygen, are one of the main themes investigated in this thesis. The embryonic origin, morphology, biochemical and physiological properties of glomus cells will be considered in more detail below. The primary site of termination of carotid body chemoreceptor afferent fibers is within the nucleus of the tractus solitarius (NTS) in the brainstem, which then integrates

chemoreceptor inputs and relays them to sites in the brainstem involved in respiratory and cardiovascular control (Housley and Sinclair, 1988; Mifflin, 1992). Although glomus cells have been studied extensively, the mechanism(s) by which these chemoreceptive cells transduce changes in blood O_2, CO_2 and pH are still controversial (Gonzalez et al., 1994).

Embryonic origin of the carotid body

In mammalian embryos, the carotid body develops in the third branchial arch next to the third arch artery, the blood vessel which eventually forms the initial portion of the internal carotid artery. The primordium of the carotid body can be distinguished as a condensation of undifferentiated cells as early as 14 days of gestation in the rat (Kondo, 1975), and 6 weeks in the human embryo (Boyd, 1937). Le Douarin and co-workers used chick-quail chimeras, formed by grafting experiments, to demonstrate the neural crest origin of glomus cells of the carotid body (Le Lievre and Le Douarin, 1975; Pearse et al., 1973). The glossopharyngeal nerve may serve as a path for the migration of neural crest cells to the third branchial artery, since it is associated with the carotid body primordium from the earliest ages. Using immunocytochemical techniques, Kondo and co-workers (1982) demonstrated that carotid body glomus cells in mammals express neuron-specific enolase, a neuroendocrine/neuronal marker.

Glomus cells share many characteristics with cell types that comprise the sympathoadrenal sublineage of neural crest derivatives (Kobayashi, 1971; Patterson, 1990; Anderson, 1993). Adrenal medullary chromaffin cells, small intensely fluorescent

(SIF) cells and sympathetic neurons are derived from this sublineage, and represent a range of cellular phenotypes from endocrine to neuronal. It seems likely that these sympathoadrenal crest derivatives develop from a committed progenitor whose developmental fate depends upon signals from the environment encountered during migration (Anderson, 1993). For example, the high local concentrations of corticosteroids surrounding the adrenal medulla maintain the endocrine phenotype of adrenomedullary chromaffin cells, while exposure of these cells to nerve growth factor (NGF) in culture is sufficient to promote their "transdifferentiation" into sympathetic neurons (Doupe et al., 1985a). The same applies to SIF cells in sympathetic ganglia, though in this case circulating corticosteroids appear to promote their endocrine differentiation (Doupe et al., 1985b). These cells are found clustered near fenestrated capillaries and therefore have free access to circulating corticosteroid (McDonald and Blewett, 1981). SIF cells can also transdifferentiate into sympathetic neurons in the presence of NGF (Doupe et al., 1985b). Similarly, the potential for neuronal differentiation may exist in their crest-derived endocrine counterparts, the glomus cells, in response to appropriate environmental signals. However, during the course of this thesis it was found that NGF did not promote neuronal differentiation in neonatal glomus cells (Jackson and Nurse, 1995a).

Structural organization of the carotid body

The carotid body is a small (1-2 mm in diameter), highly vascularized paired organ located between the internal and external carotid arteries near the bifurcation of the

common carotid artery. One or more small arteries originating in nearby branches of the common carotid artery supply the carotid body with blood via a strikingly dense network of small vessels. The specialized parenchymal tissue of these arterial chemoreceptor organs consists of clusters or glomeruli of type I (glomus) cells, which are innervated by afferent fibers of the carotid sinus nerve (CSN), a branch of the IXth cranial nerve (see below). Within each cluster, glomus cells are partially enveloped by slender cytoplasmic processes of glial-like type II (sustentacular) cells (McDonald, 1981). Individual clusters of glomus and sustentacular cells are separated by walls of connective tissue, which converge on the surface to form a capsule surrounding the whole organ.

Glomus cells are round or ovoid with a diameter of 8-15 μm, depending on the species, and often have short cytoplasmic processes (up to 40 μm long). In rat carotid body, there are an estimated 8700 glomus cells, as determined by serial sectioning (McDonald, 1981), and these comprise approximately 59% of the total volume of the organ (Laidler and Kay, 1975). Their cytoplasm has a well-developed Golgi apparatus, extensive endoplasmic reticulum, numerous mitochondria, and a variety of dense- and clear-cored synaptic vesicles that contain various putative neurotransmitters. The distribution of dense-core vesicles within the cytoplasm is not uniform; they are rare in the Golgi region and tend to accumulate in the periphery of the cells often apposed to nerve endings (McDonald, 1981; Verna, 1979). As discussed in more detail below, glomus cells form afferent and efferent chemical synapses with sensory nerve endings, and appear to be in synaptic contact with each other. In addition to chemical synapses, gap junctions are also present, suggesting the presence of electrical coupling and the

importance of intercellular communication between neighboring glomus cells (McDonald and Mitchell, 1975; Monti-Bloch and Eyzaguirre, 1990).

Innervation of the carotid body

In 1900 Kohn proposed that the carotid body, along with the adrenal medulla, belonged to a system of organs (paraganglia) that were composed of *chromaffin cells*, based on the degree of yellow staining after fixation with potassium dichromate. After some debate, Kohn's hypothesis that all glomus cells are chromaffin cells, had been supported by findings that all glomus cells exhibited at least some degree of the "chromaffin reaction" and they all contained catecholamines or indoleamines (Kobayashi, 1971; Bock and Gorgas, 1976). As a result of similarities drawn between glomus and adrenal chromaffin cells by Kohn, it was assumed that glomus cells were innervated by sympathetic axons. However, data from axonal degeneration studies and from autoradiographic studies of axoplasmic transport of radioactive amino acids, coupled with data from morphological studies of the (sensory) petrosal ganglia, indicate that the majority (~95%) of vesicle-containing nerve endings on glomus cells are part of unipolar neurons whose cell bodies lie in the petrosal ganglion (McDonald and Mitchell, 1975). These sensory neurons project axons to the carotid body via the carotid sinus nerve (CSN), which is a branch of the glossopharyngeal nerve. Using electron microscopy, McDonald (1981) estimated the CSN in rat contains a total of ~650 axons, of which approximately 50% innervate glomus cells. If the majority of glomus cells are innervated by chemoreceptive axons, each axon must branch extensively and innervate

an average of about 30 glomus cells in the rat carotid body (assuming 300 axons and 10 000 glomus cells). Using serial reconstructions from electron micrographs, Kondo (1976) successfully demonstrated this extensive branching of sensory axons by tracing the terminal arborization of a single axon and its contact with 17 glomus cells in the rat carotid body.

Nerve endings directly apposed to glomus cells are usually calyceal and bouton-shaped and range in surface area from under 1 μm^2 to about 10 μm^2 (McDonald, 1981). Nearly all sensory nerve endings are *postsynaptic* to glomus cells and most of the sensory nerves that are *presynaptic* to glomus cells are also postsynaptic to the same glomus cell; therefore, about 10% of sensory nerve endings form reciprocal synapses with glomus cells. The presence of presynaptic sensory neurons implies that terminals of sensory neurons may modulate glomus cell function by the release of neurotransmitter(s) (e.g., dopamine) or other substances at the glomus cell-nerve synapse.

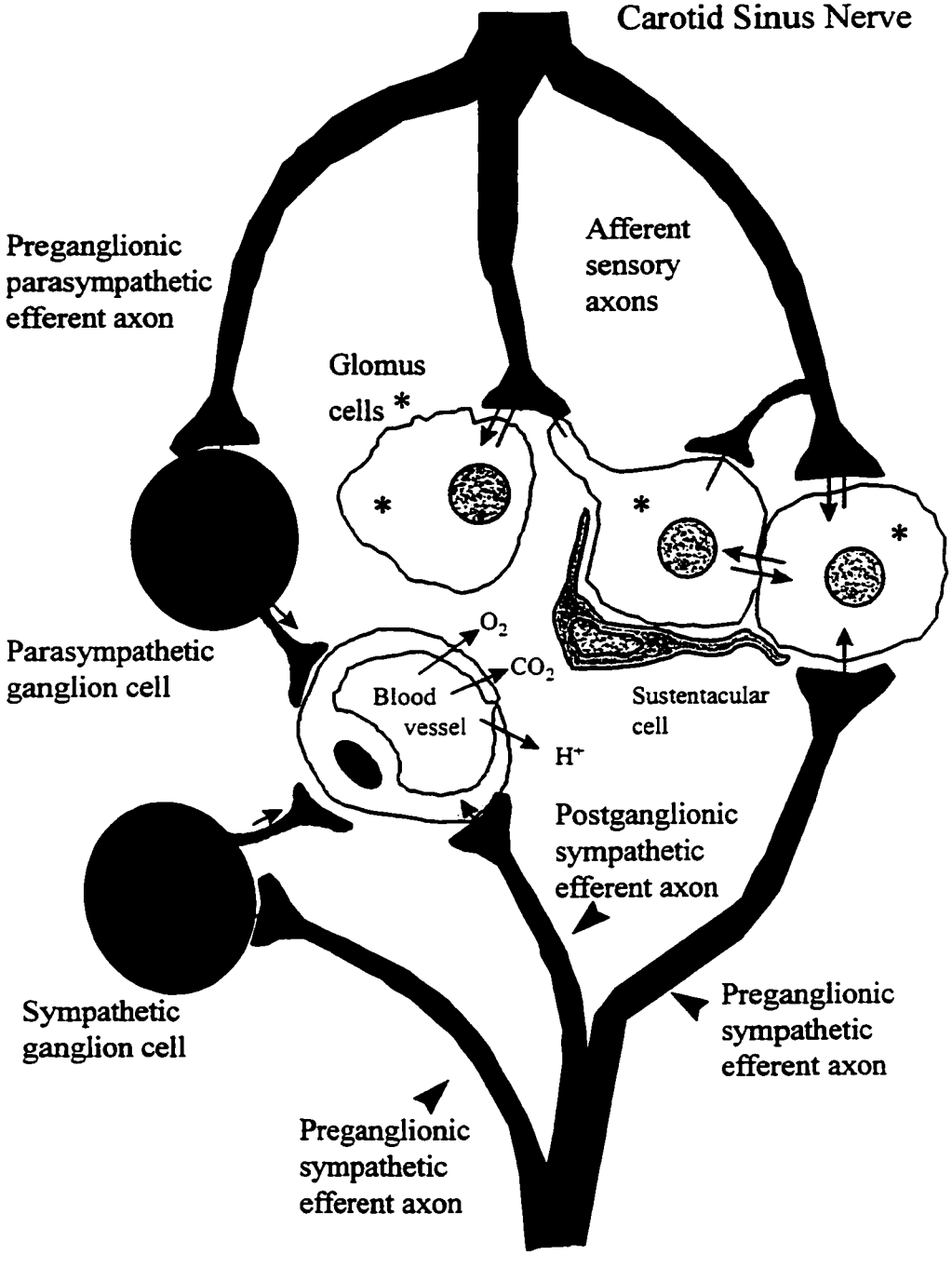
A small proportion (~5%) of nerve endings which terminate on glomus cells do not degenerate after the CSN or glossopharyngeal nerve is cut; however, they do disappear after the superior cervical sympathetic trunk is cut (McDonald, 1981). The ganglioglomerular nerve carries these sympathetic (motor) nerve fibers from the nearby superior cervical ganglion (SCG) to the carotid body, and the majority innervate carotid body vasculature. Occasionally, some glomus cells are innervated by both sensory and preganglionic sympathetic nerve endings in the rat carotid body (McDonald, 1981).

Arteries and arterioles in the carotid body are innervated by both sympathetic and parasympathetic axons. Most parasympathetic vasomotor axons derive from ganglion

cells within the carotid body that receive their preganglionic input from motor axons of the CSN. Sympathetic vasomotor axons originate mainly from neurons of the SCG, but some vasomotor axons arise from sympathetic ganglion cells within the carotid body. This dual innervation pattern, provided by sympathetic and parasympathetic axons, allows blood flow to be precisely controlled through out this highly vascularized organ by vasoconstriction and vasodilation.

The following schematic diagram (Figure 1) illustrates the complex pattern of synaptic connections found between: (1) nerve endings and glomus cells, (2) nerve endings and blood vessels, and (3) adjacent glomus cells (including gap junctions).

Cellular, neural, and vascular elements of the rat carotid body.



[Adapted from McDonald and Mitchell, 1975]

Neurotransmitters in carotid body glomus cells

It is likely that chemoreception in the carotid body involves the concerted actions of multiple neurotransmitters or neuromodulators released from glomus cells. Neurochemical and immunocytochemical studies have identified several such agents in glomus cells, including the biogenic amines dopamine, norepinephrine, and serotonin, as well as acetylcholine and the neuropeptides substance P, met-enkephalin, and atrial natriuretic peptide (Chiocchio et al., 1967; Fidone et al., 1976, 1990; Wang et al., 1991a, 1992; Gonzalez et al., 1994; Gauda and Gerfen, 1996). A variety of biochemical methods have shown that dopamine (58-86%) is the principal catecholamine of the rat, cat, rabbit, and pig carotid body, whereas norepinephrine (14-37%) and epinephrine (0-6%) are present in smaller amounts (Zapata et al., 1969; Hellstrom and Koslow, 1975; Hansen and Morgan, 1977).

Dopamine as a carotid body neurotransmitter

Dopamine is synthesized and stored principally in glomus cells, since cutting the CSN or removing the superior cervical ganglion (SCG) does not significantly alter dopamine content of the carotid body (Hanbauer and Hellstrom, 1978). In addition, all glomus cells contain tyrosine hydroxylase (TH), the rate limiting enzyme for catecholamine synthesis, as well as the enzyme dopa decarboxylase, which converts dopa to dopamine (Bolme et al., 1977). Norepinephrine, however, is stored both in glomus cells and in sympathetic nerve endings in the rat carotid body, since excision of the SCG results in a ~50% reduction in norepinephrine levels (Hanbauer and Hellstrom, 1978).

Unlike the ubiquitous expression of TH and dopa decarboxylase, the enzyme responsible for the conversion of dopamine to norepinephrine, i.e. dopamine-β-hydroxylase (DβH), is present in approximately 30% of rat glomus cells (Chen et al., 1985).

The release of dopamine from the carotid body by acute hypoxia was first *directly* measured in studies *in vitro* by Gonzalez and Fidone (1977), and later verified in several laboratories (Gonzalez et al., 1994). *In vitro* carotid body preparations, with the CSN still attached, are valuable for characterizing the release response of chemoreceptors as they permit simultaneous measurements of stimulus intensity, dopamine release, and CSN discharge. In initial studies performed with rabbit carotid bodies, the release of [^{3}H]dopamine into the perfusate increased linearly with decreasing Po_2 between 300-0 mmHg, and chemoreceptor discharge recorded simultaneously from the CSN increased in parallel with the release of dopamine (Fidone et al., 1981, 1982). In more recent studies measurement of catecholamine release using carbon-fiber microelectrodes impaled in rat carotid bodies *in vitro*, confirmed that catecholamines (probably dopamine) are released in response to hypoxia in a dose-dependent and calcium-dependent manner (Donnelly, 1993; Iturriaga et al., 1996). Similarly, high Pco_2/low pH induce release of dopamine in proportion to their intensity and in parallel with the increase in action potential frequency recorded simultaneously in the CSN (reviewed by Gonzalez et al., 1994). Since the nerve and catecholamine responses changed in parallel, some workers considered that dopamine is excitatory and its release may be causal to the increased chemosensory discharge. However, this view is challenged by recent experiments demonstrating that depletion of carotid body dopamine with reserpine pre-treatment fails to abolish the

hypoxia-induced increase in chemosensory discharge in the CSN (Donnelly, 1996; Iturriaga et al., 1996). As discussed below the effects of exogenous dopamine in the carotid body are complex.

Pharmacological studies on the effects of *exogenously* administered dopamine on carotid chemosensory activity have shown that it commonly depresses CSN discharge, but can also cause excitation or biphasic changes in discharge (Zapata and Llados, 1977; Docherty and McQueen, 1978, 1979; Fidone et al., 1991). For instance, Zapata (1975) reported that dopamine injections in the cat carotid body *in vitro* produced a range of effects, including pure excitation (31%) of CSN fibers, pure inhibition (33%), inhibition followed by excitation (18%), as well as no effect (18%). These pharmacological studies are the basis for the most popular contention that dopamine is inhibitory in the carotid body. There are, however, several findings that contest this theory: (1) intracarotid injection of high doses of dopamine always produced a delayed excitation (Docherty and McQueen, 1978; Llados and Zapata, 1978), (2) in reserpinized (catecholamine-depleted) animals superfusion of the carotid body with dopamine was always excitatory (Okajima and Nishi, 1981; Leitner and Roumy, 1986), and (3) after intravenous administration of certain doses of dopaminergic blockers the injection of dopamine augmented the CSN action potential frequency (Nishi, 1977; Zapata and Llados, 1977). In addition, the use of dopaminergic blockers and dopamine injections have produced mixed results raising questions about drug specificity and possible vascular effects (compare Donnelly et al., 1981 versus Nolan et al., 1985). For example, dopamine injections *in vivo*, acting on D_1 receptors located in the rabbit carotid body vasculature, would produce vasodilation

(Kohli and Goldberg, 1987) and secondarily tend to decrease ongoing CSN discharge (Docherty and McQueen, 1979). Commonly, dopaminergic (D_2) blockers reduce the chemodepressant effects of dopamine, but the excitatory effect of dopamine on chemoreceptors, when obtained, is generally unaffected (Mir et al., 1984). Perhaps the excitatory action of dopaminergic blockers may be the result of an increased release of endogenous dopamine, and part of the inhibitory actions of exogenous dopamine may be the result of a reduction in the release of endogenous dopamine due its actions at inhibitory D_2 receptors on glomus cells (Gonzalez-Guerrero et al., 1993). Overall, it is difficult to draw a firm conclusion from experiments using exogenous dopamine and dopaminergic blockers, since they may affect carotid body vasculature via D_1 receptors, and glomus cells and sensory nerve endings which express D_2 receptors. Therefore, dopamine can have both excitatory and inhibitory effects in the carotid body, and clarification of its physiological role in chemoreception requires further study.

Similar to other systems where neurotransmitters are actively secreted, catecholamines released by glomus cells are inactivated by re-uptake, enzymatic degradation by monoamine oxidase and catechol-O-methyltransferase, and diffusion from the synaptic cleft (Gonzalez et al., 1994). In addition, both basal and stimulus-evoked dopamine release from glomus cells is continously modulated by feedback inhibition mediated by dopaminergic (D_2) receptors located on chemoreceptor cells (Fidone et al., 1991; Mir et al., 1984).

Norepinephrine as a carotid body neurotransmitter

Early studies of norepinephrine action in carotid body demonstrated that this catecholamine produced hyperventilation (McQueen, 1983). Norepinephrine influences respiration via its actions at β-adrenergic receptors in the carotid body, since β-adrenergic antagonists and agonists are able to block and mimic, respectively, the effects of this catecholamine on respiration (Fidone et al., 1991). However, because these experiments were done *in vivo*, the possiblity that effects of these drugs were mediated by changes in local blood flow, rather than by direct actions on glomus cells or nerve endings, could not be ruled out. In more recent experiments, chronically sympathectomized rabbit carotid bodies were used to eliminate sympathetic (norepinephrine-containing) nerve terminals and the release of [^{3}H]norepinephrine (synthesized from [^{3}H]tyrosine]) was measured in response to hypoxia *in vitro* (Fidone et al., 1991). These studies showed that acute hypoxia elicited [^{3}H]norepinephrine release that was 2x baseline levels, even though norepinephrine constituted only a small percentage of the total catecholamine released from the rabbit carotid bodies. These results have been confirmed in the rat carotid body, and similar to dopamine release, norepinephrine release in response to hypoxia was dependent on extracellular calcium (Shaw et al., 1989). Although the carotid body is considered primarily a dopaminergic organ, Gomez-Nino et al. (1990) have shown that different stimuli can elicit differential release of the two catecholamines. For instance, while acute hypoxia preferentially released dopamine, nicotinic agonists preferentially released norepinephrine from the rabbit carotid body. These results suggest that different environmental stimuli may

trigger different mechanisms for the mobilization and release of catecholamine stores in the carotid body. The ability of nicotine to stimulate the preferential release of norepinephrine in rabbit carotid body leads to questions regarding the role of endogenous acetylcholine and cholinergic receptors in chemoreception. These are considered in the next section.

Role of acetylcholine in carotid body function

The significance of acetylcholine in carotid body chemoreception has been debated throughout the history of arterial chemoreceptors. Corneille Heymans, who won the Nobel prize for uncovering the role of the carotid body in cardiopulmonary control, demonstrated that nicotine and exogenous acetylcholine, acting at the level of the carotid body, can evoke respiratory excitation comparable to that produced by hypoxia (Heymans et al., 1931, 1936; also see Zapata et al., 1976). These results lead to the "cholinergic hypothesis" of carotid body chemoreception which proposed that acetylcholine was the neurotransmitter mediating the activation of sensory nerve endings of the CSN. Numerous studies have shown that glomus cells contain acetylcholine (Fidone et al, 1976; Hellstrom, 1977), express choline acetyl transferase activity (Hellstrom, 1977; Wang et al., 1985) and the degradative enzyme acetylcholinesterase (Nurse, 1987), accumulate choline by a high affinity mechanism, and exhibit a high turnover rate for acetylcholine (Fidone et al., 1977).

After decades of carotid body research the "cholinergic hypothesis" remains controversial for a number of reasons. First, even though classical nicotinic blockers

suppressed the excitatory actions of nicotinic agonists, cholinergic antagonists failed to block the normal CSN response to hypoxia (Nishi and Eyzaguirre, 1971; McQueen, 1977, 1983). Second, radioligand binding and autoradiographic studies, using [^{125}I]α-bungarotoxin (α-BGT) as ligand for nicotinic acetylcholine receptors in normal, CSN-denervated and sympathectomized carotid bodies, showed that high affinity specific binding was located in glomus cells and sympathetic endings, but not in sensory nerve endings (Dinger et al., 1981, 1985). These results should be interpreted with caution since neuronal nAChR are known to be insensitive to α-BGT blockade (Sargent, 1993; McGehee and Role, 1995; see Obeso et al., 1997). Third, the effect of acetylcholine on CSN discharge and catecholamine release are species-dependent (Fidone et al., 1991). For example, cholinergic receptors in the cat carotid body are primarily nicotinic, and nicotine (as well as acetylcholine) evokes both catecholamine release and an increase in CSN discharge. In the rabbit, however, muscarinic receptors dominate and muscarinic agonists (as well as acetylcholine) depress both catecholamine release and CSN discharge (Fidone et al., 1991). More recent studies on co-cultures of rat petrosal neurons and glomus cells indicate that ACh is likely to be an excitatory neurotransmitter released from glomus cells during hypoxia (Zhong et al., 1997), and that ACh excites the majority of rat petrosal neurons via hexamethonium-sensitive nAChR (Zhong and Nurse, 1997).

Alternative actions for ACh in carotid body function include a role as a secondary neurotransmitter which modulates the function of presynaptic glomus cells. Receptors for both nicotinic and muscarinic acetylcholine receptors are abundant in glomus cells (Dinger et al., 1985, 1986). In addition, electrophysiological studies using isolated

glomus cells from neonatal rat carotid body have demonstrated that nicotine evokes inward currents and membrane depolarization via mecamylamine-sensitive nAChR (Wyatt and Peers, 1993). More recent studies using calcium imaging techniques have shown an increase in intracellular Ca^{2-} in isolated rat glomus cells in response to both nicotinic and muscarinic agonists (Dasso et al., 1997).

Other neuromodulators and peptide neurotransmitters

Enkephalins (Met- and Leu) and substance P are also putative neurotransmitters or neuromodulators in the carotid body (Gonzalez et al., 1994). Met-enkephalin has been localized to glomus cells by immunohistochemistry (Wharton et al., 1980; Gonzalez et al., 1994) and appears to function as an inhibitory neurotransmitter, attenuating CSN discharge (McQueen and Ribeiro, 1980); however, the location of the delta-type receptor (δ-opioid receptor; Kirby and McQueen, 1986) for this opioid peptide has not been determined. Agonists to the δ-opioid receptor attenuate and antagonists augment chemosensory discharge in response to hypoxia in the cat, although under normoxic conditions these antagonists have little effect on basal CSN activity (Kirby and McQueen, 1986). Further, acute exposure to hypoxia reduces enkephalin levels by 40-50% in the rabbit carotid body (Hanson et al., 1986). On the other hand, substance P is an excitatory neuropeptide (McQueen, 1980) and immunocytochemical studies have demonstrated substance P-immunoreactivity in glomus cells and nerve fibers in the cat carotid body (Prabhakar et al., 1989a; Kusakabe et al., 1994). In rat glomus cells, however, in situ hybridization studies failed to detect mRNA encoding substance P and

the substance P receptor (Gauda and Gerfen, 1996). In the cat, exogenous administration of substance P agonist increase, while antagonists decrease, CSN discharge in response to hypoxia (Prabhakar et al., 1984, 1989a, 1993). However, Monti-Bloch and Eyzaguirre (1985) reported that substance P had a mixed excitatory/inhibitory effect (depending on dose) on CSN discharge in the response to low Po_2 in cat carotid body *in vitro*. As shown for enkephalins, acute exposure of awake rabbits to hypoxia reduces carotid body substance P by ~40% (Hanson et al., 1986). In conclusion, the mechanism of action of substance P and enkephalin in the carotid body remains uncertain; however, these peptides appear to be released from glomus cells by natural stimuli and may modulate chemosensory output. Interestingly, Prabhakar et al. (1989b) have demonstrated that substance P increases oxygen consumption of isolated mitochondria, suggesting an intracellular site of action for this peptide. Another putative transmitter, atrial natriuretic peptide (ANP), has been localized to glomus cells of the cat carotid body using immunocytochemistry and moreover ANP is a potent inhibitor of CSN discharge in response to hypoxia (Wang et al., 1991a). Finally, Prabhakar et al. (1995) reported that carbon monoxide (CO), produced by heme oxygenase (HO), appears to function as an important physiological regulator of chemosensory activity. Using immunocytochemistry, heme oxygenase 2 was localized to glomus cells in the cat and rat carotid body and inhibition of this enzyme, with zinc protoporphyrin IX, markedly increased CSN discharge. Exogenous CO reverses the stimulatory effects of zinc protoporphyrin IX. Since HO requires molecular oxygen, hypoxia might directly lower HO activity and CO formation, leading to an increase in CSN activity.

Theories of oxygen chemotransduction

It is now generally accepted that the glomus cell is the primary chemosensory element of the carotid body. The correlation between hypoxia-evoked release of neurotransmitters (particularly dopamine) and afferent chemoreceptor fiber discharge point to chemostimulus-induced transmitter release from glomus cells as being a fundamental step in carotid body chemotransduction (Fidone et al., 1990; Gonzalez et al., 1992; Peers and Buckler, 1995; Lopez-Barneo, 1996). However, as discussed above numerous studies have shown that glomus cells contain a wide array of putative neurotransmitters including catecholamines and neuropeptides. Most likely, the final chemoreceptor output results from the summed actions of *several* neuroactive agents at both presynaptic autoreceptors, and postsynaptic receptors on afferent terminals of the CSN. However, the mechanisms by which chemoreceptors initially "sense" physiological stimuli (e.g., hypoxia) remain controversial. Essentially, there are two main models proposed for O_2-chemotransduction, the *plasma membrane model* and the *mitochondrial model*. Both models propose that hypoxia raises intracellular Ca^{2+} (Ca^{2+}_i) levels in type I cells and this in turn triggers neurotransmitter secretion; however, the source of the calcium mediating the rise in Ca^{2+}_i is different in the two models.

In the *plasma membrane model*, hypoxia leads to closure of K^+ channels causing depolarization of the plasma membrane and/or an increase in action potential frequency. This in turn promotes Ca^{2+} influx through voltage-gated (L-type) Ca^{2+} channels, a rise in [Ca^{2+}i], and transmitter release from glomus cells (reviewed by Lopez-Barneo, 1996; Peers and Buckler, 1995). Buckler and Vaughan-Jones (1994) used microfluorimetric

methods to show that the hypoxia-evoked increase in $[Ca^{2+}_i]$ in isolated rat glomus cells depends almost exclusively on Ca^{2+} entry through voltage-gated ion channels in the plasma membrane. Although there is substantive evidence that the modulation of potassium channel activity by hypoxia plays a key role in oxygen sensing by glomus cells, the subtype of O_2-sensitive K^+ channel appears to differ between species. Lopez-Barneo and colleagues were the first to report that hypoxia could selectively inhibit a lower conductance (40 pS), voltage-gated, Ca^{2+}-insensitive K^+ channel (Ko_2 channel) in adult *rabbit* type I cells (Lopez-Barneo et al., 1988; Ganfornina and Lopez-Barneo, 1992). In contrast, in neonatal *rat* type I cells, the hypoxia-sensitive K^+ channel has been characterized as a high conductance (190 pS), charybdotoxin-sensitive, Ca^{2+}-activated K^+ channel (BK_{Ca}; Peers, 1990b; Wyatt and Peers, 1995). However, Buckler (1997) proposes that the initial depolarization and Ca^{2+} influx in rat glomus cells is mediated via inhibition of charybdotoxin-insensitive, low conductance "leak" K^+ channels. He suggests that this "leak" channel is important in determining resting membrane potential and initiating the depolarizing response to acute hypoxia, while BK_{Ca} channels play a secondary or modulatory role in oxygen chemoreception. Experiments performed in this thesis (Chapter 2) show that even at a relatively high Po_2 of ~160 mmHg, at least some iberiotoxin-sensitive, Ca^{2+}-dependent K^+ channels are open, and that their closure can lead to catecholamine secretion in cultured rat glomus cells.

Several groups have investigated the mechanism(s) by which hypoxia suppresses K^+ channel activity. In rabbit glomus cells, low Po_2 decreases the open probability of K^+ channels through a membrane-delimited mechanism, since the inhibitory action of

hypoxia is retained in single channel recordings from isolated membrane patches (Ganfornina and Lopez-Barneo, 1992). In addition, the inhibition of K^- channels in this species is extremely rapid and can be fully reversed by carbon monoxide, suggesting that K^- channels are closely coupled to an O_2-sensor protein (Lopez-Lopez and Gonzalez, 1992). However, K^- channel inhibition in these studies is seen over much higher Po_2 ranges (70-150 mmHg) than that required for increased CSN discharge (<60 mmHg; Ganfornina and Lopez-Barneo, 1991). The inconsistency of this dose-response relation between Po_2 and K^- channel inhibition is largely removed by the finding in rabbit glomus cells that dopamine release does not take place before a certain Ca^{2+}_i threshold is reached, and that L-type Ca^{2-} channels show a voltage-dependent inhibition by low Po_2 (Montoro et al., 1996). This inhibition is apparent at voltages more negative than -10 mV and is released at more depolarized potentials. Hence, the differential inhibition of K^- and Ca^{2-} channels by hypoxia helps to explain why the secretory response of rabbit glomus cells is displaced towards low Po_2 values (less than 60-70 mmHg) where increases in CSN activity are detected. The voltage-dependent inhibition of Ca^{2-} channels by low Po_2 appears to be species dependent since it has not been seen in rat glomus cells (Lopez-Lopez et al., 1997). Finally, other factors (e.g., cAMP and probably other second messengers) may further modulate the O_2-sensitive K^- channel within physiological ranges of tissue Po_2 (Lopez-Lopez et al., 1993).

Lopez-Lopez et al. (1989) suggested that O_2-sensitive K^- channels may be linked via a G-protein to a hemoglobin-like O_2 sensor in the plasma membrane. However, there is no direct evidence to support the involvement of G-proteins in this process. Using

membrane patches from glomus cells, the O_2-sensitive K^+ channels were uneffected by the presence of GTPγ-S (a non-hydrolyzable analog of GTP which inhibits G-protein function) at the internal surface of the plasma membrane (Ganfornina and Lopez-Barneo, 1991). Acker and colleagues, on the other hand, have presented strong evidence that the actual O_2 sensor is a membrane-bound NADPH oxidase, containing a b-type cytochrome, closely associated or linked with specific K^+ channels (Acker et al., 1992; Cross et al., 1990). Under normal Po_2, cytochrome-b_{558} (a component of the NADPH oxidase complex) is proposed to synthesize superoxide (O_2^-) which is then converted to H_2O_2 by superoxide dismutase. H_2O_2 is then further reduced to H_2O by glutathione peroxidase with consequent oxidation of glutathione (a sulfhydryl-reducing agent). In hypoxic conditions, H_2O_2 production falls and there is an increase in the reduced form of glutathione, which may reduce thiol groups of specific amino acids of the K^+ channel critical for their gating properties, i.e., K^+ channel conductance will be inhibited.

The *metabolic hypothesis*, put forward in 1963 by Anichkov and Belenkii, proposed that hypoxia, as well as metabolic poisons (e.g. cyanide), activate the chemoreceptors by decreasing ATP levels. However, the link between the presumed decrease in ATP and neurotransmitter release was not explained. More recently, Biscoe and Duchen (1990a, b) have restated the metabolic hypothesis and claim that low Po_2 slows electron transfer through the respiratory chain of chemoreceptor cells, leading to a decrease in the mitochondrial proton electrochemical gradient. As a result, mitochondria would release Ca^{2+}, leading to an increase in $[Ca^{2+}_i]$ and neurotransmitter release. Using intracellular microfluorimetry, Biscoe and Duchen (1990a, b) have shown rises in Ca^{2+}_i in

glomus cells during hypoxia; however, this rise was not mediated by depolarization-induced influx through Ca^{2-} channels, but by release from mitochondrial stores. They further demonstrated that a similar rise of $[Ca^{2-}{}_i]$ could be induced by inhibitors of mitochondrial metabolism (cyanide, rotenone and FCCP), suggesting that some of the Ca^{2-} released might originate from mitochondria (Biscoe and Duchen, 1990a, b). However, the assumption is made that mitochondria store high enough levels of Ca^{2-} to produce significant rises in $[Ca^{2+}{}_i]$. Following criticism of this assumption, Biscoe and Duchen have recently suggested that mitochondria are not the source of cytoplasmic Ca^{2-} needed for neurotranmitter release (Duchen and Biscoe, 1992a, b). In a recent series of experiments, they have demonstrated a graded depolarization of mitochondrial membrane potential and rise of NAD(P)H with hypoxia, which correlated with changes in $[Ca^{2-}{}_i]$ (Duchen and Biscoe, 1992a, b). Given that mitochondrial metabolism in glomus cells appears to be unusually sensitive to changes in Po_2, the role of mitochondria in oxygen chemotransduction remains controversial.

Lahiri (1994) suggests that cytochrome $a_3{}^{2-}$ is a plausible candidate as an intracellular O_2 sensor that functions in the low range of Po_2 <25 Torr. In this model, low Po_2 prevents the reduction of O_2 by cytochrome $a_3{}^{2-}$, diminishing the energy state and increasing Ca^{2-} release from intracellular stores, presumably through microsomal Ca^{2-}-ATPase. In these studies, carbon monoxide (CO) was used as a molecular probe that simulated hypoxia, since CO complexes with the heme group in cytochrome $a_3{}^{2-}$ and prevents its reaction with O_2. Perfusion and superfusion of cat carotid body with a medium containing CO (Pco~560 Torr) induced a strong chemosensory discharge in the

dark. Light dissociated the cytochrome a_3^{2-}-CO complex and abolished this excitatory effect.

Development of hypoxic chemosensitivity

In the fetus, where the arterial Po_2 (PaO_2) from the maternal circulation is reduced, carotid chemoreceptors are adapted to a baseline PaO_2 of ~23 mmHg and only respond to lower PaO_2 levels (Blanco et al., 1984). At birth, there is a 3-4 fold increase in PaO_2 and this relative hyperoxia silences the carotid chemoreceptors (i.e., no CSN activity can be elicited). Over the next 2-3 days the carotid chemoreceptors slowly develop a vigorous response to hypoxia, except that the range of O_2 sensitivity has shifted to the right (more sensitive) compared to the fetal response. It is widely accepted that the rise in O_2 tension at birth is the trigger for this shift of O_2 sensitivity or "resetting" of arterial chemoreceptors (Eden and Hanson, 1987a, b; Hanson et al., 1989; Hertzberg et al., 1992). During infancy the strength of this ventilatory response to hypoxia continues to increase until the sensitivity of peripheral chemoreceptors has reset to the high PaO_2 of the adult. In the rat, this postnatal increase of carotid body hypoxic sensitivity has been described at the level of ventilation *in vivo* (Eden and Hansen, 1987a), as well as at the level of CSN discharge and catecholamine release from intact carotid bodies *in vitro* (Donnelly and Doyle, 1994a, b; Pepper et al., 1995a). Interestingly, the emergence of the chemoreflex in the neonate is preceded by a drop in carotid body dopamine turnover over the first 12 h after birth (Hertzberg et al., 1990).

In addition to the ventilatory defense during hypoxic stress, carotid

chemoreceptors are essential for the development of normal breathing patterns during postnatal maturation, recovery from apnea, and arousal from sleep during hypoxic-apnea episodes or upper airway obstruction, especially in the neonate (Nakayama, 1961; Sullivan, 1980; Bowes et al., 1981; Bureau et al., 1985; Hofer, 1986; Donnelly and Haddad, 1990). Hence, abnormal development or delayed maturation of carotid chemoreceptors may put the infant at increased risk for a variety of potentially fatal neonatal disorders associated with abnormalities in the control of breathing, including sudden infant death syndrome (SIDS), apnea of prematurity, and bronchopulmonary dysplasia.

Adaptation of the carotid body to chronic hypoxia

Carotid body changes after chronic hypoxia in vivo

When humans or animals ascend to elevations where the partial oxygen pressure falls to low levels, many adaptative changes take place to maintain gas and pH homeostasis. An increase in pulmonary ventilation and in red blood cell count, a shift to the right of the hemoglobin dissociation curve, an increase in the density of capillaries in peripheral tissues, changes in oxidative cellular enzymes, and enlargement of the carotid body have been documented during adaptation or acclimatization to chronic hypoxia (Lahiri, 1977; West, 1991). Arias-Stella (1969) reported that the carotid bodies of subjects living at high altitude (4330 m, barometric pressure 460 mmHg) in the Andes are larger than those of people dwelling on the coastal plain (150 m) of Peru. Heath et al. (1973) determined in a morphometric study that carotid bodies of adults rats exposed a

barometric pressure of 380 mmHg (equivalent to an altitude of 5500 m) for 5 weeks had an average volume 3.6 times that of controls. Also, people living at high altitudes have an increased incidence of carotid body tumors or chemodectomas (Saldana et al., 1973). A lack of oxygen appears to be responsible for carotid body enlargement, since this organ is also enlarged in patients at sea level who are subjected to chronic hypoxia as a result of various pulmonary and cardiovascular diseases (Heath et al., 1970), as well as in experimental animals exposed to normobaric hypoxia (Hanbauer et al., 1981; Dhillon et al., 1984; McGregor et al., 1988). The increase in size is due to hypertrophy and hyperplasia of glomus and sustentacular cells, as well as dilatation of blood vessels and the proliferation of vascular and connective tissues (McDonald, 1981; Dempsey and Forster, 1982; Dhillon et al., 1984; McGregor et al., 1988). The mechanisms underlying these adaptive responses to chronic hypoxia are still controversial.

