STIMULUS-SECRETION COUPLING IN THE RAT CAROTID BODY

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by

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CHAPTER 1

GENERAL INTRODUCTION

1.1 EARLY STUDIES

The first description of the carotid body as the 'ganglion minutum' was by Taube in 1743 and until the mid nineteenth century, much of the literature concerned discussions as to whether the structure was a ganglion or a gland (see Pallot, 1987). Luschka (1862) was the first to refute the idea that the carotid body was a ganglion, revealing that it was a glandular structure rich in nerves and blood vessels and named it the 'glandula carotica'; he also recognized that there were morphological similarities with both the adrenal and pituitary glands (see Al-Neamy, 1983).

During the period 1926-8, Fernando de Castro of Madrid re-named the structure 'glomus caroticum' and suggested that the carotid body might represent an organ containing receptor cells very similar to those found in other sense organs. Since de Castro saw changes in the size of the carotid body following alterations in gas tensions of the blood, he concluded that the structure was an independent sensory organ able to perceive both the oxygen and carbon dioxide concentrations of the circulating blood. In addition, he inferred that as the carotid body could detect qualitative variations in the blood, the tissue could, by reflex action, have some influence on the functional activity of other organs. These suggestions were confirmed by Heymans et al. (1930), de Kock (1954) and by Heymans and Neil (1958), who stated that 'the carotid body is able to

initiate respiratory and cardiovascular reflexes in response to hypoxia (low pO_2), hypercapnia (high pCO_2) or acidosis (low pH)'.

By the early 1970's many studies were concerned with the functional significance of the carotid body following suggestions that it may have an important role in the ventilatory response to chronic hypoxia (Belenky et al., 1979). The central nervous system responds to chronic hypoxia such as occurs during exposure to high altitude (Smith et al., 1986) or in lung disease, by increasing ventilatory drive which partly results from acidification of cerebral interstitial fluid in the environment of the central chemoreceptors (Fencl et al., 1979). The central ventilatory response cannot operate independently of the carotid body and indeed it is thought that these peripheral chemoreceptors are essential for ventilatory acclimatization to chronic hypoxia and this is characterized by a progressive hyperventilation (Dempsey and Forster, 1982)

Apart from the physiological significance of the carotid body, defects in carotid body function may be clinically important since it has been suggested from histological observations that patients at risk from Sudden Infant Death Syndrome (SIDS; 'Cot Death') may have a depressed hypoxic sensitivity. The reasons for this are as yet unknown but may reflect changes in the endogenous content of putative neurotransmitters in the carotid body which then contribute towards the afferent neural response.

1.2 LOCATION

Embryologically, the carotid body is thought to have both a neural and mesochymal origin (Pearse et al., 1973; Korkala and Hervonen, 1973) and in most mammals it is found near to the bifurcation of the common carotid artery. The exact location in relation to the surrounding blood vessels is species dependent and in the rat it is associated with both the external and internal carotid arteries from which it may receive its blood supply (Biscoe, 1971). The venous outflow arising from the vascular bed joins the external or internal jugular veins (see Pallot, 1987).

The structure is innervated via the carotid sinus nerve which is a branch of the IX glossopharyngeal nerve. In addition, the organ also receives a sympathetic innervation from noradrenergic fibres of the superior cervical ganglion (Verna, 1975) and these are also thought to innervate the surrounding vasculature.

1.3 STRUCTURE

The structure of the carotid body is complex. The fundamental histological units of the tissue consist of clusters of two cell types which, together with an adjacent blood vessel, form a functional unit or 'glomoid'. The most abundant cell type has been given a wide variety of names, eg. 'chief cells' (Eyzaguirre and Uchizono, 1961) and 'glomus cells' (Al-Lami and Murray, 1968) with the most

favoured name being 'Type I cells' (Biscoe and Stehbens, 1966).

The other main cell type have been termed 'capsular cells' (Kraus and Martinek, 1967), 'sustentacular cells' (Ross, 1957), or simply 'Type II cells' and are generally smaller than the Type I cells. They are extensively branched, having long cytoplasmic processes which partially surround groups of two or three Type I cells, offering a supportive role similar to the Schwaan cells of the central nervous system (see Eyzaguirre and Fidone, 1980).

The carotid body is also rich in blood vessels and capillaries which are both fine branches of the arterial system. The tissue has a very high blood flow and oxygen consumption (Daly et al., 1954), and the capillaries are thought to account for approximately 25% of the total organ volume. The cells and blood vessels are situated in a connective tissue matrix and the entire structure is enclosed in a collagenous capsule (Figure 1.1).

Numerous myelinated ('A' type) and unmyelinated ('C' type) afferent nerve fibres penetrate the collagenous capsule. They appear to be continuous with the carotid sinus nerve and have their somata in the petrosal ganglion. Sensory nerve endings of the carotid sinus nerve are in synaptic contact with the cell clusters. Each sensory unit may consist of one axon innervating 10-20 Type I cells (Hess, 1968; Verna, 1979). These nerve endings appear to be pre-synaptic and reciprocally synaptic to the Type I cells (McDonald and Mitchell, 1975; Biscoe, 1977).

Figure 1.1: Light micrograph of the rat carotid body

The specific tissue (st) consisting of Type I cells, Type II cells and nerve endings is broken up into segments by strands of connective tissue. Carotid body tissue is characterized by the presence of numerous blood vessels (b) which are closely associated with the specific tissue-clusters to form functional units or 'glomoids' as described in the text.

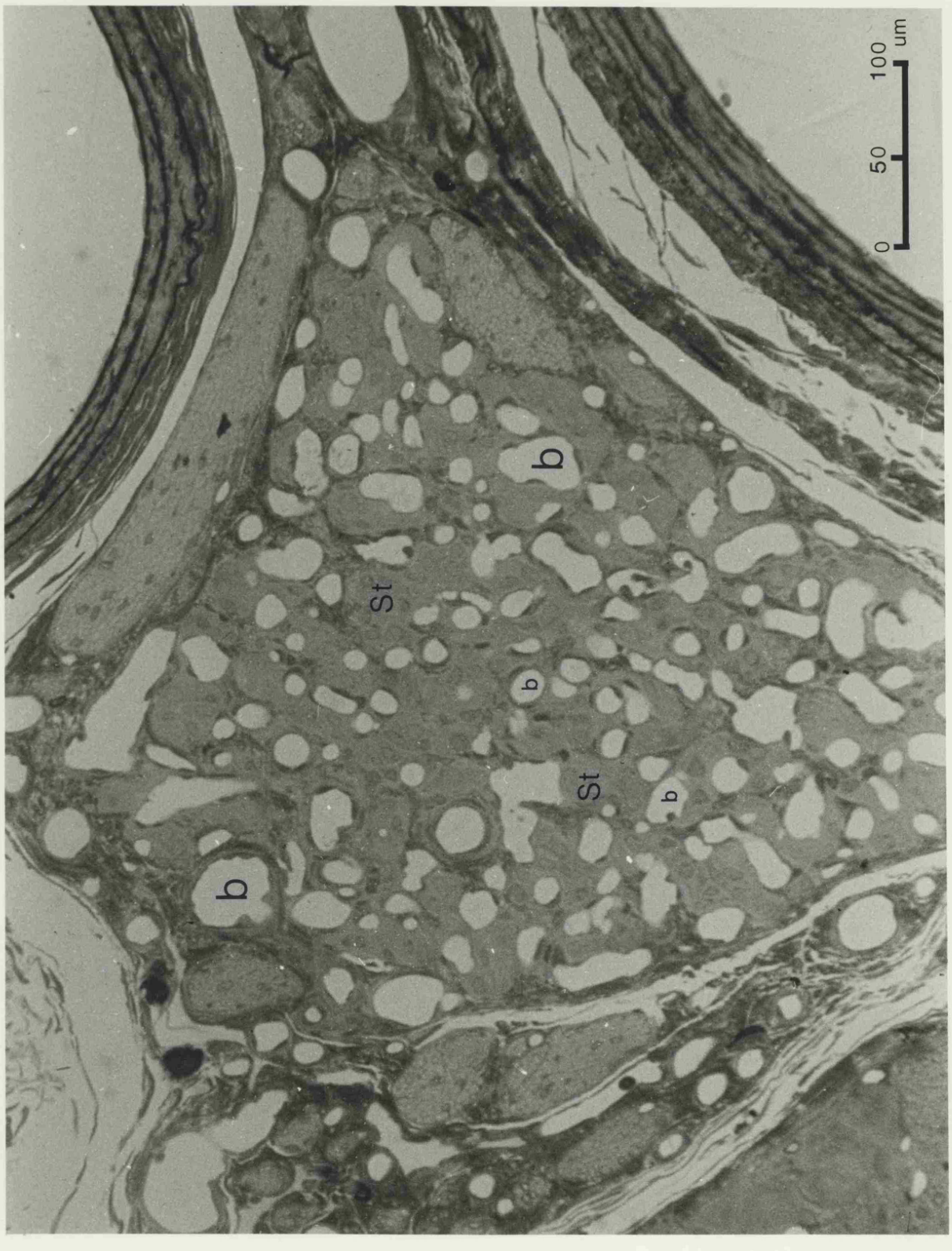


FIG 1·1

1.4 IDENTITY OF THE SENSOR

The organization of the carotid body is so complicated that there is little agreement about the functions of the array of structures that can be identified. As a result of this heterogeneity, almost every component of the tissue has been postulated as the actual chemoreceptor. The two most favoured candidates have been the sensory nerve endings (Mitchell et al., 1972; Mills and Smith, 1981) and the Type I cells (Verna et al., 1975; Zapata et al., 1976). The available evidence suggests that the afferent nerve endings can generate chemoreceptor activity when they are in a highly vascular environment but in the absence of the major cell types, the responses do not mirror those of the intact carotid body (see Pallot, 1983).

De Castro (1928) initially suggested that the Type I cells 'taste the blood' (see Eyzaguirre and Gallego, 1975), but much of the evidence in support of this came at a later stage both from re-innervation experiments (Mills and Jobsis, 1972; Acker et al., 1985) and from studies involving destruction of the Type I cells (Verna, 1975; Leitner et al., 1981).

From the wealth of information available it would seem that one or more elements may actually participate in the sensory event, although there is increasing evidence that the integrity of the Type I cell is essential for normal transduction and the discovery of several putative transmitters in these cells would seem to make them a more

favourable candidate.

1.5 ULTRASTRUCTURE OF TYPE I CELLS

The Type I cells are thought to originate from the neural crest. They are complex in shape with finger-like extensions projecting from the cell body and in close proximity to the vasculature. These cells possess many of the morphological characteristics common to neurosecretory cells (Normann, 1976), neurons (Smith, 1971), adrenal medullary chromaffin cells (Grynszpan-Winograd, 1971), pancreatic β cells (Kondo, 1981) and paraneurons (Nagasawa, 1977; Kobayashi and Fujita, 1981) and it has been suggested that the structural organization of the Type I cells may play a direct role in the chemoreceptive process (Belmonte and Gonzalez, 1983).

These cells have a large rounded nucleus with a prominent nucleolus. There are many mitochondria which are concentrated in the finger-like processes of the cells. In addition, there is a well developed rough endoplasmic reticulum and prominent Golgi apparatus together with an abundance of microtubules (Eyzaguirre and Fidone, 1980).