Ventilatory acclimatization to chronic hypoxia

Ventilatory acclimatization to hypoxia (VAH) is characterized by a time-dependent hyperventilation, involving increases in both respiratory frequency and tidal volume, followed by a persistent and slow decrease in hyperventilation upon restoration of normoxic conditions (e.g., return to sea level). Importantly, carotid body chemoreceptors are essential for ventilatory acclimatization to chronic hypoxia (Smith et al., 1986); CSN-denervated animals do not exhibit hyperventilation at high altitude, so the blood Po_2 decreases in proportion to the altitude (Weil, 1986). In addition, Busch et al. (1985) have demonstrated that carotid body alone, without systemic hypoxemia or

alkalosis, elicited ventilatory acclimatization in goats within six hours. After a given altitude and environmental Po_2 are reached, there is a second rise in ventilation that develops within hours to days, with a further reduction in blood Pco_2 and a proportional increase in blood Po_2, suggesting that ventilatory stimulation by hypoxia might be increasing during acclimatization (Dempsey and Forster, 1982). This second rise in ventilation, until the final steady-state hyperventilation is achieved, is also mediated by the carotid body (Weil, 1986). Barnard et al. (1987) have shown that the response of chemoreceptor afferents to hypoxia was significantly greater in chronically hypoxic than in control cats. Therefore, hypoxic sensitivity of carotid chemoreceptors appears to increase during chronic hypoxia, providing a time-dependent increased respiratory drive that may be involved in ventilatory acclimatization. On return to sea level, animals subjected to an acute hypoxic test also exhibit an exaggerated carotid body-mediated hyperventilation (Barnard et al., 1987; Vizek et al., 1987). Despite the importance attributed to the carotid body in the regulation of breathing during hypoxia, little is known about the cellular mechanisms operative in the chemoreceptors during chronic hypoxia.

"Blunting" of the ventilatory response in chronic hypoxia

In clinical studies, Severinghaus and associates (1966) demonstrated that people native to high altitude (e.g., Cerro de Pasco, Peru) have a diminished ventilatory response to acute hypoxia, that persists for prolonged periods, if not indefinitely, when such individuals go to sea level (Sorensen and Severinghaus, 1968). A similar blunted

ventilatory response to hypoxia is seen in chronically hypoxic adult rats (Wach et al., 1989). Interestingly, the carotid bodies of these rats also have increased levels of dopamine (and norepinephrine) content and synthesis (Hanbauer et al., 1981; Pequignot et al., 1987; Gonzalez-Guerrero et al., 1993). In addition, the ventilatory response to acute hypoxia of neonatal rats born and reared in hypoxic environments is blunted or absent (Eden and Hanson, 1987b). This weak chemoreflex in hypoxic rat pups has been correlated with high turnover rates of carotid body dopamine (Hertzberg et al., 1992). The strength of the chemoreflex was increased after chronically hypoxic pups were returned to normoxia (Hertzberg et al., 1992). Hence, increases in dopamine content and turnover in carotid body of adult mammals exposed to chronic hypoxia, and neonates born and reared in chronic hypoxia, may explain, at least in part, this reduced chemosensitivity.

Goal of the thesis

The primary goal of this thesis was to elucidate the cellular and molecular mechanisms underlying oxygen chemoreception and the adaptive responses of chemoreceptors to chronic changes in oxygen availability. The use of dissociated cell cultures of the rat carotid body and adrenal medulla permitted the *direct* exposure of putative O_2-chemoreceptors to various drugs, factors, and oxygen tensions in a controlled cellular, gaseous, and fluid environment *in vitro*. This represented a novel strategy for studying the chronic effects of low Po_2 on carotid body chemoreceptors, since almost all previous studies relied on whole animal exposure to low oxygen in a hypobaric chamber

for several days. The latter approach results in several cardiovascular adjustments, including alterations in blood Pco_2 and pH (Eden and Hanson, 1987b), as well as the hormonal status. These secondary adjustments make it difficult to isolate effects due solely to the direct effects of low oxygen on the chemoreceptor cells. In the *in vitro* model used in this thesis the carotid body cells are isolated and exposed directly to low oxygen in an incubator set at constant Pco_2, thereby eliminating the confounding effects of the circulatory adjustments present *in vivo*. A significant portion of this research utilized HPLC measurements of the final catecholaminergic output from chemoreceptor cells to delineate the cellular and molecular mechanisms which underlie the adaptive responses of mammals to different oxygen environments. Examples of such environments include the hypoxic stress associated with birth, the change in oxygen availability as the animal moves from fetal to extrauterine life, and the reduced levels of oxygen associated with high altitude dwelling and various pulmonary and cardiovascular diseases associated with chronic hypoxaemia. Essentially, knowledge of the mechanisms of oxygen chemoreception are of fundamental importance not only for the fields of cellular physiology and biology, but also for understanding our ability to survive O_2 deprivation. Abnormalities in oxygen chemoreception have been implicated in fatal conditions such as sudden infant death syndrome (SIDS), which is the major cause of death in infants between 1 month and 1 year of age. In Canada, the incidence of SIDS is about 1.2 per 1,000 live births (Becker, 1990).

The main body of this thesis is organized into 4 Chapters. Chapter 1 will address the plasticity of carotid body chemoreceptors in culture following chronic stimulation by

various environmental factors including hypoxia, and agents that cause elevation of intracellular cAMP. Chapter 2 is a detailed study of the dopaminergic properties of cultured rat carotid body chemoreceptors in normoxic and chronically hypoxic environments. High performance liquid chromatography (HPLC) is used to determine chemoreceptor dopamine stores and release *in vitro*; dopamine release is used as an assay for chemoreceptor cell function and to elucidate the role of K^- channels in oxygen sensing and adaptation to hypoxia. Chapter 3 examines the role of AChR and dopamine transporter in the regulation of extracellular dopamine in chemoreceptors exposed to chronic hypoxia. In addition, this Chapter examines carotid body chemoreceptor function following chronic treatment with nicotine, and suggest pathways by which this neuroactive drug can interfere with the protective chemoreflex response against hypoxia. In Chapter 4, a developmentally regulated O_2-sensing mechanism in rat adrenal chromaffin cells is discussed. Finally, there is a discussion of the relevance of the above results to the field of oxygen chemoreception.

CHAPTER 1

Plasticity in Cultured Carotid Body Chemoreceptors: Environmental Modulation of GAP-43 and Neurofilament

All data presented in this chapter represents work done entirely by me and has been published in *Journal of Neurobiology*, Vol. 26, pp.485-496 (1995).

SUMMARY

In this study we use dissociated cell cultures of the rat carotid body to investigate the adaptive capabilities of endogenous oxygen chemoreceptors, following chronic stimulation by various environmental factors. These oxygen chemoreceptors are catecholamine-containing glomus cells, which derive from the neural crest and resemble adrenal medullary chromaffin cells. Using double-label immunofluorescence, we found that chronic exposure of carotid body cultures to hypoxia (2% to 10% oxygen) caused a significant fraction of tyrosine hydroxylase-positive (TH^+) glomus cells to acquire detectable immunoreactivity for growth-associated protein GAP-43. The effect was dose-dependent and peaked around an oxygen tension of 6%, where approximately 30% of glomus cells were GAP-43 positive. Treatment with agents that elevate intracellular cyclic adenosine monophosphate (cAMP) (i.e., dibutyryl cAMP or forskolin) also

markedly stimulated GAP-43 expression. Since hypoxia is known to increase cAMP levels in glomus cells, it is possible that the effect of hypoxia on GAP-43 expression was mediated, at least in part, by a cAMP-dependent pathway. Unlike hypoxia, however, cAMP analogs also stimulated neurofilament (NF 68 or NF 160 kD) expression and neurite outgrowth in glomus cells, and these properties were enhanced by retinoic acid. Nerve growth factor, which promotes neuronal differentiation in related crest-derived endocrine cells, and dibutyryl cGMP were ineffective. Thus, it appears that postnatal glomus cells are plastic and can express neuronal traits *in vitro*. However, since hypoxia stimulated GAP-43 expression, without promoting neurite outgrowth, it appears that the two processes can be uncoupled. We suggest that stimulation of GAP-43 by hypoxia may be important for other physiological processes, e.g., enhancing neurotransmitter release or sensitization of G-protein-coupled receptor transduction.

INTRODUCTION

The regulation of ventilation in mammals in response to changes in environmental oxygen tension (Po_2) is mediated mainly by the carotid body, a peripheral chemosensory organ containing the oxygen-sensing glomus, or type 1 cells (Gonzalez et al., 1992). Developmentally, these cells derive from the embryonic neural crest (Pearse et al., 1973) and share many characteristics with cell types that comprise the sympathoadrenal sublineage of crest derivatives (Kobayashi, 1971; Patterson, 1990; Anderson, 1993). In particular, they synthesize and store catecholamines, which are released in a calcium-dependent manner following exposure to acute hypoxia (Fishman et al., 1985; Gonzalez et al., 1992). When animals or humans are exposed to *chronic* hypoxia, a series of adaptive changes occur in the carotid body, resulting in increased chemosensitivity (Barnard et al., 1987; Vizek et al., 1987), modifications in catecholaminergic functions (Gonzalez-Guerrero et al., 1993), and hypertrophy of the organ (e.g. McGregor et al., 1988). Although the mechanisms underlying these long-term adaptive responses to hypoxia are unknown, there is evidence supporting the involvement of cyclic adenosine monophosphate (cAMP)-mediated pathways within chemoreceptor cells. For example, recent studies from this laboratory have shown that when dissociated cell cultures of the rat carotid body are exposed to chronic hypoxia (6 % oxygen) over 1 to 3 weeks, or grown under normal O_2 tensions in the continuous presence of cAMP analogs, the

glomus cells undergo hypertrophy and display an increased density of voltage-gated sodium channels (Stea et al., 1992; Mills and Nurse, 1993). Furthermore, chronic hypoxia *in vivo* causes a dose-dependent modulation of cAMP levels in the carotid body (Perez-Garcia et al., 1990), and exposure of isolated carotid bodies to acute hypoxia causes elevation of intracellular cAMP in glomus cells (Wang et al., 1991b). Taken together, these studies suggest that cAMP-mediated intracellular pathways could be involved in the plastic responses of glomus cells during chronic chemosensory stimulation.

In the present study we investigated whether other plastic properties might be revealed in glomus cells exposed to various agents, including chronic hypoxia and cAMP analogs *in vitro*. First, using an immunocytochemical approach we examined whether expression of "growth-associated protein" GAP-43 (also known as neuromodulin, B-50, F1, pp46 and p57) might be regulated by these treatments. Although the physiological functions of GAP-43 are still unclear, expression of this protein has been associated with plastic changes elsewhere in the nervous system (Gordon-Weeks, 1989; Skene, 1990). Furthermore, GAP-43 mRNA has been localized to glomus cells in normal adult rat carotid body (Yamamoto and Kondo, 1990), and although expression of the protein was not studied, there is evidence for widespread GAP-43 immunoreactivity in the sensory and autonomic nervous system (Ramakers et al. 1992; Stewart et al., 1992). Secondly, we investigated whether glomus cell plasticity in response to environmental factors includes the potential for neuronal differentiation, as occurs when several of their crest-derived endocrine counterparts, e.g., adrenal medullary chromaffin cells (Doupe et al., 1985a;

Patterson, 1990; Herman et al., 1994), PC12 cells (Rydel and Green, 1988), small intensely fluorescent (SIF) cells (Doupe et al., 1985b), and thyroid parafollicular cells (Barasch et al., 1987; Anderson, 1993), are exposed to nerve growth factor (NGF) or cAMP analogs. In particular, we asked whether these or other agents, including hypoxia, dibutyryl cyclic guanosine monophosphate (dbcGMP) and retinoic acid (RA), can stimulate neurite outgrowth or neurofilament (NF) expression in glomus cells. These cells have been reported to be NGF-sensitive in fetal life (Aloe and Levi-Montalcini, 1980), and although they express *in situ* the neuronal/neuroendocrine markers, ubiquitin carboxyl-terminal hydroxylase (PGP 9.5, protein gene product 9.5) (Kent and Rowe, 1992) and neuron-specific enolase (Kondo et al., 1982; Nurse, 1990), they normally elaborate few or no processes and lack neurofilament (Kummer and Habeck, 1992). Some of these results were reported in a recent abstract (Jackson and Nurse, 1994).

MATERIALS AND METHODS

Cell Culture

The detailed procedures used for the isolation and culture of glomus cells by combined enzymatic and mechanical dissociation of 5- to 7-day-old rat (Wistar, Charles River, Quebec) carotid bodies were similar to those previously described (Fishman et al., 1985; Nurse, 1987). In brief, the carotid bodies were removed, cleaned of surrounding tissue, and incubated at 37°C for 45 to 50 min in an enzymatic solution containing 0.1% collagenase, 0.1% trypsin (Gibco, Grand Island, NY), and 0.01% deoxyribonuclease (Millipore Corp., Freehold, NJ). The enzyme was inactivated by a rinse in growth medium containing F12 nutrient medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 80 U/l insulin (Sigma Chemical Co.; St. Louis, Mo), 0.6% glucose, 2mM L-glutamine and 1% penicillin-streptomycin (Gibco). Individual carotid bodies were mechanically teased apart with forceps and triturated with a Pasteur pipette before plating the cell suspension on collagen-coated central wells of modified 35mm culture dishes. Normoxic cultures were grown at 37°C in a humidified atmosphere (20% oxygen/5% carbon dioxide; Forma Scientific automatic carbon dioxide incubator); hypoxic cultures were grown for the first 2 days in normoxia before transfer to a hypoxic environment (2% oxygen/5% carbon dioxide, 6% oxygen/5% carbon dioxide, or 10% oxygen/5% carbon dioxide; Forma Scientific O_2/CO_2 incubator) for up to 14 days.

The following agents were added to the growth medium as indicated: N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (Sigma) or dibutyryl cAMP (dbcAMP) at 1mM (10mM stock in F12 nutrient medium); N^2, 2'-O-dibutyrylguanosine 3':5'-cyclic monophosphate (Sigma) or dbcGMP at 1mM (10mM stock in F12 nutrient medium); all trans-RA (Sigma), dissolved in a minimal volume of ethanol (3mg/ml) and diluted to 0.1μM in growth medium; forskolin (ICN, Cleveland, OH) at 10μM added from a 10mM stock in ethanol; tetrodotoxin (TTX; Sigma) at 500nM added from a 1mM stock in citrate buffer; NGF (a gift from Dr. E. Cooper, McGill University) at 1μg/ml. The various stocks were diluted in growth medium and added to the cultures approximately 48 h after plating. Drug-treated or control growth medium was replaced every 3 to 5 days.

Immunofluorescence

For immunofluorescent detection of GAP-43, the cultures were first rinsed in phosphate-buffered saline (PBS; pH 7.2) and fixed at -20°C for 15 min in absolute methanol. The cultures were then rinsed in PBS and blocked with 0.5% bovine serum albumin (Boehringer Mannheim, Montreal) and 2% horse serum in PBS for 30 min at room temperature. The blocking solution was decanted and replaced with mouse anti-GAP-43 (9-1E12; a generous gift from Dr. D.J. Schreyer, Queen's University) and rabbit anti-tyrosine hydroxylase (TH) antibody (Chemicon, El Segundo, CA) diluted in blocking solution to final concentrations of 1:20000 and 1:900, respectively. Monospecificity of antibody 9-1E12 for GAP-43 has been previously characterized by Schreyer and Skene (Schreyer and Skene, 1991), using western blotting. After incubation

for 30 min at 37°C with primary antibodies the cultures were washed three times for 10 min each with PBS and 0.05% Tween 20 (Bio-Rad, Mississauga, Ont.). The cultures were then incubated for 30 min at room temperature with fluorescein-conjugated goat anti-rabbit IgG (Cappel, Malvern, PA) and Texas red-conjugated goat anti-mouse IgG (Jackson Immunoresearch Lab, Inc., Westgrove, PA) diluted in 1% normal goat serum in phosphate buffer to 1:50 and 1:400, respectively. Following three washes for 10 min each in PBS and 0.05% Tween 20, the cultures were covered in a solution containing a 1:1 mixture of 0.2 M sodium carbonate (pH 9.0): glycerol, to which 2 mg/10 ml p-phenylenediamine was added to reduce photobleaching.

For the detection of NF 68 kD and 160 kD by immunofluorescence, the cultures were first rinsed in PBS and fixed at -20°C for 1 h in 95% methanol/5% acetic acid. After removal of the fixative, the cultures were incubated in 0.1 M glycine in 0.1 M phosphate buffer (pH 7.2) for 5 min at room temperature. Following three washes for 5 min each in phosphate buffer, the cells were incubated in 10% fetal bovine serum (FBS) in phosphate buffer for 30 min at room temperature. The FBS solution was decanted and the cells were incubated in rabbit anti-TH antibody and mouse anti-NF 68 kD (Boehringer Mannheim) or 160 kD monoclonal antibody (clone NN18; Boehringer Mannheim) diluted in 1% normal goat serum in phosphate buffer. Anti-TH antibody, which served as a marker for glomus cells, was also used at a final concentration of 1:900. Antibodies to NF 68 kD and 160 kD were diluted to concentrations of 10 μg/ml and 5 μg/ml, respectively. After incubation at 37°C for 1 h in primary antibodies, the cultures were washed three times for 5 min each with phosphate buffer. The cells were then incubated for 30 min at room

temperature with the same battery of secondary antibodies as described above for GAP-43 and TH staining. Following three rinses for 5 min each in phosphate buffer, the cultures were covered with the anti-photobleaching reagent. In control experiments, cultures from each condition were processed as already described except that the primary antibody was omitted. In these cases no detectable staining of glomus cells above background was observed.

Visualization of the immunofluorescence and photography were done with a Zeiss (IM35) inverted microscope, equipped with epifluorescence and automatic exposure control.

RESULTS

Regulation of GAP-43 expression in glomus cells by chronic hypoxia

We used a specific monoclonal antibody (9-1E12; Schreyer and Skene, 1991) to investigate whether GAP-43 expression is regulated in cultured glomus cells by chronic hypoxia. As in previous studies (Nurse, 1990), glomus cells were positively identified by tyrosine hydroxylase (TH)-immunofluorescence and visualized with a fluorescein-conjugated secondary antibody. In control cultures, grown under normoxic (20% O_2) conditions, GAP-43 immunoreactivity was not detectable in glomus cells over a two-week period [Figs. 1(A,B) and 2]. Since the antibody was used at very dilute concentrations throughout (see Materials and Methods), we cannot exclude the presence of low levels of GAP-43 that were beyond the limits of detection by this assay. In contrast, exposure of carotid body cultures to chronic hypoxia, beginning after 2 days in the control (normoxic) environment, resulted in a significant fraction of TH-positive glomus cells expressing detectable GAP-43 immunoreactivity (visualized with a Texas red-labeled secondary antibody). For example, after 3 days exposure to 6% O_2, approximately 8% of glomus cells had acquired detectable GAP-43 immunoreactivity, and this value increased to a peak level of about 30% after 1 week in culture [see Figs. 1(C,D), 2]. In neuronal cells, GAP-43 is linked to the cytoplasmic face of the plasma membrane (Skene, 1990), and the staining pattern shown in Fig. 1D for glomus cells is

consistent with this localization.

The proportion of glomus cells that acquired detectable GAP-43 immunoreactivity was related to the degree of hypoxic exposure over a fixed time period. This dose-response is illustrated in Figure 3 for 6-day exposures to four different oxygen tensions: 2%, 6%, 10% and 20%. Maximum levels of GAP-43 expression occurred during exposure to a moderate-to-severe hypoxia of 6% oxygen, where about 30% of glomus cells showed positive GAP-43 immunoreactivity. At more severe (2% oxygen) and less severe (10% oxygen) levels of hypoxia, the percentage of GAP-43 positive glomus cells fell to about 6%, and GAP-43 was not at all detectable in cultures grown under normal oxygen tensions (20%).

Does neurite outgrowth accompany GAP-43 expression in glomus cells exposed to chronic hypoxia?

As illustrated in Figure 1(C,D), the expression of GAP-43 in glomus cells was not accompanied by any overt elaboration of neurites visualized by immunofluorescence or phase contrast microscopy (not shown); this was the case even in cultures exposed for up to 2 weeks of chronic hypoxia. Although we cannot exclude the possibility that very fine neurites might not be revealed under our staining conditions, our experience is that the GAP-43 antibody is very sensitive in revealing the presence of fine neuritic arborizations in these cultures, especially in conditions where the use of phase contrast microscopy and neurofilament antibodies proved negative (see later). Thus, the regulation of GAP-43 in glomus cells by hypoxia may be related to physiological processes, not associated with

neurite extension and growth cone functions, as appears to be the case for regenerating neurons (Gordon-Weeks, 1989). As will be discussed, this failure to display neuronal differentiation, complete with neuritic arborization, is not due to an irreversible loss of this function in postnatal glomus cells. However, it is noteworthy that this stimulus, i.e., chronic hypoxia, also fails to induce neurofilament expression (NF68 or 160 kD), a neuronal marker associated with process formation, even after 25 days exposure (not shown).

GAP-43 regulation in glomus cells by agents that elevate intracellular cAMP

Chemosensory stimulation of the carotid body *in vivo* by acute (Wang et al., 1991b) and chronic (Perez-Garcia et al., 1990) hypoxia is known to cause elevation of cAMP, particularly in glomus cells (Wang et al., 1991b). Since intracellular cAMP is a potential regulator of GAP-43 expression in chromaffin-derived cells (Costello et al., 1990), it is possible that the above effects of hypoxia on GAP-43 expression in glomus cells is mediated via the cAMP-signal transduction pathway. To test this, we treated normoxic cultures chronically with agents known to elevate intracellular cAMP. As illustrated in Figures 1(G,H) 2, chronic treatment of normoxic cultures with 1 mM dbcAMP was very effective in inducing GAP-43 expression in glomus cells. During the first week of treatment, there was a progressive, time-dependent increase in the percentage of GAP-43 positive glomus cells; after the first week, nearly 75% of glomus cells were GAP-43- positive, and almost the entire population was positive after 2 weeks of treatment (Fig. 2). Thus, it appears that almost all glomus cells have the capacity for

GAP-43 expression and conceivably the level of intracellular cAMP could be a controlling agent. This level could in turn be determined physiologically, for example, by the degree of the hypoxic challenge during chronic exposure (Perez-Garcia et al., 1990).

We also tested whether activation of adenylate cyclase, the membrane-associated enzyme that normally converts adenosine triphosphate to cAMP, could induce GAP-43 expression in glomus cells. As illustrated in Table 1, treatment with 10 μM forskolin (an activator of adenylate cyclase) for 10 days, caused more than 60% of glomus cells to acquire detectable GAP-43 immunoreactivity. Thus, these studies together with those demonstrating elevation of cAMP levels in the carotid body following hypoxic stimulation (Perez-Garcia et al., 1990; Wang et al., 1991b), point to intracellular cAMP as a strong candidate for the mediation of GAP-43 up-regulation during prolonged hypoxic stimulation of carotid body chemoreceptors.

Neuronal differentiation of glomus cells induced by dbcAMP in the presence or absence of retinoic acid

As already discussed, chronic hypoxia triggered a variety of plastic changes in glomus cells that partly resembled neuronal differentiation, but there was a paucity of neuritic processes and a virtual absence of neurofilament (NF) expression, similar to control cultures [Fig. 4(A,C)]. In contrast, treatment of control cultures with 1 mM dbcAMP resulted in detectable NF expression in a population of glomus cells, as well as significant neurite outgrowth [Fig. 4(D,I)]. As shown in Figure 5, the proportion of TH-positive glomus cells that were positive for NF 68 kD immunoreactivity increased

between day 5 and 14 of treatment from less than 2% to 20%; control untreated cultures were almost always NF-negative. Similarly, the higher molecular weight NF (160 kD) was also detectable in about 20% glomus cells after 10 days exposure to dbcAMP, but not in control cultures (Fig. 6). However, when dbcAMP-treated cultures were exposed to a prepulse of retinoic acid (0.1 μM) for 2 to 3 days, the proportion of NF 160 kD-positive glomus cells increased significantly to about 30% [Figs. 1(E,F) and 6), whereas treatment with RA alone was ineffective (Fig. 6; Table 1).

In general, conditions that enhanced NF expression in glomus cells were also effective in promoting neurite outgrowth. Although some control glomus cells extend short TH-positive neurites (30 to 40 μm; see Fig. 7), treatment with dbcAMP strikingly enhanced neuritic processes, which often appeared to interconnect neighboring and distant glomus cell clusters [see Fig. 1(G,H)]. Quantification of these processes revealed that their lengths in dbcAMP-treated cultures (with or without RA) often exceeded five times the values seen in control cultures or those treated with RA alone (Fig. 7). However, immunostained processes (whether stained for NF or GAP-43) were far more numerous in dbcAMP-treated cultures which were pre-pulsed with RA [see Fig. 1(E,F)], compared to those that were not prepulsed, resulting in larger sampling (see Fig. 7). In general, staining of dbcAMP-treated glomus cells for GAP-43 revealed a far more elaborate network of neurites compared to staining for NF (not shown), probably because NF staining was difficult to detect in small diameter neurites. These data suggest that elevated cAMP levels can induce NF expression and neurite outgrowth in glomus cells and that this property is enhanced by RA, a metabolite of vitamin A that is known to

induce neuronal differentiation in embryonic carcinoma (EC) cell lines (Kubo, 1989) and in mutant PC12 cell lines deficient in cAMP-dependent protein kinase (Scheibe et al., 1991).

The ability of dbcAMP to induce NF and GAP-43 in glomus cells (see before) was not due secondarily to its previously described role in increasing functional Na^+ channels (Stea et al., 1992), since tetrodotoxin (TTX, a blocker of voltage-gated Na^+ channels) failed to prevent these inductive effects of dbcAMP (Table 1).

Agents that failed to induce GAP-43 and NF 160 kD

Although NGF induces a neuronal phenotype in sympathoadrenal (SA)-derived adrenal medullary chromaffin cells and small intensely fluorescent (SIF) cells (Doupe et al., 1985a, b), it failed to induce detectable immunoreactivity for GAP-43 or NF 160 kD in SA-derived glomus cells (Table 1). In a previous study (Stea et al., 1992), we showed that NGF, unlike chronic hypoxia and dbcAMP, also failed to induce Na^+ channels and hypertrophy in glomus cells as it does during phenotypic conversion of other SA-derivatives (Kalman et al., 1990). Similarly, RA alone failed to induce either marker (see Table 1), although, as discussed, it appeared to enhance the frequency of process-bearing glomus cells when given as a prepulse prior to dbcAMP treatment. Finally, chronic treatment of the cultures with dibutyryl cGMP (dbcGMP; 1 to 2 mM) for up to 10 days failed to induce either NF 160 kD or GAP-43 in glomus cells (Table 1), indicating that the positive results obtained with dbcAMP were not a generalized response to elevated

cyclic nucleotides.

DISCUSSION

These studies demonstrate a novel plasticity in postnatal carotid body chemoreceptors in response to chronic stimulation *in vitro* via a physiological stimulus, hypoxia, or treatment with agents that elevate intracellular cAMP. A significant finding was that whereas hypoxia appeared to up-regulate GAP-43 expression in chemoreceptor glomus cells, treatment with agents that elevate cAMP had additional effects that resulted in a conversion of many of these cells from an endocrine to a neuronal phenotype. In particular, a substantial population elaborated neurites and expressed neurofilament immunoreactivity under the latter conditions. The apparent increase in GAP-43 protein expression *without* concomitant neurite outgrowth, which occurred with a chronic hypoxic stimulus, is of interest, since such examples illustrate that these two processes can be uncoupled, at least *in vitro*. In the nervous system, an increase in GAP-43 expression is frequently associated with neuronal plasticity involving axonal outgrowth and regeneration (Gordon-Weeks, 1989; Skene, 1990). Thus, the presence of GAP-43 mRNA in normal adult rat glomus cells (Yamamoto and Kondo, 1990), together with our immunocytochemical evidence for regulation of the protein by hypoxia, would support alternative functions for the protein, unrelated to neurite outgrowth. Conceivably, GAP-43 could play a role in the chemoreceptive properties of glomus cells by modulating calcium-dependent neurotransmitter release (see Dekker et al., 1989, 1990; Hens et al.,

1993; Ivins et al., 1993) or by enhancing the sensitivity of G protein-coupled receptor transduction (Strittmatter et al., 1993). For example, dopamine release is inhibited in a related endocrine cell type, PC12, transfected with a recombinant expression vector coding for antisense human GAP-43 cRNA (Ivins et al., 1993). These properties of GAP-43 could well contribute to the enhanced chemosensitivity of the carotid body that occurs during ventilatory acclimatization to chronic hypoxia (Barnard et al., 1987; Nielson et al., 1988; Vizek et al., 1987).

Is the effect of chronic hypoxia on GAP-43 expression in glomus cells mediated by intracellular cAMP?

Factors that control GAP-43 mRNA in related chromaffin-derived PC12 cells include NGF and dbcAMP (Costello et al., 1990). Although NGF failed to induce detectable GAP-43 immunoreactivity in glomus cells, several lines of evidence are consistent with the intracellular second messenger cAMP as a potential mediator of the hypoxia-stimulated GAP-43 expression in glomus cells. First, chronic treatment of carotid body cultures with agents that elevate intracellular cAMP, including a membrane-permeable analog (dbcAMP) or an activator of adenylate cyclase (forskolin), caused almost the entire glomus cell population to express detectable GAP-43 immunoreactivity after two weeks in culture. Second, hypoxia is known to cause a dose-dependent regulation of cAMP levels in the carotid body (Perez-Garcia et al., 1990), and the response appears to involve primarily the glomus cells (Wang et al., 1991b). Furthermore, the dose-response curve that describes cAMP levels in the whole carotid

body following acute exposure of the organ to oxygen tensions between 2 and 20% (see Fig. 2 of Perez-Garcia et al., 1990), roughly parallels the relation between GAP-43 expression and oxygen tension in chronically treated glomus cells in culture (Fig. 3). The fact that only 30% of the glomus cells expressed detectable GAP-43 after two weeks in moderate hypoxia (6% oxygen) compared to almost the entire population after treatment with cAMP analogs, may reflect: (i) a heterogenity in the sensing mechanisms among glomus cells, resulting in variable levels of intracellular cAMP reached for any given O_2 tension; (ii) the fact that hypoxia triggers other responses in glomus cells, e.g., elevation of intracellular calcium (Gonzalez et al., 1992), that may antagonize the positive effects of cAMP elevation on GAP-43 expression; (iii) the possibility that even an optimal hypoxic stimulus is incapable of producing levels of intracellular cAMP, as high as those reached with the pharmacological agents used; and (iv) our inability to detect low levels of GAP-43 at the very dilute concentration of antibody used in this study (see Materials and Methods).

Neuronal differentiation of glomus cells by cAMP analogs

In addition to enhancing GAP-43 expression, treatment with cAMP analogs revealed that at least a population of glomus cells has the potential for neuronal differentiation. In particular, some cells acquired the ability to express neurofilament proteins (NF 68 and NF 160 kD), which they do not normally express *in situ* (Kummer and Habeck, 1992; also our unpublished observations), or in dissociated cell culture (this study). The fact that only 20% of glomus cells, cultured from early postnatal pups,

acquired NF-immunoreactivity (compared with almost 100% for GAP-43 immunoreactivty) when treated for up to 2 weeks with dbcAMP, may indicate that: (i) not all cells are competent to respond, (ii) this property may be gradually lost during postnatal development, or (iii) the immunofluorescence assay was not sufficiently sensitive to detect low levels of NF expression. Furthermore, in response to dbcAMP treatment glomus cells extended neurites which reached uncharacteristic lengths of several hundred microns. This ability to extend neurites in the presence of dbcAMP appeared to be enhanced by a pretreatment with RA. These data suggest that chronic elevation of the intracellular second messenger cAMP stimulates expression of some neuronal traits in postnatal glomus cells *in vitro*. Similar effects occur among related neural crest derivatives, e.g., chromaffin, SIF, PC12, and thyroid parafollicular cells, in response to NGF or agents that elevate intracellular cAMP (Doupe et al., 1985a, b; Barasch et al., 1987; Rydel and Green, 1988; Patterson, 1990; Anderson, 1993; Herman et al., 1994). However, in the case of *postnatal* glomus cells NGF alone does not appear to be a mediator of neuronal differentiation, since it was incapable of inducing neurite outgrowth or NF expression (see also Stea et al., 1992). Since exposure of rat fetuses to anti-NGF has been reported to interfere with the normal development of glomus cells (Aloe and Levi-Montalcini, 1980), the role of NGF and other growth factors in the differentiation of *embryonic* glomus cells requires further investigation. Taken together, our data indicate that postnatal glomus cells are plastic and like their sympathoadrenal counterparts can be triggered to express neuronal traits *in vitro* in response to appropriate environmental signals.

Figure 1. Immunofluorescence labelling of cultured glomus cells under different environmental conditions. Micrographs show pairs of the same field immunostained for tyrosine hydroxylase, TH (green fluorescence) and either GAP-43 or NF (red fluorescence). (a, b) Control glomus cell cluster grown for 12 days in normoxia and stained for TH and GAP-43, respectively; (c, d) glomus cell cluster grown for 11 days in hypoxia (6% oxygen) and stained for TH and GAP-43, respectively; (e, f) glomus cell cluster, exposed to a 3-day prepulse of retinoic acid (RA; 0.1μM) followed by 7 days in dbcAMP (1mM), and stained for TH and NF 68 kD, respectively; (g, h) glomus cells, treated for 10 days with dbcAMP, and stained for TH and GAP-43, respectively. Note stained neuritic processes radiating from large and small glomus cell clusters in (f) and (h). Scale bar = 20 μm.

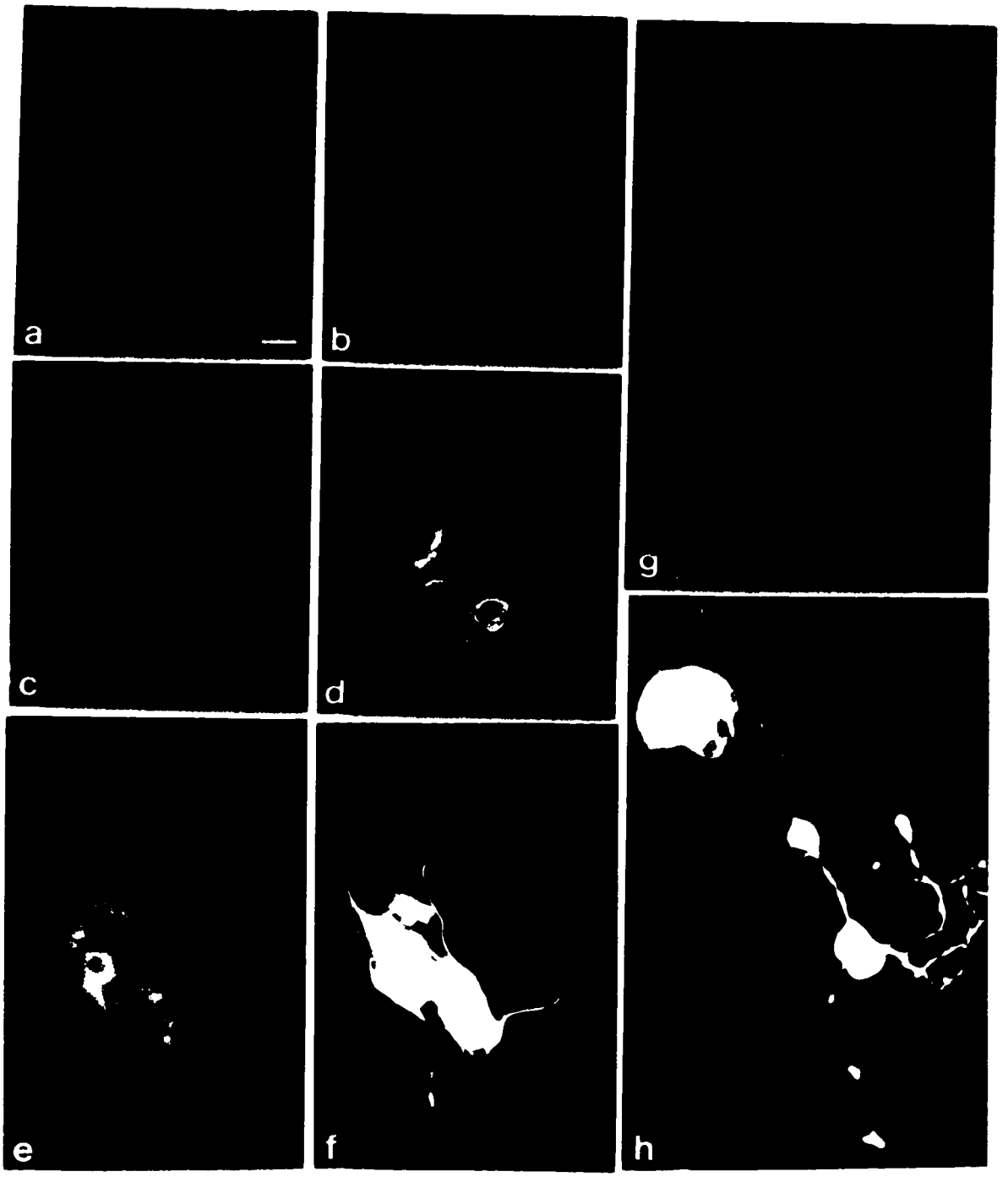

a
b
c
d
e
f
g
h

Figure 2. Time course of the effects of chronic dbcAMP and hypoxia on the percentage of glomus cells expressing GAP-43 immunoreactivity. From the second day *in vitro*, carotid body cultures were either treated with dbcAMP (1mM), exposed to hypoxia (6% oxygen) or maintained in control growth medium until fixation and staining for GAP-43 and TH immunoreactivity at the indicated time points. The vertical axis shows the percentage of TH-positive glomus cells (visualized with fluorescein-conjugated secondary antibody) that were also positive for GAP-43 (visualized with a Texas red-conjugated secondary antibody). At all time points, GAP-43 was not detectable above background levels in control glomus cells. Treatment with dbcAMP rapidly induced immunoreactivity for GAP-43 in 95% of glomus cells by 12 days in culture. Chronic exposure to hypoxia (6% oxygen) induced detectable GAP-43 expression in about 32% of the glomus cell population by 8 days in culture. "n" = the number of cultures scored.

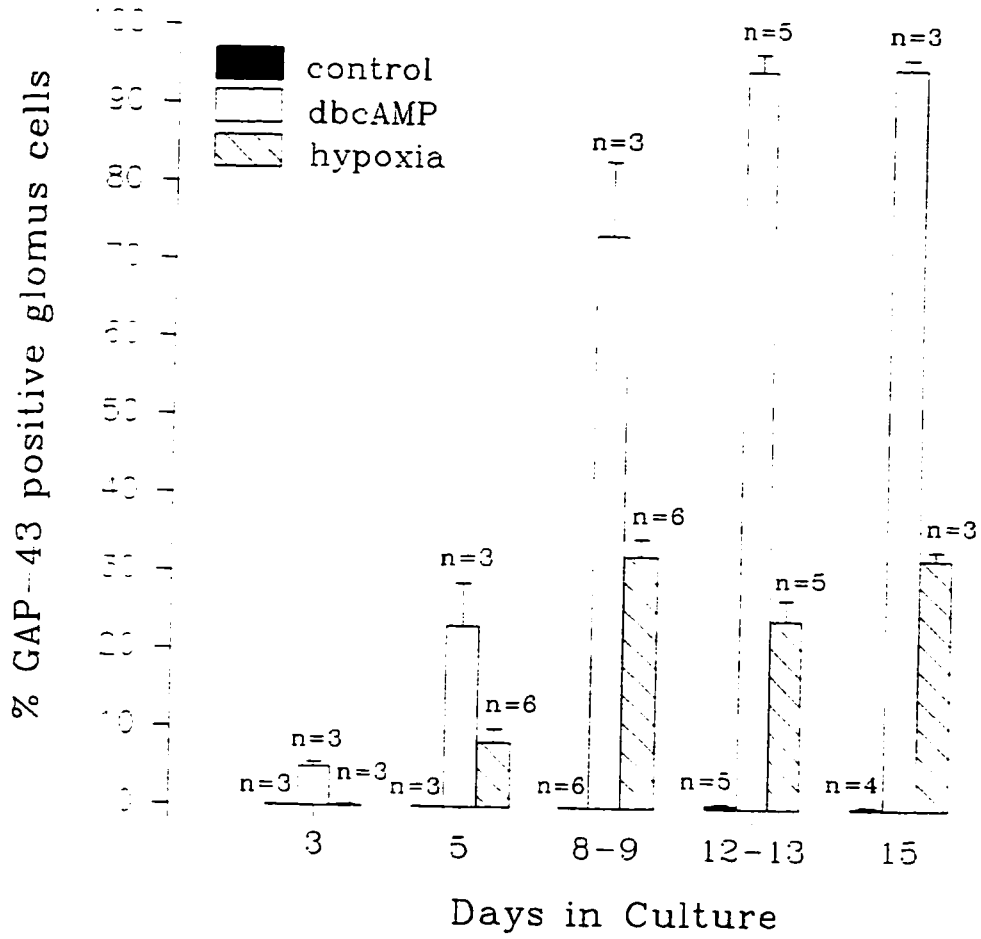

% GAP-43 positive glomus cells
control
dbcAMP
hypoxia
n=3
n=3
n=3
n=3
n=6
n=3
n=6
n=3
n=5
n=5
n=3
n=4
3
5
8-9
12-13
15
Days in Culture

Figure 3. Dependence of the frequency of GAP-43-positive glomus cells on the degree of chronic hypoxia maintained over a fixed time period. Dissociated cell cultures of carotid bodies from 5-day-old pups were exposed to 20% oxygen for the first 2 days and then transferred to either 2% O_2, 6% O_2, or 10% oxygen, or maintained in 20% oxygen for an additional 6 days. After a total of 8 days in culture, the cells were fixed and stained for GAP-43 and TH immunofluorescence. Vertical axis indicates percentage of TH-positive glomus cells that were GAP-43 positive. "n" = the number of cultures scored from at least two separate platings.

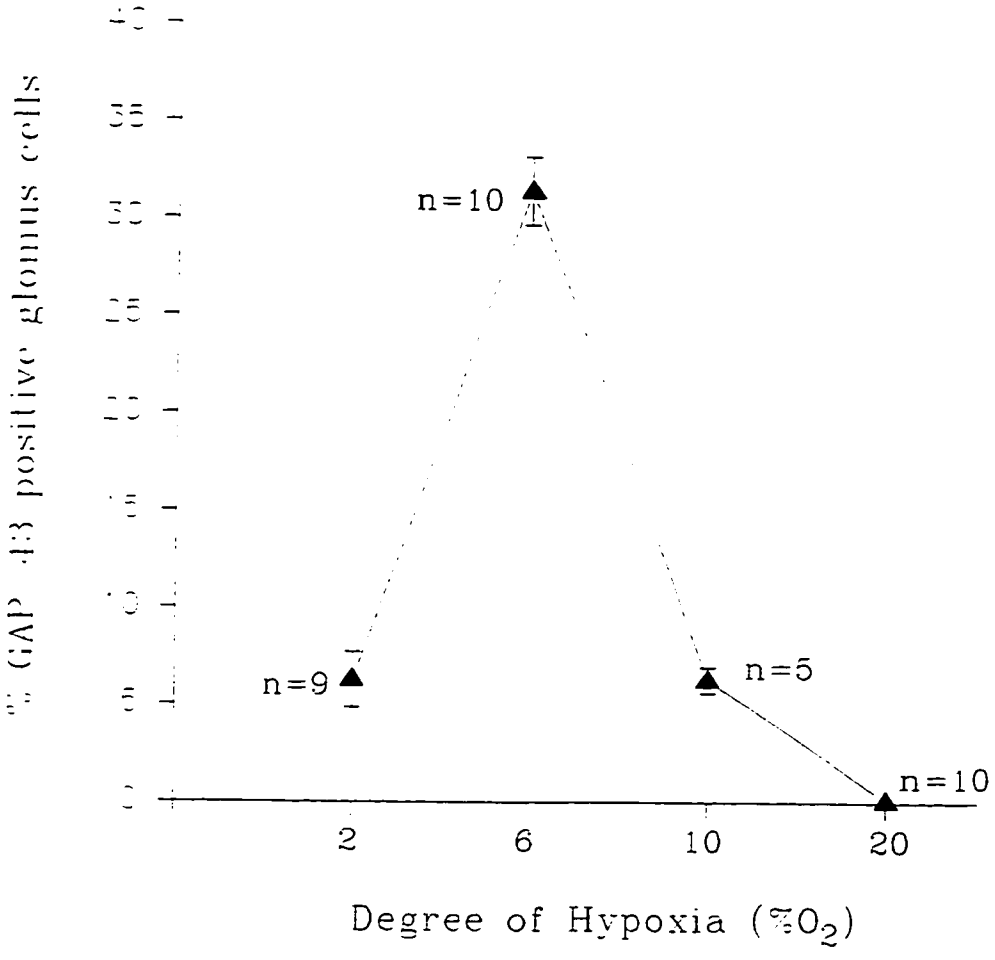

n=10
n=9
n=5
n=10
2
6
10
20
Degree of Hypoxia (%O_2)

Figure 4. Induction of NF immunoreactivity in carotid body cultures treated chronically with dbcAMP. Each row of photomicrographs represents the same microscopic field with the phase-contrast image on the left (a, d, g), the corresponding TH immunofluorescence (fluorescein-labeled) of glomus clusters in the middle (b, e, h), and corresponding NF immunofluorescence (Texas red labeled) on the right (c, f, i). Clusters were maintained for 14 days in either control (growth) medium (a, b, c) or 2 days in control medium plus 12 days in control medium supplemented with 1mM dbcAMP (d, e, f and g, h, i). Cultures were stained for NF 68 kD immunoreactivity (c, f) and for NF 160 kD immunoreactivity (i). Note control cultures are NF negative (c), in contrast to dbcAMP-treated cultures, which are NF positive (f, i). Scale bar = 10 μm.

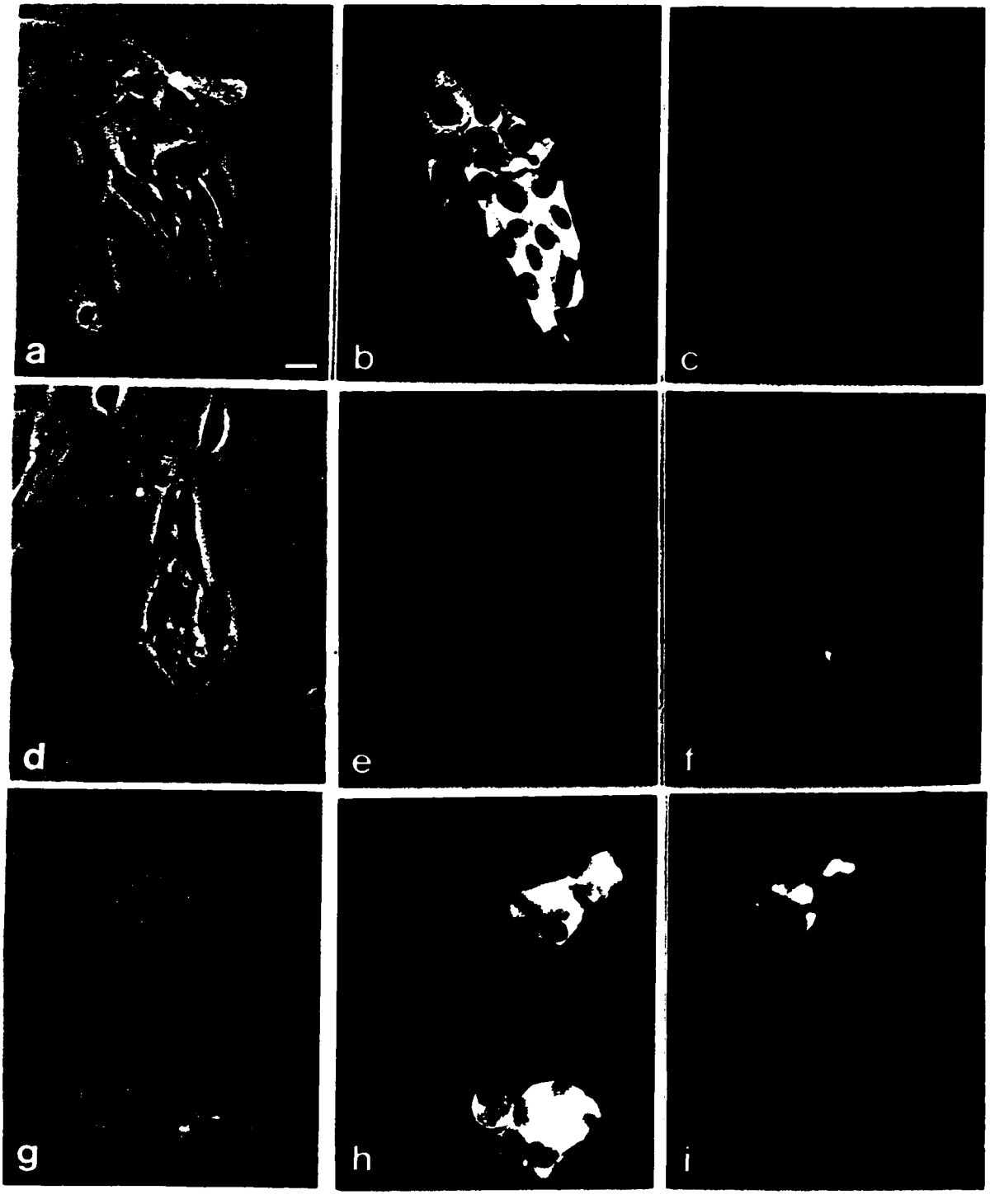

a
b
c
d
e
f
g
h
i

Figure 5. Time course of the induction of NF 68 kD immunoreactivity in glomus cells in carotid body cultures treated chronically with dbcAMP. At the indicated time points, control and dbcAMP-treated carotid body cultures were processed simultaneously for NF 68 kD and TH immunoreactivity, and visualized with Texas red- and fluorescein-labeled secondary antibodies, respectively. Treatment with dbcAMP (1mM) began after 2 days in control media. After a total of 5 to 7, 8 to 11, 12 to 13, and 14 to 16 days in culture, an average of 0.4%, 14.1%, 15.7%, and 19.4% TH-positive glomus cells chronically exposed to dbcAMP stained positively for NF 68 kD, respectively. The gradual increase in NF 68 kD immunoreactivity following dbcAMP treatment was significant (*$p<0.01$) when compared to controls by 8 to 11 days culture (Student's *t* test). At each time point, glomus cells in control cultures were virtually devoid of NF 68 kD immunoreactivity. Each bin represents mean (±S.E.); "n" = the number of cultures sampled from at least 2 separate platings.

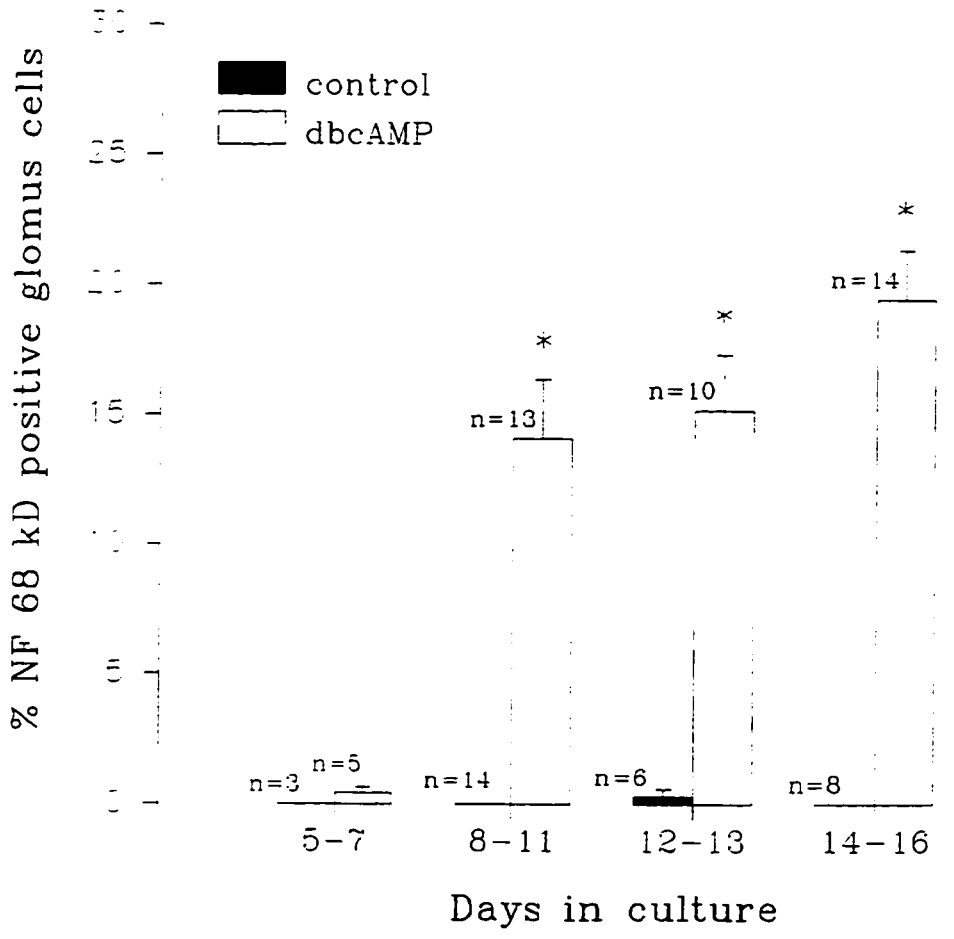

% NF 68 kD positive glomus cells
control
dbcAMP
n=3
n=5
n=14
n=13
n=6
n=10
n=8
n=14
*
*
*
5-7
8-11
12-13
14-16
Days in culture

Figure 6. Induction of NF 160 kD immunoreactivity in dbcAMP-treated glomus cells with or without pretreatment with RA. Treatment of carotid body cultures with growth medium alone or with medium containing 0.1 μM RA failed to induce detectable NF 160 kD immunoreactivity after 12 days in culture. However, chronic exposure to dbcAMP for 10 days significantly (*$p<0.01$) induced NF 160 kD immunoreactivity in approximately 19% of TH-positive glomus cell population. Furthermore, a 2- or 3-day prepulse of RA followed by an additional 7 to 8 days of treatment with dbcAMP significantly (**$p<0.01$ when compared with dbcAMP alone) increased the percentage of NF 160 kD positive glomus cells to 32.5%. Bins represent mean (±S.E.) fraction of double-labeled (TH/NF 160 kD) cells expressed as a percentage of total TH-positive population; "n" = number of cultures scored from two to six separate platings.

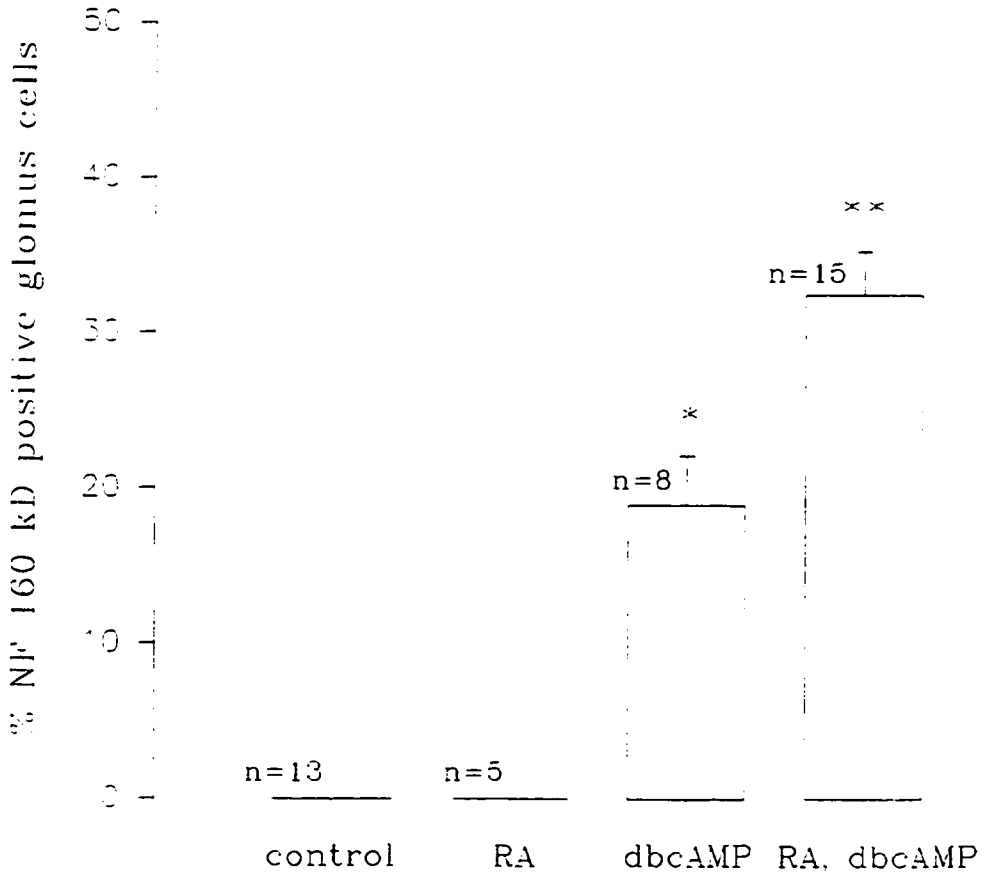

% NF 160 kD positive glomus cells
n=13
n=5
n=8
n=15
*
**
control
RA
dbcAMP
RA, dbcAMP

Figure 7. Lengths of neuritic processes elaborated by glomus cells under various culture conditions. Average process lengths (mean±S.E.) were measured in 12-day-old cultures grown in control medium, control medium plus RA, control medium plus dbcAMP, or control medium plus a 2- or 3-day prepulse of RA followed by 7 or 8 days in dbcAMP. Processes radiating from glomus cells were visualized and measured in cultures stained for TH and NF 68 kD or NF 160 kD immunoreactivity. Infrequently, glomus cells from control and RA-treated cultures extended short TH-positive (NF-negative) processes up to 4 cell diameters (average glomus cell diameter is 10 μm). Treatment with dbcAMP alone or RA followed by dbcAMP significantly (*$p<0.01$) induced the elaboration of processes that were on average 140 μm long and frequently stained for NF. The frequency of glomus cells bearing processes was considerably higher in cultures exposed to a 2- or 3-day prepulse of RA followed by dbcAMP than in any other condition. This accounts for the larger sample size ("n") in cultures with RA prepulse followed by dbcAMP.

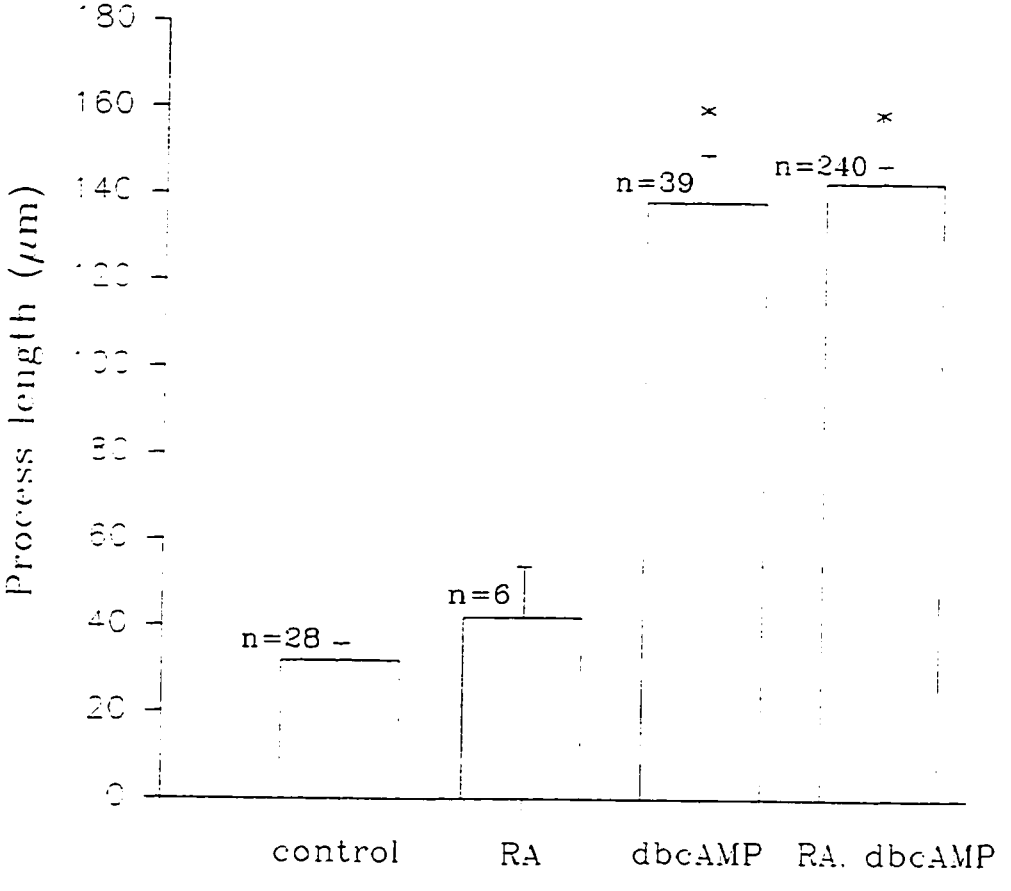

Process length (μm)
180
160
140
120
100
80
60
40
20
0
n=28
n=6
n=39
n=240
*
*
control
RA
dbcAMP
RA. dbcAMP

Table 1. Comparison of various treatments with respect to their ability to induce immunoreactivity for GAP-43 and neurofilament 160 kD in glomus cells.

Carotid body cultures were grown in control growth medium or medium treated with nerve growth factor (NGF), forskolin, dibutyryl cGMP (dbcGMP), dbcAMP + TTX (500 nM), or retinoic acid. After 12 days in culture the cells were fixed and stained for TH and either GAP-43 or NF 160 kD. The fraction of GAP-43 or NF 160 kD immunoreactive glomus cells is expressed as a percentage of the total TH-positive population; "n" = the number of culture dishes sampled for each condition.

Treatment	GAP-43/ Total TH	GAP-43 (%)	NF160/ Total TH	NF160 (%)
Control	28/5801 n=5	0.5	1/12363 n=16	0.0
NGF	0/3842 n=3	0.0	0/4043 n=3	0.0
Forskolin	1522/2479 n=4	61.4	364/3331 n=6	10.9
dbcGMP	0/4734 n=3	0.0	1/6421 n=3	<0.1
dbcAMP + TTX	1097/1256 n=3	87.3	154/778 n=3	19.8
Retinoic acid	2/2988 n=3	<0.1	0/1630 n=4	0.0

CHAPTER 2

Dopaminergic Properties of Cultured Rat Carotid Body Chemoreceptors Grown in Normoxic and Hypoxic Environments

All data presented in this chapter represents work done entirely by me and has been published in *Journal of Neurochemistry*, Vol. 69, pp. 645-654 (1997).

SUMMARY

Using dissociated carotid body (CB) cultures prepared from neonatal (postnatal days 5-7; P7) or juvenile (postnatal days 19-20; P20) rats, we compared catecholaminergic properties and mechanisms of O_2 sensing in glomus cells grown in normoxic (Nox; 20% O_2) and chronically hypoxic (CHox; 6% O_2) environments for up to 2 weeks. In Nox cultures, basal dopamine (DA) release, determined by HPLC and normalized to the number of tyrosine hydroxylase-positive glomus cells present, was similar for P7 and P20 cultures (~0.3 pmol/1,000 cells/15min) and was unaffected by culture duration (2 vs 12 days). Acute hypoxia (5 and 10% O_2) caused a dose-dependent stimulation (6x and 3x basal, respectively) in DA release, that was inhibited by nifedipine (10 μM). DA release was also stimulated by high extracellular K^+ (30 mM) and iberiotoxin (200 nM), a selective blocker of Po_2-regulated, Ca-dependent K^+ channel

in glomus cells. The stimulatory effect of iberiotoxin was similar to 5% O_2 in P20 cultures, but substantially less (about one-half) in P7 cultures. In contrast, in CHox cultures, basal DA release was substantially elevated, ~8x Nox levels, although this did not correlate with significant differences in stores. Further, whereas acute hypoxia (5%O_2) and high K^+ also stimulated DA release in CHox cultures(~2x and ~3x basal), iberiotoxin (200 nM) did not. Thus after chronic hypoxia *in vitro*, there is an enhanced basal catecholamine release and an apparent down-regulation of functional Ca-dependent K^+-channels in CB chemoreceptors. These cellular adaptations may relate to changes in CB chemosensitivity during chronic hypoxemia.

INTRODUCTION

In mammals, the increase in ventilation after acute hypoxia is mediated by the carotid body (CB), whose sensory afferents project to the nucleus tractus solitarius in the brainstem via the carotid sinus nerve or CSN (Eyzaguirre and Zapata, 1984; Weil, 1986; Gonzalez et al., 1994). Although an increase in CSN discharge during hypoxia is correlated with an increase in catecholamine (CA) secretion from CB chemoreceptors, i.e., glomus or type 1 cells (Fidone et al., 1982; Gonzalez et al., 1994; Donnelly and Doyle, 1994a), the role of CA in oxygen chemoreception remains controversial (Gonzalez et al., 1994). Nevertheless, catecholamines have been implicated in several aspects of carotid body function, including resetting of chemoreceptor sensitivity after birth (Hertzberg et al., 1990), ventilatory acclimatization to chronic hypoxia (Bisgard et al., 1987), and "blunting" of the ventilatory response in high-altitude dwellers or neonates born and reared in hypoxia (Dempsey and Forster, 1982; Eden and Hansen, 1987b; Hertzberg et al., 1990; see also Wach et al., 1989). In several biochemical studies, exposure of animals to chronic hypoxia *in vivo* was found to increase carotid body CA content and turnover (Hanbauer et al., 1981; Pequignot et al., 1987; Gonzalez-Guerrero et al., 1993), and up-regulation of tyrosine hydroxylase (TH), the rate-limiting enzyme in CA biosynthesis (Hanbauer et al., 1977; Czyzyk-Krzeska et al., 1992).

Despite these findings, however, the mechanisms underlying the adaptive changes

in CB catecholamines during chronic hypoxia *in vivo* are poorly understood. For example, these changes may result directly from chronic stimulation of CB chemoreceptor cells by low oxygen and/or indirectly, from secondary cardiovascular adjustments. Moreover, separation of these effects *in vivo* are difficult for the following reasons. First, whole-animal exposure to chronic hypoxia also results in secondary changes in Pco_2 and pH (Eden and Hansen, 1987b; Aaron and Powell, 1993), thus making it difficult to isolate changes due to Po_2 alone. Second, effects on CB catecholamines may be due to release into the circulation of stress-related factors, e.g., glucocorticoid, a known regulator of TH. Third, measurements of CA stores and release are often normalized to whole CB or milligrams of protein (Hanbauer et al., 1981; Pequignot et al., 1987; Gonzalez-Guerrero et al., 1993), an approach that does not consider any accompanying glomus cell hyperplasia, nor the relative contribution of other tissues to CB hypertrophy (see Dhillon et al., 1984). Fourth, although sympathetic nerve endings are a recognized source of total CB catecholamines (see, e.g., Hanbauer and Hellstrom, 1978), contributions from afferent sensory nerve endings are usually ignored. However, the latter are now known to be a potential source of dopamine (DA) in the rat CB, and importantly, the level of this CA pool may increase after chronic hypoxia, as a result of activity-dependent mechanisms (Finley et al., 1992; Hertzberg et al., 1995).

A major goal of the present study was to use established cell cultures of dissociated rat CB to investigate adaptive changes in catecholamine properties of glomus cells, grown in either normoxic (Nox; 20% O_2) or chronically-hypoxic (CHox; 6% O_2) environments, using HPLC. With this approach, the chronic effect of O_2 tension alone, on

glomus cell function, could be assessed in the absence of several of the confounding variables present *in vivo*. Moreover, our ability to obtain absolute counts of glomus cells using TH immunofluorescence (Nurse 1990; Jackson and Nurse, 1995a) allowed, for the first time, normalization of CA release to the number of amine-producing cells, thereby avoiding the potential complication of glomus cell hyperplasia, reported *in vivo* (Dhillon et al., 1984).

A second goal was to test whether maturation of catecholaminergic properties in isolated glomus cells is influenced by O_2 tension. To this end, we compared the effects of chronic hypoxia *in vitro* on DA secretion in cultures derived from rats of two age groups; i.e., neonatal (postnatal ages 5-7 days; P7), where CB catecholaminergic functions are developing *in vivo*, and juvenile (ages 19-20 days; P20), where these functions are thought to be fully mature (Hertzberg et al., 1990: Donnelly and Doyle, 1994a). Finally, because chronic hypoxia *in vivo* has recently been reported to alter expression of O_2-sensitive, Ca-dependent K^+ channels in rat glomus cells (Wyatt et al., 1995), we tested, with aid of the specific blocker iberiotoxin (IbTx; Galvez et al., 1990), whether inhibition of these channels differentially affect DA release in cultures reared in Nox and CHox environments. Some of the present findings have been reported in a recent abstract (Jackson and Nurse, 1995b).

MATERIALS AND METHODS

Cell Culture

Primary cultures of dissociated rat CBs were prepared as described elsewhere (Fishman et al., 1985; Nurse, 1990). In brief, CBs were dissected from 5-7- and 19-20-day-old rats (Wistar, Charles River, Quebec, Canada), cleaned of surrounding tissue, and incubated at 37°C for 45 to 50 min in an enzymatic solution containing 0.1% collagenase, 0.1% trypsin (Gibco, Grand Island, NY, USA), and 0.01% deoxyribonuclease (Millipore, Freehold, NJ, USA). The enzyme was inactivated by a rinse in growth medium containing F12 nutrient medium (Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco), 80 U/L insulin (Sigma Chemical Co., St. Louis, MO, USA), 0.6% glucose, 2mM L-glutamine, and 1% penicillin-streptomycin (Gibco). Individual CBs were mechanically dissociated and the resulting cell suspension was plated on collagen-coated central wells of modified 35 mm culture dishes. For the first 2 days, all cultures were grown in a control Nox environment (95% air/5% CO_2; Forma Scientific automatic CO_2 incubator) at 37°C in a humidified atmosphere, before separation into two groups. One group remained in this environment, whereas the other was transferred to a hypoxic environment (6% O_2/89% N_2/5% CO_2; Forma Scientific O_2/CO_2 incubator) for up to 13 days. Routinely, the medium was replaced on day 2, 7, and 12 *in vitro* with fresh growth medium.

Catecholamine analysis using HPLC

CA released from live cultures or extracted from cellular stores were separated by HPLC (Waters, model 510) with a Spherisorb-ODS2 column (10 x 0.46 cm, 3 μm particle size; Chromatography Sciences Company, Montreal, Quebec, Canada), coupled with an electrochemical detector (Coulochem II detector, model 5200; ESA, Inc., Bedford, MA, USA). The detection system consisted of a conditioning cell (model 5021) and an analytical cell (model 5011) containing dual coulometric working electrodes made from porous graphite. The first detector in the analytical cell was set at 0.05 V to reduce interference by contaminating electroactive compounds at the second detector which was set at -0.3 V, the potential required for electro-reduction of (-)-arterenol (noradrenaline, NA), DA and the internal standard, di-3,4-hydroxybenzylamine hydrobromide (DHBA). Stock solutions of DA, NA, and DHBA (Sigma) were prepared in 0.1 M $HClO_4$ at a concentration of 1.0 mM and were stored at 4°C. On the day of the assay, stock solutions were diluted in water to produce 25 nM CA standards. Examples of HPLC peaks for these standards are shown in Fig. 1A. The mobile phase consisted of NaH_2PO_4 (6.9 g/L; Sigma), Na_2EDTA (80 mg/L; BDH Chemicals, Toronto, Ontario, Canada), and heptanesulfonic acid (250 mg/L; Sigma) in water and 5% methanol; pH was adjusted to 3.5 with concentrated H_3PO_4. Water used in mobile phase and CA standards was distilled and deionized (NANOpure 11, D3700 series, cartridge deionization system, Barnstead, Newton, MA, USA); mobile phase was filtered (20 μm, 47 mm Nylon-66 disc filters; S.P.E., Limited, Concord, Ontario, Canada) and degassed before each assay. The flow rate was 1 ml/min at approximately 2,000 psi. Chromatograms were analyzed with

the aid of a Waters 740 Data Module (Millipore, Milford, MA, USA) and quantified using the peak area ratio method. The peak area of a known amount of external CA standard (see Fig. 1A) was used to calculate the amount of CA in a sample from its integrated peak area. A known amount of internal standard, DHBA, was added to each sample to correct for variations in injection volume.

For measurements of CA stores, the cultures were rinsed briefly three times with Hanks Balanced Salt Solution (HBSS; Gibco) before the cells were extracted with 150 μl of 0.1 M $HClO_4$ containing 2.7 mM Na_2EDTA. The cell extracts were immediately stored at -80°C until analysis by HPLC.

In studies of CA release, CB cultures were first rinsed and incubated for 10 min in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (50:50) or DMEM/F-12 supplemented with 1.2 % glucose. Then, a basal release sample was collected after a 15 min incubation of the culture at 37°C in 55 μl bicarbonate-buffered salt solution (BBSS), under an atmosphere of 5% CO_2/20% O_2 (normoxia), using a Forma Scientific O_2/CO_2 incubator. The BBSS was added to the central (~0.8-1 cm diameter) circular well, where the cells were confined in the culture dish. The BBSS contained (mM): NaCl, 116; KCl, 5; $NaHCO_3$, 24; $CaCl_2$, 2; $MgCl_2$, 1.1; Hepes, 10; glucose, 5.5; at pH 7.4. Basal release samples were collected (in Nox) by withdrawing 50 μl of BBSS from the well, and this measurement provided the baseline for comparing stimulus-evoked release. The stimulus was applied following a "recovery" period of 10 min, during which the culture were re-incubated in DMEM/F-12 medium. Except for the nature of the stimulus, evoked release was performed for the same duration and under similar conditions as basal release.

Samples of evoked release were collected after exposure of the cultures for 15 min to (1) hypoxia, 5 or 10 % O_2 ; (2) high extracellular K^+ (30 mM K^+; 25mM NaCl was replaced by equimolar KCl); or (3) iberiotoxin (IbTx, 200 nM; Alomone Labs, Jerusalem, Israel) in BBSS; in a few experiments the L-type calcium channel blocker nifedipine (10 μM; Sigma), was included in the release medium. Repetition of the basal release measurement meant that each "evoked" release sample was flanked by a basal release sample collected under normoxia before and after the stimulus. Release samples were mixed 1:1 with 0.1 M $HClO_4$ containing 2.7 mM Na_2EDTA and stored at -80°C until HPLC analysis.

Immunofluorescence

Following CA release experiments, glomus cells were positively identified by tyrosine hydroxylase (TH) immunoreactivity and visualized with a fluorescein-conjugated secondary antibody (Fig. 1B) as previously described (Nurse, 1990; Jackson and Nurse, 1995a). The cultures were first rinsed in phosphate-buffered saline (PBS; pH 7.2) and fixed at -20°C for 20 min in 95% methanol/5% acetic acid. After removal of the fixative, the cultures were incubated in 0.1 M glycine in 0.1 M phosphate buffer (pH 7.2) for 5 min at room temperature. Following three washes for 5 min each in phosphate buffer, the cells were incubated in 10% FBS in phosphate buffer for 30 min at room temperature. The FBS solution was decanted and the cells were incubated in rabbit anti-TH antibody (Chemicon, El Segundo, CA, USA) diluted to 1:900 in 1% bovine serum albumin (BSA; Sigma) in PBS. After incubation for 45 min at 37°C with primary antibody, the cultures were washed three times for 5 min each with phosphate buffer. The

cells were then incubated for 30 min at room temperature with fluorescein-conjugated goat anti-rabbit IgG (Cappel, Malvern, PA, USA) diluted in 1% BSA in PBS to 1:50. Following three rinses for 5 min each in phosphate buffer, the cultures were covered with an antiphotobleaching reagent (1,4-diazabicyclo[2.2.2]octane; Lancaster Synthesis Inc., Windham, NH, USA).

Visualization and quantification of immunofluorescent TH-positive glomus cells was done with a Zeiss (IM35) inverted microscope, equipped with epifluorescence.