The most striking aspect of the Type I cells is the presence of electron-dense cored vesicles in their cytoplasm, which were first identified by Lever and Boyd in 1957. These vesicles are 30-120nM in diameter and are similar to, but smaller than, those of the adrenal medulla chromaffin cells (Verna, 1979; McDonald, 1981). They have

been identified as the principal site of neurotransmitter storage in the carotid body (Dearnaley et al., 1968; Chen et al., 1969; Zapata et al., 1969; Chiocchio et al., 1971).

1.6 PUTATIVE TRANSMITTERS IN THE CAROTID BODY

In 1898, Stilling described a 'chromaffin reaction' in the carotid body and this was thought to be a specific technique for the histochemical demonstration of amine-like substances. In the 1950's, Eranko developed a fluorescence method to detect amine-storing cells and this was used in the study of the carotid body in different species (see Al-Neamy, 1983). Later studies more specifically identified the presence of dopamine, noradrenaline and small amounts of adrenaline in the carotid body of a wide range of species (Chiocchio et al., 1966; Dearnaley et al., 1968; Hellstrom and Koslow, 1975).

In addition to the monoamines, several authors have reported the presence of acetylcholine or acetylcholine-like substances in the carotid body (Fidone et al., 1977; Hellstrom, 1977). Although acetylcholine may be released from the carotid body, the actual structure responsible for their manufacture has yet to be established. Serotonin (5-HT) and a multitude of peptide hormones including the enkephalins (Wharton et al., 1980; Smith et al., 1988), Substance P (Cuello and McQueen, 1980; Monti-Bloch and Eyzaguirre, 1985) and vasoactive intestinal polypeptide (VIP; Fitzgerald et al., 1981) have also been found in the

carotid body. Pearse (1969) was the first to suggest that the Type I cell may secrete a low molecular weight polypeptide which he called 'glomin', and there is histological evidence for polypeptide or protein containing granules in the carotid body Type I cells (Capella and Solcia, 1971). It has been suggested that these peptides coexist with the amines in the granules of the Type I cells (Wang et al., 1988), but the functions of these substances in the carotid body have yet to be determined.

1.7 THEORIES OF CHEMORECEPTOR FUNCTION

The carotid body sensors are thought to mediate their effects on the central nervous system via sensory discharges of the carotid sinus nerve. The biogenic amines have been implicated in the chemosensory response (Zapata, 1975; Gonzalez and Fidone, 1977; Llados and Zapata, 1978).

Two theories for the sensor mechanism of the carotid body have been postulated and in both hypotheses, catecholamines function as neurotransmitters in an inhibitory feedback pathway activated by natural stimuli.

Osborne and Butler (1975) favour the idea that the Type I cells are the actual chemosensor. They propose that during unstimulated conditions, there is continuous release of the inhibitory neurotransmitter dopamine from the Type I cells at the afferent nerve synapses. The discharge frequency of these nerves is continually depressed under resting conditions. During stimulation of the Type I cells, the

secretion of dopamine is reduced and sensory nerves increase their activity when no longer inhibited by dopamine. This increase in firing frequency causes the release of another transmitter, possibly acetylcholine, which further suppresses dopamine release from the Type I cells resulting in an even greater discharge frequency. Thus, these authors envisage a positive feedback loop.

McDonald and Mitchell (1975) have postulated that the afferent nerve endings are the chemoreceptors and that they interact with the Type I cells through reciprocal synapses that form an inhibitory feedback loop. They suggest that the sensory nerves release an excitatory transmitter (eg. acetylcholine) when stimulated; the transmitter causes the Type I cells to release dopamine and dopamine inhibits these sensory nerves.

Obviously these hypotheses are based on the authors' individual opinions as to the nature of the actual chemosensor and consequently there is evidence for and against both postulated mechanisms.

1.8 CATECHOLAMINES AND THE CAROTID BODY

There are a number of criteria which must be satisfied in order to establish a particular substance as a neurotransmitter. The substance must be synthesized and stored within the cell from which it is released and all enzymes involved in its synthesis must also be present in the same cell (see Kruk and Pycock, 1983).

1.8.1. CATECHOLAMINE BIOSYNTHESIS

Catecholamine-containing cells synthesize their endogenous amine content from the amino acid L-tyrosine (Figure 1.2) which is present at a concentration around 10^{-4}M in the plasma. L-Tyrosine is formed in the liver by the hydroxylation of the amino acid L-phenylalanine (Blaschko, 1939) and it actively enters the cell via a low affinity uptake mechanism before being rapidly converted into L-3,4-dihydroxyphenylalanine (L-DOPA) by the soluble cytoplasmic enzyme, tyrosine hydroxylase. This enzyme requires molecular O_2, Fe^{2+} and a tetrahydropteridine cofactor. Since tyrosine hydroxylase is inhibited by the presence of catecholamines in the cytoplasm, the hydroxylation of tyrosine represents the rate-limiting step in their biosynthesis (Nagatsu et al., 1964). Tyrosine hydroxylase activity has been measured in the rat carotid body by Hanbauer et al. (1977), and by Gonzalez et al. (1979b).

A very active but relatively non-specific cytoplasmic enzyme, DOPA decarboxylase converts DOPA into dopamine (Holtz et al., 1938) and this has been located in the Type I cells of the rat carotid body (Hanbauer et al., 1977; Bolme et al., 1977). Dopamine is actively taken up into the dense-cored storage vesicles where it may be converted into noradrenaline by way of dopamine-β-hydroxylase (DBH), which is a vesicle bound enzyme and has been located in the bovine carotid body (Thorn and Schon, 1981) and in that of the cat

Figure 1.2: Pathway of catecholamine biosynthesis

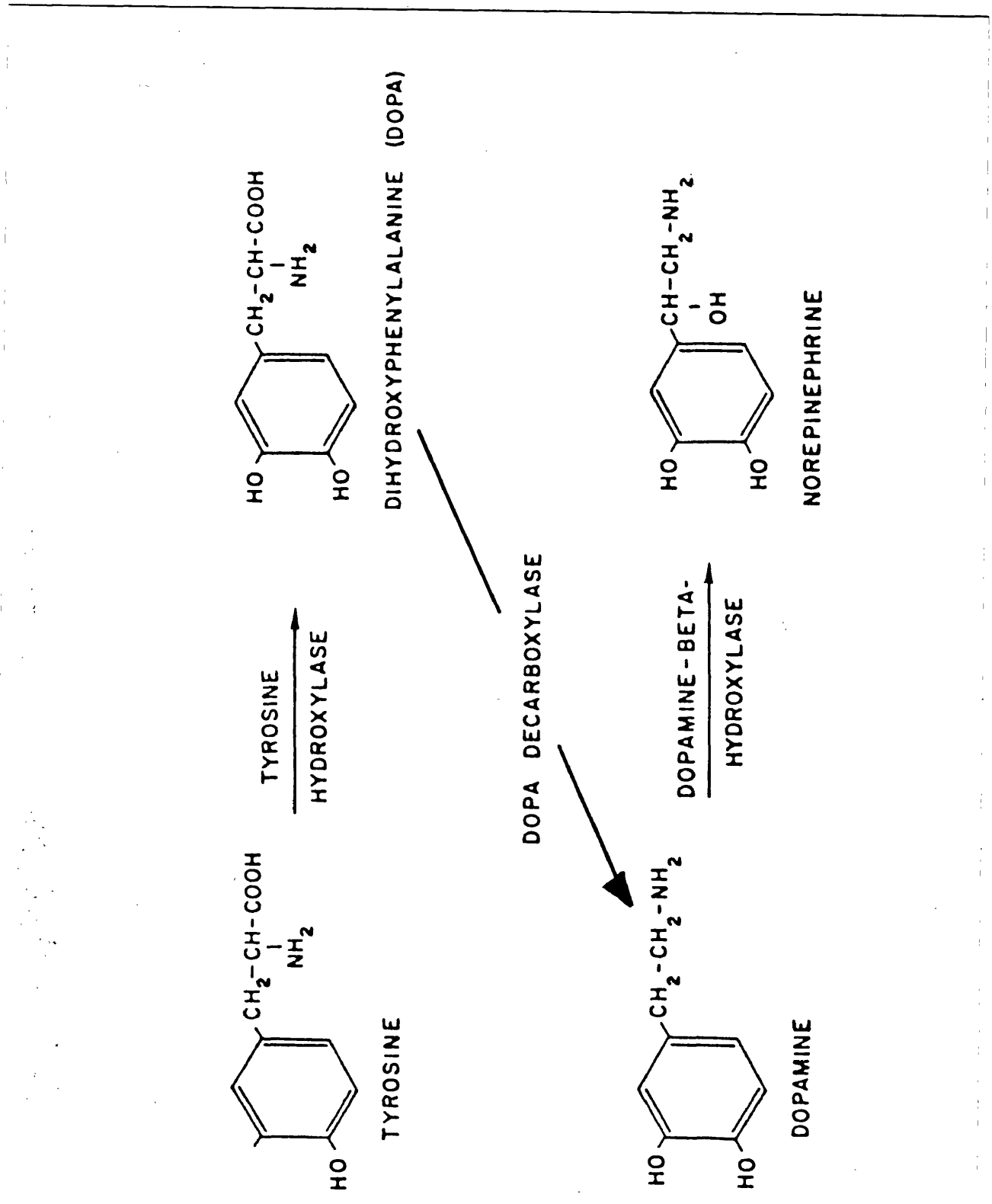

TYROSINE
TYROSINE HYDROXYLASE
DIHYDROXYPHENYLALANINE (DOPA)
DOPA DECARBOXYLASE
DOPAMINE
DOPAMINE-BETA-HYDROXYLASE
NOREPINEPHRINE
HO
NH2
OH

FIG 1·2

(Belmonte et al., 1977). In the adrenal medulla, uptake of the catecholamines into granules is known to occur by active transport involving a specific carrier which is driven by an electrochemical proton gradient (Winkler et al., 1987).

1.8.2 CATECHOLAMINE STORAGE

Vesicles containing catecholamines in tissues such as the adrenal medullary chromaffin cells have also been shown to store variable but small quantities of nucleotides, calcium, ascorbic acid, proteins and peptides (Serck-Hanssen and Helle, 1972). These substances are thought to constitute 27% of the vesicle content (Winkler et al., 1987).

There appears to be some degree of controversy surrounding the storage of the catecholamines in the carotid body. It has been suggested that noradrenaline and dopamine are stored in different Type I cells since the distribution of dopamine-β-hydroxylase seems to be limited (Morgado et al., 1976; Chen and Yates, 1981). In addition, the relative concentrations of the amines vary from species to species and within the same species from author to author (see Mills et al., 1978 and Mir et al., 1982).

In the central nervous system there are thought to be two forms of subcellular storage of the amines; a particulate (vesicular) and a soluble component (cytoplasmic). More than 60% of noradrenaline in the rat brain has been recovered from the particulate fractions of homogenates, whereas less than 40% of the dopamine content has been recovered from the

particulate fraction (see Glowinski, 1970). These observations would seem to suggest that most of the synthesized dopamine is present in a free form in the cytoplasm although it is unknown how the soluble amine fraction resists the action of endogenous monoamine oxidases. An alternative explanation for the relatively larger proportion of dopamine being associated with the soluble fraction is a direct result of the homogenization process since it is possible that catecholamine-containing vesicles are not resistant to homogenization. Clearly the question of how the catecholamines are stored both in peripheral tissues and the central nervous system requires further investigation.