RESULTS

Given the relatively low yield of glomus cells obtained from dissociated rat carotid bodies (Fishman et al., 1985; Nurse, 1990), and the inherent variability in cell number among different platings, we developed a procedure that permitted multiple CA release samples to be obtained from the same culture, interspersed by 10 min recovery periods, over a duration of 75 min (see Materials and Methods). It was found that 15 min samples of both basal and evoked release contained sufficient dopamine (DA) to allow ready detection and quantification by HPLC. In some experiments, data are expressed as ratios of stimulus-evoked/basal DA release, obtained from successive 15 min samples. Further, in order to "normalize" release measurements, in many experiments an estimate of glomus cell number was obtained from the *same* culture used to monitor release, by immunostaining for TH after sampling was complete (Fig. 1B; see also Nurse, 1990; Jackson and Nurse, 1995a). In all experiments, basal release measurements were obtained under Nox conditions, i.e., 20% O_2.

Normoxic Cultures

Stability of basal dopamine release in cultured CB cells.

Basal or spontaneous DA release was detected in all samples from rat CB cultures, regardless whether they originated from neonatal (P7) or juvenile (P20) rats. As

illustrated in Fig. 2A, basal DA release/15 min, sampled at three intervals from a single culture, remained relatively constant over a period of 75 min in control Nox (20% O_2) conditions. The normalized basal DA release (~0.3 pmol/ 1,000 TH^- cells/15 min) was similar for P7 and P20 cultures when tested soon after isolation, i.e., 2 days *in vitro* (Fig. 2B). These data suggest that basal DA release rate by glomus cells is mature by the end of the first postnatal week. In addition, basal DA release after only 2 days *in vitro* was similar to that after 12-14 days for both P7 and P20 cultures (Fig. 2B), suggesting that culture duration had no effect on at least this aspect of dopaminergic function.

Regulation of DA release in P7 cultures by O_2 tension.

To investigate whether glomus cells retain their ability to sense oxygen after long-term isolation *in vitro*, DA release was monitored in ~12 day-old Nox cultures exposed for 15 min intervals to different O_2 tensions (Fig. 3A). As shown in Fig. 3B, DA release rate/1,000 TH^- cells showed a steep dose-dependence on O_2 tension; this rate in 5% and 10% O_2 was ~6x and ~3x higher, respectively, than that in 20% O_2. Further, whereas DA release in 5% O_2 was substantially inhibited (~60 %) by the L-type calcium channel blocker nifedipine, release in 20% O_2 was unaffected (Fig. 3B; ▽). These results suggest that in moderate-to-severe hypoxia (i.e. 5% O_2), cultured glomus cells are sufficiently depolarized to open voltage gated, L-type calcium channels that facilitate calcium entry and neurotransmitter release. Presumably, DA release in 20% O_2 (i.e., basal) is controlled mainly by the resting level of intracellular calcium.

Blockade of O_2-sensitive, Ca-dependent K^+ channels by IbTx stimulates DA release in normoxic cultures.

In the rat carotid body, closure of large conductance, Ca-dependent K^+ channels by hypoxia is thought to be an important step in O_2 chemotransduction (Wyatt and Peers, 1995; Wyatt et al. 1995; see however, Cheng and Donnelly, 1995; Buckler, 1997). To test whether a sufficient number of these channels are open under Nox conditions, we investigated whether selective blockade with the scorpion venom IbTx (Galvez et al., 1990) could evoke DA release. This was the case as shown in Fig. 4A. A comparison of the effect of IbTx (200 nM) on DA release is shown in Fig. 4B for neonatal (P7) and juvenile (P20) cultures; in each case, stimulus-evoked release (per 15 min interval) is expressed as a ratio, relative to basal release collected during the first 15 min sample (see Materials and methods). Although IbTx stimulated DA release significantly above basal in both P7 and P20 cultures, the effect was much more pronounced in P20 cultures. This point is considered further below.

Is hypoxia-evoked DA release tightly coupled to the activity of Ca-dependent K^+ channels at all developmental ages?

Whole-cell and single channel recordings from rat glomus cells indicate that more than one K^+ channel subtype can be regulated by Po_2 (Wyatt and Peers, 1995; Wyatt et al., 1995; Buckler, 1997). Although there is recent evidence that one of these subtypes, i.e., the Ca-dependent K^+ channel, undergoes postnatal maturation (Peers, 1996), little is known about the developmental regulation of the other subtypes. This raises the question

of whether the hypoxia-evoked DA release from glomus cells is tightly coupled to the activity of Ca-dependent K^+ channels at all developmental stages. Interestingly, we found that the ratio of hypoxia-evoked DA release relative to basal was similar for P7 and P20 cultures (Fig. 4B). As the normalized basal release was not significantly different between the two age groups (Fig. 2B), it follows that hypoxia (5% O_2) had quantitatively similar effects on DA secretion in P7 and P20 cultures. In marked contrast however, blockade of Ca-dependent K^+ channels with IbTx (200 nM) stimulated DA release to different extents at the two ages, i.e., >6x in P20 compared to 2-3x in P7 cultures. A comparison with the hypoxia-evoked release reveals that whereas IbTx stimulated DA release to a similar extent as 5% O_2 in P20 cultures (Fig. 4B; open bars), it was significantly less effective in P7 cultures (Fig. 4B; closed bars).

Chronically-hypoxic Cultures

To test the long-term effects of chronic hypoxia on the secretory functions of glomus cells, DA release was investigated in cultures grown in 6% O_2 for 9-11 days. Before monitoring release, cultures were briefly returned to normoxia (20% O_2) for ~12-16 h at the termination of the hypoxic period.

Exaggerated basal DA release after chronic hypoxia in vitro.

The normalized basal or spontaneous DA release was measured in CB cultures, derived from P7 and P20 rats, and grown for 9-11 days in low oxygen (6% O_2). The rate of DA release/ 1,000 TH^+ cells was elevated significantly in CHox cultures derived from

the two age groups. Comparison of the data in Figs. 5A and 2B reveals that basal DA release in CHox cultures is higher than that in age-matched Nox ones by ~8x for P7, and ~5x for P20 cultures. In contrast to Nox cultures (Fig. 2B), where the normalized basal release was similar for the 2 age groups, after chronic hypoxia basal DA release was significantly higher (~1.6x) in neonatal compared with juvenile cultures (Fig. 5A).

As we previously reported, electrical excitability of glomus cells is increased after chronic hypoxia *in vitro*, due to increased Na^- and Ca^{2-} currents (Stea et al., 1995; Nurse, 1995); thus it is possibile that the exaggerated basal DA release in CHox cultures is due to increased spontaneous electrical activity. However, such an explanation appears unlikely since basal DA release in neonatal CHox cultures was unaffected by 10 μM nifedipine (compare Fig. 5A and B; 20% O_2), which blocks >70% of the calcium current in CHox glomus cells (Stea et al., 1995).

Differential effects of acute hypoxia and IbTX on DA release in CHox cultures.

Given the enhanced basal DA secretion after chronic hypoxia (see above), we wondered whether CHox glomus cells could still sense an acute hypoxic stimulus by augmenting DA release above basal. As shown in Fig. 6, this was the case for both neonatal (P7) and juvenile (P20) CHox cultures, where a 15 min exposure to hypoxia (5% O_2) resulted in a doubling of DA release over basal (20% O_2). As for Nox cultures, the hypoxia (5% O_2)-evoked DA release in CHox cultures was dependent on entry of extracellular calcium via voltage-gated, L-type calcium channels, since it was almost completely abolished by 10 μM nifedipine (Fig. 5B; 5% O_2).

In contrast to results on Nox cultures (see Fig.4), blockade of Ca-dependent K^+ channels by 200 nM IbTx had no effect on DA release in CHox cultures. As shown in Fig. 6, for both P7 and P20 CHox cultures, the ratio of stimulus-evoked/basal DA release was ~1 with IbTx as the stimulus, implying the latter did not stimulate DA release significantly. Thus, it appears that the large conductance Ca-dependent K^+channels become inactive or are down-regulated following direct exposure of glomus cells to chronic hypoxia *in vitro*.

High K^+-evoked DA release in Nox vs CHox cultures.

Since chronic hypoxia *in vitro* appeared to modify the pathway coupling O_2 sensing to particular K^+ channel subtypes in glomus cells, we compared DA secretion evoked by a different depolarizing stimulus, i.e., high extracellular K^+. As shown in Fig. 7, the normalized DA secretion rate after exposure to 30 mM K^+ was about three fold higher for CHox (P7) cultures compared with Nox. When compared with basal release (see Fig. 2B), high K^+ had a pronounced stimulatory effect on secretion in each case; DA release was stimulated ~10x basal in Nox cultures and ~4x basal in CHox cultures.

Dopamine stores in Nox and CHox cultures.

An increase in CB catecholamine content occurs in response to long-term hypoxia *in vivo* and may involve both the induction of TH as well as hyperplasia in glomus cells (Hanbauer et al., 1981; Czyzyk-Krzeska et al., 1992). Therefore, it was of interest to investigate whether the increased basal DA release in CHox cultures occurred in parallel

with increased DA stores. To examine DA stores, neonatal (P7) CB cultures were exposed to normoxia or chronic hypoxia (6%O_2) for up to 13 days (15 days *in vitro*), rinsed to remove any extracellular CA, and then lysed before the cell extract was collected for HPLC analysis. It was noteworthy that normalized DA stores were comparable in the two culture conditions between 3 and 12 days *in vitro* (Fig. 8). In fact, after 13 days in hypoxia, CHox cultures appeared to contain *smaller* DA stores than Nox controls, although as described above, basal release was ~8x higher. Estimates of glomus cell number for each condition were obtained from counts in "sister" cultures plated at approximately the same initial density and stained for TH immunoreactivity at the corresponding age. In both Nox and CHox cultures, however, the normalized DA stores appeared to increase during the second week in culture, suggesting this property was developing rapidly *in vitro* in P7 glomus cells.

DISCUSSION

The present study provides a quantitative comparison of the effects of acute and chronic hypoxia on catecholaminergic properties of cultured rat CB chemoreceptors, i.e., glomus cells, and the role of Ca-dependent K^+ channels in stimulus-secretion coupling. A novel aspect of the study was that CA release was monitored in CB cultures, grown under different O_2 tensions, but at constant Pco_2 and pH, and measurements were normalized to the actual number of CA-secreting cells determined by TH immunofluorescence. The latter approach meant that the confounding effect of glomus cell hyperplasia reported during whole-animal exposure to chronic hypoxia (Dhillon et al., 1984) was not a contributing factor in this study. In addition, since glomus cells were, in effect, denervated in culture, CA contributions from sensory and sympathetic endings were excluded.

During the initial phase of the study, it was necessary to validate that long-term culture per se did not overtly alter the secretory functions of glomus cells nor their ability to sense oxygen. Measurements of basal DA release, normalized to glomus cell number, revealed that this property was similar for both 2-day-old and ~2-week-old cultures, suggesting that culture duration had no obvious effect on basal secretion rate. Moreover, in 12-day-old cultures, acute hypoxia caused a dose-dependent stimulation of DA release that appeared to require entry of extracellular calcium through L-type Ca^{2+} channels.

Thus, glomus cells retain their ability to sense oxygen, even after 2 weeks in the foreign culture environment, and do so by mechanisms similar to their counterparts in freshly isolated whole CBs *in vitro* (Hanbauer and Hellstrom, 1978; Fidone et al., 1982; Shaw et al., 1989; Doyle and Donnelly, 1994a; Gonzalez et al., 1994), or in short-term dissociated cell culture (Fishman et al., 1995; Lopez-Barneo, 1996).

Perhaps surprisingly, we did not observe any significant difference in "normalized" basal DA release or hypoxia-evoked (5% O_2) DA release relative to basal, between P7 and P20 CB cells cultured for 2-12 days. Previous measurements of tissue CA from whole CB *in vitro*, using carbon fiber electrodes, revealed significantly less baseline and hypoxia-evoked CA levels in CBs from 6-day-old, compared to 10- and 20-day-old rats (Donnelly and Doyle, 1994a). However, the presence of sensory "dopaminergic" terminals in the latter preparation (see Finley et al., 1992), precludes a direct comparison with the present study. Also, it is possible that our P7 cultures continued a normal course of CA maturation *in vitro* and acquired some adult traits before release measurements began.

Comparison of Normoxic and Chronically-hypoxic Glomus Cells

(i) Dopaminergic properties.

One of the most striking differences between Nox and CHox glomus cells is the exaggerated basal DA release after chronic hypoxia *in vitro*. We found that the rate of basal DA release for P7 cells was almost 10 fold higher after ~9 days in chronic hypoxia, compared to controls grown in normoxia. An enhancement of basal DA release was also

seen in P20 cells after a similar hypoxic exposure, though the effect was less pronounced (about five-fold). Since measurements were normalized to glomus cell number, these data suggest that the modification in basal secretion was induced at the level of individual chemoreceptor cells and resulted from the direct stimulatory effects of low oxygen. The fact that chronic hypoxia had a more pronounced effect on basal release in P7 compared with P20 cultures may reflect a greater plasticity in neonatal glomus cells or the involvement of other developmental factors. In another study, baseline tissue CA was significantly elevated in CB of rats reared in a CHox environment (Donnelly and Doyle, 1994b); however, CA contributions from glomus cell hyperplasia and sensory nerve terminals to these measurements were unknown in this preparation.

It should be noted that in the present study all basal release measurements were done in Nox (room air plus 5% CO_2) conditions, i.e., 20% O_2 or a Po_2 of ~160 mm Hg. For an animal breathing normal room air, these values are higher than CB tissue Po_2 which, although controversial, appears to be ~70 mmHg (see Gonzalez et al., 1994). Extension of this argument leads to the conclusion that our Nox growth conditions likely represent a hyperoxic environment for these cells from a physiological viewpoint; however, chronic exposure to 6% O_2 likely reflects cell or tissue hypoxia. Thus, it is possible that in our studies the observed alterations in secretion were due to changes in O_2 availability rather than the absolute Po_2.

Interestingly, the elevated basal release in CHox cultures was unaffected by nifedipine (10 μM), suggesting it was not mediated by voltage-dependent, L-type calcium channels. Thus, these data suggest that the exaggerated basal DA release in

CHox glomus cells is mediated by mechanisms other than an increase in spontaneous electrical activity (see Nurse, 1995; Stea et al., 1995). For example, chronic hypoxia may lead to an increase in resting intracellular calcium, or modulation of dopamine D2 autoreceptors (see Gonzalez-Guerrero et al., 1993), or modulation of other CB neurotransmitters and/or their receptors, which secondarily regulate DA secretion. The greater calcium current seen in the CHox glomus cells *in vitro* (Stea et al., 1995) is, however, the likely explanation of our finding that the normalized high K^+-evoked DA was ~3x higher in CHox compared with Nox cultures. Presumably, the depolarizing action of high K^+ (30 mM) resulted in larger calcium influx, and consequently greater neurotransmitter release in CHox cells.

It is noteworthy that we found no evidence *in vitro* that DA stores were elevated in CHox glomus cells relative to normoxic ones, as expected from studies on whole-animal exposures (see Gonzalez et al., 1994). Although we did observe an exaggerated basal release after chronic hypoxia, consistent with increased DA turnover as reported *in vivo*, direct comparisons with data on DA stores are tentative, due to the presence of glomus cell hyperplasia and contributions from sensory nerve endings *in vivo*. We are unaware of *in vivo* studies which take both these variables into account.

(ii) Expression of functional Ca-dependent K^+ channels.

Closure of Ca-dependent K^+ channels by acute hypoxia is thought to be an important step in chemotransduction, and secretion of catecholamines from rat glomus cells (Wyatt and Peers, 1995). However, it is unclear whether these channels are open at

the resting potential of glomus cells (at CB tissue Po_2), or whether other K^+ channels mediate the initial depolarization during hypoxia (Buckler, 1997). In the present study, attempts to block large-conductance, Ca-dependent K^+ channels with a relatively specific blocker, IbTx (200 nM), resulted in a pronounced stimulation in DA release in Nox (Po_2= ~160 mmHg) cultures; in P7 and P20 cultures the stimulation was ~2x and 6x basal, respectively. It therefore appears that in these cultures, at least some IbTx-sensitive Ca-dependent K^+ channels are open, even at the relatively high normoxic Po_2, and that their closure can initiate DA release. Given that basal release was similar in P7 and P20 cultures, the significantly higher IbTx-evoked release observed in the latter is consistent with a postnatal maturation of Ca-dependent K^+ current in glomus cells, as recently reported (Peers, 1996). It is interesting that in P20 cultures, DA release evoked by 5% O_2 was similar to that obtained by blockade of Ca-dependent K^+ channels with IbTx. In contrast, only approximately one-half of the release can be attributed to these channels in P7 cultures. Thus, additional pathways may mediate hypoxia-evoked DA release in neonatal glomus cells. In other studies on whole rat CB *in vitro*, attempts to block Ca-dependent K^+ channels with charybdotoxin had no effect on basal nerve activity (Cheng and Donnelly, 1995; see also Pepper et al., 1995a), although another non-specific K^+ channel blocker, 4-aminopyridine, increased significantly both basal nerve activity and tissue catecholamines (Doyle and Donnelly, 1994). Differences in drug concentration and lack of information on drug penetration in whole CB preparations preclude a direct comparison with our studies. However, the different results we obtained in Nox and CHox cultures (see following discussion) argue against a non-specific action of IbTx on

DA release, at the concentration (200 nM) used.

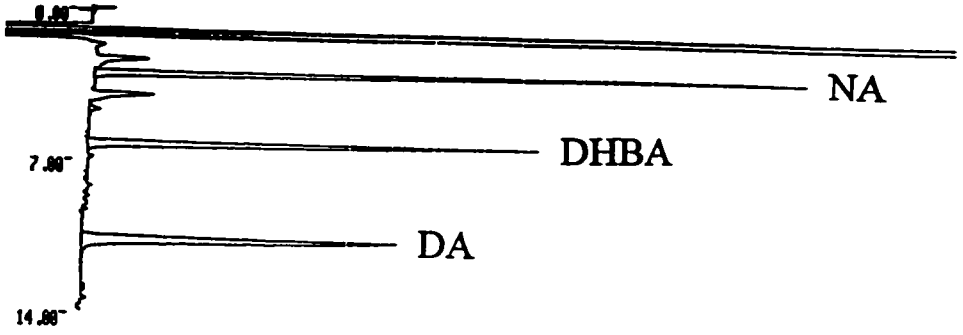
In pronounced contrast to our results in Nox cultures, IbTX did not stimulate DA release in CHox cultures. This was unlikely due to a masking effect of the elevated basal release after CHox *in vitro*, since other stimuli, e.g., acute hypoxia (5% O_2) and high extracellular K^+, were still effective in enhancing DA release by 2-3x basal. The simplest explanation is that chronic hypoxia *in vitro* caused a down-regulation or decreased functional expression of Ca-dependent K^+ channels in glomus cells, and that sensing of acute hypoxia in CHox cells was mediated through a different pathway. A similar conclusion was reached by Wyatt et al. (1995), who examined the properties of glomus cells acutely isolated from rat pups that were born and reared for 9-14 days in a CHox chamber (10 % O_2). Their results suggest that after chronic hypoxia from birth, Ca-dependent K^+ channels are down-regulated, and hypoxic sensing by glomus cells involves closure of a different K^+ channel subtype. Our findings *in vitro* further suggest that this modulation in K^+ channel expression is likely due to a direct effect of low oxygen on glomus cells, and not a secondary response to other cardiovascular changes during chronic hypoxia, e.g., Pco_2 or pH. Moreover, our findings that chronic hypoxia abolished the IbTx-induced DA secretion in P20 cultures, derived from animals with fully mature Ca-dependent K^+ currents (Peers, 1996), implies a functional down-regulation of the underlying channels by low Po_2 rather than a failure of their development. The latter may well occur in animals born and reared in chronic hypoxia (see Wyatt et al., 1995).

Glomus cell plasticity during chronic hypoxia

The present findings add to a growing list of adaptive responses displayed by carotid body chemoreceptors during chronic hypoxia, and imply that manipulation of a single variable, i.e., O_2 tension, can trigger a wide array of cellular responses. We have previously identified several such "plastic" properties at the cellular level, and these are likely to play important roles in glomus cell physiology during adaptation to hypoxia. Among these are (1) altered ion channel function, leading to an increase in electrical excitabilty (Stea et al., 1995); (2) glomus cell hypertrophy with an increase in both surface area and three dimensional cell volume (Stea et al., 1992; Mills and Nurse, 1993); and (3) up-regulation of GAP-43 (Jackson and Nurse, 1995a), a calmodulin-binding protein implicated in neurotransmitter release. Whether the latter relates to the present finding that basal DA release is markedly elevated after CHox remains to be determined. Most likely, the observed modifications in basal and stimulus-evoked DA release, and probably other excitatory or inhibitory neurotransmitters (Gonzalez et al., 1994), in glomus cells exposed to chronic hypoxia, provide for fine control of the final chemoreceptor output and activity in the carotid sinus nerve. As such, they likely contribute to the peripheral mechanisms controlling the hypoxic ventilatory response after long-term, whole animal exposure to low Po_2, e.g., at high altitude or during cardiovascular and pulmonary disease states associated with chronic hypoxemia (Dempsey and Forster, 1982; Weil, 1986; Barnard et al., 1987; Eden and Hanson, 1987b; Nielsen et al., 1988).

Figure 1. A, HPLC chromatogram of external catecholamine standards (10 nM) from detector 2 at -0.3V. Noradrenaline (NA), internal standard DHBA, and dopamine (DA) peaks represent 160, 110, and 95 pg of each compound, respectively (vertical scale readings in minutes). B, an example of rat glomus cell clusters in a 10-day-old culture that was stained for tyrosine hydroxylase (TH) immunoreactivity, and visualized with a fluorescein-conjugated secondary antibody; TH-positive cell counts were used to normalize DA release to glomus cell number in any given culture. Scale bar = 20 μm.

A



B

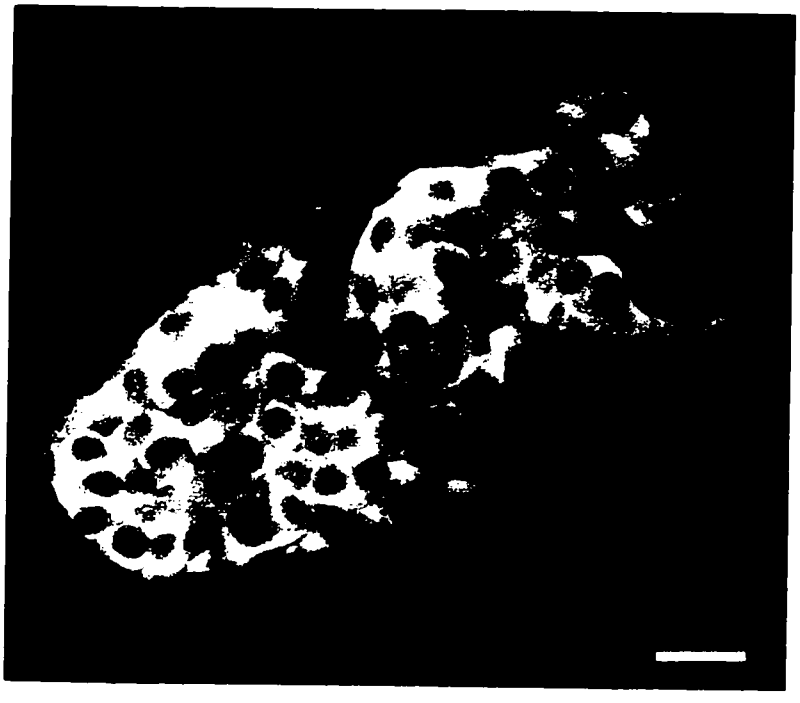


Figure 2. Comparison of normalized basal dopamine (DA) release in neonatal (P7) and juvenile (P20) cultures. A, HPLC peaks (inverted) of DA release measured in three consecutive 15 min samples (b_1, b_2, b_3) collected from a single culture under basal (20% O_2/5% CO_2) conditions; cultures were kept in a serum-free DMEM/F12 medium for 10 min periods between sampling. Note the relative stability of basal release (area under peaks) over entire sampling period. B, histogram illustrating the similarity in normalized basal DA release in both neonatal and juvenile CB cultures after 2 and 12-14 days *in vitro*. To normalize DA release, collected over 15 min intervals, the number of glomus cells present in the culture was estimated after sampling using TH immunofluorescence. Bars in B represent mean ± SEM values for the number of cultures indicated.

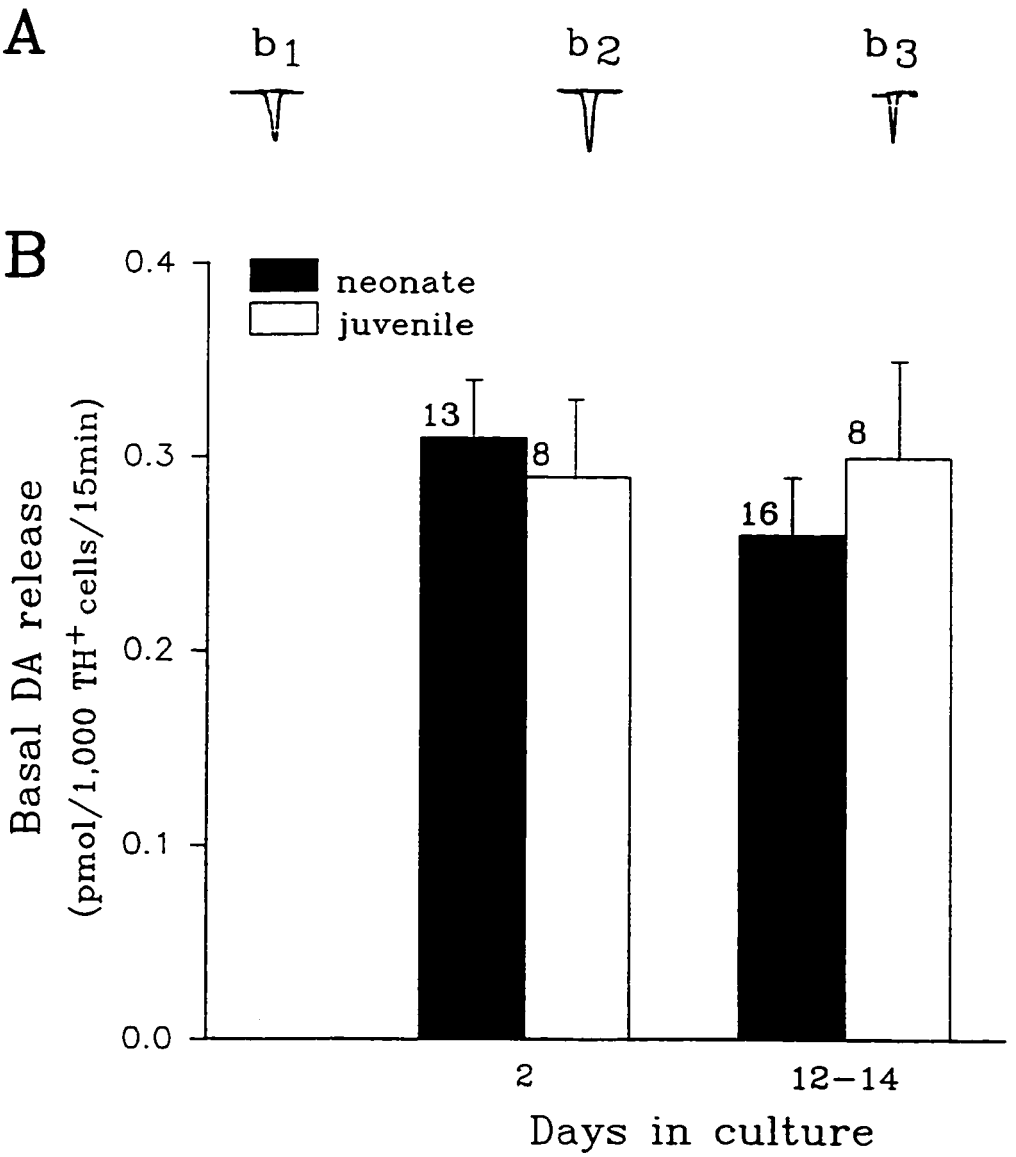

A
b1
b2
b3
B
neonate
juvenile
Basal DA release
(pmol/1,000 TH+ cells/15min)
0.4
0.3
0.2
0.1
0.0
13
8
16
8
2
12–14
Days in culture

Figure 3. Effect of O_2 tension and L-type calcium channel blockers on DA release from neonatal (P7) cultures. A, HPLC peaks of DA release from a single culture under basal (b_1 and b_2; 20% O_2/5% CO_2) and stimulated (10% O_2/5% CO_2) conditons. Each peak obtained from 15 min release samples was separated by a 10 min recovery period as in Fig. 2A. B, acute hypoxia caused a dose-dependent stimulation in DA release above basal; exposure to 5% and 10% O_2 stimulated DA release significantly by ~6x and ~3x basal (20% O_2), respectively (●;**$p<0.01$, Student's *t* test). DA release evoked by 5% O_2, but not 20% O_2 (basal), was significantly (**$p<0.01$) inhibited by the L-type calcium channel blocker nifedipine (10 μM; ▽). Data points represent mean ± SEM for the number of cultures indicated.

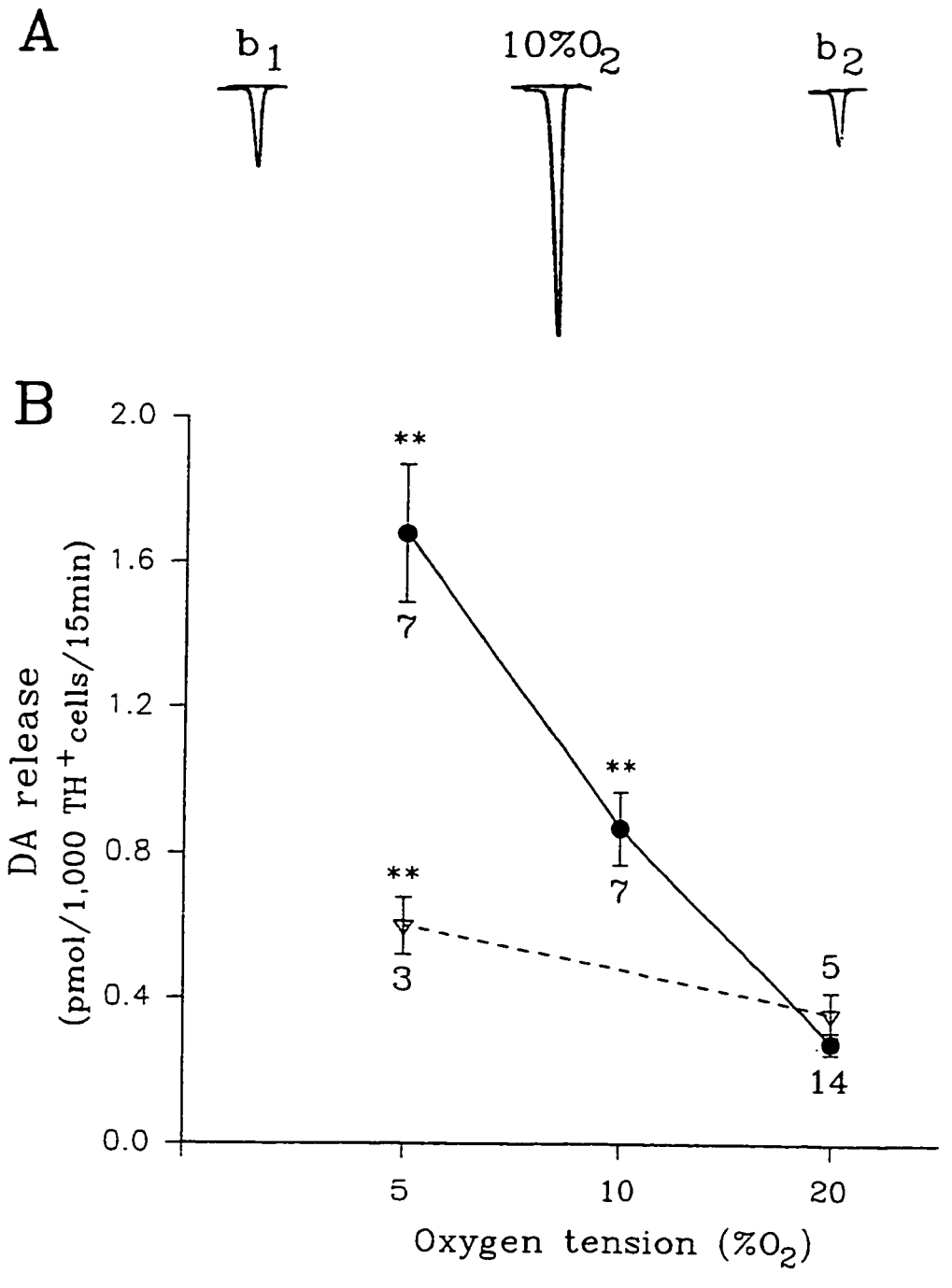

A
b1
10%O2
b2
B
DA release
(pmol/1,000 TH+cells/15min)
2.0
1.6
1.2
0.8
0.4
0.0
**
**
**
7
7
3
5
14
5
10
20
Oxygen tension (%O2)

Figure 4. Comparison of the effects of acute hypoxia and IbTx on DA release in neonatal (P7) and juvenile (P20) cultures. A, HPLC peaks of DA release evoked by a 15 min exposure to IbTx (200 nM; middle trace) is compared with basal release samples taken before (b_1) and after (b_2) IbTx over the same time period. Experimental details are similar to Fig. 2A. B, stimulus-evoked release is expressed as a ratio relative to basal release, obtained during the first 15 min sample. Exposure to acute hypoxia (5% O_2) or IbTx (200 nM) significantly stimulated DA release above basal for both age groups. With IbTx as the stimulus, DA release was elevated significantly (*$p<0.05$) more in P20 compared with P7 cultures. Each bin represents mean (±SEM) for the number of cultures indicated.

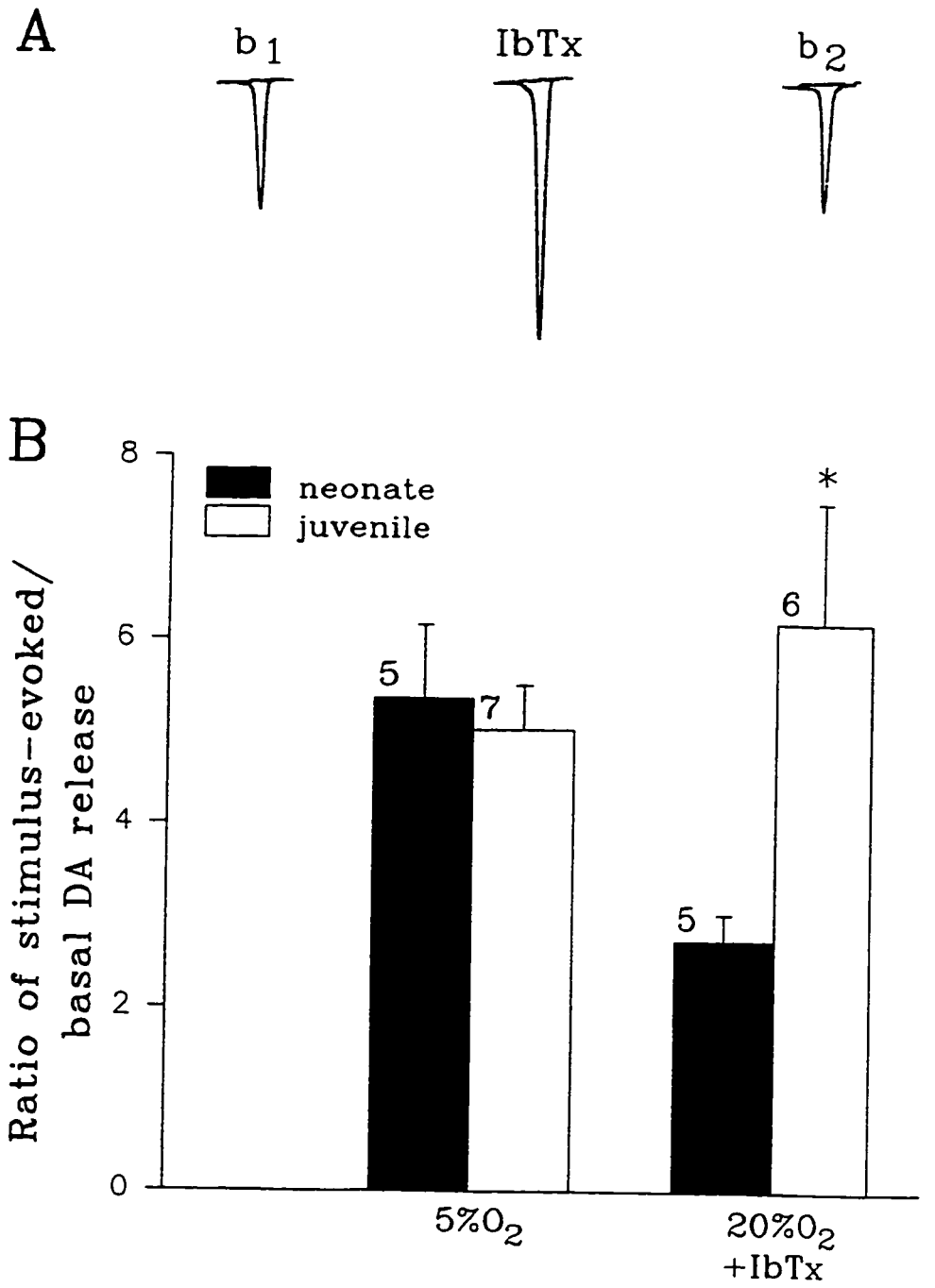

A
b1
IbTx
b2
B
neonate
juvenile
Ratio of stimulus-evoked/
basal DA release
0
2
4
6
8
5
7
5
6
*
5%O2
20%O2
+IbTx

Figure 5. Effects of chronic hypoxia and the role of voltage-gated L-type calcium channels on basal and evoked DA release from CB cultures. Neonatal (P7) and juvenile (P20) cultures were grown in 6% O_2 for 9-11 days and then returned briefly to normoxia (20% O_2) for 12-16 h before monitoring release. A, when compared with Nox cultures (see Fig. 2B), the normalized DA release (ordinate) was significantly ($p<0.01$) elevated in CHox cultures derived from the two age groups. After chronic hypoxia, basal DA release was significantly higher (~1.6x; *$p<0.05$) in neonatal compared with juvenile cultures. B, the exaggerated basal DA release after chronic hypoxia was unaffected by 10 μM nifedipine (compare left columns in A and B), suggesting it was not dependent on entry of extracellular calcium through L-type channels. In contrast, DA release evoked by 5% O_2 in CHox cultures was significantly (**$p<0.01$) reduced by 10 μM nifedipine (right columns in B). Each bin represents mean (± SEM) for the number of cultures indicated.

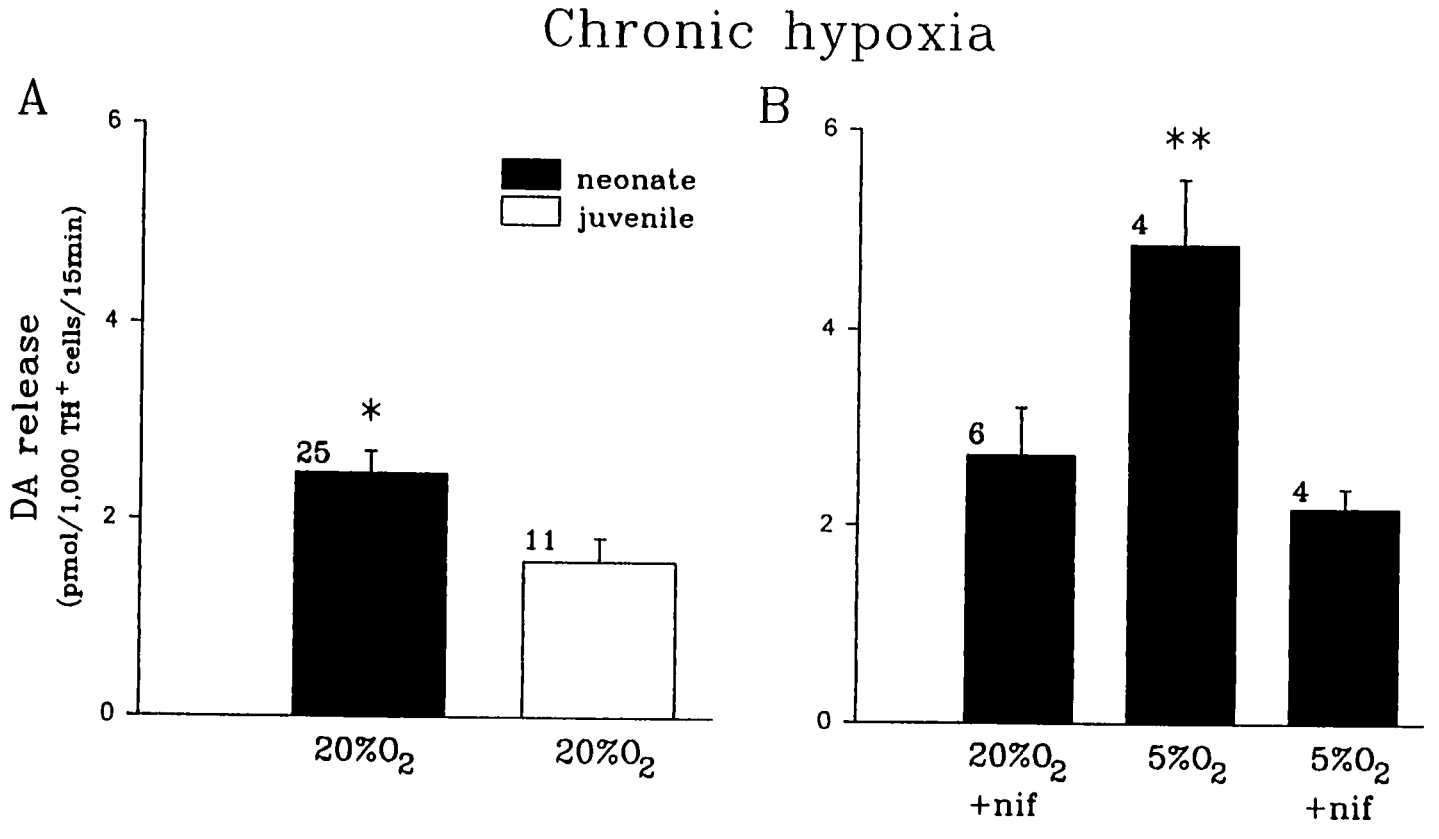

Chronic hypoxia
A
B
DA release
(pmol/1,000 TH+ cells/15min)
neonate
juvenile
6
4
2
0
*
**
25
11
6
4
4
20%O2
20%O2
20%O2
+nif
5%O2
5%O2
+nif

Figure 6. Differential effects of acute hypoxia and IbTx on DA release in CHox cultures. Cultures were grown in 6% O_2 for 9-11 days and then returned briefly to normoxia (20% O_2) for 12-16 h before monitoring release. On the ordinate, stimulus-evoked release is expressed relative to basal as in Fig. 4B. Acute hypoxia (5% O_2) stimulated DA release by ~2x basal in both P7 and P20 cultures. In contrast to Nox cultures (Fig. 4B), IbTx (200 nM) failed to stimulate DA release significantly above basal (ratio = ~1) in both P7 and P20 CHox cultures. Each column represents mean (± SEM) for the number of cultures indicated.

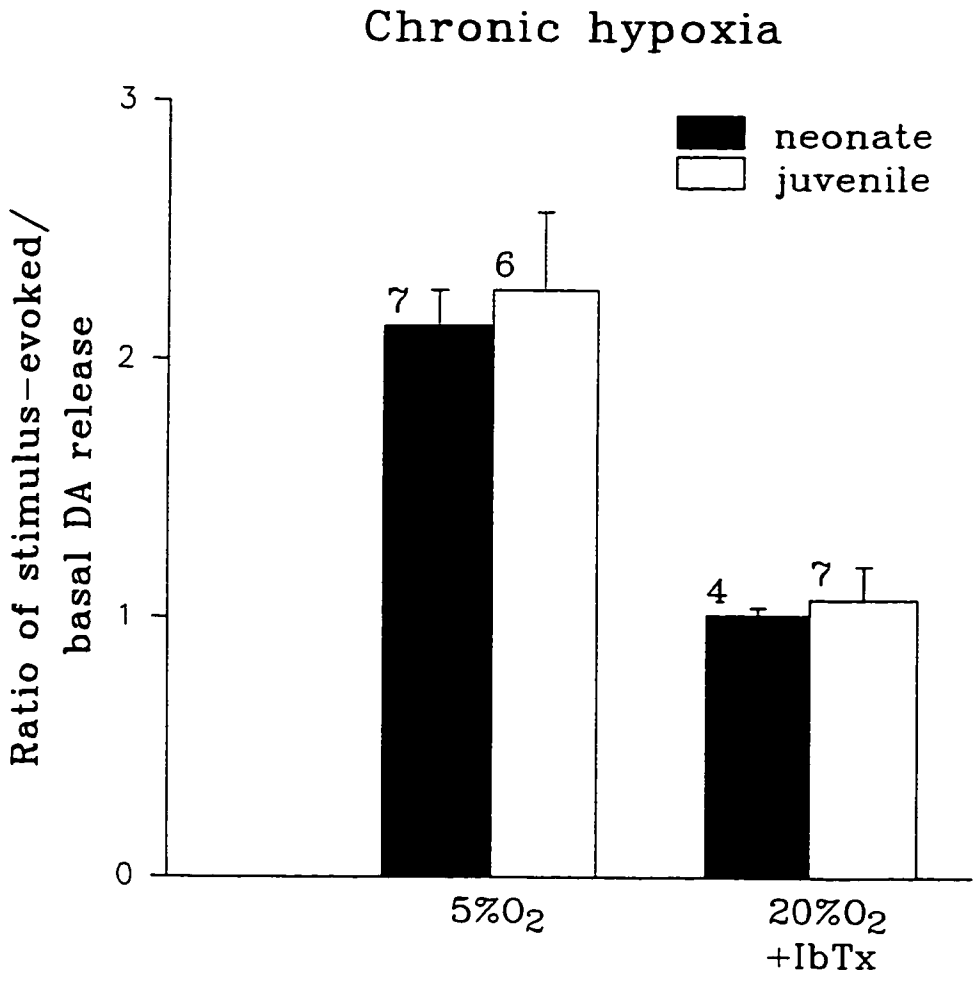

Chronic hypoxia
neonate
juvenile
Ratio of stimulus-evoked/
basal DA release
3
2
1
0
7
6
4
7
5%O2
20%O2
+IbTx

Figure 7. High K^+-evoked DA release in Nox and CHox cultures. P7 cultures were grown *in vitro* for 12-15 days in normoxia or chronic hypoxia; CHox cultures were returned to normoxia 12-16 h before release samples were collected. Although high extracellular K^+ (30 mM) stimulated DA release in both Nox and CHox cultures, the effect was significantly (**$p<0.01$) higher in CHox cultures. Each bin represents mean (±SEM) for the number of cultures indicated.

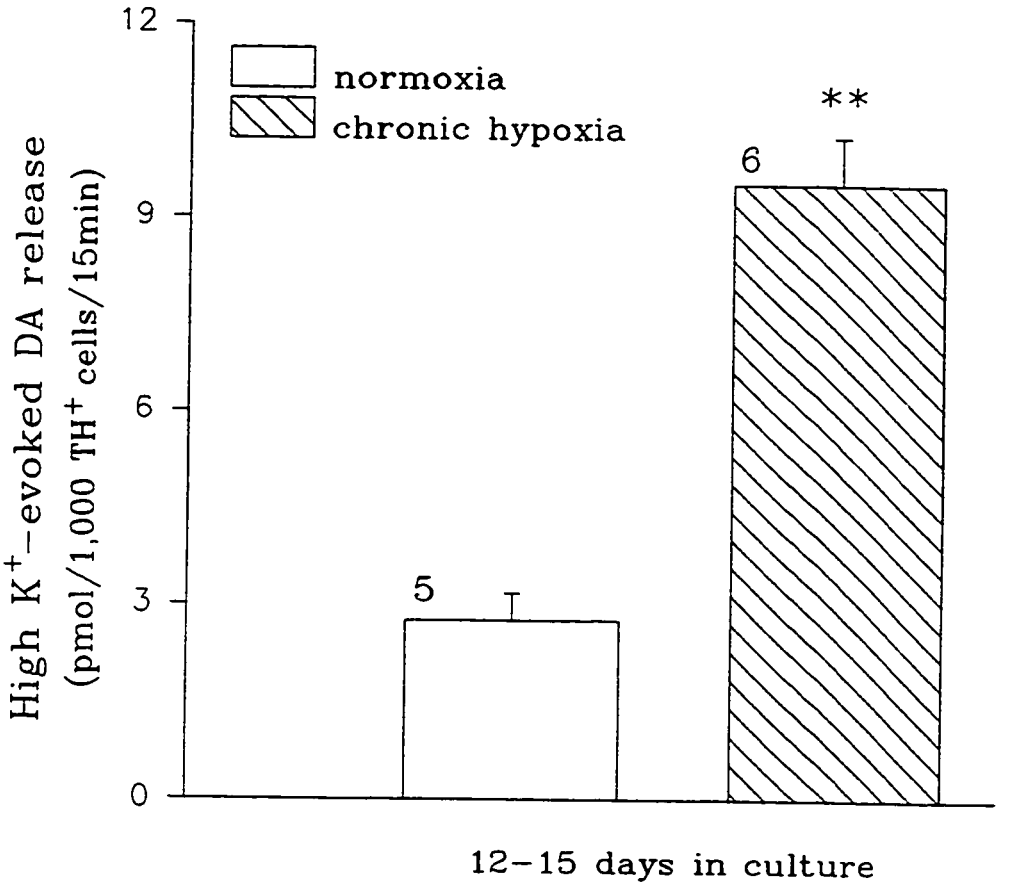
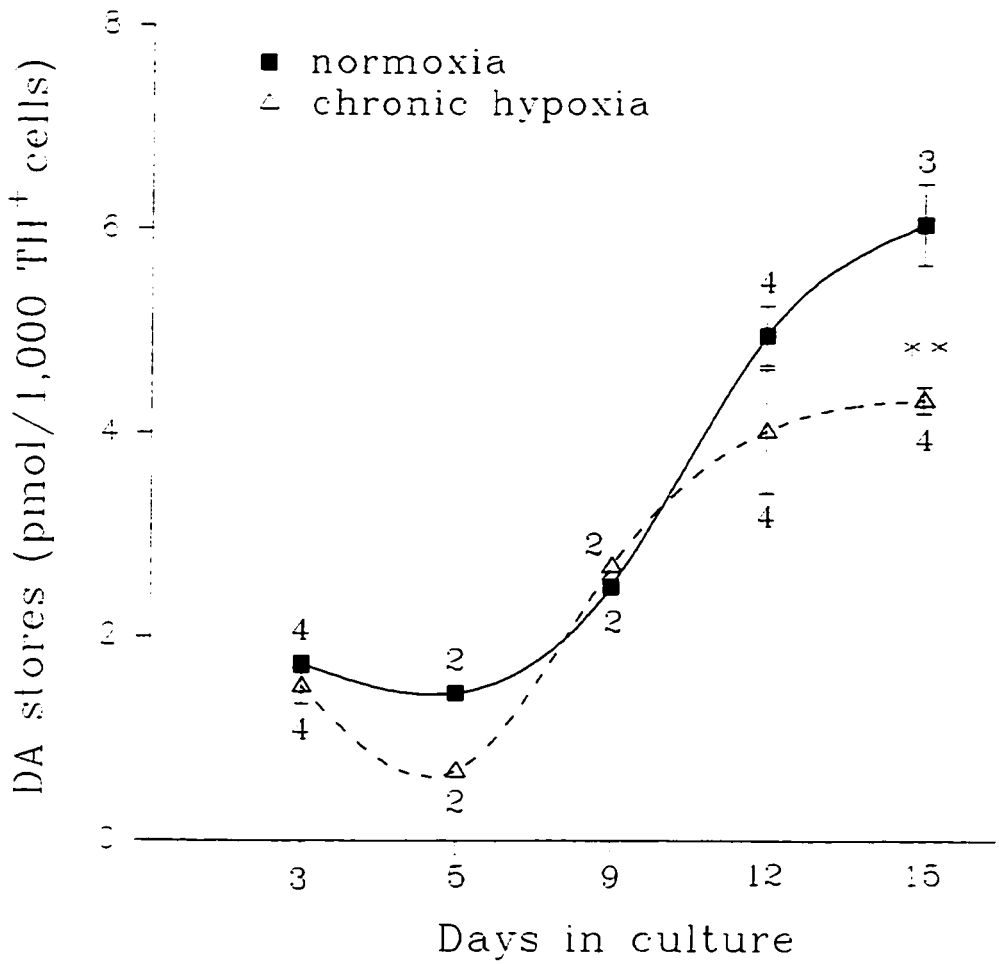

12
9
6
3
0
normoxia
chronic hypoxia
**
6
5
High K+-evoked DA release
(pmol/1,000 TH+ cells/15min)
12–15 days in culture

Figure 8. Dopamine stores in Nox and CHox cultures. After 2 days in normoxia, cultures were transferred to hypoxia (6% O_2) or maintained in normoxia (20% O_2) until the indicated time points, when cellular stores were collected and analyzed using HPLC. The vertical axis shows mean DA stores (± SEM) per 1,000 glomus cells; estimates of glomus cell counts were obtained by TH immunofluorescence from sister cultures plated at approximately the same initial density and maintained in either 20% O_2 (Nox) or 6% O_2 (CHox). In both Nox and CHox cultures, DA stores increased with time in culture after an initial lag. After 15 days *in vitro*, CHox cultures had significantly (**$p<0.01$) smaller DA stores compared with Nox controls. The number of cultures, from at least two separate platings, are indicated.


normoxia
chronic hypoxia
DA stores (pmol/1,000 TH+ cells)
Days in culture
3
5
9
12
15
4
2
2
4
4
2
3
4
x x

CHAPTER 3

Role of Acetylcholine Receptors and Dopamine Transporter in Regulation of Extracellular Dopamine in Rat Carotid Body Cultures Grown in Chronic Hypoxia or Nicotine

All data presented in this chapter represents work done entirely by me and has been accepted for publication in the *Journal of Neurochemistry*, July 29, 1997.

SUMMARY

Using dissociated rat carotid body (CB) cultures, we compared levels of extracellular dopamine (DA) around oxygen-sensitive glomus cells grown for ~12 days in normoxia (Nox; 20% O_2), chronic hypoxia (CHox; 6% O_2), or chronic nicotine (CNic; 10 μM nicotine, 20% O_2), with or without acetylcholine receptor (AChR) agonists/antagonists and blockers of DA uptake. In Nox cultures, extracellular DA, determined by HPLC and normalized to the number of tyrosine hydroxylase (TH)-positive glomus cells present, was augmented by acute (~15 min) exposure to hypoxia (5% O_2; ~6x basal; see Jackson and Nurse, 1997a), high extracellular K^+ (30 mM, ~10x basal), nomifensine (1 μM; ~3x basal), a selective DA uptake inhibitor, and nicotine (100 μM; ~5x basal), but not methylcholine (300 μM), a non-selective muscarinic agonist. In contrast, in CHox cultures, where basal DA release is markedly elevated (~9x

control), the stimulatory effect of high K^+ (3-4x basal) and acute hypoxia (~2x basal) on DA release persisted, but nicotine and nomifensine were no longer effective, and methylcholine had a partial inhibitory effect. In CNic cultures, basal DA levels were also elevated (~9x control), similar to that in CHox cultures; however, though acute hypoxia had a stimulatory effect on DA release (~2x basal), nicotine, nomifensine and high K^+ were ineffective. The elevated basal DA in both CHox and CNic cultures was attenuated by acute or chronic treatment with mecamylamine (100 μM), a nicotinic AChR (nAChR) antagonist. In addition, long-term (16 h), but not acute (15 min), treatment with the muscarinic antagonist, atropine (1 μM), produced an additional enhancement of basal DA levels in CHox cultures. Thus, after chronic hypoxia or nicotine *in vitro*, extracellular DA levels around CB chemoreceptor cell clusters appear to be set by a variety of factors including, released ACh, positive and negative feedback regulation via nAChRs and mAChRs respectively, and modulation of DA transporters. These results provide insight into roles of endogenous transmitters in the adaptation of CB chemoreceptors to chronic hypoxia, and suggest pathways by which neuroactive drugs, e.g. nicotine, can interfere with the protective chemoreflex response against hypoxia.

INTRODUCTION

The mammalian carotid body (CB) consists of innervated clusters of chemoreceptor cells which sense and regulate blood Po_2, Pco_2, and pH via reflex control of ventilation (Eyzaguirre and Zapata, 1984; Gonzalez et al, 1994). Though an increase in discharge of CB afferent sensory fibers during acute hypoxia is correlated with enhanced neurosecretion from the chemoreceptors, i.e. glomus or type 1 cells (Fidone et al., 1991; Donnelly and Doyle, 1994a; Gonzalez et al., 1994), the role of released neuroactive agents in oxygen chemoreception remains controversial (Gonzalez et al., 1994). The presence of chemical synapses between contiguous glomus cells (McDonald and Mitchell, 1975), together with the occurrence of multiple endogenous neurotransmitters, allow for complex modulation of the final chemoreceptor output, afferent nerve activity, and ultimately, the hypoxic ventilatory response. Among the CB neurotransmitters present in glomus cells, dopamine (DA) and acetylcholine (ACh) have received much attention, though their roles in hypoxic chemoreception remain arguable (Gonzalez et al., 1994). Since numerous studies have demonstrated hypoxia-evoked DA release from the CB of several mammalian species (Shaw et al., 1989; Fidone et al., 1991; Donnelly and Doyle, 1994a; Gonzalez et al., 1994), as well as from singly-isolated (Lopez-Barneo, 1996) and clusters of cultured glomus cells (Jackson and Nurse, 1997a), understanding the factors that regulate DA secretion provides insight into the mechanisms of CB

neurotransmission and function. Further, DA has been implicated in long-term CB adaptive responses, including ventilatory acclimatization to hypoxia (Bisgard et al., 1987) and "blunting" of the ventilatory response in high-altitude dwellers or neonates born and reared in hypoxia (Dempsey and Forster, 1982; Eden and Hanson, 1987b; Wach et al., 1989; Hertzberg et al., 1990).

We are interested in the cellular and molecular mechanisms underlying CB adaptation to prolonged hypoxia. Recently, we showed that exposure of rat CB cultures to chronic hypoxia *in vitro* results in an apparent down-regulation of O_2-sensitive, Ca-dependent K^+ channels in glomus cells, and a greatly exaggerated basal DA release (Jackson and Nurse, 1997a). These *in vitro* results, obtained at the cellular level, parallel those obtained following whole animal exposure to chronic hypoxia (Donnelly and Doyle, 1994b; Wyatt et al., 1995), though the underlying mechanisms remain unresolved. The enhanced basal DA secretion seen in CB cultures after chronic hypoxia *in vitro* was unaffected by nifedipine (Jackson and Nurse, 1997a), suggesting that activation of voltage-dependent, dihydropyridine-sensitive, L-type calcium channels was not involved. Since the majority of glomus cells that survive long-term culture remain in isolated clusters (3 to 30 cells), the result of incomplete dissociation of CB tissue at the time of initial plating, it is likely that some functional synapses remain intact among neighboring glomus cells (see Nurse, 1987; Nurse et al.,1993), and are therefore targets for long-term modulation by chronic hypoxia.

In the present study we considered the possibility that the CB neurotransmitter ACh and its receptors, together with DA uptake mechanisms, might participate in the

regulation of CB dopamine during adaptation to chronic hypoxia. Indeed, radioligand binding, autoradiographic, and electrophysiological studies have demonstrated the presence of both nicotinic and muscarinic acetylcholine receptors (AChR) on CB glomus cells in different species (Chen and Yates, 1984; Dinger et al., 1985, 1986, 1991; Wyatt and Peers, 1993; Dasso et al. 1997). In addition, pharmacological studies have revealed that nicotinic agonists excite CB chemoreceptors, stimulate CA release, and potentiate the response to hypoxia (Eyzaguirre and Zapata, 1984; Dinger et al., 1985; Gomez-Nino et al., 1990; Wyatt and Peers, 1993). In contrast, muscarinic agonists have been shown to inhibit both the release of CA and the increased carotid sinus nerve (CSN) activity evoked by nicotine stimulation in rabbit CB *in vitro* (Dinger et al., 1991). Since AChR density is known to be regulated by chronic hypoxia in other systems, e.g. cardiac muscle (Kacimi et al., 1993), it is plausible that a similar regulation may contribute to glomus cell physiology after chronic hypoxia both *in vivo* and *in vitro*. In this study, we tested the latter possibility by measuring extracellular DA levels in the presence of cholinergic agonists and antagonists after growing rat CB cultures in a low oxygen environment. Further, we compared the effects of chronic stimulation of nAChR *in vitro*, by treating the cultures with chronic nicotine, on extracellular DA levels. The potential role of the DA transporter was studied by measuring extracellular DA in the presence and absence of nomifensine, a blocker of DA uptake. Our results suggest that ACh and its receptors, as well as the DA transporter, are key participants in the regulation of CB dopamine levels after both acute and chronic hypoxia, and following nicotine exposure.

MATERIALS AND METHODS

Cell Culture

Primary cultures of dissociated rat carotid bodies (CBs) were prepared as described in detail elsewhere (Nurse, 1990; Jackson and Nurse, 1997a). Briefly, CBs were dissected from 5-7 day-old rats (Wistar, Charles River, Quebec), cleaned of surrounding tissue, and mechanically dissociated after a 40 to 45 min incubation in an enzymatic solution containing 0.1% collagenase, 0.1% trypsin (Gibco, Grand Island, NY), and 0.01% deoxyribonuclease (Millipore, Freehold, NJ). The enzyme was inactivated by a rinse in growth medium containing F12 nutrient medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 80 U/L insulin (Sigma Chemical Co., St. Louis, MO), 0.6% glucose, 2mM L-glutamine and 1% penicillin-streptomycin (Gibco). Dispersed cells and cell clusters were plated on collagen-coated central wells (diameter, 8-10 mm) of modified 35mm culture dishes. For the first 2 days, all cultures were grown in a control normoxic environment (95% air:5% CO_2; Forma Scientific automatic CO_2 incubator) at 37°C in a humidified atmosphere, before separation into one of three groups. Two groups remained in this environment, except that one of them was treated for an additional 10 days with 10 μM (-)-nicotine (Sigma). The third group was transferred to a hypoxic environment (6% O_2/89% N_2/5% CO_2; Forma Scientific O_2/CO_2 incubator) for an additional 9 days and then transferred to normoxia for the final 24 h. Some of these cultures were treated with mecamylamine (100 μM; Research

Biochemicals International or RBI, Natick, MA) or atropine sulfate (1 μM; RBI) for varying periods as indicated in the text. Routinely, the medium was replaced on day 2 and 7 *in vitro* with fresh growth medium plus the appropriate additives.

Catecholamine determination using HPLC

Released dopamine (DA) from living cultures was analyzed by HPLC as described previously (Thompson et al., 1997; Jackson and Nurse, 1997a), using a Waters model 510 pump with a Spherisorb-ODS2 column (10 x 0.46 cm, 3 μm particle size; Chromatography Sciences Company, Montreal, Quebec), and an electrochemical detector (Coulochem II detector, model 5200; ESA, Inc., Bedford, MA). The detector potential was set at -0.3 V and the flow rate was 1 ml/min at ~ 2000 psi. Using the peak area ratio method, and a known amount of external DA standard (3-hydroxytyramine; Sigma), the actual amount of DA in a given sample could be determined from the integrated peak area. For each run, a known amount of internal standard, di-3,4-hydroxybenzylamine hydrobromide (DHBA; Sigma), was added to each sample to correct for variations in injection volume. The mobile phase consisted of NaH_2PO_4 (6.9 g/L; Sigma), Na_2EDTA (80 mg/L; BDH Chemicals, Toronto, Ontario), and heptanesulfonic acid (250 mg/L; Sigma) in water and 5% methanol; pH was adjusted to 3.5 with concentrated H_3PO_4.

Before collection of release samples, CB cultures were first rinsed and incubated for 10 min in Dulbecco's Modified Eagle's Medium/F-12 medium or DMEM/F-12 (50:50; Gibco) supplemented with 1.2 % glucose. Then, a basal release sample was collected after a 15 min incubation of the culture at 37°C in 55 μl bicarbonate-buffered

salt solution (BBSS), under an atmosphere of 5% CO_2, 20% O_2 (normoxia). The BBSS was added to the central circular well and contained (mM): NaCl, 116; KCl, 5; $NaHCO_3$, 24; $CaCl_2$, 2; $MgCl_2$, 1.1; Hepes, 10; glucose, 5.5; at pH 7.4. Basal release samples were collected by withdrawing 50 µl of BBSS from the well; this measurement provided a baseline for comparing release evoked by the stimulus, which was applied after a 10 min 'recovery' period during which the culture was re-incubated in DMEM/F-12. Except for the nature of the stimulus evoked release was carried out under similar conditions as basal release. Samples of evoked release were collected after exposure of the cultures for 15 min to: (i) hypoxia (5 % O_2); (ii) (-)-nicotine (100 µM); (iii) high extracellular K^+ (30 mM K^+; 25mM NaCl was replaced by equimolar KCl); (iv) nomifensine maleate (1µM; RBI); or (v) acetyl-β-methylcholine chloride (300 µM; Sigma) in BBSS. In some experiments, to block muscarinic or nicotinic receptors respectively, atropine (1 µM) or mecamylamine (100 µM) was added to BBSS, as well as during the DMEM/F-12 preincubation. Similarly, to inhibit the dopamine transporter, nomifensine was added to both the preincubation and release (BBSS) medium. Release samples were mixed 1:1 with 0.1 M $HClO_4$ containing 2.7mM Na_2EDTA, and stored at -80°C until HPLC analysis.

Immunofluorescence

Following catecholamine (CA) release experiments, glomus cells were positively identified by tyrosine hydroxylase (TH) immunoreactivity and visualized with a fluorescein-conjugated secondary antibody (see Fig. 1) as previously described (Nurse,

1990; Jackson and Nurse, 1995a, 1997a). Some cultures were immunostained for both TH and nicotinic acetylcholine receptors (nAChR), which were visualized with a Texas red-conjugated secondary antibody (see Fig. 1). The cultures were first rinsed in phosphate-buffered saline (PBS; pH 7.2) and fixed at -20°C for 30 min in 95% methanol/5% acetic acid. After removal of the fixative, the cultures were incubated in 0.1 M glycine in 0.1 M phosphate buffer (pH 7.2) for 5 min at room temperature. Following three washes for 5 min each in phosphate buffer, the cells were incubated in concentrated mouse anti-nAChR monoclonal antibody (mAb 27.35.74; a generous gift from Dr. Bruce Chase), and rabbit anti-TH antibody (Chemicon, El Segundo, CA) diluted to 1:900 in 1% bovine serum albumin (BSA; Sigma) in PBS. The antibody mAb 27.35.74, which was raised against nAChR from the *Torpedo* electric organ, recognizes an epitope on the main immunogenic region of the α subunit and is known to cross react with neuronal nAChR in different species (Chase et al., 1987). After incubation for 24 h at 4°C with primary antibodies, the cultures were washed three times for 5 min each with phosphate buffer. The cells were then incubated for 1 h at room temperature with fluorescein-conjugated goat anti-rabbit IgG (Cappel, Malvern, PA) and Texas red-conjugated goat anti-mouse IgG (Jackson Immunoresearch Lab, Inc., Westgrove, PA) diluted in 1% BSA in PBS to 1:50 and 1:200, respectively. Following three rinses for 5 min each in phosphate buffer, the cultures were covered with an anti-photobleaching reagent (1,4-Diazabicyclo[2.2.2] octane; Lancaster Synthesis Inc., Windham, NH).

Visualization and quantification of immunofluorescent TH- and AChR-positive glomus cells was done with a Zeiss (IM35) inverted microscope, equipped with

epifluorescence.

RESULTS

As in our recent studies (Jackson and Nurse, 1997a; see Materials and Methods), basal and evoked (or drug-treated) release samples were collected from each culture during 15 min incubation intervals, separated by a 10 min recovery period. Released DA, determined by HPLC, was expressed as a ratio of stimulus-evoked/ basal or normalized to glomus cell number, determined in the *same* culture by immunostaining for tyrosine hydroxylase (TH) after sampling was complete (Fig. 1B, E, H; see also Nurse, 1990; Jackson and Nurse, 1995a, 1997a). In all experiments basal release measurements were obtained under normoxic conditions, i.e. 20% O_2.

Expression of nicotinic AChR on glomus cells after long-term culture

Studies in various species indicate that neuronal nicotinic AChR (nAChR) are expressed on glomus cells *in situ* and soon after isolation *in vitro* (Eyzaguirre et al., 1990; Wyatt and Peers, 1993; Dasso et al. 1997; Obeso et al., 1997). To test whether nicotinic AChRs are still expressed on glomus cells after long-term culture under different O_2 tensions or after chronic exposure to nicotine, ~ 2 week-old cultures were immunostained with antibodies against TH and nAChR, and visualized with fluorescein and Texas red-conjugated secondary antibodies respectively (see Materials and Methods). As shown in Fig. 1, TH-positive glomus cells grown under normoxic (Nox; 20% O_2), chronically

hypoxic (CHox; 6% O_2), or chronic nicotine-treated (CNic; 10 μM) conditions displayed positive-immunoreactivity for nAChR. The antibody used, i.e. mAb 27.35.74, was raised against nAChR from *Torpedo* electric organ and recognizes an epitope on the α subunit of the nAChR in various species (Chase et al., 1987).

Nicotine and high K_e^+ differentially stimulate DA release in normoxic, chronically hypoxic and chronic-nicotine cultures

Nicotinic receptors have been shown to modulate the release of catecholamines from the CB of several species (Dinger et al., 1985; Gomez-Nino et al., 1990; Obeso et al., 1997). Since nAChR appeared to be expressed on glomus cells grown in different O_2 tensions and in chronic nicotine (CNic; Fig. 1), we anticipated that acute exposure to nicotine would stimulate DA release due to its depolarizing action (see Wyatt and Peers, 1993). Indeed, exposure of Nox cultures to nicotine (100 μM) resulted in a significant increase in DA release (~5x basal; Fig. 2). Surprisingly however, in CHox and CNic cultures, acute nicotine failed to stimulate DA release significantly above basal after 10 days of treatment (Fig. 2). Though interpretation of these data is complicated by the substantial elevation in basal DA release in both CHox (8-10x Nox values; Jackson and Nurse, 1997a) and CNic cultures (see later), these results may be due to: (1) a reduction in the density of nAChRs expressed in CHox glomus cells; (2) nAChR desensitization in CNic and CHox cultures (with the latter arising from chronic ACh release in low oxygen); and/or (3) modulation of stimulus-secretion coupling downstream from nAChR stimulation.

Since in a recent study we showed that the depolarizing agent, high extracellular K^+ (K_e^+; 30 mM), stimulated DA release in CHox cultures by 3-4x basal (Jackson and Nurse, 1997a; see also, Fig. 2), it appears that the pathways coupling membrane depolarization, and entry of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels, to DA release are functional in CHox cultures. To test whether the same applies to CNic cultures, we compared the effects of high K_e^+ on DA secretion. Interestingly, unlike CHox cultures, high K_e^+ failed to stimulate DA release significantly above basal in CNic cultures (Fig. 2).

Basal and hypoxia-evoked DA release in cultures treated with chronic nicotine

Recently, we reported that in CHox CB cultures, basal DA release was substantially augmented (8-10x Nox values), though an acute hypoxic stimulus was still effective in stimulating DA release above basal (~2x ; Jackson and Nurse, 1997a; see also, Figs. 2, 3). This elevated DA release persisted in the presence of nifedipine (10 μM), a blocker of L-type calcium channels, suggesting that voltage-dependent Ca^{2+} entry through these channels was not involved (Jackson and Nurse, 1997a). Conceivably, chronic activation of neurotransmitter/ receptor systems on glomus cells, e.g. continuous stimulation of surface AChR by hypoxia-evoked ACh release within glomus cell clusters (see Fig. 1), could contribute to the elevated basal release in CHox cultures. To test whether chronic stimulation of nAChR might be a contributing factor, we exposed Nox cultures to chronic nicotine and measured basal DA release, as well as the response to acute hypoxia.

Treatment of Nox cultures with chronic nicotine (10 μM; CNic) for 10 days dramatically stimulated basal DA release (~9x control), and the effect was comparable to that of chronic hypoxia (6% O_2) over the same culture duration (Fig. 2). However, in contrast to CHox cultures where the elevated basal release was virtually unaffected if the cultures were returned to normoxia during the final 24 h *in vitro*, there was a significant ($p<0.01$; Student's t-test) reduction in basal DA release to ~3x control (n =6; data not shown), if nicotine was withdrawn from CNic cultures during the final 24 h. Similar to CHox cultures, however, CNic cultures retained the ability to sense oxygen. For example, as shown in Fig. 3, exposure of CNic cultures to acute hypoxia (5% O_2 for 15 min) resulted in ~2x stimulation in DA release relative to basal. These results are consistent with the hypothesis that endogenous ACh, acting via nAChR, may be involved in the mechanisms leading to the marked elevation in basal DA release after chronic hypoxia. Further evidence supporting this idea is presented below.

Blockade of nAChR suppresses the elevated basal DA release in CB cultures treated chronically with hypoxia or nicotine

If the enhanced basal DA release in CHox cultures, and presumably that in CNic cultures, are mediated by signaling pathways initiated at nAChRs on glomus cells, then blockade of these receptors with mecamylamine should abolish this response. As shown in Fig. 4, chronic (10 days) treatment of both CHox and CNic cultures with mecamylamine (100 μM) significantly reduced basal DA release. Representative HPLC traces of DA release from control and mecamylamine-treated (day 2-12) CHox "sister"

cultures, containing similar numbers (475 vs 470) of glomus cells, are compared in Fig. 4A. It is noteworthy that chronic mecamylamine had no effect on basal DA release in Nox cultures (Fig. 4B), suggesting its inhibitory effect is more readily detectable following stimulation of the chemoreceptors by low oxygen. However, this inhibition does not require the continuous presence of the antagonist during the chronic treatment, since brief (15 min) exposures to mecamylamine (during collection of the HPLC sample) were sufficient to prevent the elevation in basal release in both CHox and CNic cultures (Fig. 4B).

Muscarinic AChR inhibit DA release after chronic hypoxia

Muscarinic AChR (mAChR) are present on CB glomus cells (Dinger et al., 1986; 1991; Dasso et al. 1997) and muscarinic agonists suppress basal carotid sinus nerve (CSN) activity (Docherty and McQueen, 1979; Monti-Bloch and Eyzaguirre, 1980; see, however, Dinger et al., 1991), and inhibit both DA release and the increased CSN activity evoked by nicotine stimulation in the rabbit CB (Dinger et al., 1991). In addition, some glomus cells of the rat CB express both nicotinic and muscarinic receptors (Dasso et al., 1997), raising the question of whether activation of mAChR, by ACh released during chronic hypoxia, also participates in the regulation of DA release, as discussed above for nAChR. We therefore examined, in both Nox and CHox cultures, the effects of a muscarinic agonist, methylcholine (300 μM), and the non-selective muscarinic antagonist, atropine (1 μM), on DA release. Acute (15 min) methylcholine and long-term (16 h) atropine failed to alter DA release in Nox cultures (Fig. 5). In contrast, however, in

CHox cultures, acute methylcholine significantly reduced basal DA release, while chronic (16 h), but not acute (15 min), atropine augmented even further the elevated basal DA levels characteristic of these cultures (Fig. 5). These findings suggest that mAChR on rat glomus cells function to suppress DA release during chronic hypoxia.

Are DA transporters down-regulated in cultures exposed chronically to hypoxia or nicotine?

Dopaminergic transmission is initiated by the presynaptic release of DA and terminated largely by re-uptake through a specific, sodium-dependent dopamine transporter (DAT). Thus, the effective concentration of DA at the synapse, and therefore, the level of DA receptor stimulation is regulated by DAT activity. In the carotid body, the concentration of extracellular DA could be regulated by a similar re-uptake mechanism, which itself may undergo long-term modulation by chronic hypoxia or nicotine. To test whether the enhanced basal DA secretion seen in CHox and CNic cultures was influenced by modulation of this re-uptake pathway, released DA was compared in Nox, CHox and CNic cultures, treated with nomifensine (1 μM), a potent inhibitor of DA uptake (Meiergerd and Schenk, 1994). Interestingly, in Nox cultures, acute (15 min) exposure to this drug caused ~3x increase in basal DA levels (Fig. 6), suggesting that under normoxia, a nomifensine-sensitive DA re-uptake mechanism helps limit the accumulation of extracellular DA. In marked contrast, however, nomifensine had no effect on DA accumulation in CHox and CNic cultures (Fig. 6; ratio of stimulated to basal DA release was ~1), suggesting that DATs are down-regulated or inactivated by

these chronic treatments, or that there is a change in their sensitivity to the drug.