1.8.3 CATECHOLAMINE SECRETION

The exocytotic release of catecholamines is generally thought to follow two pathways; one that is apparently unregulated and occurs continuously, but at a low rate, and another that is triggered by a second messenger system (see Section 1.9). Triggered exocytosis leads to a rapid release of the amines, with associated nucleotides, proteins and calcium (Knight and Baker, 1987).

Release of carotid body catecholamines has been indirectly demonstrated *in vitro* and a number of stimulatory agents have been shown to cause a decrease in the endogenous amine content. In addition, physiological and pharmacological agents have been shown to modify amine turnover.

Morphological studies have shown a decreased fluorescence of rat carotid body Type I cells after strong hypoxic or hypercapnic stimuli (Blumcke et al., 1967; Hervonen et al., 1972; Hansen, 1981). Observations on the density, number and disposition of the dense-cored vesicles following natural stimulation have also been interpreted in favour of increased release (Hoffman and Birrell, 1958).

Biochemical evidence for a reduction in the catecholamine content of the cat carotid body following stimulation has been provided by Fitzgerald et al. (1983). Mills and Slotkin (1975) and Gonzalez and Fidone (1977) reported a reduction in the amine content of the rabbit carotid body whilst Hellstrom et al. (1976) reported a selective depletion of dopamine from the rat carotid body following severe hypoxia.

Direct release studies were performed by Fidone et al. (1982) and they were able to demonstrate the selective release of [^{3}H] dopamine, newly synthesized from [^{3}H] tyrosine, following superfusion of the rabbit carotid body in vitro with hypoxic medium. In addition, sodium cyanide, which induces histotoxic hypoxia, high extracellular potassium concentrations (Delpiano and Acker, 1988) and acetylcholine or applications of its analogues have also been shown to depolarize Type I cell membranes (Eyzaguirre et al., 1975) and these are thought to induce catecholamine secretion. The dopamine content of the rat carotid body has been found to be reduced by methacholine administration (a cholinergic agonist) and the effect has been blocked specifically by the antagonist, methylatropine (Hellstrom

and Hanbauer, 1981). These authors attributed the decrease in dopamine content to the actual release of the amine.

The dopamine that is released from the Type I cells may have excitatory or inhibitory actions at the afferent nerve endings in order to subsequently influence sensory discharge, but the mechanism by which the stimulus is transduced throughout the Type I cell remains largely unknown.

1.9 STIMULUS-SECRETION COUPLING

The term stimulus-secretion coupling was first coined to describe the series of events that occurred between the arrival of an excitatory message at the cell membrane and the discharge of the secretory contents of the same cell (Douglas and Rubin, 1963). A number of functional links between the arrival of the message and the cellular response have been identified and these are termed the 'messenger systems'. A large number of hormones, neurotransmitters, growth factors and other 'first messengers' are able to exert their effects via specific membrane receptors (see Fisher and Agranoff, 1987) yet less than a dozen second messenger systems have been established. These second messenger systems are able to determine the rate, magnitude and duration of exocytotic secretion either directly or via even higher order messengers (Lichtstein and Rodbard, 1987).

1.9.1 INTRACELLULAR CALCIUM AS A SECOND MESSENGER

Most intracellular calcium is non-ionized, being associated with various cellular compartments and only a very small proportion is free and ionized within the cytoplasm. In the resting state, the level of free ionized calcium in the cytoplasm of a wide range of cell types has been estimated to be approximately 5×10^{-8}M (eg. see Lazarus, 1976).

All cells possess mechanisms to regulate their cytoplasmic calcium concentration. The plasma membranes of most cells have Ca^{2+}-ATPase pumps and a Na^{+}/Ca^{2+} exchange mechanism for calcium extrusion. In addition, calcium may be sequestered in organelles such as the endoplasmic reticulum and mitochondria by way of high affinity Ca^{2+}-ATPase pumps and respiration-driven $[H^{+}]$ gradients respectively. In addition, there are usually a variety of calcium-binding proteins and other compounds which contribute to the intracellular buffering of the free calcium (Naylor and Merrillees, 1971).

During cell excitation, the cytosolic free calcium concentration may rise to 10^{-6}M as a result of an influx of calcium through voltage-dependent calcium channels of which there are three sub-types: long, transient and neuronal (Schwartz and Triggle, 1984; Nowycky et al., 1985). Long or 'L' type channels are ubiquitous and are the only type which are susceptible to the classical calcium channel antagonists (the dihydropyridines, verapamil and dilitiazem analogues;

Tsien, 1983).

The cytosolic calcium concentration may also rise as a result of its mobilization from intracellular stores. Other messengers may be responsible for this mobilization and in this respect, calcium may influence secretion by its action as a 'third' messenger.

There is both morphological and biochemical evidence to implicate calcium ions in the chemosensory transduction process of the carotid body. The chemoreceptor response to natural or chemical stimulation, low pH or electrical stimulation has been found to be influenced by the extracellular calcium concentration (McDonald, 1977a; Gronblad et al., 1979). In the rabbit carotid body for example, stimulation of catecholamine release by hypoxia is known to be dependent on the extracellular calcium concentration since the release of this amine falls by 90% in the absence of calcium (Fidone et al., 1982). In addition, an increased uptake of calcium has been demonstrated in carotid body Type I cells stimulated with either acetylcholine or hypoxia (Pietruschka, 1985). In parallel studies, some authors have reported a decrease in the extracellular calcium concentration following exposure of the tissue to hypoxia (Acker, 1980). These results suggest that a decrease in pO_2 triggers calcium influx with the possibility that this may evoke catecholamine secretion.

Attempts have been made to localize the effects of calcium on the receptor complex and results have shown that calcium may act directly on the Type I cell membranes (Hess,

1977b). Cultured carotid body Type I and Type II cells do not have the same requirement for calcium during stimulation (Pietruschka, 1985). The possible involvement of intracellular calcium stores in the secretory process has been studied by Roumy and Leitner (1977) who were able to block the uptake of calcium by the mitochondria using ruthenium red and lanthanum and this resulted in an increased release of neurotransmitter. However, the presence of more than one calcium store has more recently been suggested (Valdeolmillos et al., 1988).

The most recent studies on the involvement of calcium in the transduction process have been performed by Valdeolmillos et al. (1988) and Koyano et al. (1988) who have followed changes in the cytosolic calcium concentration of Type I cells loaded with the calcium-sensitive fluorescent dye, Fura-2. The cells were then subjected to both natural and chemical stimulation. Decreases in fluorescence provided a quantitative estimate of the cytosolic calcium concentration under variable conditions and were taken to represent an increased calcium release. These studies are valuable but need to be extended in order to study calcium efflux directly to provide an overview of the calcium movements following cell stimulation.

1.9.2 CYCLIC NUCLEOTIDES AS INTRACELLULAR SIGNALLING MOLECULES

Cellular excitation by a variety of first messengers may generate changes in the intracellular levels of adenosine 3',5'-cyclic monophosphate (cyclic AMP) or guanosine 3',5'-cyclic monophosphate (cyclic GMP). There is evidence that in some cellular processes, these two nucleotides promote opposite responses (Goldberg et al., 1975). Increases in the cytosolic cyclic AMP concentration are known to occur in response to a wide variety of agonists acting at multiple types of receptor, eg. activation of β-adrenergic receptors by the catecholamines, noradrenaline and adrenaline, activation of V_2 vasopressin receptors, H_2 histamine receptors and S_1 serotonin receptors. Conversely, decreases in cyclic AMP may occur by activation of α_2-adrenergic receptors and at M_2 muscarinic cholinergic receptors. The various responses are known to be mediated by stimulatory or inhibitory 'G' proteins. These proteins directly influence the activity of the enzyme adenylate cyclase which is responsible for the formation of cyclic AMP from endogenous ATP (see Exton, 1987).

The involvement of the cyclic nucleotides as intracellular messengers in the chemosensory transduction mechanism of the carotid body has been studied to a limited extent. Folgering et al. (1982) first suggested that hypoxia may exert its effects on the carotid body chemoreceptors via activation of the β-adrenergic receptors. Mir et al. (1983)

investigated this possibility by following the effects of the biogenic amines and hypoxia on the formation of cyclic AMP. Although β-adrenoceptor agonists were able to promote cyclic AMP production, hypoxia was without effect and thus it was suggested that activation of β-adrenoceptors was not involved in transduction of the hypoxic stimulus.

These studies were reasonably conclusive. However, hypoxia-induced cyclic GMP has never been studied in the carotid body. Cyclic GMP is known to be produced in response to stimulation of muscarinic receptors and these have been indirectly demonstrated in the carotid body (Monti-Bloch and Eyzaguirre, 1981). In view of this, and the fact that in taste chemoreception (by the gustatory cells), cyclic GMP has been implicated in the transduction of membrane-mediated events (Tonosaki and Funakoshi, 1988; Avenet et al., 1988), a systematic study of agonist-induced cyclic GMP formation in the carotid chemoreceptors is required before the involvement of the cyclic nucleotides can be effectively ruled out.

1.9.3 CONTRIBUTION OF PHOSPHOINOSITIDES TO CELLULAR SIGNALLING

As previously mentioned (Section 1.9.1), some intracellular messengers are known to influence the intracellular calcium concentration. Recent work has greatly clarified the actions of calcium-mobilizing agonists and it is now known that calcium-mediated first messengers may operate by

enzymatic breakdown of specific phospholipids in the plasma membrane (Berridge, 1984). The phospholipids involved are termed phosphoinositides and they consist of phosphatidyl-inositol (PI) which represents 5-10% of total membrane phospholipid and its two minor phosphorylated derivatives, phosphatidylinositol-4-phosphate (PIP) and phosphatidyl-inositol 4,5-biphosphate (PIP_2). PIP and PIP_2 are formed from PI by successive enzymatic phosphorylations involving ATP (Downes and Michell, 1982).

The interaction of first messengers with their specific receptors at the plasma membrane activates a specific phospholipase 'C' enzyme. This enzyme catalyses the breakdown of membrane PIP_2 to a water soluble product, inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3; IP_3), and a lipid product, 1,2-diacylglycerol (DAG) which remains in the membrane. There is evidence that IP_3 and DAG are second messengers since they are formed very rapidly, are quickly degraded by specific enzymes and act at very low concentrations. IP_3 has been shown to couple the receptor-mediated event to the liberation of calcium from the endoplasmic reticulum (Streb et al., 1983; Joseph et al., 1984), or specialized 'calciosomes' (Volpe et al., 1988) causing a rapid increase in the cytosolic calcium concentration which may be independent of the influx of extracellular calcium. The response is short-lived due to the small size of the calcium stores and following its release it is pumped out of the cell. This efflux can be measured by pre-loading tissue with ^{45}Ca (Burgess et al.,

1984; Suematsu et al., 1984). The transient rise in cytosolic calcium may trigger exocytosis and this response has been shown in a wide variety of endocrine, exocrine and neuroendocrine tissues. In the central nervous system, receptors linked to phosphoinositide metabolism have been demonstrated to be involved in neurotransmission, and in the retinal photoreceptors, light itself is a sufficient stimulus for membrane phospholipid cleavage (Anderson, 1986).