DISCUSSION

This study provides novel insights into the cellular and molecular mechanisms underlying the elevated basal DA release in rat carotid body (CB) cultures after chronic hypoxia *in vitro* (Jackson and Nurse, 1997a), and suggests a modulatory role for ACh and involvement of the DA transporter in the regulation of extracellular DA (see Fig. 7). In addition, we show that an ingredient of cigarette smoke, i.e. nicotine, which has previously been reported to attenuate the protective CB arterial chemoreflex response when administered chronically in mammals (Holgert et al., 1995), mimicks the effect of chronic hypoxia in causing elevation of basal DA release in CB cultures. In general, the regulation of extracellular DA is likely to be important for CB function since the prevailing evidence suggests that released DA, acting via pre- and post-synaptic D_2-receptors, inhibits the CB hypoxic ventilatory response (see Gonzalez et al., 1994; Holgert et al., 1995) and appears to be involved in the resetting of CB chemosensitivity in the perinatal period (Hertzgberg et al., 1990). In a recent study (Jackson and Nurse, 1997a), we found that "basal release" of DA, determined by HPLC, was substantially elevated in rat CB cultures after chronic hypoxia *in vitro*, though the effect did not involve entry of extracellular Ca^{2-} through L-type calcium channels. This "basal release" however, actually represents the level of extracellular DA around secretory glomus cell clusters and is set by a combination of factors including : (i) DA released by the direct

effect of the prevailing Po_2 on oxygen-sensitive glomus cells (see Jackson and Nurse, 1997a); (ii) the secondary action of released neurotransmitters (including ACh, DA, etc) acting within glomus cell clusters in an autocrine and paracrine fashion so as to faciltitate or inhibit release; and (iii) re-uptake into glomus cells by DA transporters. In the present study, the use of pharmacological blockers of nicotinic and muscarinic AChR, and of the DA transporter, allowed us to investigate whether the latter two factors play significant roles in the regulation of extracellular DA during normoxia, and after chronic exposure of CB cultures to hypoxia and nicotine.

Regulation of extracellular DA in normoxic CB cultures

We previously showed that in normoxic (Nox) cultures DA release can be stimulated by : (i) acute hypoxia in a dose-dependent fashion; (ii) blockade of oxygen-sensitive Ca-dependent K^- channels with iberiotoxin; and (iii) exposure to high extracellular K^- (Jackson and Nurse, 1997a). In these experiments stimulus-evoked DA release was compared to basal release, determined at a Po_2 of ~ 160 mmHg, and though evoked release appeared to involve Ca^{2-} entry through voltage-dependent L-type calcium channels, the factors that controlled basal release levels were unknown. In the present study we considered the possibility that under normoxic conditions activity of the DA transporter may actually limit the accumulation of extracellular DA in the immediate vicinity of the glomus cell clusters, and therefore contribute to the magnitude of the "basal release" measurement. The DA transporter may be considered part of the normal biochemical machinery for terminating the action of released DA at pre- and post-

synaptic receptors during dopaminergic neurotransmission (Meiergerd et al., 1993). Interestingly, we found that inhibition of the DA transporter with nomifensine caused ~3x increase in basal DA release. Since nomifensine is known to increase synaptic DA by a mechanism that requires exocytosis from actively secreting dopaminergic cells (Carboni et al., 1989), it appears that in Nox cultures extracellular DA concentration is regulated partly by reuptake of released DA. This process may even be facilitated by a feedback mechanism involving D_2 autoreceptors on glomus cells (Gonzalez et al. 1994), since agonist stimulation of these receptors can up-regulate the function of the DA transporter by increasing its V_{max} (Meiergerd et al., 1993).

Regulation of extracellular DA in chronically hypoxic CB cultures

In the present study we obtained evidence that regulation of at least three factors, i.e. nicotinic and muscarinic AChR as well as the DA transporter, during chronic hypoxia *in vitro* may contribute to marked elevation in extracellular DA observed in chronically hypoxic (CHox) CB cultures (see Jackson and Nurse, 1997a). The potential role of each factor, and their possible interactions, is considered below.

(i) *Role of nicotinic AchR*

Since functional nAChR are expressed on rat glomus cells (Wyatt and Peers, 1993; Dasso et al., 1997; see also Figs. 1, 2), and ACh is thought to be a neurotransmitter released from glomus cells during hypoxia (Eyzaguirre and Zapata, 1984; Gonzalez et al., 1994), then chronic activation of nAChR in CHox cultures may lead to long term changes in membrane properties. Conceivably, increased spontaneous ACh release during chronic

hypoxia and accompanying agonist-induced up-regulation of nAChR on glomus cells could lead to an enhanced basal DA release, via augmented calcium entry through the cation non-selective receptor channels (see Mollard et al., 1995). However, the mechanisms by which nicotinic agonists and antagonists regulate nAChR density and function during chronic stimulation are complicated by receptor desensitization and turnover rate; the end result may be either receptor up- or down- regulation, depending on AChR subtype, central vs peripheral location, as well as pattern of delivery and dose of agonist/antagonist (Lapchak et al., 1989; Lukas, 1991; Rowell and Li, 1997). Nevertheless, it is noteworthy that blockade of neuronal nAChR with mecamylamine led to an almost complete inhibition of the exaggerated DA release present in CHox cultures. The fact that acute (15 min) exposures of the cultures to mecamylamine, i.e. during the time of HPLC sampling, were as effective as chronic exposures in preventing the exaggerated basal release, suggests that there is a persistent increase in nAChR activity in CHox cultures and this acts in a positive feedback manner to increase extracellular DA. However, the status of the nAChR in these experiments is rendered uncertain by the observation that whereas acute nicotine significantly stimulated DA release in Nox cultures, it failed to do so in CHox ones. An additional possibility is that stimulation of nAChR may also have an inhibitory action on the function of the DA transporter (see below), and therefore on the level of extracellular DA (Yamashita et al., 1995).

(ii) *Role of DA transporter*

As discussed above, it appears that the activity of the DA transporter normally

limits the accumulation of extracellular DA in Nox cultures, by re-uptake (presumably into glomus cells) via a nomifensine-sensitive pathway. Since in CHox cultures the basal levels of extracellular DA were unaffected by nomifensine the possibility is raised that the DA transporters are down-regulated or functionally inhibited after chronic hypoxia *in vitro*. A similar mechanism could also operate *in vivo* where tissue catecholamine is also elevated in the CB of rats exposed chronically to low oxygen in a chamber (Donnelly and Doyle, 1994b). The implications are there is little or no re-uptake of released DA in CHox cultures or that re-uptake occurs via an alternative pathway. The signaling mechanisms leading to possible down-regulation and/or inhibition of the DA transporter during chronic hypoxia are unknown. Given the similarity of responses of glomus cells grown chronically in hypoxia and nicotine, with respect to the loss of nomifensine sensitivity, the possibility is raised that chronic stimulation of nAChR indirectly regulates activity of the DA transporter. This point is discussed further during consideration of nicotine-treated cultures.

(iii) *Role of muscarinic AchR*

Muscarinic ACh receptors (mAChR) regulate a number of important basic physiologic functions by modulating the activity of several effector enzymes and ion channels (reviewed by Felder, 1995). Reports of mAChR function in the CB and the receptor subtypes involved are varied and species dependent. In the rabbit CB, muscarinic agonists depressed chemosensory discharge (Monti-Bloch and Eyzaguirre, 1980), and inhibited both the release of CA and the increased discharge evoked by

nicotine (Dinger et al., 1991). However, in *singly-isolated* rat glomus cells the muscarinic agonist, methylcholine, evoked a biphasic increase in intracellular Ca^{2+}, that arose from both intracellular and extracellular sources (Dasso et al., 1997). Though this response might be expected to facilitate DA release, we observed that in Nox rat CB cultures, containing predominantly *clusters* of glomus cells, neither the muscarinic agonist, methylcholine, nor the antagonist, atropine, had any effect on basal DA release. These results contrast with the profound effects of these same agents in CHox cultures. In particular, whereas methylcholine strongly suppressed the exaggerated basal DA release in CHox cultures, atropine produced an *additional* elevation in this release. These results suggest that at least in CHox cultures mAChR have an inhibitory function on DA release, and ACh released under these conditions can reduce extracellular DA by a negative feedback mechanism. Indeed, the apparent low activity of mAChR in Nox compared to CHox cultures raises the possibility that mAChR may become up-regulated in glomus cells after chronic hypoxia *in vitro*. Interestingly, in the rat, chronic hypoxia *in vivo* causes an increase in both muscarinic M_2-receptor affinity and density in cardiac tissue (Kacimi et al., 1993). Thus it appears that both mAChR and nAChR (see above) may play a more active role in CHox cultures, and that activation of this dual receptor system on the same or different glomus cells (see Dasso et al., 1997) can lead respectively to both negative and positive regulation of extracellular DA, and presumably the level of CB inhibitory drive after chronic hypoxia.

Regulation of extracellular DA in cultures treated with chronic nicotine

Rat CB cultures grown in the presence of chronic nicotine (CNic) displayed some of the features of those grown in chronic hypoxia (CHox). In particular, there was a substantial elevation in basal DA release that was considerably reduced when the nicotinic antagonist, mecamylamine, was present either chronically, or acutely, i.e. during the 15 min period when the HPLC samples were collected. Moreover, the presence of the inhibitor of the DA transporter, nomifensine, was without effect on extracellular DA levels in both CNic and CHox cultures. The similarity of these findings suggests that direct stimulation of nAChR per se, by nicotine in CNic cultures and by synaptic ACh release between neighboring glomus cells in CHox cultures, leads to down-regulation or inhibition of the DA transporter. Interestingly, in related studies a mechanism involving cross-talk between the nAChR and DA transporter, has been described in PC 12 cells, where nicotine regulates extracellular DA by an indirect inhibition of DA uptake (Yamashita et al., 1995).

CNic cultures also resembled CHox ones in other ways. For example, in both sets of cultures whereas acute hypoxia significantly stimulated DA release (~2x basal), acute nicotine was ineffective. These observations raise the question whether some functional inactivation of nAChR occurred on glomus cells exposed to these chronic treatments (see Lukas, 1991). Though nAChR were present on glomus cells under *all* culture conditions (Fig. 1), as revealed by binding of monoclonal antibody mAb 27.35.74, which appears to recognize an epitope on the main immunogenic region (MIR) of the α subunit (Chase et al., 1987), the functional state of the receptors with respect to activation by nicotine may have been altered. The fact that the neuronal nAChR antagonist mecamylamine, when

present either acutely or chronically, largely abolished the elevation in basal DA release in both CNic and CHox cultures, suggests that at least functional nAChR were present.

Despite the above similarities between CNic and CHox cultures there was one notable difference. Whereas high extracellular K^+ markedly stimulated DA release (3-4x) in CHox cultures, presumably by its depolarizing action on glomus cells and stimulation of calcium influx through voltage-dependent L-type Ca^{2+} channels (Stea et al., 1995; Jackson and Nurse, 1997a), it had no significant effect on extracellular DA levels in CNic cultures. These findings suggest that chronic nicotine may also have an indirect effect on calcium channel regulation, though this possibility remains to be tested. In another study, high K^+-evoked release of ACh was selectively suppressed in the striatum of rats treated chronically with nicotine for 10 days (Lapchak et al., 1989).

Clinical implications

Our finding that exposure of rat CB arterial chemoreceptors to chronic nicotine *in vitro* can modify their physiological and biochemical properties has clinical implications. Nicotine is an active ingredient of cigarette smoke, and maternal smoking has been identified as a key risk factor in the development of Sudden Infant Death Syndrome (SIDS; see Holgert et al., 1995; Slotkin et al., 1995). Since nicotine can be readily transferred to the infant via the placenta prenatally, and in breast milk postnatally (for refs. see Holgert et al., 1995), the possibility of continuous nicotine exposure in the perinatal period is quite high for the offspring of smoking mothers. Previous studies suggest that chronic nicotine administration to perinatal rats induces release and synthesis

of carotid body DA, which acts on local D_2-receptors to produce inhibition of the hypoxic drive (Holgert et al., 1995). Also, in the latter study it was suggested that nicotine could also interfere with the normal postnatal resetting of CB chemosensitivity, and collectively, these mechanisms could act to attenuate the protective chemoreflex response. Our studies, which examined the *direct* effects of nicotine on CB chemoreceptors *in vitro*, also indicated a marked elevation in released DA. However, this response appeared to involve multiple factors, some of which may underlie the "blunting" effect of sustained hypoxia on the CB ventilatory response in the neonatal period (Dempsey and Forster, 1982; Eden and Hanson; 1987b). In particular, our studies suggest that chronic nicotine may cause elevation in extracellular DA in part by inhibiting the activity of the DA transporter via nAChR on chemoreceptor cells. Chronic nicotine *in vitro* also appeared to interfere with mechanisms underlying high K^+ -evoked DA release from these cells. It will be of interest to test whether similar mechanisms also affect chemoreceptor physiology during chronic nicotine administration *in vivo*.

Figure 1. Immunofluorescence labeling of glomus cells cultured under different environmental conditions. Each horizontal row of micrographs shows the *same* microscopic field viewed under phase-contrast (left), with a fluorescein filter set to reveal positive (green) immunofluorescence for tyrosine hydroxylase (TH; middle), and with a rhodamine filter set to reveal positive (Texas red) immunofluorescence for nAChR (right). Upper row (a, b, c) represents a control glomus cell cluster grown for 12 days in normoxia; middle row (d, e, f) represents a glomus cell cluster exposed chronically to nicotine (10 μM) for 10 days; bottom row (g, h, i) represents a glomus cell cluster grown for 9 days in chronic hypoxia (6% O_2). Scale bar = 20 μm.

a
d
g

Figure 2. Comparison of the effects of acute nicotine and high extracellular K^+ on normalized dopamine (DA) release in CB cultures grown in normoxia, chronic hypoxia, or chronic nicotine. Cultures were grown in normoxia (Nox; 20% O_2) for 12 days, chronic hypoxia (CHox; 6% O_2) from day 2-11, or normoxia plus nicotine (10 μM; CNic) from day 2-12. CHox cultures were returned to normoxia 12-16 h before release experiments began. Compared with Nox cultures, basal DA release (ordinate), normalized to the number of TH^+ cells present, was significantly ($p<0.01$; Student's t test) elevated by ~9x, in both CNic and CHox cultures . In Nox cultures, acute nicotine (100 μM) and high extracellular K^+ (30 mM) stimulated DA release by ~5x and 10x basal, respectively. In contrast, acute nicotine failed to stimulate DA release significantly above basal in both CNic and CHox cultures. Interestingly, although high K^+ stimulated DA release in CHox cultures (3-4x basal; right column), it was without effect in CNic cultures. Bars represent mean ± S.E.M. for the number of cultures indicated.

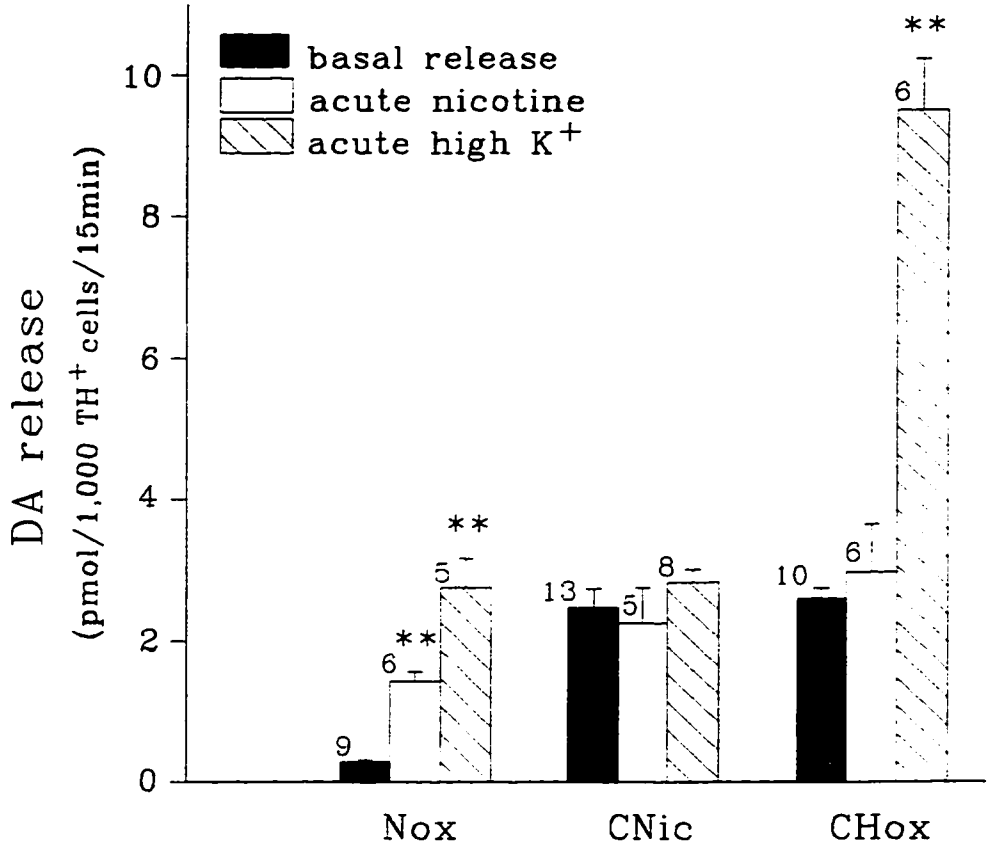

basal release
acute nicotine
acute high K+
DA release
(pmol/1,000 TH+ cells/15min)
0
2
4
6
8
10
Nox
CNic
CHox
9
6
5
13
5
8
10
6
6
**
**
**

Figure 3. Comparison of the effects of acute hypoxia on DA release in cultures treated chronically with nicotine (CNic) or hypoxia (CHox). Hypoxia-evoked release is expressed as a ratio (ordinate) relative to basal; release samples were collected during 15-min exposures separated by a 10 min recovery period (see Materials and Methods). Exposure to acute hypoxia (5% O_2) stimulated DA release by ~2x basal in both CNic and CHox cultures. Bars represent mean ± S.E.M. for the number of cultures indicated.

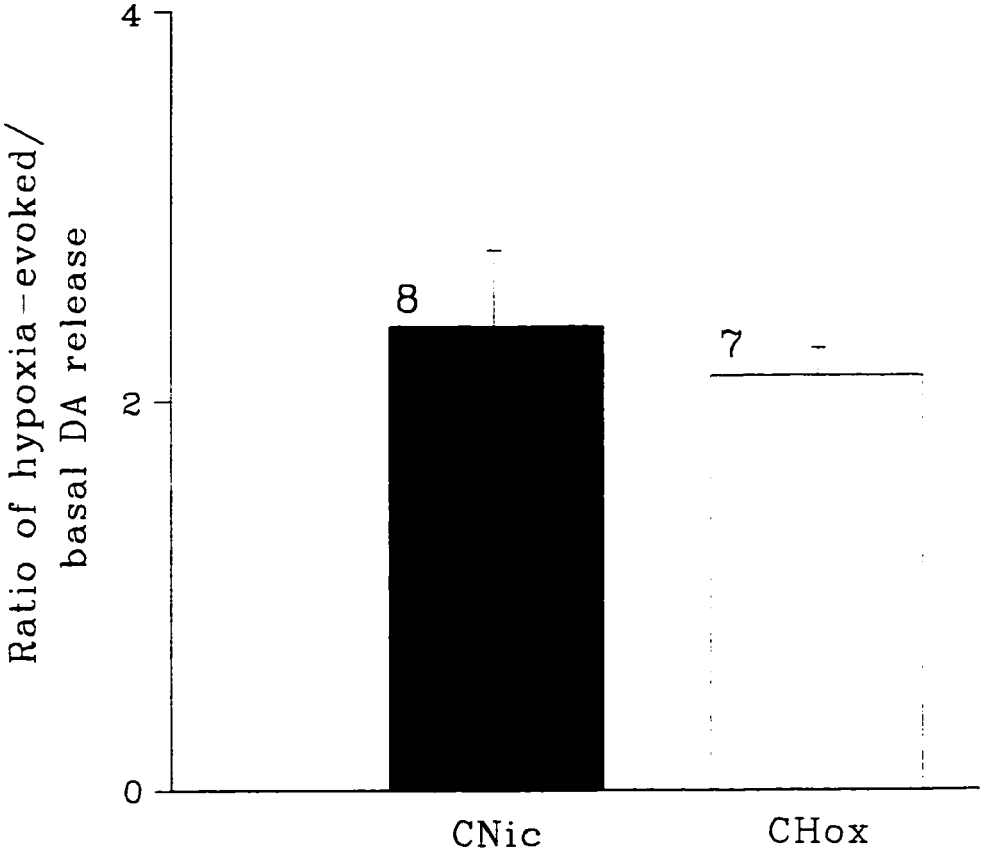

Ratio of hypoxia-evoked/
basal DA release
4
2
0
8
7
CNic
CHox

Figure 4. Effects of chronic and acute blockade of nAChR on basal DA release in CHox and CNic cultures. **A.** HPLC peaks of basal DA release from two CHox "sister" cultures, one of which was treated with chronic mecamylamine (100 μM). Cultures were returned to normoxia (20% O_2) during the final 16 h before release experiments began. Note the mecamylamine-treated CHox culture had a dramatically reduced basal DA release (bottom peak), even though both cultures had similar numbers of TH-positive glomus cells (475, top; 470, bottom peak). **B.** Comparison of the effect of mecamylamine on basal DA release in Nox, CNic and CHox cultures. Acute (15 min) and chronic (10 days) exposure to mecamylamine (100 μM) significantly (**$p<0.01$; Student's t test) reduced basal DA release in both CNic and CHox, but not Nox, cultures. Bars in B represent mean (± S.E.M.) for the number of cultures indicated.

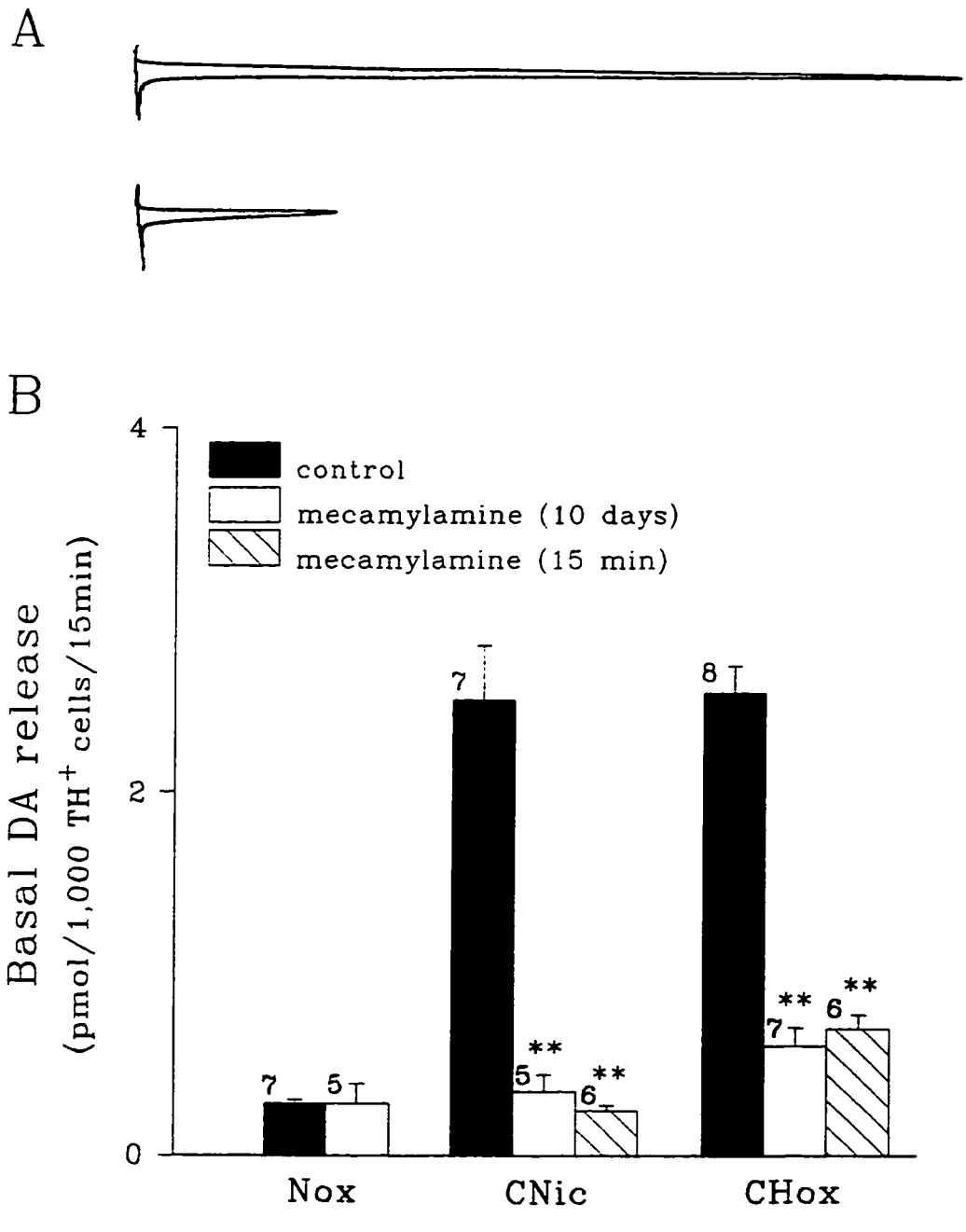

A
B
control
mecamylamine (10 days)
mecamylamine (15 min)
Basal DA release
(pmol/1,000 TH+ cells/15min)
4
2
0
7
5
7
5
**
6
**
8
7
**
6
**
Nox
CNic
CHox

Figure 5. Comparison of the effects of muscarinic agonists and antagonists on DA release in Nox and CHox cultures. In Nox cultures, neither acute (15 min) methylcholine (300 μM) nor long-term (16 h) atropine (1 μM) altered basal DA release. In contrast, in CHox cultures acute methylcholine significantly (**$p<0.01$) reduced basal DA release, while *long-term* atropine caused an additional enhancement of DA release. However, *acute* atropine was without effect in CHox cultures. Each bin represents mean (± S.E.M.) for the number of cultures indicated.

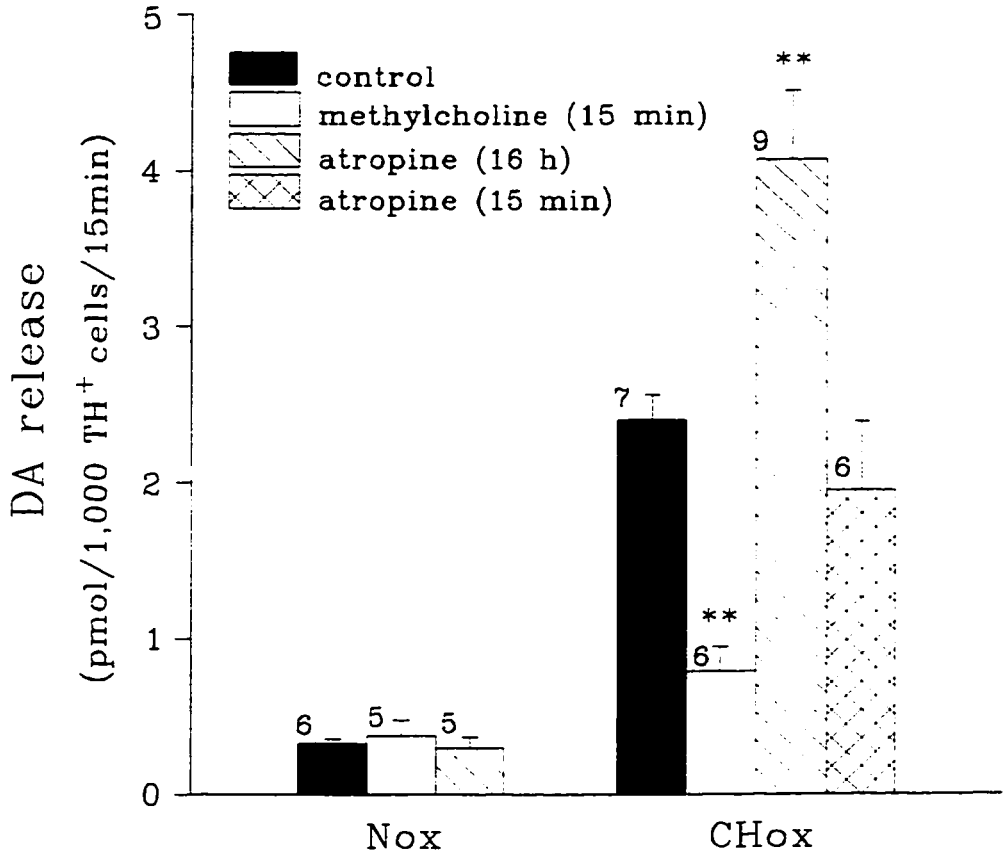

DA release
(pmol/1,000 TH+ cells/15min)
5
4
3
2
1
0
control
methylcholine (15 min)
atropine (16 h)
atropine (15 min)
6
5
5
7
**
6
9
**
6
Nox
CHox

Figure 6. Effect of blockade of DA transporters on extracellular DA in normoxic (Nox), chronic nicotine (CNic), and chronically hypoxic (CHox) cultures. Cultures were exposed (15 min) to 1 μM nomifensine, a blocker of the DA transporter, and extracellular DA expressed as a ratio relative to control (without blocker present), collected during first (15 min) sample (see Materials and Methods). Acute nomifensine caused ~3x increase in extracellular DA in Nox cultures, but was *without* effect in CNic and CHox cultures (ratio = ~1). Each bin represents mean (± S.E.M.) for the number of cultures indicated.

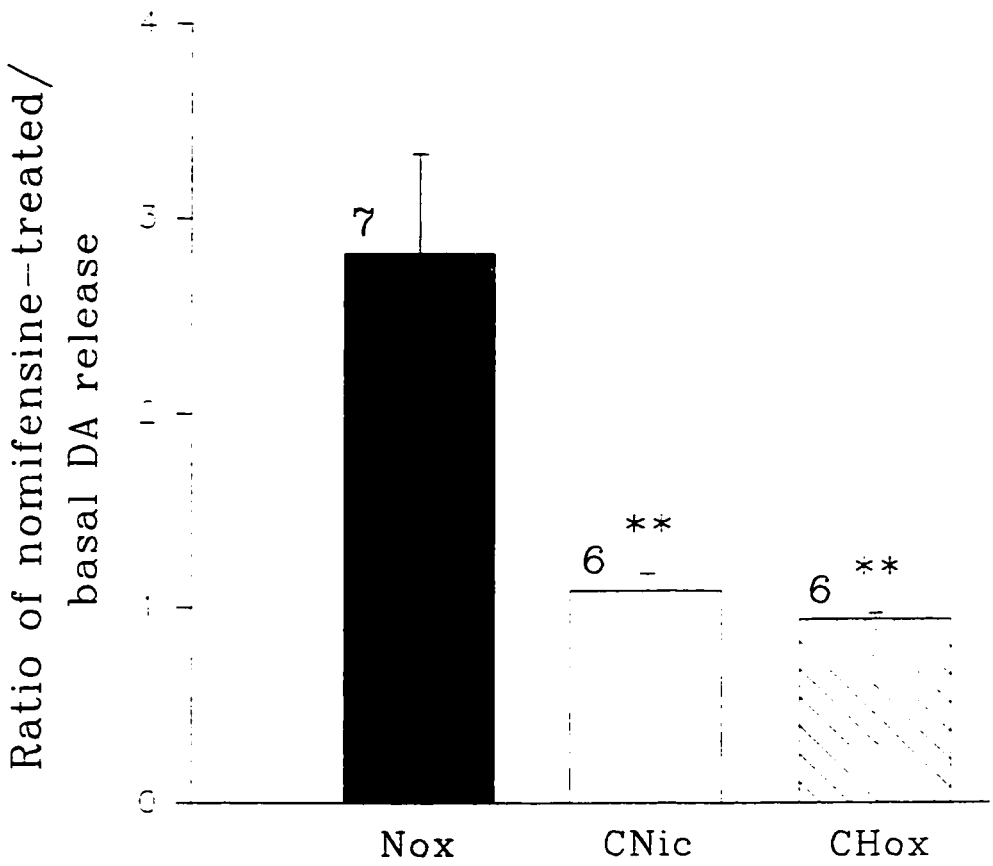
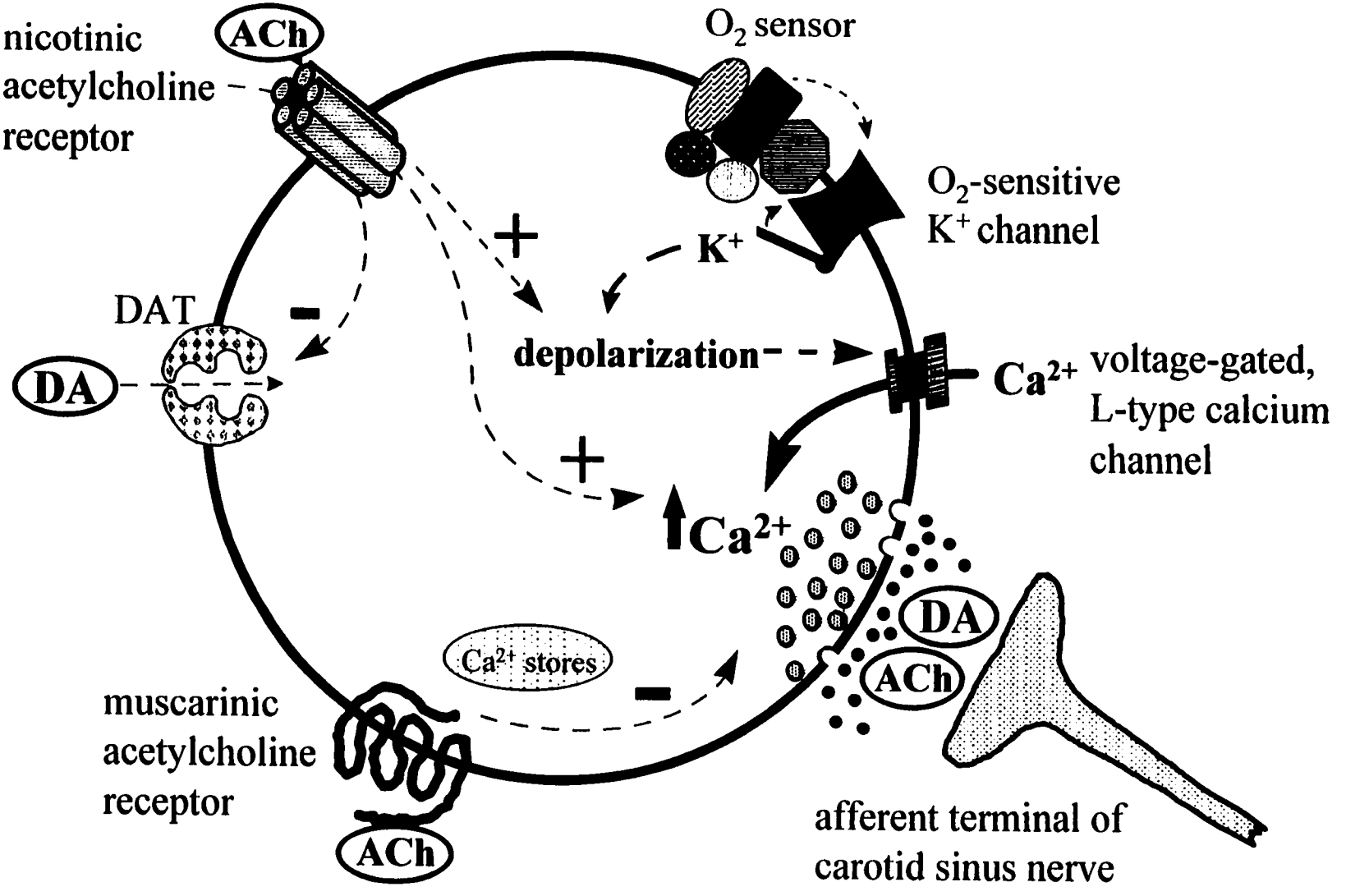

Ratio of nomifensine-treated/
basal DA release
7
6
**
6
**
Nox
CNic
CHox

Figure 7. Modulation of rat carotid body DA by ACh in a chronically hypoxic environment. ACh, released together with DA from glomus cells, binds to both muscarinic and nicotinic AChR receptors located on the same or nearby glomus cells within the cluster. On binding to nAChRs, ACh can stimulate DA by at least three potential mechanisms : (i) triggering Ca^{2-} influx and catecholamine secretion via nicotinic receptor cationic channels; (ii) depolarizing the plasma membrane and opening of voltage-activated Ca^{2-} channels; and (iii) inhibiting DA re-uptake by an indirect mechanism involving cross-talk between nAChR and the DA transporter. Muscarinic AChR (mAChR), likely acting via second messengers, may provide negative feedback regulation of DA to counterbalance the positive effects of nAChR. Hence, ACh can act at two different sites on glomus cells to regulate the concentration of extracellular DA (and other potential neurotransmitters) to provide fine control of the final chemoreceptor output after chronic hypoxia.


nicotinic
acetylcholine
receptor
ACh
O_2 sensor
O_2-sensitive
K^+ channel
K^+
DAT
DA
depolarization
Ca^{2+} voltage-gated,
L-type calcium
channel
Ca^{2+}
Ca^{2+} stores
DA
ACh
muscarinic
acetylcholine
receptor
ACh
afferent terminal of
carotid sinus nerve

CHAPTER 4

Developmental loss of hypoxic chemosensitivity in rat adrenomedullary chromaffin cells.

The majority of this work has been published as a co-authored paper by R.J. Thompson, A. Jackson, and C.A. Nurse in *J. Physiol. (Lond.)* Vol. 498.2, pp. 503-510 (1997). All data presented in this chapter represents work done entirely by me.

SUMMARY

Using short-term (1-2 days) cultures prepared from neonatal (P1-P2) and juvenile (P13-P20) rat adrenal medullae, we investigated whether adrenomedullary chromaffin cells (AMC) express O_2-chemoreceptive properties. Exposure of *neonatal* AMC cultures to a moderate (10% O_2) or severe (5% O_2) hypoxia for 1 hr caused a dose-dependent stimulation (~ 3x or 6x normoxia (20% O_2), respectively) in catecholamine (CA) release, mainly epinephrine, determined by High Performance Liquid Chromatography (HPLC). This hypoxia-evoked CA release was dependent on the entry of extracellular Ca^{2+}, since it was abolished by the L-type calcium channel blocker, nifedipine (10 μM). CA release in neonatal AMC cultures was also stimulated by high extracellular K^+ (30 mM) and iberiotoxin (200 nM), a known blocker of oxygen-sensitive, Ca^{2+}-dependent K^+ channels

in a related cell type, i.e. glomus cells of the carotid body. This stimulatory effect of iberiotoxin on CA release was comparable to that evoked by 10% O_2 in neonatal AMC cultures. Exposure of neonatal AMC to normoxia, acute hypoxia (10% and 5% O_2) and iberiotoxin resulted in preferential release of epinephrine (E) over norepinephrine (NE; ratio of E/NE≈6-7). However, CA release evoked by high extracellular K^+ was less selective for epinephrine (i.e., ratio of E/NE≈2-3). In contrast to the above results on *neonates*, hypoxia (10% and 5% O_2) had no significant effect on CA release in cultures of *juvenile* AMC. This was not due to a loss or impairment of secretory function in culture, since CA release was stimulated in juvenile cultures by high extracellular K^+. The ratio of E/NE released by high K^+ in juvenile AMC cultures was ~2-3, a value similar to that obtained for basal release over the full range of O_2 tension (5-20% O_2) in response to acute hypoxia, high extracellular K^+, and normoxia. These results suggest that rat adrenal chromaffin cells possess a developmentally-regulated O_2-sensing mechanism, similar to carotid body type I cells, and the activity of Ca^{2+}-dependent K^+ channels may also play an important role in this process.