Ins 1,4,5-P_3 may be metabolized by two independent pathways, both of which yield compounds that do not release calcium from intracellular stores. In one pathway, Ins 1,4,5-P_3 may undergo successive dephosphorylations to form Ins 1,4-P_2 (IP_2) and Ins, 4-P (IP) with the eventual production of free myo-inositol (Downes et al., 1982). The enzyme responsible for this latter conversion, inositol monophosphatase, is inhibited non-competitively by Li+ (Allison and Stewart, 1971; Hallcher and Sherman, 1980) and consequently Li+ may be used to amplify the agonist-induced accumulation of inositol phosphates. The accumulation of the inositol phosphates represents a sensitive assay for the detection of receptor-mediated events which operate through phosphoinositide catabolism. Tissues in which accumulation of the inositol phosphates has been reported in the presence of lithium include superior cervical ganglion (Bone et al., 1984), hippocampal slices (Labarca et al., 1987), pancreatic β cells (Montague et al., 1985) and adrenal medullary chromaffin cells (Eberhard and Holz, 1987).

In the alternative pathway of IP_3 metabolism, a kinase enzyme may convert IP_3 into inositol 1,3,4,5-tetrakis-phosphate (Ins 1,3,4,5-P_4; IP_4; Batty et al., 1985). It has been proposed that IP_4 functions as a signal in its own right either by stimulating calcium influx into cells (Irvine and Moor, 1986) or by enhancing calcium movements between cellular compartments (Morris et al., 1987).

Nishizuka (1984) was the first to suggest that DAG may also function as a second messenger. Following receptor-stimulated PIP_2 cleavage, the concentration of DAG rises slowly and this is thought to activate a cytosolic kinase enzyme which requires the presence of calcium and a phospholipid for maximum activity. This enzyme, termed protein kinase 'C' (PKC) is thought to phosphorylate specific proteins that are involved in the secretory response. As a result of the slow rise in the DAG concentration, PKC activity is prolonged and therefore this enzyme may be responsible for mediating other long-term effects of calcium-mediated first messengers.

An additional feature of the phosphoinositide mechanism is that the two cellular messenger systems may interact in a synergistic or inhibitory manner (Zawalich, 1983; Berridge, 1984; Nishizuka, 1986). There are also complex negative and positive interactions between the cyclic nucleotide signalling systems and the IP_3/DAG systems, the nature of which varies from cell to cell (Nishizuka, 1986).

The aim of the present study was to undertake a systematic investigation into the chemosensory transduction process of the carotid body. A number of intracellular mechanisms are studied which may be involved in the transfer of information from the carotid body sensors to the generation of neural messages in afferent nerves. Studies on the stimulus-secretion coupling mechanism should enhance our understanding of the physiological functions of the carotid body.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

In this chapter, the reagents and experimental methods that were used to study carotid body function are described. The first part of the experimental section details the methods used to study the synthesis and secretion of the catecholamines, noradrenaline and dopamine. The second and third parts deal with the methods employed to investigate aspects of intracellular calcium mobilization and polyphosphoinositide metabolism respectively.

2.2 REAGENTS

L-[2,6-^{3}H] Tyrosine (specific activity, 1.5 - 2.2 TBq/mmol; radioactive concentration 37 MBq/ml), [7,8-^{3}H] Dopamine (specific activity, 1.5 - 2.2 TBq/mmol; radioactive concentration 37 MBq/ml), DL-[7-^{3}H] Noradrenaline hydrochloride (specific activity 370-740 GBq/mmol; radioactive concentration, 37 MBq/ml), myo-[2-^{3}H] Inositol (specific activity, 703 GBq/mmol; radioactive concentration, 37 MBq/ml) and, ^{45}Calcium (specific activity, 0.37 - 1.5 GBq/mg Ca; radioactive concentration, 37 MBq/ml) were all purchased from Amersham, Bucks., U.K.

myo-[2-^{3}H] Inositol 1,4,5-trisphosphate and myo-[2-^{3}H] Inositol 1,3,4,5-tetrakisphosphate, were gifts from Dr. J. Baird, Department of Pharmacology and Experimental Therapeutics, University of Leicester.

All inorganic salts used in buffer solutions and incubation/ superfusion media were of analytical grade.

Acid-washed aluminium oxide (alumina) was obtained from Anachem, Luton, Beds., U.K.
Tetramethylammonium chloride was purchased from Aldrich Chemical Co. Ltd., Dorset, U.K.
Mecamylamine hydrochloride, ethyl carbamate, Trizma base, L-tyrosine hydrochloride, carbamylcholine chloride, atropine sulphate, 3-hydroxy tyramine (dopamine), hexamethonium chloride, (-) arterenol bitartrate (noradrenaline), ethyleneglycol bis-(β aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA), and N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) were all obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.
Dowex AG 50W-X4 cation-exchange resin and Dowex AG 1-X8 anion exchange resin were purchased from Bio-Rad, Watford, Herts., U.K.
Nitrendipine was obtained as a gift from Dr. R. I. Norman, Department of Medicine, University of Leicester.
Citric acid, diaminoethanetetra-acetic acid (EDTA), trichloroacetic acid (TCA), 1-Octane sulphonic acid, sodium salt (SOS) and all other organic and inorganic solvents of ANALAR grade were purchased from Fisons, Loughborough, Leics., U.K.

2.3 TISSUE ISOLATION

In all studies, tissue was isolated from male WISTAR rats weighing 175-250g. The rats were obtained from a colony bred in the Biomedical Services Unit of Leicester University and were housed in groups of 4-6 under controlled conditions of light (14:10, LD), temperature (21^{o}C) and humidity (40%). They were provided with food and water ad libitum.

Rats were anaesthetized with ethyl carbamate (Urethane) at a dose of 1.25g/Kg body weight intraperitoneally. The neck was opened with a ventral midline incision and, under a dissecting microscope, the common carotid artery was exposed in the region of the carotid bifurcation. The superior cervical ganglion and its associated ganglioglomerular nerves were carefully detached from the dorsal side of the bifurcation and discarded. Local bleeding was controlled by way of a bipolar coagulator (Downs Surgical Ltd., U.K.), enabling the bifurcation to be removed prior to terminal anaesthesia. The tissue was immediately placed into ice-cold, physiological Tyrodes medium at pH 7.42 (see Section 2.4).

2.4 CATECHOLAMINE SYNTHESIS

Studies on the synthesis of the catecholamines noradrenaline and dopamine were performed using radio-isotopic techniques similar to those of Fidone and Gonzalez, (1982) in which radiolabelled tyrosine was used in

conjunction with cold tyrosine as a precursor.

Initially, it was necessary to determine the optimum conditions for labelling the catecholamines in the rat carotid body since the studies of Fidone and Gonzalez (1982) were performed on rabbit carotid bodies. In particular, it was necessary to determine the concentration of precursor and the length of the incubation period required to give optimum prelabelling of the catecholamine pools.

2.4.1 EFFECT OF PRECURSOR CONCENTRATION

A stock incubation medium of tyrosine hydrochloride was prepared at a concentration of 120μM in modified Tyrodes medium, pH 7.42. The Tyrodes medium had the following composition: NaCl (112mM), KCl (4.7mM), $MgCl_2$ (1.1mM), $CaCl_2$ (2.2mM), sodium glutamate (42mM), HEPES (5mM) and glucose (5.6mM). The stock solution was added to a tube containing 14.8 MBq of [^{3}H] tyrosine which had been previously freeze-dried to avoid dilution. The stock solution containing the isotope was then diluted with an appropriate volume of Tyrodes medium to obtain a range of concentrations from 10μM-80μM. Pairs of carotid bodies selected at random were incubated at 37^{o}C in 1ml of each tyrosine concentration for an initial time period of two hours. Throughout the incubation period, the medium was constantly gas-equilibrated with 100% O_2. At the end of the incubation period, the catecholamines were extracted from the tissue and separated as described below.

2.4.2 TIME COURSE OF SYNTHESIS

Randomly sampled pairs of carotid bodies were incubated at 37°C in 1ml of incubation medium containing 50μM [^{3}H] tyrosine (14.8 MBq). The tissue was incubated for periods of 30, 60, 120 or 180 minutes. The medium was constantly gas-equilibrated with 100% O_2 during these periods. At the end of the incubation, the catecholamines were extracted from the tissue and separated as described below.

2.4.3 EXTRACTION OF [^{3}H] CATECHOLAMINES

At the end of the incubation period, the medium was aspirated from the tissue which was washed with 2 volumes of ice-cold Tyrodes medium. The pairs of carotid bodies were then transferred to glass homogenization tubes containing 1ml 0.1M HCl and kept on ice until the homogenization could be performed manually. Homogenized extracts were transferred to 1.5ml capacity centrifuge tubes and spun at 12,000g for 3 minutes (MSE Microfuge). The pellets were discarded and the supernatants were decanted into clean tubes and kept on ice.

2.4.4 SEPARATION PROCEDURES

Following tissue extraction, the procedures described below were employed to separate labelled catecholamines from [^{3}H] tyrosine and from each other.

2.4.4.1 Alumina Adsorption

Following an adaptation of a method from Crout (1961), the supernatant samples were brought to pH 7.8 - 8.2 with a buffer mixture consisting of 1M Trizma base and 0.02M EDTA, adjusted to pH 8.6 with 0.5M NaOH. Approximately 25mg of acid-washed aluminium oxide was added to each sample and the tubes were slowly mixed by continuous rotation (Rotator, Scientific Industries Ltd., U.K.) for 10 minutes. The alumina was allowed to settle and the supernatants containing the unmetabolized tyrosine were aspirated.The alumina was then washed with 3 successive volumes of distilled water which were discarded. After the final wash, the catecholamines were eluted with 0.6ml of 1M perchloric acid. The eluate was kept on ice until required for determination of radioactivity or HPLC analysis (see below). The recovery of catecholamines from the alumina was determined using the pure [^{3}H] standards and was found to be approximately 85% for both noradrenaline and dopamine.

2.4.4.2 Cation-exchange Chromatography

Dowex AG 50W-X4, 200-400 mesh cation exchange resin was used. It was converted to the sodium-salt form by pre-treatment of the commercially available hydrogen form with sodium hydroxide (Appendix I).

Polypropylene chromatography columns with a capacity of 11ml (Econo-columns, Watford, Herts., U.K.), were loaded

with 1ml Dowex resin and washed with 3x 10ml distilled water. Columns were kept moist and cold until use.

(i) Standardization of Dowex Columns

Three separate 1mM standard solutions were prepared consisting of tyrosine hydrochloride, dopamine hydrochloride and noradrenaline bitartrate. These solutions were added to 0.37 MBq of their respective freeze-dried radioisotope, [^{3}H] tyrosine, [^{3}H] dopamine or [^{3}H] noradrenaline, and diluted to a final concentration of 100µM with 0.1M HCl.

In addition, a mixed standard solution was prepared containing all three labelled components in equimolar concentrations. All solutions were neutralized with 100µl 2M K_2CO_3 and 0.5ml 1M HEPES before application onto the resin.

Aliquots of each solution were taken for determination of their radioactivity before application to the columns. The columns were then eluted by a modification of the method of Paden et al. (1976). All columns received 4x 5ml distilled water, 20x 2ml 1M HCl and 20x 2ml 2M HCl in an initial stepwise elution regime. All fractions were retained and aliquots were sampled for a determination of their radioactivity. The elution profiles are shown in Figure 2.1 and from this information, it was decided to use the elution volumes as shown in Table 2.1. Recoveries of noradrenaline and dopamine using these conditions were approximately 95%.

The supernatants resulting from extraction of the tissue (0.8 - 1.0ml) were neutralized using 100µl 2M K_2CO_3 and

Figure 2.1: Separation of [^{3}H] tyrosine from [^{3}H] catecholamines by cation-exchange chromatography

A mixed solution containing authentic tyrosine, noradrenaline and dopamine standards in equimolar concentrations, was prepared as described in section 2.4.4.2(i) and the solution was added to a chromatography column containing Dowex-50 cation exchange resin. The radioactively labelled standards were eluted from the resin following the initial stepwise regime as described in Table 2.1. Each fraction was sampled for a determination of its radioactivity.

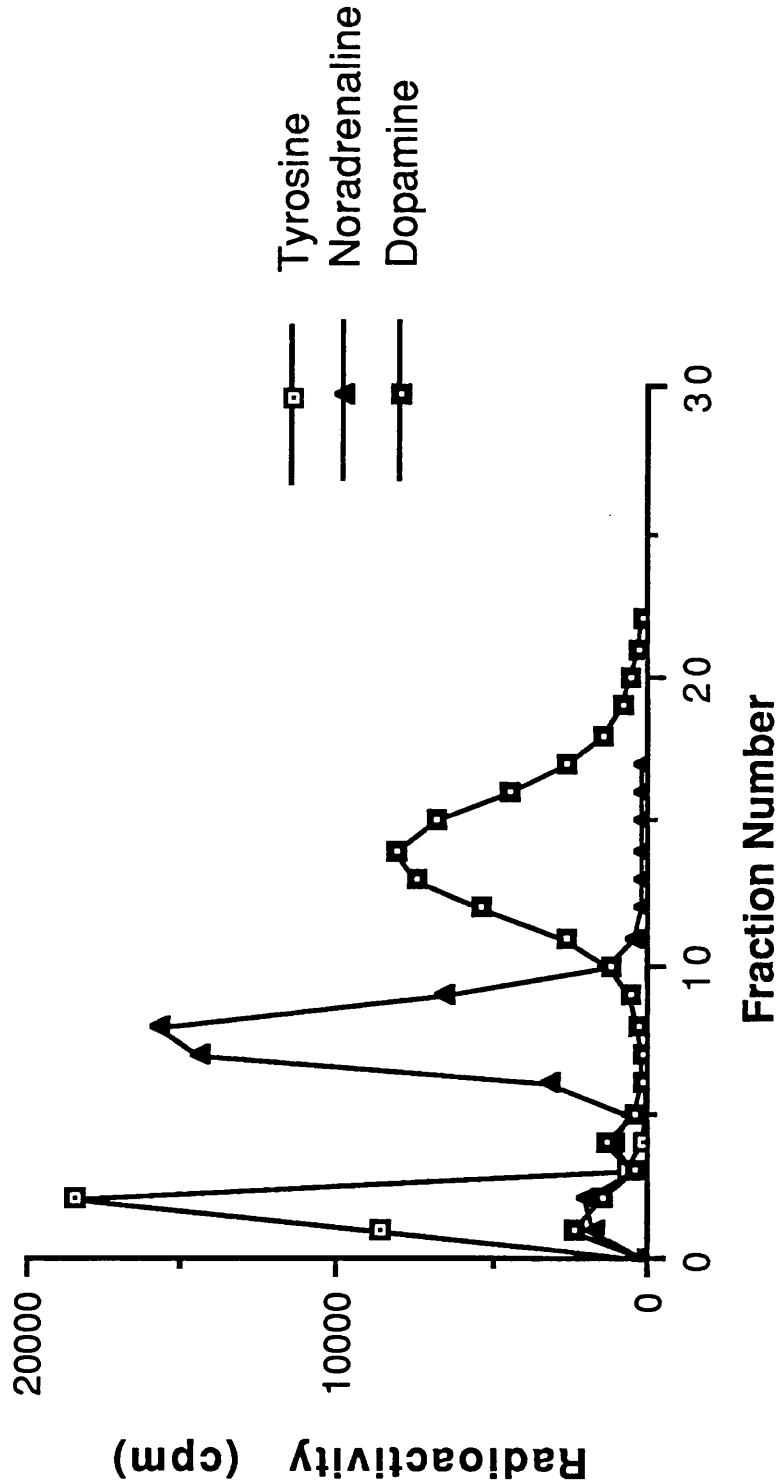

Tyrosine
Noradrenaline
Dopamine
Radioactivity (cpm)
20000
10000
0
0
10
20
30
Fraction Number

Figure 2·1

Table 2.1: Elution regime for the separation of [^{3}H] catecholamines on Dowex AG 50-X4 cation-exchange columns

ELUANT	VOLUME	ELUATE
Distilled water	10ml	Tyrosine
1M HCl	4ml	Tyrosine/Noradrenaline
1M HCl	10ml	Noradrenaline
2M HCl	10ml	Dopamine

0.5ml 1M HEPES. The mixtures were agitated to liberate carbon dioxide and placed onto Dowex columns. The columns were then eluted following the regime as described in Table 2.1. The noradrenaline and dopamine fractions were retained and aliquots were taken for a determination of their radioactivity.

2.4.4.3 High Performance Liquid Chromatography

(i) HPLC-ED Components

The major components of the HPLC-ED system consisted of a high pressure pump (Altex Model 110A, Beckman Instruments, USA, or an LKB Model 2150 pump, Bromma, Sweden), set at a flow rate of 0.9ml/min and operational at a pressure of 200-300psi. Samples were manually injected via a 200µl loop onto a C-18, ion-pair, reversed phase column; 5µm particle size and 4.6x 250mm internal diameter (Altex Ultrasphere, Beckman, USA). The electrochemical detector was either an ESA Coulochem detector (Model 5100, Mass., USA) or an LKB Electrochemical detector (Model 2143, Bromma, Sweden). The operating range was 20-100nA at 0.5v oxidation potential. Both detectors were capable of pMole sensitivity. The systems were linked to an integrator, (Shimadzu C-RI Chromatopac, Japan) or chart recorder (Linseis, GMBH, Model 6512). In some cases, radioactive eluate from the column was collected over 1 minute periods using a fraction collector (LKB Ultrorac II; Model 2027, Bromma, Sweden).

(ii) Solvent System

The mobile phase comprised a 0.5M citrate-acetate buffer at pH 5.2 containing 100µM SOS and 10% methanol (Appendix II). The ratio of SOS to methanol could be varied to alter the elution times of the amines. Before and during use, the solvent was degassed with helium to minimize baseline noise.

(iii) Standard Preparation

Individual standards were prepared containing 50pM noradrenaline bitartrate or dopamine hydrochloride in 100µl 0.1M perchloric acid which had been pre-filtered under vacuum. Mixed standards were also prepared containing both noradrenaline and dopamine in equimolar concentrations. Standards were stored at -20^{o}C as a stock solution and were used at a final concentration of 5pM.

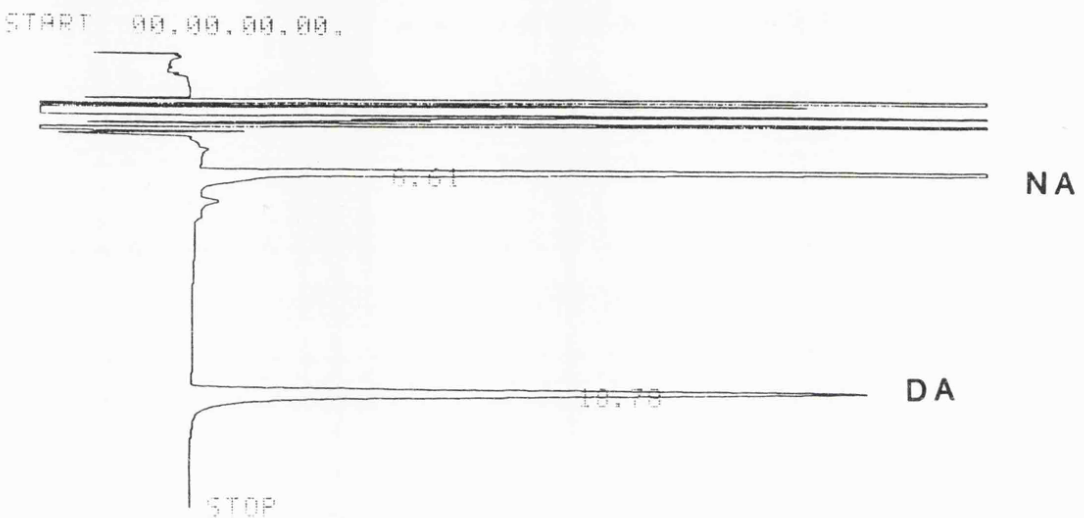
(iv) Sample Preparation

Tissue extracts were pre-treated by alumina adsorption in order to remove unmetabolized tyrosine and eliminate protein from the sample. Amines were eluted from the alumina using 0.6ml pre-filtered perchloric acid and 200µl of this was injected onto the column manually. Retention times for the amine standards were approximately 6 minutes for noradrenaline and 18 minutes for dopamine (Figure 2.2) using the solvent system described above. Analyses were performed

Figure 2.2: Separation of carotid body catecholamines by HPLC-ED

Authentic standards containing both noradrenaline and dopamine in equimolar concentrations were prepared as described in Section 2.4.4.3(iii). Rat carotid body extract was found to contain both amines with retention times of approximately 6 minutes for noradrenaline and 18 minutes for dopamine using a citrate-acetate solvent system at pH 5.2 (Appendix II).

STANDARDS



CAROTID BODY EXTRACT

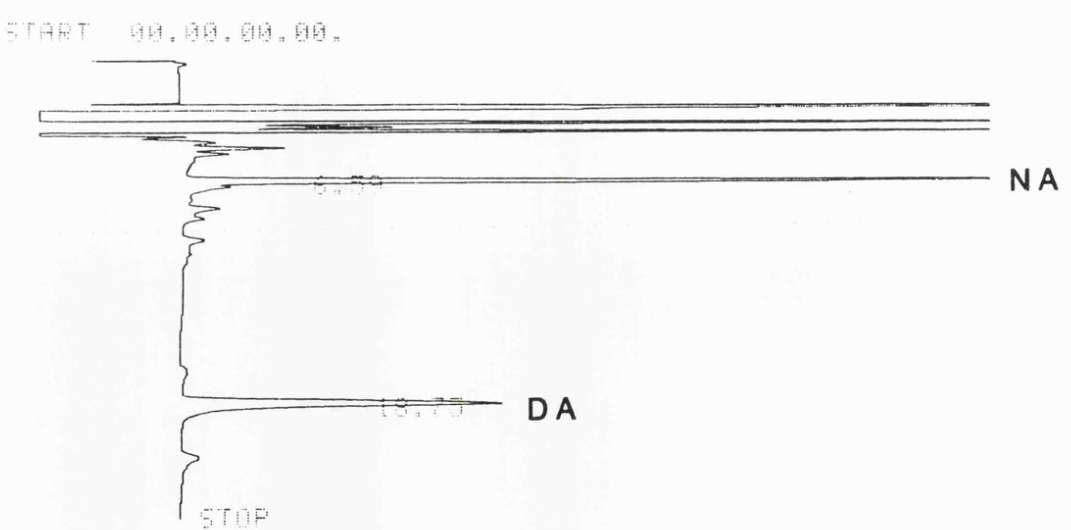


FIG 2·2

either on line by the integrator or calculated by comparing the peak areas of samples and standards. Peak heights were not used since there were often changes in detector sensitivity after successive sample runs causing a broadening of peaks. For this reason, standards were regularly interspersed with samples.