INTRODUCTION

In the perinatal period, catecholamine (CA) release from adrenomedullary chromaffin cells (AMCs) is critical for the animal's ability to survive stresses associated with delivery and the transition to extrauterine life. This release plays a vital role in the modulation of cardiovascular, respiratory and metabolic responses to stressors such as hypoxia (Lagercrantz and Slotkin, 1986; Slotkin and Seidler, 1988). In some species such as rat and man, sympathetic innervation of the adrenal medulla is immature or absent in the neonate, yet the animal can still elicit the vital catecholamine surge in response to hypoxic challenge (Seidler and Slotkin, 1985). Seidler and Slotkin (1985) showed that in the newborn rat, acute hypoxia reduces adrenal catecholamines through a "non-neurogenic" mechanism, which disappears postnatally with a rough correlation to the maturation of the sympathetic innervation of the adrenal medulla. Furthermore, they demonstrated that sympathetic denervation of the adrenal medulla in mature animals causes a gradual re-appearance of this non-neurogenic mechanism (Seidler and Slotkin, 1986).

The mechanisms underlying the non-neurogenic response of the adrenal medulla to hypoxia are unknown. One possibility is that it is mediated indirectly, via humoral factors released into the circulation during hypoxia. Alternatively, adrenomedullary chromaffin cells might themselves possess O_2-sensing mechanisms, similar to their

neural crest counterparts in the carotid body, i.e. glomus or type I cells, which are the prototype for O_2 chemoreceptors in mammals (Gonzalez et al., 1994). These cells respond to hypoxia by suppression of an outward K^+ current (Lopez-Barneo et al., 1988; Delpiano and Hescheler, 1989; Peers, 1990b; Stea and Nurse, 1991), membrane depolarization and/or increased action potential frequency (Lopez-Barneo et al., 1988; Buckler and Vaughan-Jones, 1994), leading to entry of extracellular Ca^{2-} and enhanced CA release, predominantly dopamine (Buckler and Vaughan-Jones, 1994; Montoro et al., 1996). In this study, using HPLC determination of CA release, the hypothesis was tested that newborn rat AMC share similar O_2-sensing properties to carotid body type I (glomus) cells, and that these properties are lost with postnatal maturation.

METHODS AND MATERIALS

Pregnant or lactating Wistar rats (Charles River, Quebec, Canada) and pups were housed in our animal facility under constant 12 h light-12 h dark cycle, according to the guidelines of the Canadian Council on Animal Care (CCAC). All procedures for animal handling and tissue removal were carried out according to CCAC guidelines. Animals were rendered unconscious by a blow to the head (1- to 14-day-old pups) or by inhalation of the anaesthetic Somnothane (Wyeth-Ayerst Canada Inc., Montreal, Quebec, Canada; 15- to 20-day-old pups). The pups were then immediately killed by decapitation or cervical dislocation and the adrenal glands removed.

Cultures

Primary cultures enriched in dissociated rat adrenomedullary chromaffin cells (AMCs) were prepared by a modification of methods previously described (Doupe et al., 1985a). Briefly, adrenal glands were dissected from rat pups of two age-groups, i.e. neonatal (postnatal day (P)1-P2) or juvenile (P13-P20). Most of the surrounding cortical tissue was trimmed and discarded, whereas the remaining central medulla was incubated in an enzymatic solution, containing 0.1% trypsin, 0.1% collagenase (Gibco, Grand Island, NY), and .01% deoxyribonuclease (Millipore Corp., Freehold, NJ) for 1 h at 37 °C. Following incubation, most of the enzyme was removed with a pipette and the

remainder was inactivated with growth medium consisting of F-12 nutrient medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 80 U/l insulin (Sigma), 0.6% glucose, 2 mM glutamine, 1% penicillin and streptomycin (Gibco), and 0.01% dexamethasone (Sigma). In most experiments, after mechanical dissociation of the tissue with forceps and trituration with a Pasteur pipette, the resulting cell suspension was preplated for 24 h on collagen to remove most of the cortical cells. The non-adherent chromaffin cells were then replated on the central wells of modified culture dishes (Nurse, 1990), coated with Matrigel (Collaborative Research, Bedford, MA, USA). The cells were grown at 37 °C in a humidified atmosphere of 95% air-5% CO_2 for 1-2 days before they were used for determination of CA release.

Catecholamine determination by HPLC

Catecholamines (CA) released from living cultures, were separated by HPLC (Waters, model 510) with a Spherisorb-ODS2 column (10x 0.46 cm, 3 μm particle size; Chromatography Sciences Co., Montreal, Quebec, Canada), coupled with an electrochemical detector (Coulochem II detector, model 5200; ESA, Inc., Bedford, MA, USA). The first detector in the analytical cell was set at 0.05 V to reduce interference by contaminating electroactive compounds at the second detector which was set at -0.3 V, the potential required for electroreduction of (-)-arterenol (norepinephrine), (-)-adrenaline (epinephrine), 3-hydroxytyramine (dopamine) and the internal standard, di-3,4-hydroxybenzylamine hydrobromide (DHBA). The mobile phase consisted of NaH_2PO_4 (6.9 g/L; Sigma), Na_2EDTA (80 mg/L; BDH Chemicals, Toronto, Ontario, Canada), and

heptanesulfonic acid (250 mg/L; Sigma) in water and 5% methanol; pH was adjusted to 3.5 with concentrated H_3PO_4. Chromatograms were analyzed with the aid of a Waters 740 Data Module (Millipore, Milford, MA, USA) and quantified by the peak area ratio method, using known external standards (25 nM) and the internal standard, DHBA.

In studies of CA release, cultures, grown on a circular area of ~8 mm diameter, were first rinsed in 1:1 Dulbecco's modified Eagle's medium/F-12 medium before a 1 h incubation at 37°C in 100 µl bicarbonate-buffered salt solution (BBSS), under an atmosphere of 5% CO_2 plus either normoxia (Po_2 ~ 20% O_2 or 160 mmHg), moderate hypoxia (Po_2 ~10% O_2 or 75 mmHg), or severe hypoxia (Po_2 ~5% O_2 or 35 mmHg), using a Forma Scientific O_2/CO_2 incubator. The BBSS contained (mM): NaCl, 116; KCl, 5; $NaHCO_3$, 24; $CaCl_2$, 2; $MgCl_2$, 1.1; HEPES, 10; glucose, 5.5; at pH 7.4; for high K^+ experiments 25 mM NaCl was replaced by equimolar KCl. In a few experiments the calcium channel blocker, nifedipine (10 µM; Sigma), and the Ca^{2+}-dependent K^+ channel blocker, iberiotoxin (200 nM; Alomone Laboratories, Jerusalem, Israel) was added to the BBSS. Each release sample was mixed with an equal volume of 0.1 M $HClO_4$ containing 2.7 mM Na_2EDTA and then stored at -80°C prior to HPLC analysis. CA release was compared between different treatments using Student's *t* test with the level of significance set at $p < 0.05$. Results are presented as mean±S.E.M.

Immunofluorescence

At the end of release experiments cultures were processed for tyrosine hydroxylase (TH) immunofluorescence , to obtain an absolute count of the number of

chromaffin cells present. Procedures for TH immunostaining were similar to those described previously (Nurse, 1990; Chapter 2, this thesis); the primary TH antibody (rabbit; Chemicon, El Segundo, CA, USA) was visualized with a fluorescein-conjugated goat anti-rabbit IgG secondary antibody (Cappel, Malvern, PA, USA).

RESULTS

Evidence that neonatal rat AMC sense oxygen

The potential for neonatal rat AMC to sense oxygen was investigated using catecholamine (CA) release as a functional assay. Using High Performance Liquid Chromatography (HPLC) with electrochemical detection, CA secretion was measured (Fig. 1A) in neonatal AMC cultures, following 1 h exposure to moderate (10 % O_2) or severe (5% O_2) hypoxia. The use of *anoxia* (0% O_2) was avoided since this stimulus is known to deplete CA from adult bovine chromaffin cells (e.g. Dry et al., 1991), conceivably by a different mechanism involving calcium release from intracellular compartments, e.g. mitochondria (see Duchen and Biscoe, 1992b). Further, to aid quantitation the number of chromaffin cells was estimated at the end of release experiments using tyrosine hydroxylase (TH) immunofluorescence (Fig. 1B). As shown in Fig. 2A, in *neonatal* cultures CA release per h, normalized to 10,000 AMC, was stimulated ~3 and 6 times above normoxic (20% O_2) basal release when cultures were exposed to 10% and 5% O_2, respectively. These results imply that neonatal AMC possess a mechanism for sensing oxygen. The CA release induced by both hypoxic stimuli was abolished by the L-type calcium channel blocker, nifedipine (10 μM; Fig. 2A), suggesting a requirement for the entry of extracellular calcium through voltage-gated L-type Ca^{2+} channels. Though epinephrine (E) was the major CA released (Fig. 2A),

hypoxia also stimulated norepinephrine (NE) and dopamine (DA) release (Fig. 2A). CA release was also stimulated ~2x basal when neonatal AMC cultures were exposed to high extracellular K^+ (30 mM; Fig 2A). The relative amounts of E to NE released with these different stimuli are discussed below.

Neonatal AMCs secrete preferentially more epinephrine than norepinephrine.

In neonatal AMC cultures, epinephrine was the predominant CA secreted under all of the conditions tested, i.e., normoxia, hypoxia, high K^+, and IbTx (Fig. 3A). Interestingly, the ratio of epinephrine (E) to norepinephrine (NE) released by normoxia and acute hypoxia (10% and 5% O_2) was 6-7, suggesting that hypoxia was equally effective in evoking CA secretion from both epinephrine- and norepinephrine-containing neonatal AMCs. Neonatal AMC cultures released significantly more norepinephrine in response to high extracellular K^+ in comparison to other stimuli (Fig. 3A). The E/NE ratio for high K^+ was 2-3 versus 6-7 for acute hypoxia.

Blockade of Ca^{2+}-dependent K^+ channels by IbTx stimulates epinephrine and norepinephrine release in neonatal AMC cultures.

In the rat carotid body, closure of large conductance, Ca-dependent K^+ channels by hypoxia is thought to be an important step in O_2 chemotransduction (Wyatt and Peers, 1995; Wyatt et al., 1995; see, however, Cheng and Donnelly, 1995; Buckler, 1997). Selective blockade of this channel with the scorpion venom IbTx stimulated dopamine release significantly above basal in both neonatal and juvenile carotid body cultures

(Chapter 2, this thesis). In a recent study (Thompson and Nurse, 1997), three types of O_2-sensitive K^+ currents have been described in neonatal AMC, and one of them is carried by IbTx-sensitive Ca^{2+}-dependent K^+ channels. To test whether these K^+ channels are open at the resting potential of neonatal chromaffin cells under normoxic conditions, we investigated whether IbTx (200 nM) could evoke CA release in neonatal AMC cultures. This was the case as shown in Fig. 4, where the secretion of both epinephrine and norepinephrine was significantly increased above basal. The magnitude of CA secretion was similar to that evoked by 10%O_2 (compare Figs. 2A and 4). In addition, the E/NE ratio for IbTx was the same as that for acute hypoxia (Fig.3A). Hence, at least some Ca^{2+}-dependent K^+ channels are open at the resting potential of neonatal AMC and their closure can initiate CA secretion.

Loss of hypoxic chemosensitivity in juvenile AMC

In contrast to the above results in neonates, exposure of *juvenile* AMC cultures to either hypoxic stimulus (5% or 10 % O_2) had no effect on the normalized CA release relative to basal conditions (Fig. 2B). In addition, CA release in juvenile AMC cultures was unaffected by nifedipine at the oxygen tensions tested (Fig. 2B). These results indicate that the ability of AMCs to secrete CA in response to an acute hypoxic stimulus is lost by 2 weeks of postnatal life. This is probably due to a developmental loss of hypoxic chemosensitivity in AMCs, rather than a failure of the secretory machinery, since in juvenile AMC cultures CA release could be stimulated 2-3x basal by a different stimulus, i.e. high extracellular K^+ (30 mM; Fig. 2B). The ratio of E to NE released by

normoxia, hypoxia (10% and 5% O_2), and high K^+ (30 mM) was 2-3 in juvenile AMC, which was much lower than in neonatal AMC under these conditions.

DISCUSSION

O_2-chemoreceptive properties is present in neonatal but not juvenile AMC

In this study we demonstrate that acute hypoxia stimulates catecholamine (CA) release in neonatal (P1-P2) chromaffin cells of the rat adrenal medulla and that this mechanism is lost with postnatal maturation. In addition, work also carried out in this laboratory (Thompson et al.,1997) has shown that acute hypoxia suppresses a voltage-dependent K^+-current and causes membrane depolarization. More recent studies suggest that at least 3 types of K^+ channels can be regulated by Po_2 in neonatal AMC (Thompson and Nurse, 1997), one of which is the Ca^{2+}-dependent K^+ channel. The fact that in the present study, a blocker of these channels, IbTx, also evoked CA release from these cells, suggests that some of these channels are open at the resting membrane potential of AMC. Therefore, the combined findings from these studies suggest that acute hypoxia causes closure of probably several K^+ channel subtypes (e.g., Ca-dependent K^+ channel and voltage-sensitive K^+ channel), which leads to membrane depolarization , influx of extracellular Ca^{2+} and CA release in neonatal AMCs. Significantly however, these properties were virtually absent in *juvenile* AMC, suggesting that the mechanisms for sensing oxygen in these cells are transiently expressed in the perinatal period. In this respect, they differ from chromaffin-like (type 1) cells of the carotid body, a major O_2-chemosensory organ which, throughout postnatal life, continues to sense oxygen and

regulate arterial Po_2 via reflex input to the respiratory centre (Gonzalez et al., 1994). The fact that the hypoxia-evoked CA release in neonatal AMC was abolished by the L-type calcium channel blocker, nifedipine (this study), and that the depolarization was often sufficient to trigger action potentials (Thompson et al., 1997), suggests that the hypoxia-sensing mechanism in these cells is similar to that described in other cell types including, carotid body type 1 cells (Lopez-Barneo et al., 1988; Delpiano and Hescheler, 1989; Peers, 1990b; Stea and Nurse, 1991; Buckler and Vaughan-Jones, 1994; Gonzalez et al., 1994; Montoro et al., 1996), and neuroepithelial bodies of the lung (Youngson et al., 1993).

In neonatal rats, the proportion of epinephrine to norepinephrine released into the circulation by stressors which act through a non-neurogenic mechanism is similar to that in the immature adrenal gland (Slotkin and Seidler, 1988). In many species, these two CAs arise from morphologically distinct cells that secrete one or the other (Coupland, 1965; Grynszpan-Winograd, 1974). The findings in this study suggest that hypoxia elicits secretion from both epinephrine- and norepinephrine-containing AMCs, such that the E/NE ratio remains unchanged in normoxic conditions. Therefore, hypoxia appears to enhance the rate of basal secretion from both types of AMCs, without preference for epinephrine- versus norepinephrine-containing cells. However, the reduction in E/NE ratio by high K^+ suggests that some stimuli are capable of stimulating the preferential release of one CA over the other. For instance, stimulation with high extracellular K^+ evoked the secretion of substantially more norepinephrine than epinephrine in cultured bovine AMCs, suggesting that the stimulus-secretion coupling mechanism or the

exocytotic machinery in the 2 cell types are different (Marley and Livett, 1987).

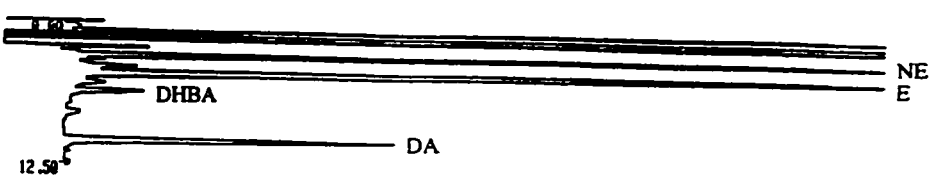
Importance of hypoxic chemosensitivity in neonatal animals

In the context of neonatal physiology, these results suggest a plausible 'non-neurogenic' mechanism for adrenal catecholamine release during hypoxic stress in the newborn rat, where sympathetic innervation of the adrenal medulla is immature or absent (Slotkin and Seidler, 1988; Lagercrantz and Slotkin, 1986; see also Cheung, 1990). This catecholamine release is crucial for survival of the neonate, producing multiple systemic effects that facilitate the transition from fetal to extrauterine life. Among these are absorption of lung fluid, secretion of surfactant (a process mediated by β_2-receptors), and regulation of cardiac function via stimulation of α- adrenergic receptors (Slotkin and Seidler, 1988). Thus, our data indicate that the increase in plasma catecholamine associated with birth could arise from the hypoxic-sensing mechanism we have uncovered in neonatal chromaffin cells, leading to membrane depolarization and increased action potential frequency, entry of extracellular calcium and catecholamine release. Interestingly, our results also account for the observation that this 'non-neurogenic' mechanism disappears in the rat during the first few weeks of postnatal life (Slotkin and Seidler, 1988). At this time adrenal catecholamine release in response to hypoxic stress is abolished by blockers of cholinergic transmission or by short-term denervation of the adrenal medulla (Slotkin and Seidler, 1988). Thus our finding that the hypoxic-sensing mechanism, present in the neonate, disappears in juvenile (P13-P20) chromaffin cells is consistent with a model where O_2 sensing in these cells is a

developmentally regulated process. It remains to be determined whether preganglionic sympathetic innervation can directly modulate O_2 chemosensitivity in adrenal chromaffin cells.

Figure 1. **A**, HPLC record of a release sample from a neonatal AMC culture (exposed to normoxic conditions, i.e. 20% O_2/5% CO_2) indicating peaks corresponding to the catecholamines: norepinephrine (NE), epinephrine (E), dopamine (DA), as well as to the internal standard DHBA (vertical scale readings in minutes). **B**, an example of rat chromaffin cells (diameter ~10 μm each) in a 2-day-old culture that was stained for tyrosine hydroxylase (TH) immunoreactivity, and visualized with a fluorescein-conjugated secondary antibody; cultures prepared in this way were used to obtain absolute chromaffin cell counts at the end of the release experiments, and for normalizing release to 10,000 TH^- cells in Figures 2A , 2B and 4.

A



B

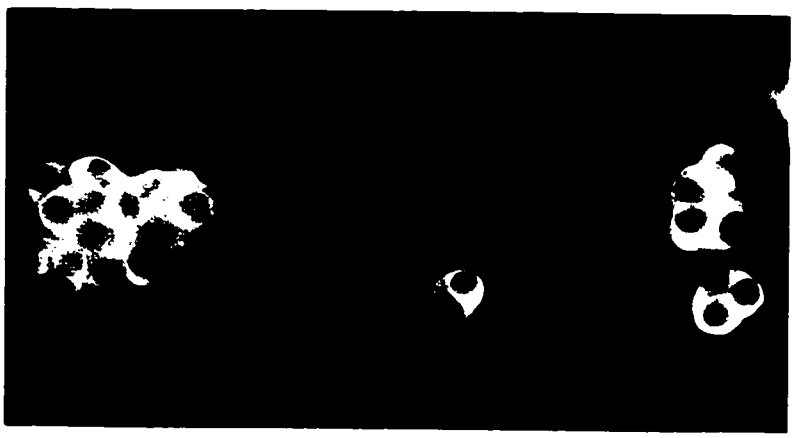


Figure 2. Comparison of normalized catecholamine release in neonatal and juvenile chromaffin cell cultures. **A**, histogram illustrating the stimulatory effects of moderate (10% O_2) and severe (5% O_2) hypoxia, and high extracellular K^+ (30 mM) on catecholamine (NE, norepinephrine; E,epinephrine; and DA, dopamine) release in *neonatal* AMC cultures after 1 h exposure; basal release is represented by 20% O_2 (normoxia). Hypoxia-induced catecholamine release is abolished by the L-type calcium channel blocker, nifedipine (Nif, 10 μM). **B**, histogram illustrating the lack of effect of moderate and severe hypoxia on catecholamine release in *juvenile* AMC cultures; in these cultures, however, high extracellular K^+ (30 mM) significantly stimulated catecholamine release. Bars in A and B represent mean ± S.E.M. for the number of cultures indicated; **P<0.01 and *P<0.05, release is significantly different from basal release (Student's *t* test).

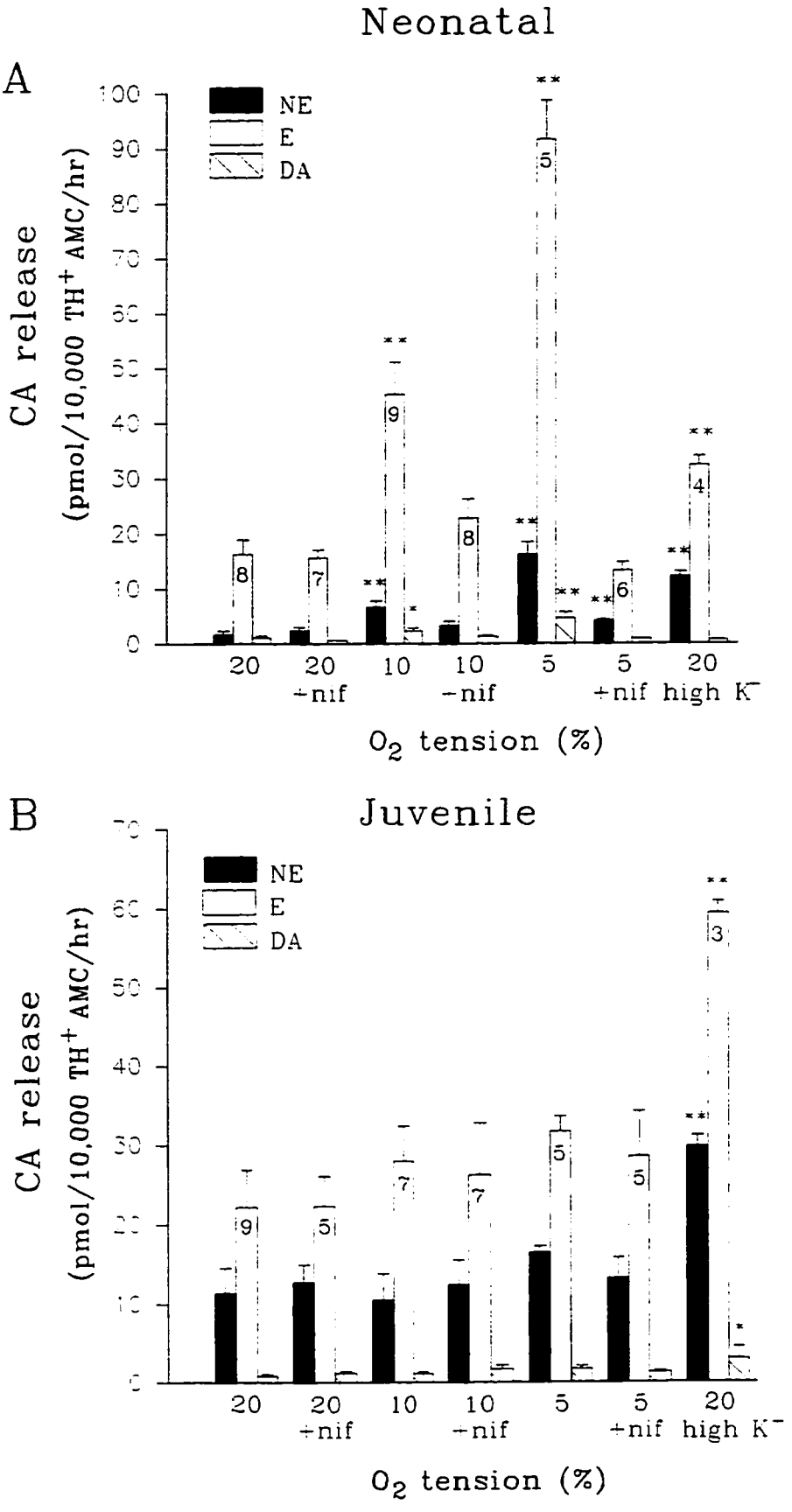
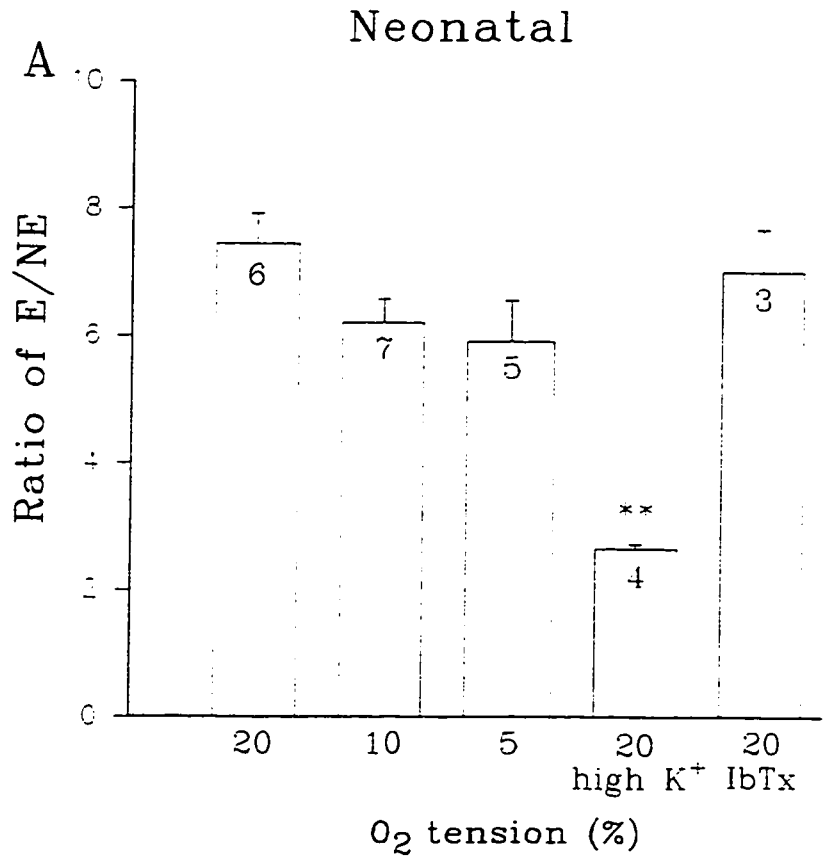

A
Neonatal
CA release
(pmol/10,000 TH+ AMC/hr)
NE
E
DA
100
90
80
70
60
50
40
30
20
10
0
20
20
+nif
10
10
−nif
5
5
+nif
20
high K+
O2 tension (%)
B
Juvenile
CA release
(pmol/10,000 TH+ AMC/hr)
NE
E
DA
60
50
40
30
20
10
0
20
20
+nif
10
10
−nif
5
5
+nif
20
high K+
O2 tension (%)

Figure 3. Comparison of the ratio of epinephrine (E) to norepinephrine (NE) release from neonatal (**A**) and juvenile (**B**) AMC cultures in response to different environmental factors. AMC cultures were exposed to normoxia (20% O_2), acute hypoxia (10% and 5% O_2), high extracellular K^+ (30 mM), or iberiotoxin (200 nM) for 1 h before monitoring catecholamine release; the ratio of epinephrine (E) to norepinephrine (NE) release is expressed on the vertical axis. In general, the ratio of E/NE release was higher in neonatal compared to juvenile cultures. Within each age group, the ratio of E/NE release in response to acute hypoxia was similar to that measured in normoxia. In juvenile AMC cultures, the E/NE ratio in response to high K^+ was also the same as observed in normoxia; however, in neonatal cultures, the ratio of E to NE release evoked by high K^+ was significantly reduced (**$P<0.01$, Student's *t* test) when compared with normoxia. IbTx evoked epinephrine and norepinephrine release from neonatal AMCs in the same ratio as that measured during basal (20% O_2) release. Bars in A and B represent mean±S.E.M. for the number of cultures indicated.


Neonatal
A
Ratio of E/NE
6
7
5
**
4
3
20
10
5
20
high K+
20
IbTx
O2 tension (%)

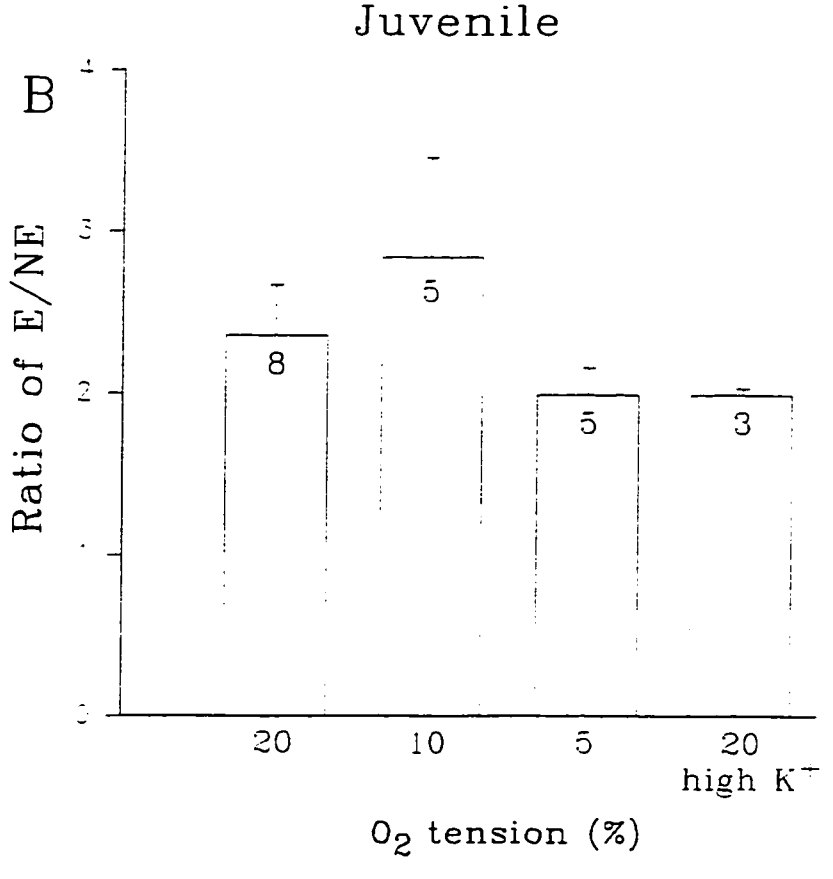
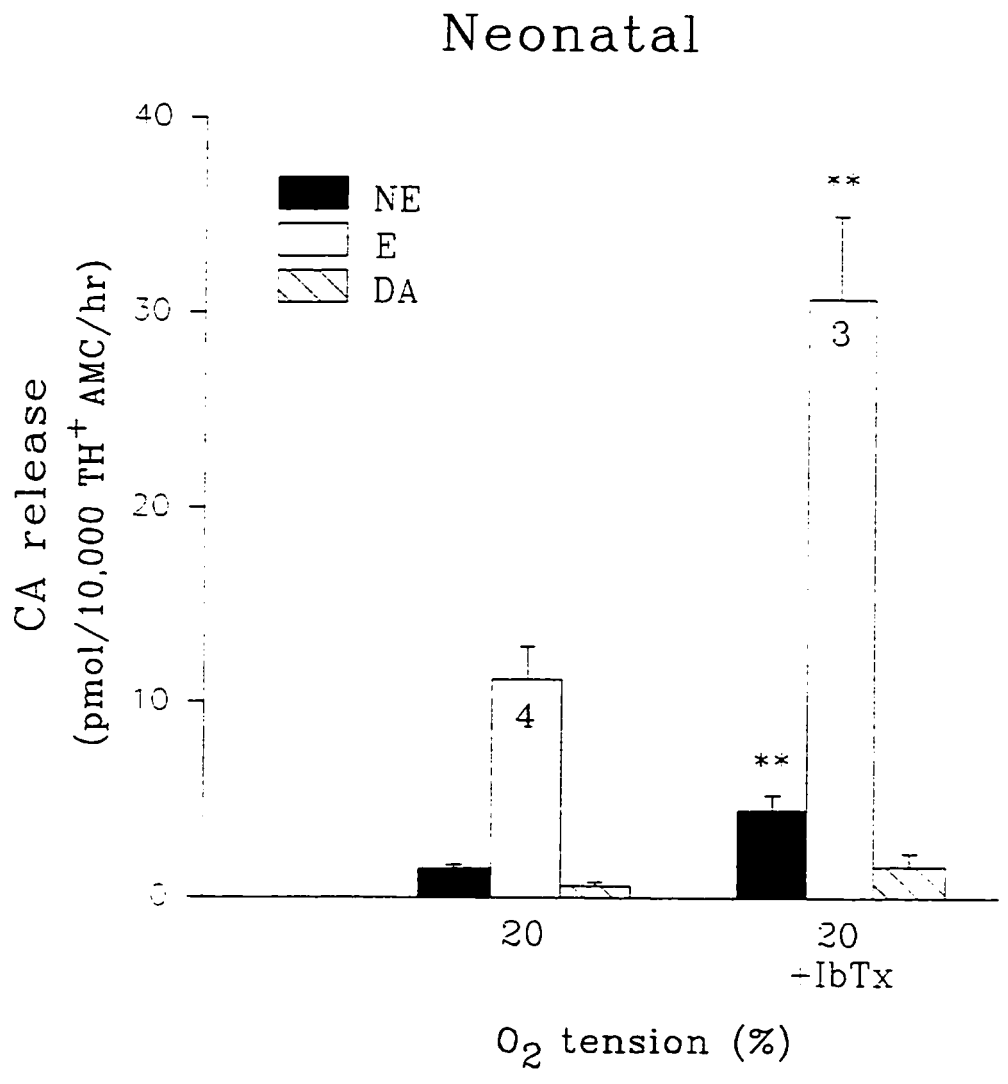

Juvenile
B
Ratio of E/NE
8
5
5
3
20
10
5
20
high K+
O2 tension (%)

Figure 4. A histogram illustrating the stimulatory effects of IbTx on epinephrine and norepinephrine release in neonatal AMC cultures. When compared with normoxic (20% O_2) or basal release, the normalized epinephrine (E) and norepinephrine (NE) release (ordinate) was significantly ($p<0.01$) greater following a 1h exposure to IbTx (200 nM). Bars represent mean±S.E.M. for number of cultures indicated.


Neonatal
40
30
20
10
0
NE
E
DA
**
3
4
**
20
20
+IbTx
CA release
(pmol/10,000 TH+ AMC/hr)
O2 tension (%)

DISCUSSION OF THESIS

It is now generally accepted that the glomus cell is the primary chemosensory element of the carotid body, and relays chemosensory information to the brainstem via neurotransmitter release at synaptic contacts with afferent terminals of the carotid sinus nerve (CSN; see however, Sun and Reis, 1994). The correlation between hypoxia-evoked neurotransmitter release and afferent chemoreceptor fiber discharge suggests that chemostimulus-induced transmitter release from glomus cells is a fundamental step in carotid body chemotransduction (Fidone et al., 1990; Gonzalez et al., 1992; Peers and Buckler, 1995). However, glomus cells contain multiple neuroactive agents (e.g., catecholamines, acetylcholine and neuropeptides), several of which are potential candidates for initiating or modulating CSN discharge during chemosensory signaling (Gonzalez et al., 1994). Most likely, the final chemoreceptor output results from the summed actions of *several* neuroactive agents at both presynaptic autoreceptors and postsynaptic receptors on afferent terminals of the CSN. Furthermore, carotid body catecholamines have been implicated in the resetting of chemoreceptor sensitivity after birth (Hertzberg et al., 1990), ventilatory acclimatization to chronic hypoxia (Bisgard et al., 1987), and "blunting" of the ventilatory response in high-altitude dwellers or neonates born and reared in hypoxia (Dempsey and Forster, 1982; Eden and Hanson, 1987b;

Hertzberg et al., 1990). The cellular and molecular mechanisms which underlie these adaptive responses of mammals to different oxygen environments are largely unknown.

In this thesis, the use of partially dissociated cell cultures of the rat carotid body and adrenal medulla, and HPLC measurements of the final catecholaminergic output from putative O_2-chemoreceptors, have provided valuable information regarding: (1) the role of K^+ channels in oxygen sensing; (2) cellular and molecular mechanisms which underlie the adaptative responses of glomus cells to chronic hypoxia and nicotine, and (3) developmental changes in O_2 transductive mechanisms in both glomus and adrenomedullary chromaffin cells isolated from neonatal and juvenile rats. The advantages of studying chemotransductive mechanisms in established cell cultures versus whole-animal or organ preparations include: (1) the growth of chemoreceptor cells in a controlled cellular, gaseous, and fluid environment where one factor, e.g., oxygen tension, can be manipulated without interference from secondary cardiovascular adjustments known to be associated with chronic hypoxia *in vivo*, e.g., acidosis and changes in stress-related factors such as glucocorticoids; (2) the lack of sympathetic and sensory innervation in carotid body and adrenal medulla cultures, eliminates potential sources of transmitter present *in vivo*; (3) the effects of drugs can be tested *directly* on glomus and chromaffin cells, whereas *in vivo*, the drug may have secondary effects via interactions with receptors on nerve endings or vasculature; and (4) measurements of catecholamine release *in vitro* can be normalized to the number of chemoreceptor cells present in the *same* culture using TH immunofluorescence, an approach which avoids the potential complications of glomus cell hyperplasia, reported in chronic hypoxia *in vivo*

(Dhillon et al., 1984). Furthermore, it is noteworthy that this *in vitro* approach also permitted some of the signaling pathways involved in oxygen chemotransduction and glomus cell plasticity during chronic hypoxia to be explored in ways not possible, or difficult *in vivo*. On the other hand, as is the case for all cell culture approaches, caution should be exercised in extrapolating *in vitro* results to the actual *in vivo* situation. In this study all cultures were grown in fetal calf serum, which represents an unusual environment for rat cells, and this could potentially reveal abnormal responses. Nevertheless, it is reassuring that several of the adaptive properties uncovered in glomus cells after chronic hypoxia *in vitro* have been observed following whole animal exposure to hypobaric hypoxia. These include glomus cell hypertrophy (McGregor, 1988), changes in Na, K and Ca ion channel function (Wyatt et al., 1995; Hempleman, 1995, 1996), as well as augmented basal CA secretion (Donnelly and Doyle, 1994b), studied in detail in this thesis.

Oxygen sensing in chromaffin cells of rat carotid body and adrenal medulla in a normoxic environment.