2.4.4.4 Comparison of Noradrenaline and Dopamine Peaks as Identified by HPLC and Cation-exchange Chromatography

Following preparation of the tissue sample as described in Section 2.4.3, the supernatant obtained was treated by alumina adsorption and divided into two aliquots. One aliquot was neutralized as described in Section 2.4.4.1 and placed onto a Dowex column while the other sample was manually injected onto the HPLC column. Eluant from the HPLC column was collected at 1 minute intervals and the fractions were sampled for a determination of their radioactivity.

The Dowex column was eluted following the regime described in Table 2.1 and the noradrenaline and dopamine fractions were sampled for a determination of their radioactivity. The retention times of the catecholamines were found to correspond with the peaks of radioactivity associated with noradrenaline and dopamine fractions eluted from the HPLC column. This indicated that the newly synthesized catecholamines were incorporated into the endogenous amine pool.

The radioactivity in the amine peaks obtained from HPLC was quantitatively compared with those obtained from the Dowex column. The recovery of catecholamines by cation-exchange chromatography was 85.4% of that obtained by HPLC and this confirmed the viability of the Dowex method.

2.4.5 ANALYSIS OF THE DATA

In the prelabelling experiments, results were expressed as the amount of radioactivity (cpm) associated with total catecholamine accumulation as a function of precursor concentration or length of incubation period. HPLC results were expressed as pMole noradrenaline or dopamine/carotid body.

2.5 CATECHOLAMINE SECRETION

Following isolation, carotid bodies were incubated under the optimal labelling conditions which were established in the preliminary experiments.

Groups of eight carotid bodies were incubated at 37^{o}C for two hours in an incubation vial containing 50µM L-tyrosine hydrochloride and 14.8 MBq of [^{3}H] tyrosine in a total volume of 1ml modified Tyrodes medium. Throughout the incubation, the medium was constantly gas-equilibrated with 100% O_2. At the end of the labelling period, the radioactive incubation medium was aspirated and the tissue was washed with 3 volumes of ice-cold Tyrodes medium in order to remove

extracellular label. The carotid bodies were kept on ice ready for transfer to the superfusion apparatus.

2.5.1 SUPERFUSION APPARATUS

Following incubation, the tissue was mounted in a small polypropylene chamber which was positioned upstream of a peristaltic pump, calibrated to a flow rate of 1ml/min (Gilson Minipuls 2, Anachem, Luton). The chamber was connected to reservoirs of superfusion media via lengths of iso-versinic tubing (Anachem, Luton, U.K.), in order to minimize oxygen exchange. A two-way tap in the line upstream from the tissue chamber permitted a choice of superfusion media. Reservoirs of modified Tyrodes medium at pH 7.42 were maintained at 37^{o}C in a water bath whilst the remainder of the apparatus was housed in an insulated aluminium chamber maintained at 37^{o}C by way of a servo-controlled heating system. Throughout the superfusion regime, the Tyrodes medium was constantly equilibrated with appropriate gases, pre-warmed to 37^{o}C. A pO_2 electrode (Model E5046, Radiometer, Copenhagen) was employed to monitor the oxygen tension of the medium at source and also in the tissue chamber. Tyrodes medium in the tissue chamber could be maintained at normoxic levels by equilibrating with 100% O_2 in the reservoirs, resulting in a pO_2 of approximately 400 mmHg. When necessary, a hypoxic environment (<40 mmHg) could be produced in the tissue chamber by equilibrating the Tyrodes medium in the reservoirs with 100% N_2.

2.5.2 RELEASE OF [^{3}H] CATECHOLAMINES

The superfusion regime was divided into 'basal', 'stimulus' and 'recovery' release phases. The peristaltic pump served to draw the medium over and through the tissue, and the superfusate was collected directly onto pre-cooled Dowex AG 50W-X4 cation-exchange columns. The entire superfusion regime lasted a total of 70 minutes from the start and collections were made over 10 minute periods.

The basal or resting release phase was reached after 40 minutes of superfusion. A 10 minute stimulus phase followed, where it was possible to introduce a potential secretagogue into the medium. The stimulus period was followed by a 20 minute recovery phase, when the medium was identical to that during the basal release phase. After each 10 minute collection period, the Dowex columns were capped and placed on ice ready for elution. At neutral pH, the catecholamines were found to be stable on the Dowex resin for up to 2 hours.

2.5.3 SEPARATION OF [^{3}H] CATECHOLAMINES

Cation-exchange chromatography was used to separate the catecholamines noradrenaline and dopamine from each other and also from any unmetabolized tyrosine. Columns were eluted as described in Table 2.1. Samples of the noradrenaline and dopamine fractions were taken for a determination of their radioactivity. Results were expressed

as cpm/carotid body/10 minutes.

2.5.4 ANALYSIS OF THE DATA

Results were expressed as the percentage increase in radioactivity associated with the stimulus period when compared to the basal release phase or when compared to a corresponding control experiment. The basal release period was taken as the average of the catecholamine-associated radioactivity release during the two 10 minute periods immediately before and after the stimulation period.

2.6 INTRACELLULAR CALCIUM MOBILIZATION

The efflux of ^{45}calcium from prelabelled carotid bodies was studied using a method modified from that of Frankel *et al*. (1978).

2.6.1 TISSUE INCUBATION

Groups of four carotid bodies were incubated for 30 minutes in 1.5ml conical incubation vials containing 90µl of calcium-free Tyrodes medium and 0.37 MBq $^{45}CaCl_2$. The final ^{45}Ca concentration of the medium was approximately 50µM. At intervals of 10 minutes during the incubation period, the tissue was briefly agitated with 100% O_2. Following pre-loading, the tissue was washed with 3 volumes of calcium-free Tyrodes medium and the tissue was transferred to the

superfusion apparatus as described in section 2.5.1.

2.6.2 ^{45}CALCIUM EFFLUX

Pre-loaded carotid bodies were superfused at a flow rate of 1ml/min at 37^{o}C for an initial period of 40 minutes prior to any alteration in the composition of the superfusion medium. Superfusions were performed using Tyrodes medium containing the normal concentration of ^{40}calcium (2.2mM). The 40 minute basal release phase was followed by a 10 minute stimulus period and a 20 minute recovery period. The superfusion regime was identical to that of the catecholamine release experiments. Throughout the experiment, the effluent medium was collected over successive two minute periods using a fraction collector (Model 2070 Ultrorac II, LKB, Sweden). Each superfusion experiment lasted a total of 70 minutes. The ^{45}Ca content of the fractions were determined by liquid scintillation analysis.

2.6.3 ANALYSIS OF THE DATA

The results were calculated as the percentage increase in radioactivity during the stimulus period as compared with the basal release phase or when compared to a corresponding control experiment. The basal release phase was taken as the average ^{45}Calcium efflux during the two 10 minute periods immediately before and after the stimulation period.

2.7 POLYPHOSPHOINOSITIDE METABOLISM

Following a method modified from that described by Simpson et al. (1987), agonist-induced polyphosphoinositide breakdown was monitored directly by measuring either the efflux of radiolabelled inositol from prelabelled carotid bodies or the inositol phosphate content of the carotid bodies.

2.7.1 TISSUE PRELABELLING

Groups of eight carotid bodies were incubated at 37^{o}C for two hours in a vial containing 1mM unlabelled myo-inositol and 0.925 MBq of myo-[2-^{3}H] inositol, in a total volume of 1ml 100% O_2 equilibrated Tyrodes medium. At intervals of 15 minutes during the incubation, the tissue was briefly agitated with 100% O_2. At the end of the incubation period, the radioactive medium was aspirated and the tissue was washed with 3x 1ml ice-cold Tyrodes medium.

2.7.2 [^{3}H] INOSITOL EFFLUX

Following incubation, the tissue was transferred to the superfusion apparatus in order to follow the release of [^{3}H] inositol from the prelabelled tissue. The superfusion apparatus, superfusion regime and collection periods were exactly the same as for the ^{45}Ca experiments. Aliquots of each fraction were taken for a determination of their

radioactivity, and results were expressed as cpm/carotid body/2 minutes. The data were analysed according to the procedure described in section 2.6.3.

2.7.3 INOSITOL PHOSPHATE CONTENT

Following the two hour incubation as described earlier, the tissue was washed with 3x 1ml Tyrodes medium containing 1mM unlabelled myo-inositol and 10mM lithium chloride. The tissue was re-incubated for 40 minutes at 37°C in 0.45ml of medium having the same composition as the washing medium and 50µl of a 10-fold concentration of agonist or 50µl of the normal incubation medium were added. The incubation was continued for a further 10 minutes and the reaction was terminated by the addition of 0.5ml 1M trichloroacetic acid.

2.7.4 TISSUE EXTRACTION

The contents of the incubation vial were transferred to a glass homogenization tube and left on ice for 20 minutes. After this time, the tissue was homogenized and transferred to a 1.5ml polypropylene centrifuge tube. The extract was spun for 5 minutes at 12,000g in an MSE microfuge. Following centrifugation, the supernatant was decanted into a clean glass-stoppered tube and washed with 5x 2ml water-saturated diethyl ether. The top etherized layer was discarded after each wash and after the final wash, the sample was neutralized with 0.55ml of 50mM $NaHCO_3$, ready for separation

of the inositol phosphates.

2.7.5 SEPARATION OF [^{3}H] INOSITOL PHOSPHATES

Following a method originally devised by Ellis et al. (1963), the water soluble inositol phosphates were separated by anion-exchange chromatography.

Polypropylene chromatography columns were loaded with 1ml Dowex AG 1-X8 resin of the formate form and the resin bed was washed with 3x 10ml distilled water before use.

2.7.5.1 Standardization of Dowex Columns and Sample Preparation

The method used to separate the inositol phosphates was based on that of Berridge et al. (1983) and Simpson et al. (1987).

1ml of neutralized extract containing the inositol phosphates was applied to the column and a preliminary elution profile was performed using the solvent mixtures as described in Table 2.2. Each 2ml fraction was sampled for a determination of its radioactivity.

The complete elution profile is shown in Figure 2.3 and from this, it was decided to use the final elution volumes as shown in Table 2.2.

A separate column standardization was performed using [^{3}H] IP_3 and [^{3}H] IP_4 in order to check that the tissue sample IP_3 and IP_4 fractions were being eluted at the

Table 2.2: Elution regime for the separation of [^{3}H] inositol phosphates on Dowex AG 1-X8 anion-exchange columns

ELUANT SOLVENT MIXTURE	ELUANT VOLUME (ml) Initial	Final	ELUATE
Distilled water	10x 2	20	Inositol (Ins)
5mM $Na_2B_4O_7$/60mM Ammonium formate	10x 2	20	Glycerophospho-inositol (GroIns)
0.1M Formic acid/ 0.2M Ammonium formate	10x 2	15	Inositol mono-phosphate (IP)
0.1M Formic acid/ 0.5M Ammonium formate	10x 2	15	Inositol bis-phosphate (IP_2)
0.1M Formic acid/ 0.8M Ammonium formate	10x 2	15	Inositol tris-phosphate (IP_3)
0.1M Formic acid/ 1.0M Ammonium formate	10x 2	15	Inositol tetra-kisphosphate (IP_4)

Figure 2.3: Separation of [^{3}H] inositol phosphates by anion-exchange chromatography

Carotid body tissue prelabelled with [^{3}H] inositol was homogenized in order to extract the inositol phosphates as described in section 2.7.4. The extract was centrifuged and the supernatant was placed onto a column containing Dowex-AG 1 anion exchange resin. The inositol phosphates were eluted from the resin using the stepwise regime as described in Table 2.2. Each fraction was sampled for a determination of the radioactivity associated with each inositol phosphate and any unmetabolized myo-inositol.

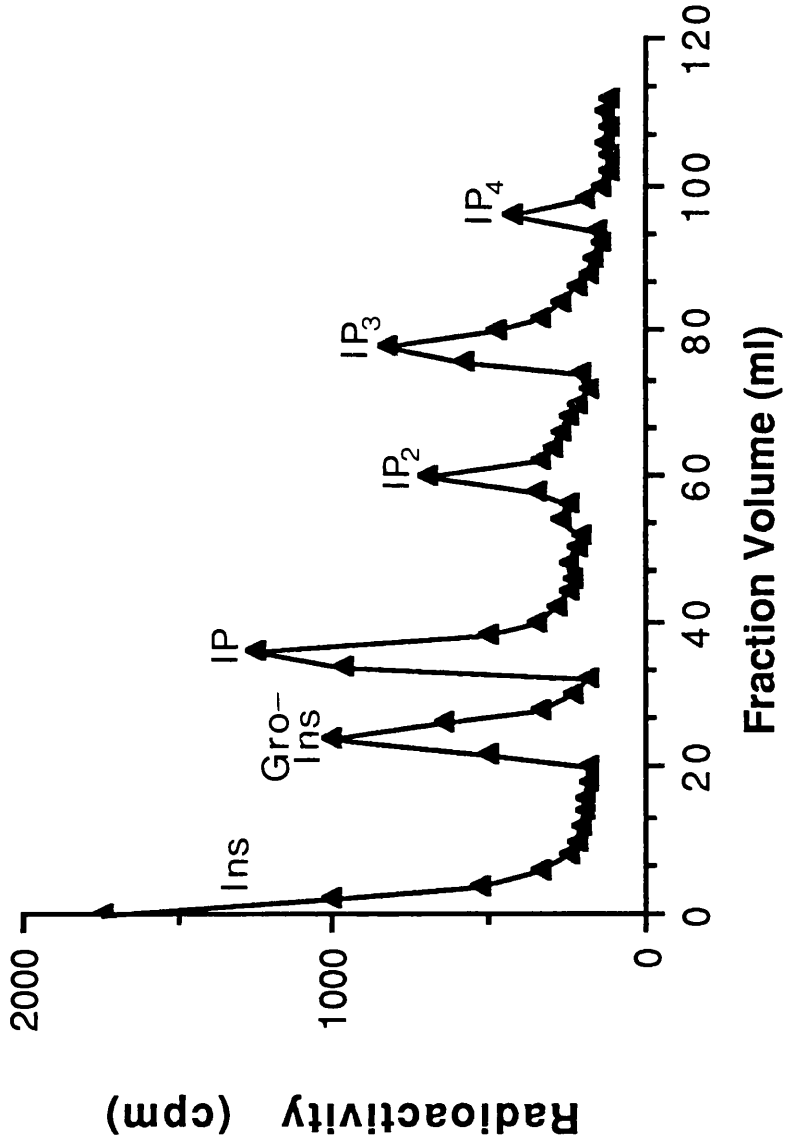

Radioactivity (cpm)
2000
1000
0
Ins
Gro-Ins
IP
IP2
IP3
IP4
0
20
40
60
80
100
120
Fraction Volume (ml)

Figure 2·3

recommended solvent concentrations. Recovery of IP_3 and IP_4 by this method was approximately 65%.

2.7.6 ANALYSIS OF THE DATA

Results were expressed as the percentage increase in radioactivity associated with each inositol phosphate following stimulation as compared with a corresponding control experiment.

2.8 DETERMINATION OF RADIOACTIVITY

Samples were mixed with a toluene-based scintillation cocktail (OptiPhase T, Fisons, Loughborough), in a ratio of 1:10, sample to scintillant, in polypropylene scintillation vials. The samples were cooled to $2^{o}C$ and analysed using a Packard 2000CA liquid scintillation spectrometer.

2.9 STATISTICAL ANALYSIS OF RESULTS

Where appropriate, the data were evaluated using Student's t-test.

CHAPTER 3

SYNTHESIS OF CATECHOLAMINES IN THE RAT CAROTID BODY IN VITRO

3.1 INTRODUCTION

A wide variety of techniques have been employed in order to attempt to overcome the technical problems involved in analysis of the endogenous amine content of tissue as small as the carotid body. Gas chromatography, mass spectrometry and radioenzymatic assays have provided quantitative estimates of both noradrenaline and dopamine (Hellstrom and Koslow, 1975; Mir et al., 1982), but by far the most sensitive method to date has been high performance liquid chromatography (HPLC) with electrochemical detection (HPLC-ED: Hansen and Christie, 1981). Current literature suggests that catecholamines can be detected by HPLC-ED most reliably when the concentration of each amine is above 0.1 pMol per carotid body. Levels of noradrenaline and dopamine in the rat carotid body have previously been found to range from 2.5-14.6 and 5.1-15.0 pMol per carotid body respectively (see Mir et al., 1982), but variations between individual carotid bodies are substantial (sometimes below or at the level of detection by HPLC) and thus pooling of samples is required to give reliable data.

In contrast to the wealth of information on the endogenous catecholamine content under both resting and stimulated conditions, there has been a relatively limited number of studies on the actual synthesis of the amines in the rat carotid body. The turnover of endogenous amines has been studied by Hellstrom and Hanbauer (1981) and also by Brokaw et al. (1985). Other indirect studies involving

enzyme assays on rat carotid body tissue have also been performed (Gonzalez et al., 1979b). By far the most comprehensive study on the kinetics of catecholamine synthesis was presented by Fidone and Gonzalez (1982), using the rabbit carotid body. Catecholamine synthesis was monitored in vitro using [^{3}H] tyrosine as a precursor for the formation of both noradrenaline and dopamine. Some workers have questioned the viability of such experiments, eg. Herdon et al. (1985), but in catecholaminergic neurons of the central nervous system, it has been shown that labelled amino acid precursors for catecholamine synthesis are taken up and mix freely in the same cellular compartments as the cold endogenous precursor which is taken up from the plasma pool (see Glowinski, 1970). Provided that the necessary enzymes for synthesis are available, the synthetic machinery of the cell is unable to distinguish between labelled or unlabelled tyrosine.

Fidone and Gonzalez (1982) followed a time course for catecholamine synthesis by incubating the tissue in a tyrosine concentration which was the same as that of rabbit plasma. Once the length of the incubation period had been determined, this was kept constant and they incubated the tissue in various [^{3}H] tyrosine concentrations in order to determine the optimum specific activity for labelling of noradrenaline and dopamine.

In this study, it was necessary to repeat the preliminary experiments of Fidone and Gonzalez, (1982) to follow the synthesis of these amines in the rat carotid body since the

calculated turnover times suggest that the synthesis kinetics are different to the carotid body of the rabbit (Hanbauer and Hellstrom, 1978). In addition, the effect of tetrahydrobiopterin (6-MPH_4) a cofactor for the enzyme tyrosine hydroxylase, on the synthesis of the amines was followed, since the availability of 6-MPH_4 is known to be a limiting factor for synthesis in other catecholaminergic tissues (eg. Kettler et al., 1974).

In some studies unilateral sympathectomy (removal of the superior cervical ganglion and its associated nerve fibres on one side of the animal), or unilateral section of the carotid sinus nerve (see Hess, 1977a) were performed in order to determine the effect on catecholamine synthesis. It has been suggested that neural input to the carotid body may influence synthesis by altering the activity of tyrosine hydroxylase or contribute to the actual carotid body amine content directly (Hanbauer et al., 1977; Gonzalez et al., 1979a).

It was hoped that these preliminary studies would provide a foundation in order to begin to study the release of catecholamines in response to natural or chemical stimulation (Chapter 4).

3.2 METHODS

The endogenous amine content of the rat carotid body was determined using HPLC with electrochemical detection as described in Chapter 2. In addition, the synthesis of [^{3}H]

noradrenaline and [^{3}H] dopamine was studied in rat carotid bodies which had been prelabelled with [^{3}H] tyrosine as described in detail in Chapter 2.

In some experiments, the incubation medium also contained a synthetic analogue of the cofactor for tyrosine hydroxylase, 6-methyltetrahydropteridine (6-MPH_4), at a concentration of 100μM. Groups of carotid bodies incubated without the cofactor served as controls.

In studies involving removal of the superior cervical ganglion (sympathectomy) or section of the carotid sinus nerve (denervation), surgical procedures were performed under aseptic conditions and the animals were allowed to recover for 1 week before the carotid bodies were removed under terminal anaesthesia. Sympathectomy or denervation experiments were performed unilaterally, following intramuscular injection of the neuroleptic analgesic 'Immobilon' (Reckitt and Colman, Hull, UK), at a dose of 0.05ml/100g body weight, and after surgery, anaesthesia was reversed using 'Revivon' (Reckitt and Colman, Hull, UK). Following the recovery period, the animals were anaesthetized with 'Urethane' as described in detail in Chapter 2 and the carotid bodies were removed. Experimental tissue was prelabelled separately from the contralateral controls. At the end of all experiments, it was possible to check that the labelled products formed were noradrenaline and dopamine using HPLC as described in detail in Chapter 2.

3.3 RESULTS

The endogenous noradrenaline content of the freshly isolated carotid body was found to range from 1.3-10.3 pMole with a mean of 3.6 ± 0.3 pMol/carotid body and the endogenous dopamine content was in the range 1.2-18.5 pMole with a mean of 4.2 ± 0.6 pMole/carotid body. These data were obtained from 42 individual observations.

The effect of incubating carotid body tissue in various [^{3}H] tyrosine concentrations for an initial period of 2 hours is shown in Figure 3.1. The data describe the accumulation of radioactivity associated with both [^{3}H] noradrenaline and [^{3}H] dopamine as a function of the precursor concentration.

The results shown in Figure 3.1 indicate that the carotid body is able to synthesize both [^{3}H] noradrenaline and [^{3}H] dopamine from [^{3}H] tyrosine.

The relationship between [^{3}H] noradrenaline synthesis and precursor concentration was linear over the range 10-40μM with synthesis increasing by approximately 200% over this range of concentrations. At concentrations above 40μM, the rate of [^{3}H] noradrenaline synthesis did not increase significantly.

The relationship between [^{3}H] dopamine synthesis and precursor concentration was linear over the range 10-60μM with synthesis increasing by approximately 270% over this range of tyrosine concentrations. At concentrations greater than 60μM tyrosine, there were no further increases in the

Figure 3.1: Effect of precursor concentration on the synthesis of [^{3}H] catecholamines

Pairs of carotid bodies were incubated for 2 hours in Tyrodes medium containing various concentrations of tyrosine as shown. At the end of the incubation period, the tissue was homogenized in 0.1M HCl and the homogenate centrifuged for 3 minutes. Supernatants containing the labelled amines were neutralized with 2M K_2CO_3 and 1M HEPES before being placed onto Dowex-50 cation exchange columns from which the amines were subsequently eluted (Chapter 2). Fractions containing [^{3}H] noradrenaline and [^{3}H] dopamine were then sampled for a determination of their radioactivity.

Each point represents the mean ± SEM of 5 observations.