Developmental regulation of O_2-sensitive K^+ channel subtypes

The carotid body is well known to undergo postnatal development (Eden and Hanson, 1987a, b; Kholwadwala and Donnelly, 1992). For instance, O_2 sensitivity increases markedly from the fetus to the adult (Blanco et al., 1984; Pepper et al., 1995b), catecholamine secretion and CSN discharge evoked by hypoxia increases with postnatal age (Donnelly and Doyle, 1994), and the rise in intracellular Ca^{2+} in response to hypoxia

increases with age (Sterni et al., 1995). As newborns mature and adapt to the higher Po_2 in extrauterine life, a similar development or resetting of carotid body chemoreceptors can be seen at the level of K^+ channel expression (Hatton et al., 1997). Since K^+ channels are affected early during the signaling cascade initiated by low Po_2, the expression of different subtypes of K^+ channel may account, at least in part, for the postnatal development in carotid body chemosensitivity. Similarly, the expression of different types of K^+ channels in neurons varies substantially at different stages of development, regulating the excitability of neurons and the release of transmitter from nerve terminals. The time of expression, cellular abundance and distribution of specific K^+ channels are important factors that have been shown to influence the physiological properties of a number of cell types (Spitzer, 1991; Ribera and Spitzer, 1992).

In neonatal and mature rats it is well established that a major O_2-sensitive current is the Ca^{2+}-dependent K^+ current (Peers, 1990b; Hatton et al., 1997; Lopez-Lopez et al., 1997). Based on findings in Chapter 2, there appears to be a developmental shift in the subtype of K^+ channel coupled to hypoxia-evoked dopamine (DA) release in rat carotid body. The normalized basal DA release as well as the ratio of hypoxia-evoked relative to basal release (~5 fold) was similar for cultures prepared from neonatal (postnatal day 5 to 7) and juvenile (postnatal day 19-20) rats. However, blockade of Ca^{2+}-dependent K^+ (K_{Ca}) channels with IbTx in neonatal cultures stimulated DA release by only 2-3x basal, but the same concentration of IbTx stimulated DA release in juvenile cultures by >6x (see Chapter 2, this thesis). These findings are consistent with a postnatal maturation of functional Ca^{2+}-dependent K^+ channels in glomus cells. Recent electrophysiological

studies by Hatton et al. (1997) have confirmed a prominent postnatal maturation of Ca^{2+}-dependent K^+ channels (between days 4 and 10) in rats. In addition, our finding that only ~one-half of DA release can be attributed to IbTx-sensitive K^+ channels in neonatal glomus cells, suggests that alternative pathways are likely involved in mediation of hypoxia-evoked DA release. Conceivably, this additional signaling pathway could be mediated by IbTx-insensitive K^+ channels that are preferentially regulated by Po_2 in the neonatal period. It is possible that this channel could be similar to the charybdotoxin-insensitive, low conductance K^+ "leak" channel described by Buckler (1997).

Although there is no direct evidence, it is plausible that the increased sensitivity of the chemoreceptor response to hypoxia with postnatal development is a direct result of the type of K^+ channel that is coupled to the "O_2 sensor". Lopez-Barneo (1994) and Hatton et al. (1997) have suggested that the O_2 sensor is a distinct component which may couple with different K^+ channels. Perhaps, the increased availability of oxygen that occurs at birth is a signal which triggers *de novo* expression of K^+ channel subtypes which couple more effectively or preferentially with the O_2-sensor (e.g. NADPH oxidase). Low Po_2-induced closure of these "newly-coupled" K^+ channels may aid depolarization of the plasma membrane to a greater extent, and in turn, activate more voltage-dependent Ca^{2+} channels, thereby facilitating Ca^{2+} influx and transmitter release. The same idea may apply to changes that occur in glomus cell sensitivity upon exposure to chronic hypoxia (see below).

Physiological importance of Ca^{2+}-dependent K^+ channels in hypoxia-evoked dopamine

release from glomus cells.

Many studies have demonstrated contrasting results with regard to the nature of the O_2-sensitive K^+ channel in rabbit and rat glomus cells. Hypoxia selectively inhibits a voltage-gated, Ca^{2+}-independent K^+ channel (Ko_2) in rabbit cells (Ganfornina and Lopez-Barneo, 1991, 1992) and a Ca^{2+}-dependent K^+ channel (K_{Ca}) in neonatal rat cells (Peers, 1990a, b; Wyatt and Peers, 1995). Recent studies by Hatton et al. (1997) show that differences in the nature of the K^+ channel inhibited by hypoxia in rat glomus cells compared with those characterized in adult rabbit cells is not due to the fact that rat glomus cells are immature, as has been speculated (see Gonzalez et al., 1994). Instead the differences in K^+ channel subtype described in rats and rabbits reflect a genuine species difference (see also Lopez-Lopez et al., 1997).

Recently, the physiological importance of K_{Ca} in O_2 chemotransduction in rat glomus cells has also been questioned. Previous studies by Peers and co-workers have suggested that high conductance K_{Ca} channels are active (open) at the resting membrane potential of the cells, since charybdotoxin (a blocker of K_{Ca}) and hypoxia depolarize glomus cells *in vitro* (Wyatt and Peers, 1995; Wyatt et al., 1995). However, pharmacological compounds which inhibit Ko_2 and K_{Ca} channels (e.g., TEA, 4-AP and charybdotoxin) fail to excite the intact rat carotid body under normoxic conditions (Donnelly, 1995; Pepper et al., 1995a). Similarly, Buckler (1997) has shown that the same K-channel inhibitors failed to evoke a significant rise in $[Ca^{2+}]_i$, and TEA plus 4-AP failed to evoke a significant depolarization in rat glomus cells *in vitro*. Even though these pharmacological agents have low selectivity, these inconsistencies suggest that K_{Ca}

channels may not contribute to the resting membrane potential under normoxic conditions, and therefore closure of these channels cannot account for the depolarization, and neurotransmitter release, seen in hypoxia. Alternatively, Buckler (1997) has proposed that a new O_2-sensitive K^+-current, carried by charybdotoxin-insensitive, low conductance "leak" K^+ channels, is important in determining the resting membrane potential and in *initiating* the membrane depolarization seen in hypoxia. Presumably, this channel would serve to initiate electrical activity and Ca^{2+} influx, which could then be further controlled by the modulation of K_{Ca} channels.

In order to test the physiological importance of K_{Ca} channels in O_2 chemotransduction, it is necessary to establish whether a sufficient number of them are open at rest, such that their closure by hypoxia can trigger membrane depolarization and neurotransmitter release. This thesis investigated the contribution of K_{Ca} channels by pharmacologically blocking these channels with a highly specific scorpion venom, iberiotoxin, and then measuring DA release in cultures of rat glomus cells. Significantly, it was found that blockade of K_{Ca} channels in this way was sufficient to evoke DA release (~3x basal) *in vitro* (Jackson and Nurse, 1997a), suggesting that at least some IbTx-sensitive, Ca^{2+}-dependent K^+ channels are open at rest. In the future, it would be of interest to know whether suppression of the hypoxia-sensitive "leak" channel described by Buckler (1997) is sufficient to trigger catecholamine release from rat glomus cells. Based on the discussion in the previous section, it would not be surprising if closure of other types of K^+ channels by hypoxia led to catecholamine secretion from glomus cells.

In other cell types, K_{Ca} channels usually require either a depolarization and/or a

rise in intracellular Ca^{2+} in order to be activated. This suggests that the probability of K_{Ca} channels being open is low in resting glomus cells and as a result contribute little to the resting membrane potential (assuming a resting potential of ~ -50 mV; Buckler, 1997). However, glomus cells *in vivo*, as well as in our dispersed carotid body cultures, exist in clustered arrangements which permit intercellular communication (e.g. Ca^{2+} signaling and exchange of second messengers) via gap junctions and chemical synapses. Thus, there is the potential for paracrine/autocrine effects of released neurotransmitters on glomus membrane potential, via activation or inactivation of different ionic channels, or on intracellular Ca^{2+}, via liberation from intracellular stores. Therefore, glomus cells in their natural clustered arrangement may be spontaneously more active or have higher levels of intracellular Ca^{2+} than isolated cells used in most patch-clamp studies *in vitro*. If so, this could allow K_{Ca} channels to play an important role in chemotransduction in the rat. In addition, K_{Ca} channels have been shown to contribute to the resting potential of other cell types, such as vascular and smooth muscle cells where the resting membrane potential is between -55 and -70 mV, values similar to those of rat glomus cells used in patch clamp experiments (Trieschmann and Isenberg, 1989; Peers, 1990b; Stea et al., 1995).

Developmental regulation of oxygen sensing in adrenomedullary chromaffin cells.

Like their sympathoadrenal counterparts in the carotid body, rat adrenomedullary chromaffin (AMC) cells possess an O_2-sensing mechanism and more than one subtype of K^+ channel appears to transduce this stimulus in the neonate (Thompson and Nurse,

1997). Electrophysiological recordings from neonatal AMCs have shown that hypoxia suppresses a voltage-dependent K^+ current and causes membrane depolarization (Thompson et al., 1997). Results from this thesis have demonstrated a significant increase in catecholamine (CA) release (predominantly epinephrine) following hypoxia as well as after blockade of Ca^{2+}-dependent K^+ channels with iberiotoxin. Importantly, exposure of neonatal AMC cultures to moderate or severe hypoxia caused a dose- and Ca^{2+}-dependent stimulation in CA release (see Chapter 4). Taken together, these results suggest that in neonatal AMC acute hypoxia causes closure of various K^+ channels, including Ca^{2+}-dependent K^+ channels, leading to membrane depolarization and increased action potential frequency, entry of extracellular Ca^{2+} and CA release.

Significantly however, unlike glomus cells, these O_2-sensitive properties were virtually absent in juvenile AMCs. This suggests that the mechanism for adrenal CA release during hypoxic stress is only transiently expressed in the perinatal period, when sympathetic innervation of the adrenal medulla is absent or immature (Lagercrantz and Slotkin, 1986; Slotkin and Seidler, 1988). The temporal restriction of O_2-chemosensitivity in AMCs to the period around birth appears strategically designed to ensure the surge of adrenal CAs into the bloodstream at a critical period, i.e. during the hypoxia associated with delivery. This CA release is crucial for survival of the neonate, since it promotes the absorption of lung fluid, secretion of surfactant (a process mediated by β_2-receptors), and regulation of cardiac function via α-adrenergic receptors (Slotkin and Seidler, 1988). Further studies are required to determine whether AMCs possess an O_2 sensor (e.g., NADPH oxidase), similar to the one proposed to function in glomus

cells, and if so, whether its expression is limited to the neonatal period.

Adaptation of glomus cells to chronic hypoxia.

Chronic hypoxia, as occurs in humans and animals living at high altitude (Arias-Stella, 1969) or in patients with various pulmonary and cardiovascular diseases (Heath et al., 1970), causes enlargement or hypertrophy of the carotid body, a time-dependent sensitization of the carotid body hypoxic ventilatory response (Barnard et al., 1987; Nielsen et al., 1987), as well as a reduction or "blunting" of the hypoxic ventilatory response in long-term hypoxia (Dempsey and Forster, 1982). The above physiological and morphological changes may be mediated via a direct action of low arterial Po_2 on carotid body chemoreceptors (i.e., glomus cells), or indirectly via secondary circulatory factors. Relevant to the first possibility, this thesis examined the adaptive capabilities of glomus cells following chronic exposure to low Po_2 *in vitro*, and uncovered several "plastic" properties and possible underlying cellular and molecular mechanisms.

Plasticity in cultured glomus cells induced by environmental factors.

Developmentally, glomus cells derive from the embryonic neural crest (Pearse et al., 1973) and share many characteristics with cell types that comprise the sympathoadrenal (SA) sublineage of neural crest derivatives (Kobayashi, 1971; Patterson, 1990; Anderson, 1993). These adrenergic crest derivatives can display a neuronal or endocrine phenotype and consist of three major cell types: sympathetic neurons, adrenal chromaffin cells, and small intensely fluorescent (SIF) cells. Various experiments *in vitro* and *in vivo* have

shown that the local environment of crest derivatives during development plays an important role in determining their final fate. Environmental factors can also influence the phenotype of SA members, such as adrenal chromaffin cells and PC12 cells (a clonal cell line isolated from a rat pheochromocytoma or adrenal chromaffin tumor; Greene and Tischler, 1976). For instance, exposure to NGF promotes the "transdifferentiation" of chromaffin and PC 12 cells into sympathetic neurons, based on the following criteria: (1) increased cell size and loss of intense catecholamine fluorescence in their cell bodies (Doupe et al., 1985a); (2) acquisition of characteristic neuronal ultrastructure, including neuronal processes with morphologically specialized synapses (Doupe et al., 1985a; Pollock et al., 1990); (3) cessation of cell division; (4) acquisition of neuronal markers, including immunoreactivity to neurofilament protein (Doupe et al., 1985a) and up-regulation of GAP-43 gene expression (Costello et al., 1990); and (5) electrical excitability due to an increase in Na^+ channel density (Pollock et al., 1990). NGF evokes many of the above neuronal properties via increases in the second messenger, cAMP (Costello et al., 1990; Kalman et al., 1990; Pollock et al., 1990).

Similar to their SA-counterparts, glomus cells displayed considerable plasticity in response to environmental factors, though there were noticeable differences in the ones that were effected. Surprisingly, NGF had no effect on glomus cells, but prolonged exposure (i.e. 2-3 weeks) to low Po_2 triggered a wide array of cellular responses, which may be relevant to carotid body adaptation *in vivo*. These include (1) glomus cell hypertrophy, with an increase in both surface area and three-dimensional cell volume (see Stea et al., 1992; Mills and Nurse, 1993); (2) altered ion channel function (e.g.,

enhanced Na^+ channel density and Ca^{2+} current; see Stea et al., 1992, 1995); and (3) up-regulation of growth-associated protein GAP-43 (see Chapter 1), a calmodulin-binding protein implicated in neurotransmitter release (see below). An increase in Na^+ channel density in glomus cells may contribute to the observed sensitization of the carotid body afferent response to chemosensory stimuli (Barnard et al., 1987; Nielsen et al., 1987), since Na^+ channels play a major role in controlling the excitability in a variety of neurons and neuroendocrine cells. Consequently, a selective increase in Na^+ channel density would amplify the depolarization (and subsequent transmitter release) in glomus cells resulting from a given chemostimulus. Similarly, glomus cell hypertrophy and the increase in calcium currents may result in increased transmitter release for a given chemosensory stimulus. Interestingly, all of these adaptive responses to chronic hypoxia are expected to modulate the final chemoreceptor output or neurotransmitter release (see below) which, in turn, regulates activity in the carotid sinus nerve. Depending on the nature of the released neurotransmitter(s), e.g. excitatory vs inhibitory, these adaptations may lead to enhanced or reduced chemosensitivity during chronic hypoxia.

Chronic hypoxia *in vivo* causes a dose-dependent modulation of cAMP levels in the carotid body (Perez-Garcia et al., 1990) and exposure of isolated carotid bodies to acute hypoxia causes a dose-dependent increase in cAMP levels (Perez-Garcia et al., 1990; Delpiano and Acker, 1991), particularly in glomus cells (Wang et al., 1991b). These findings, together with the well-documented ability of intracellular cAMP to induce GAP-43 gene expression in PC 12 cells (Costello et al., 1990) and to promote short- and long-term modulation of ion channels in a variety of systems (Barres et al.,

1989; Offord and Catterall, 1989; Hess, 1990; Kalman et al., 1990; Dolphin, 1992; Ansanay et al., 1995), suggested that this second messenger is a potential mediator of the effects of chronic hypoxia. Consistent with this idea, we found that chronic treatment of control (normoxic) carotid body cultures with agents that elevate intracellular cAMP (i.e., dibutyryl cAMP or forskolin) stimulated an increase in proportions of glomus cells that were immunoreactive for GAP-43 (Jackson and Nurse, 1995a), enhanced both Na^+ and Ca^{2+} currents, as well as surface area measured by whole-cell capacitance (Stea et al., 1992, 1995). Thus it is plausible that the second messenger cAMP mediates, at least in part, some of the plastic responses of glomus cells following chronic exposure to low Po_2.

Unlike chronic hypoxia, treatment with agents that elevated intracellular cAMP had additional effects that resulted in a conversion of many of these cells from an endocrine to a neuronal phenotype. A substantial population of glomus cells elaborated neurites and expressed immunoreactivity to neurofilament under the latter conditions (Jackson and Nurse, 1995a). The fact that only a subpopulation of glomus cells were responsive to cAMP analogs in this way, suggests that the carotid body may not contain a homogeneous cell population. Interestingly, chronic treatment with cAMP analogs was also significantly more effective than chronic hypoxia in promoting GAP-43 immunoreactivity in glomus cells. Possibly even an optimal hypoxic stimulus is incapable of producing levels of intracellular cAMP attained with pharmacological agents. Alternatively, hypoxia may trigger other responses in glomus cells, e.g., elevation of intracelluar Ca^{2+} (Gonzalez et al., 1994), that could antagonize the ability of cAMP to

promote GAP-43 expression (see Chapter 1).

Although the physiological functions of GAP-43 are still unclear, a large body of evidence implicates this neuronal protein in synaptic plasticity processes in the brain, including neurite outgrowth in development and regeneration, hippocampal long-term potentiation (LTP), and neurotransmitter release (reviewed by Liu and Storm, 1990; Strittmatter et al., 1992; De Graan and Gispen, 1993). This protein is linked reversibly to the cytoplasmic side of the plasma membrane and binds to calmodulin (CaM) with higher affinity in the absence of Ca^{2+} than in the presence of Ca^{2+}. It has been proposed that GAP-43 may function as a "CaM-sponge" by binding and concentrating CaM at specific sites (e.g., growth cones of neurons) underneath the plasma membrane, and releases free CaM *locally* in response to phoshorylation by protein kinase C (Alexander et al., 1987). CaM dissociates from GAP-43 in response to a depolarization-induced Ca^{2+} influx or to receptor-mediated PKC activation. Once free, CaM subsequently activates one or more Ca^{2+}/CaM-dependent enzymes, which are implicated in the process of Ca^{2+}-induced neurotransmitter release. These enzymes include CaM-kinase II (Llinas et al., 1985; Nichols et al., 1990) and calcineurin, also known as Ca^{2+}/CaM-dependent protein phosphatase 2B (De Grann et al., 1992). Evidence for the importance of GAP-43 in neurotransmitter release includes: (1) the inhibition of high K^{+}- and calcium ionophore-evoked DA release in PC12 cells transfected with a recombinant expression vector coding for antisense human GAP-43 cRNA (Ivins et al., 1993); (2) the inhibition of noradrenaline release from rat cortical synaptosomes by antibodies which interfere with GAP-43 phosphorylation; and (3) decreased Ca^{2+}-induced noradrenaline release from

permeated synaptosomes, prepared from the rat cerebral cortex, after treatment with polymyxin B, a CaM antagonist affecting the interaction between GAP-43 and CaM, and anti-CaM antibodies, which inhibit Ca^{2-}/CaM-dependent protein kinase II autophosphorylation and calcineurin phosphatase activity (Hens et al., 1996).

Given the above functions of GAP-43, a potential role in the modulation of neurotransmitter release in chronically hypoxic glomus cells appears the most attractive for several reasons. First, chronic hypoxia appears to induce the expression of GAP-43 without promoting neurite outgrowth, suggesting a function unrelated to neurite outgrowth. Second, carotid body catecholamine release, which is augmented in cultures of chronically hypoxic glomus cells, has been implicated in the ventilatory acclimatization to chronic hypoxia (Bisgard et al., 1987) and "blunting" of the ventilatory response in high-altitude dwellers or neonates born and reared in hypoxia (Dempsey and Forster, 1982; Eden and Hanson, 1987b; Hertzberg et al., 1990). Third, GAP-43 may play an important role in depolarization-induced transmitter release, since the elevation of intracellular Ca^{2+} after depolarization triggers the activation of protein kinase C, which subsequently phosphorylates GAP-43 in rat cortical synaptosomes (Dekker et al., 1990). Lastly, GAP-43 may also regulate the increased electrical excitability (Ca^{2-} spikes) observed in chronically hypoxic glomus cells (Stea et al., 1995), since a GAP-43-deficient, NGF-treated PC12 cell line was found to have lowered electrical excitability, due to reduced functional expression of voltage-dependent Ca^{2-} channels (Gribkoff et al., 1995). Therefore, GAP-43 may be an important modulator of neurotransmitter release from carotid body chemoreceptors, and could well contribute to the altered

chemosensitivity that occurs during ventilatory acclimatization to chronic hypoxia (Barnard et al., 1987; Vizek et al., 1987; Nielson et al., 1988).

Evidence for down-regulation of Ca^{2+}-dependent K^+ channels in chronically hypoxic glomus cells.

A significant difference between normoxic (Nox) and chronically hypoxic (CHox) cultures was the failure of IbTx to evoke DA release above baseline levels in CHox cultures (see Chapter 2; Jackson and Nurse, 1997a). The simplest explanation is that chronic hypoxia *in vitro* caused a down-regulation or decreased functional expression of Ca^{2+}-dependent K^+ channels. However, CHox glomus cells can still respond to acute hypoxia with enhanced DA release, suggesting that sensing of hypoxia was mediated through a pathway different from closing of Ca^{2+}-dependent K^+ channels, as occurs in normoxic rat glomus cells (Peers, 1990a, b). Alternatively, a resetting of the membrane potential and/or levels of Ca^{2+}_i in chronically hypoxic cells, could have resulted in a closure of these channels at the Po_2 (~160 Torr) where measurements were done. Interestingly, Wyatt et al. (1995) have shown that glomus cells, acutely isolated from 9-14 day old rats reared in chronic hypoxia (10% O_2) from birth, possess O_2-sensitive K^+ channels; however, these cells lack Ca^{2+}-dependent K^+ channels expressed in rats reared in normal O_2 tension (20% O_2). It is plausible that chronic hypoxia may cause a switch or reversion in the K^+ channel subtype linked with the O_2-sensor to one that is more effective at signaling over the lower Po_2 range. One possibility is that chronic hypoxia triggers the down-regulation (or prevents the development) of Ca^{2+}-dependent

K^+ channels, while retaining or even up-regulating the density of the IbTx-insensitive K^+ channel type that appear to be expressed in neonatal glomus cells (see Chapter 2). Another possibility is that the subtype(s) of K^+ channel expressed in CHox glomus cells is not as efficient as Ca^{2+}-dependent K^+ channels in stimulus-secretion coupling. However, a direct comparison of the ratio of hypoxia-evoked DA release can be misleading, since basal DA release is greatly exaggerated in CHox cultures (Chapter 2; see below).

Glomus cells adapt to chronic hypoxia in vitro by dramatically increasing basal dopamine release.

The most striking difference between Nox and CHox glomus cells is the exaggerated basal DA release after chronic hypoxia *in vitro* (Jackson and Nurse, 1997a). Because these measurements were normalized to glomus cell number, this adaptation was induced at the level of individual chemoreceptor cells and resulted from direct stimulatory effects of chronic low Po_2. Therefore, hyperplasia of glomus cells associated with chronic hypoxia *in vivo* was not a confounding factor in these *in vitro* experiments (Dhillon et al., 1984). Similarly, the increase in basal DA release was not simply a reflection of increased DA stores in CHox glomus cells, since normalization of DA stores to glomus cell number in sister cultures revealed that DA stores in CHox glomus cells were similar to those in Nox cells. These studies, however, did suggest that DA turnover may be enhanced in CHox glomus cells. Also, basal DA release in CHox cultures was unaffected by nifedipine, a blocker of voltage-dependent, dihydropyridine-sensitive, L-

type calcium channels. Hence, increased spontaneous electrical activity and augmented Ca^{2-} currents (see Stea et al., 1995) were unlikely to be the underlying cause of the elevated basal release. Alternatively, chronic hypoxia may lead to an increase in resting intracellular calcium or modulation of other carotid body neurotransmitters and/or their receptors, which secondarily regulate DA secretion. For instance, activation of nicotinic acetylcholine receptors (nAChRs) triggered secretion from chromaffin cells in the absence of membrane depolarization (Mollard et al., 1995). Thus, Ca^{2-} influx through nAChRs and contributions from thapsigargin-sensitive Ca^{2-} sequestering stores was sufficient to trigger transmitter release in these cells.

Mechanisms underlying elevated basal dopamine release in chronically hypoxic cultures.

Prevailing evidence suggests that DA release from carotid chemoreceptors, acting via pre- and post-synaptic D_2-receptors, inhibits the carotid body ventilatory response (see Gonzalez et al., 1994; Holgert et al., 1995) and appears to be involved in the resetting of carotid body chemosensitivity in the perinatal period (Hertzberg et al., 1990). As discussed above, the most dramatic effect of exposing carotid body cultures to chronic hypoxia *in vitro* was an exaggerated increase in basal levels of extracellular DA, referred to as "basal release". An understanding of the underlying cellular and molecular mechanisms should aid understanding of the processes involved in the adaptation of mammals to prolonged hypoxia. As outlined in Chapter 3, the enhanced DA levels around cheomoreceptor cell clusters during chronic hypoxia *in vitro* appear to be set by several factors including, released acetylcholine (ACh), positive and negative feedback

regulation via nicotinic AChRs (nAChRs) and muscarinic AChRs (mAChRs) respectively, and modulation of DA transporters.

(i) Role of acetylcholine receptors.

Significantly, the finding that basal DA release was also dramatically augmented in normoxic cultures treated with chronic nicotine (CNic), a nAChR agonist, suggested that continuous stimulation of nAChRs by hypoxia-evoked ACh release within glomus cells clusters could contribute to elevated basal DA release in CHox cultures. Support for this hypothesis came from the use of a mecamylamine, a nAChR antagonist. Acute and chronic treatment of both CNic and CHox cultures with mecamylamine significantly reduced basal DA release.

However, in addition to nAChRs, mAChRs are also expressed on glomus cells (Chen and Yates, 1984; Dinger et al., 1985, 1986, 1991; Eyzaguirre et al., 1990; Wyatt and Peers, 1993; Dasso et al., 1997; Obeso et al., 1997). Whereas pharmacological studies have demonstrated that nicotinic agonists excite carotid body chemoreceptors, stimulate CA release, and potentiate the response to hypoxia (Dinger et al., 1985; Eyzaguirre et al., 1965; Eyzaguirre and Zapata, 1968; Gomez-Nino et al., 1990), muscarinic agonists have been shown to suppress basal CSN activity (Docherty and McQueen, 1979; Monti-Bloch and Eyzaguirre, 1980; see, however, Dinger et al., 1991), and inhibit both DA release and the increased CSN activity evoked by nicotine stimulation in rabbit carotid body (Dinger et al., 1991). These studies suggest that ACh, released along with DA during chronic hypoxia, may activate mAChRs on glomus cells

and suppress DA release. Our findings that elevated basal DA release in CHox cultures was strongly suppressed by the muscarinic agonist methylcholine, and that atropine, a muscarinic antagonist, augmented resting DA levels even further, indicate that mAChRs have an inhibitory function on DA release. Importantly, the apparent lack of effect of muscarinic agonists and antagonists on DA release in Nox cultures, raises the possibility that chronic hypoxia may up-regulate the density and/or increase the affinity of a particular subtype of mAChR in glomus cells. Hence, both muscarinic and nicotinic AChRs appear to play modulatory roles in CHox cultures, and activation of this dual receptor system on the same or different glomus cells (see Dasso et al., 1997) can lead respectively, to both negative and positive regulation of extracellular DA. In CHox cultures, positive feedback via nAChRs appears to be more dominant than negative feedback via mAChRs, with respect to ACh regulation of DA release.

(ii) Role of dopamine transporters.

In addition to DA secretion, the effective concentration of DA surrounding glomus clusters may also be regulated by re-uptake via DA transporter (DAT). DATs appear to function and limit the accumulation of DA in Nox cultures, since a potent inhibitor of DA uptake, nomifensine, caused a significant increase in basal DA levels. However, nomifensine had no effect on DA accumulation in CHox and CNic cultures, suggesting that activation of nAChR by nicotine in CNic cultures and by ACh, released between neighboring glomus cells in CHox cultures, leads to inhibition of DATs (Chapter 3). Interestingly, Yamashita et al. (1995) demonstrated a "cross-talk" between

nAChR and DAT, based on findings that nicotine regulates extracellular DA in PC 12 cell cultures by an indirect inhibition of DA uptake. Accordingly, in Nox cultures, where DA and presumably ACh are released in small amounts under basal conditions, there may be little "cross-talk" between nAChRs and DATs, permitting DATs to function effectively in the re-uptake of extracellular DA. However, in CHox cultures, where the rate of DA and presumably ACh secretion is enhanced by hypoxia, nAChRs are continuously activated leading to inhibition of DA re-uptake into glomus cells. Hence, the level of extracellular DA around CHox glomus cells appears to be set by a combination of factors including: (1) DA released by the direct effect of low Po_2 on O_2-sensitive glomus cells; (2) the secondary/modulatory action of released neurotransmitters (e.g., ACh and DA) acting within glomus cell clusters in an autocrine and paracrine fashion so as to facilitate or inhibit release; and (3) re-uptake into glomus cells by DA transporters.

Summary: Glomus cell adaptation to chronic hypoxia.

The manipulation of a single variable, i.e., O_2 tension, can trigger a wide array of adaptive responses in carotid body chemoreceptors. The *in vitro* studies performed in this thesis have uncovered some of the long-term adaptations of glomus cells to chronic hypoxia, all of which have the potential to modify the level of released transmitter, and ultimately, activity in the carotid sinus nerve and the hypoxic ventilatory response. These "plastic" responses include: (1) altered ion channel function, leading to an increase in electrical excitability (see Stea et al., 1992, 1995); (2) glomus cell hypertrophy (Stea et

al., 1992); (3) up-regulation of GAP-43 immunoreactivity (Chapter 1; Jackson and Nurse, 1995); (4) down-regulation of functional Ca^{2+}-dependent K^+ channels (Chapter 2; Jackson and Nurse, 1997a); (5) enhanced basal extracellular DA, without a significant increase in DA stores (Chapter 2; Jackson and Nurse, 1997a); this extracellular DA appears to be set by positive and negative feedback regulation via nicotinic and muscarinic AChRs, respectively, and inhibition of DA transporters (Chapter 3; Jackson and Nurse, 1997b). Mechanistically, an increase in intracellular levels of cAMP may be involved in the first three adaptations to chronic hypoxia, and ACh appears to be an important autocrine/paracrine modulator of dopaminergic function.

Clinical implications of chronic chemoreceptor stimulation by nicotine and hypoxia.

Since DA modulates peripheral arterial chemoreceptor activity in most species (Gonzalez et al., 1994) and is likely involved in the perinatal resetting of O_2 chemosensitivity (Eden and Hanson, 1987a, b; Hertzberg et al., 1990, 1992), the exaggerated levels of DA following chronic hypoxia or nicotine may interfere with postnatal development of receptor function and may attenuate the protective chemoreflex response to hypoxia. Dysfunctional arterial chemoreceptors in the neonate, compounded by other abnormalities in the autonomic control of breathing, may increase the risk for diseases such as sudden infant death syndrome (SIDS), during vulnerable periods of sleep apnea or airway obstruction which are common occurrences in neonates (Czegledy-Nagy et al., 1993; Willinger et al., 1991; Hoffman et al., 1988). Significantly, epidemiological studies have demonstrated a dose-dependent relationship between

maternal cigarette smoking during pregnancy and SIDS (Lichtensteiger et al., 1988; Taylor and Sanderson, 1995). In addition, chronic administration of nicotine in mammals attenuates the protective CB arterial chemoreflex (Holgert et al., 1995) and increases mortality during exposure of neonatal rats to hypoxia (Slotkin et al., 1995). Holgert et al. (1995) have suggested that chronic nicotine administration in perinatal rats induces the release and synthesis of carotid body DA, which acts on local D_2-receptors to produce inhibition of the hypoxic drive. The findings in Chapter 3 suggest that chronic nicotine may cause an increase in extracellular DA, in part by inhibiting the activity of the DA transporter via nAChRs on glomus cells. Hence, chronic elevation of "resting" levels of extracellular DA at the synapse between glomus cells and afferent terminals of the carotid sinus nerve may produce inhibition of the hypoxic drive by acting on postsynaptic D_2-receptors. Collectively, these results suggest mechanisms by which nicotine can interfere with the first line of defense against hypoxia and may explain why maternal smoking during pregnancy is a prevailing risk factor for SIDS.

Future Research

Characterization of the O_2-sensitive K^+ channel subtypes which trigger hypoxia-evoked DA release from both glomus and chromaffin cells has been limited to the postnatal period in this thesis. It would be of interest in the future to extend these developmental studies into the embryonic period. From previous *in vivo* studies, we anticipate that embryonic glomus and chromaffin cells can sense low Po_2; however, the subtype(s) of K^+ channel responsible for transmitter release during acute hypoxia is

unknown.

Another direction for future studies would involve testing the importance of GAP-43 in DA release in chronically hypoxic glomus cells. Does GAP-43 function to enhance the efficiency of DA secretion by sequestering calmodulin close to the plasma membrane where neurotransmitter secretion takes place? This can be tested by transfecting cultures of chronically hypoxic glomus cells with a recombinant expression vector coding for antisense GAP-43 cRNA. The effect of "knocking down" the expression of GAP-43 on DA release from chronically hypoxic carotid body cultures may reveal whether this plasticity protein contributes to chemoreceptor adaptation in this environment.

Finally, future studies could address the physiological importance of NADPH oxidase in O_2 chemoreception. Although, spectrophotometric studies and immunohistochemistry have localized the subunits of the NADPH oxidase complex to glomus cells (Cross et al., 1990; Kummer and Acker, 1995), its role as the putative O_2 sensor in the carotid body is controversial. The recent development of a strain of mice with a nonfunctional allele for a specific subunit ($gp91^{phox}$) of NADPH oxidase (Pollock et al., 1995) provides an opportunity to test the O_2-sensing capabilities of glomus cells which express an non-functional NADPH oxidase. Preliminary results have shown that carotid body cultures derived from $gp91^{phox}$ "knock-out" mice failed to respond to acute hypoxia with enhanced dopamine release (unpublished observation). This suggests that NADPH oxidase may function as an O_2 sensor in the carotid body. Similarly, it would be of interest to test the O_2-sensing capabilities of neonatal chromaffin cells cultured from the adrenal medulla of this mouse model.

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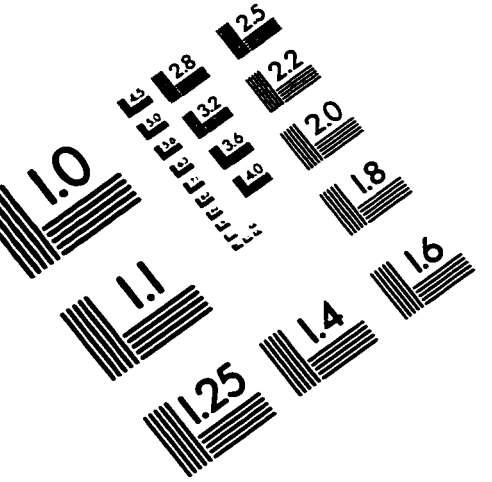
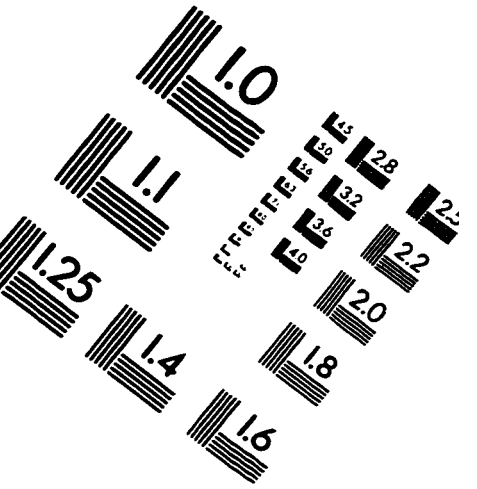
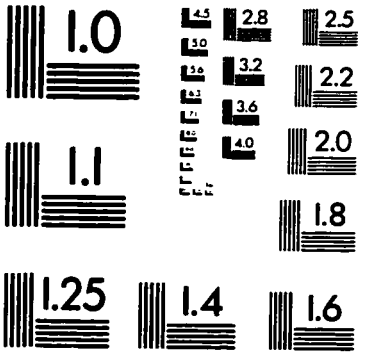
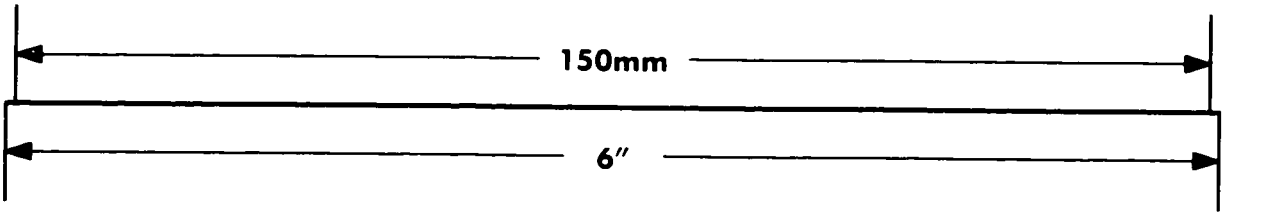
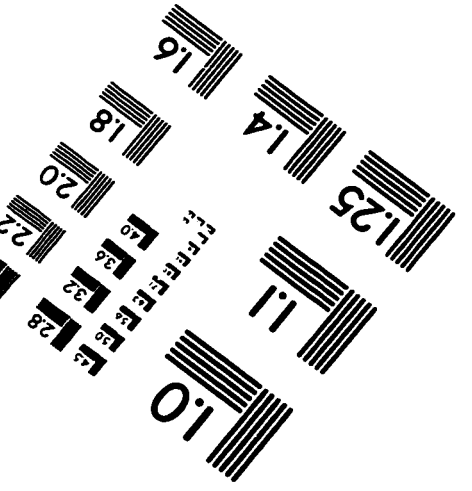
IMAGE EVALUATION TEST TARGET (QA-3)

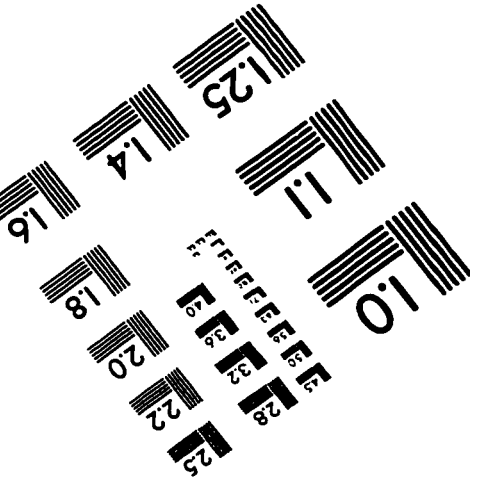












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