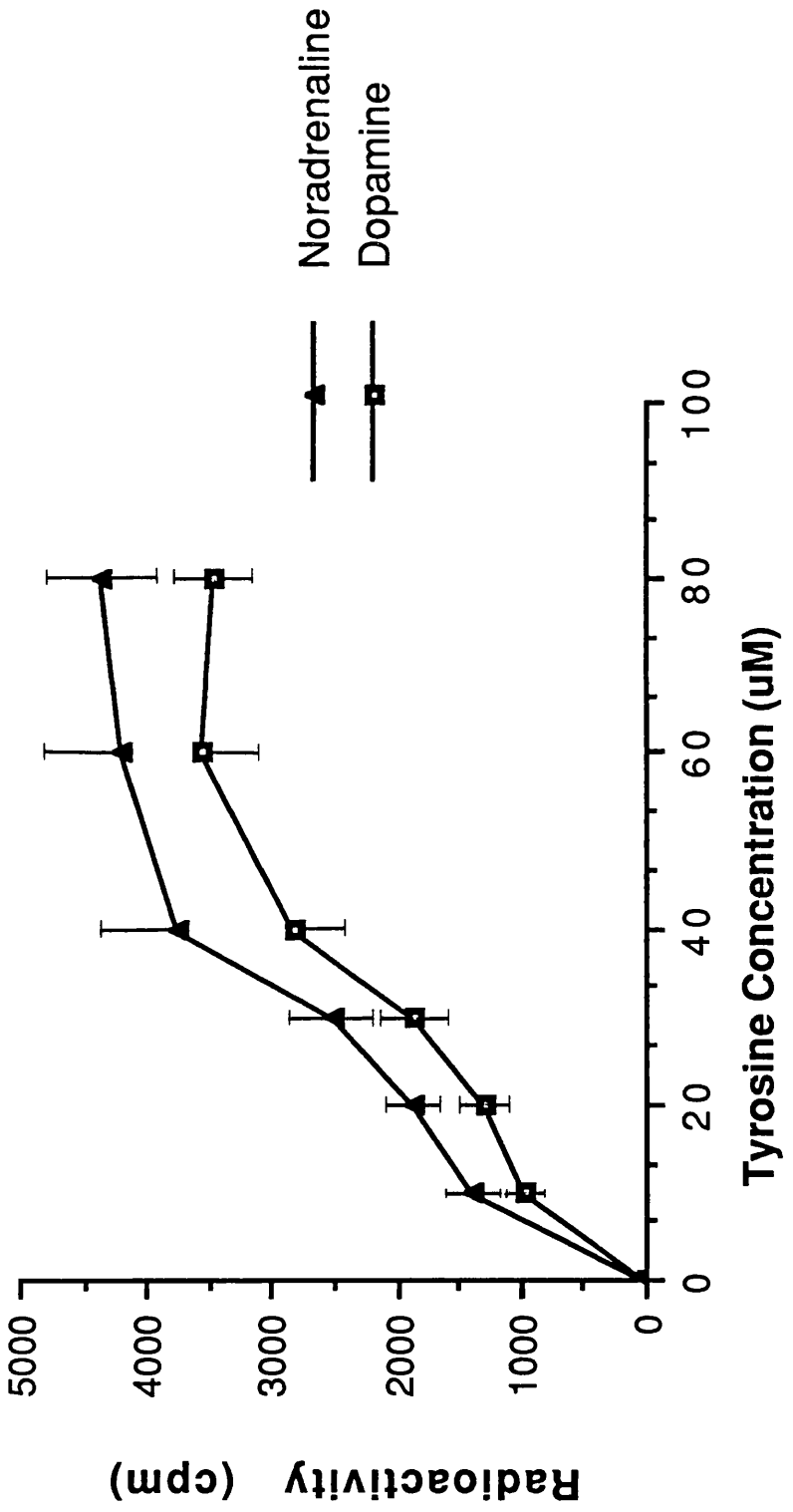

Noradrenaline
Dopamine
Radioactivity (cpm)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Tyrosine Concentration (uM)

Figure 3·1

rate of dopamine synthesis.

The effect of incubating carotid body tissue in media containing 50μM [^{3}H] tyrosine for variable time periods is shown in Figure 3.2. The data describe the accumulation of radioactivity associated with both [^{3}H] noradrenaline and [^{3}H] dopamine as a function of incubation time.

Carotid body tissue incubated in the presence of 50μM tyrosine was found to rapidly synthesize [^{3}H] noradrenaline within the first 60 minutes of incubation. The relationship between [^{3}H] noradrenaline synthesis and incubation period was linear up to 60 minutes but longer incubation periods resulted in no further increases in synthesis. The relationship between [^{3}H] dopamine synthesis and incubation period was linear up to 90 minutes. Incubation times greater than 90 minutes resulted in no further increases in synthesis.

The effect of incubating pairs of carotid bodies in the presence or absence of the tyrosine hydroxylase cofactor 6-methyltetrahydropteridine (6-MPH$_4$), the effects of carotid sinus nerve section or removal of the superior cervical ganglion on the synthesis of catecholamines from [^{3}H] tyrosine under standard prelabelling conditions (Chapter 2) are described in Table 3.1.

The results in Table 3.1 demonstrate that addition of the tyrosine hydroxylase cofactor 6-MPH$_4$ to the incubation medium has no significant effect on the synthesis of either [^{3}H] noradrenaline or [^{3}H] dopamine. Section of the carotid sinus nerve one week prior to tissue isolation appears to have no significant effect on either the accumulation of

Figure 3.2: Effect of the length of incubation period on the synthesis of [^{3}H] catecholamines from [^{3}H] tyrosine

Pairs of carotid bodies were incubated in 50μM [^{3}H] tyrosine for various incubation periods as shown. At the end of each incubation period, the catecholamines were extracted from the tissue and separated as described for Figure 3.1.

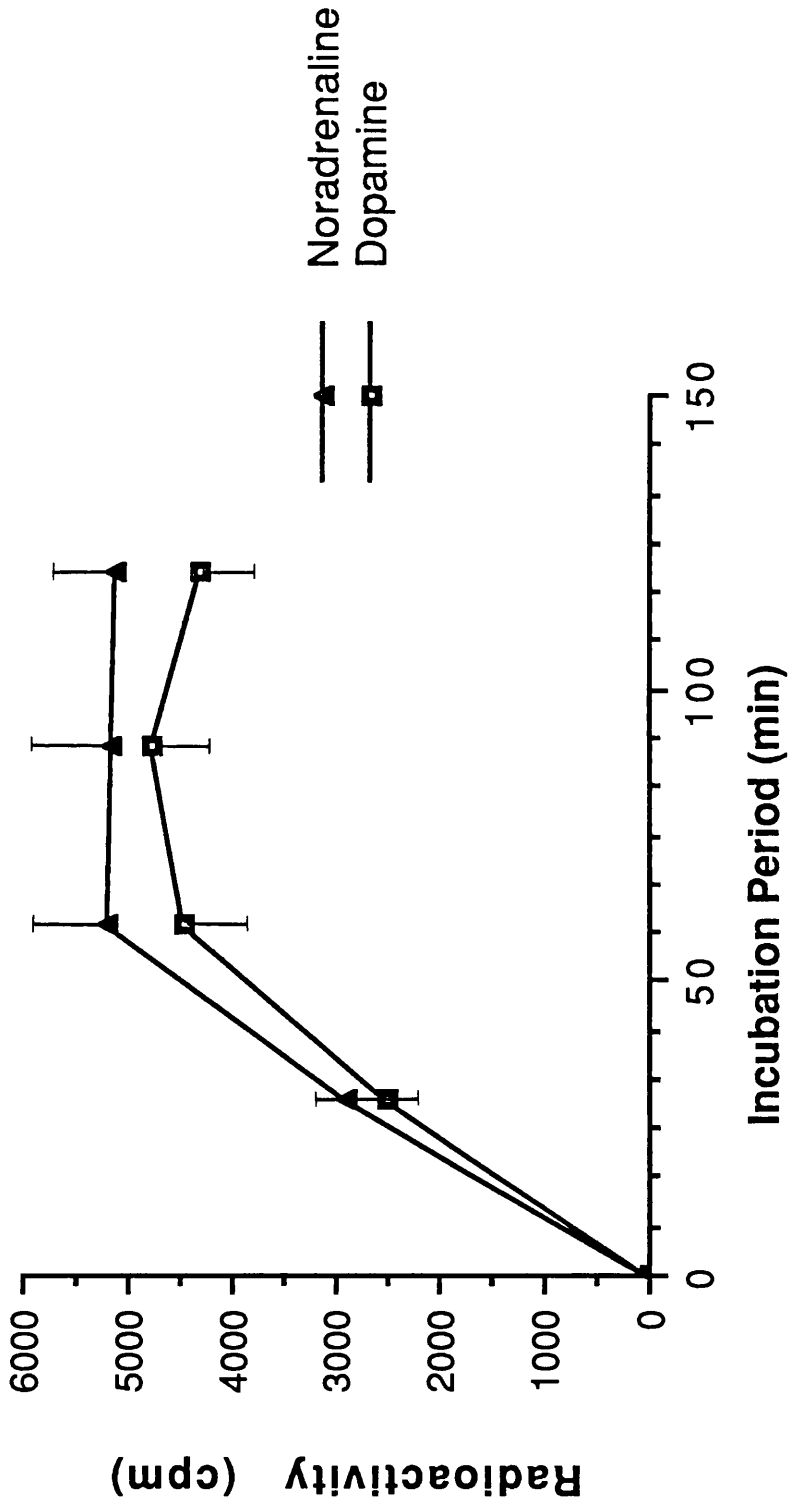

Noradrenaline
Dopamine
Radioactivity (cpm)
6000
5000
4000
3000
2000
1000
0
0
50
100
150
Incubation Period (min)

Figure 3.2

Table 3.1

In the first series of experiments, pairs of carotid bodies were incubated in 50μM [^{3}H] tyrosine for 2 hours in the absence or presence of the tyrosine hydroxylase cofactor, 6-MPH$_4$. In the second and third series of experiments, carotid bodies were isolated from rats approximately one week after unilateral denervation or sympathectomy. Experimental carotid bodies were incubated separately from their contralateral controls in 50μM [^{3}H] tyrosine for 2 hours (Chapter 2).

In all cases, at the end of the 2 hour incubation period, the catecholamines were extracted from the tissue and separated as described for Figure 3.1.

Results are expressed as radioactivity associated with either [^{3}H] noradrenaline or [^{3}H] dopamine (counts per minute) per pair of carotid bodies ± SEM. The number of observations are shown in parentheses.

* indicates values significantly different from corresponding control ($p < 0.05$).

Table 3.1: Effects of 6-MPH_4, denervation or sympathectomy on the synthesis of [^3H] catecholamines

Conditions	Radioactivity (cpm/pair carotid bodies)			
	Noradrenaline		Dopamine	
	Control	Experiment	Control	Experiment
6-MPH_4 (100μM) (6)	2319.24 ± 124.85	2347.05 ± 136.28	1035.45 ± 71.90	1335.82 ± 88.65
Denervation (15)	1381.86 ± 93.24	1289.01 ± 71.04	943.44 ± 49.83	936.76 ± 52.11
Sympathectomy (15)	1663.50 ± 112.08	1348.05* ± 97.34	848.43 ± 35.32	896.04 ± 66.91

[^{3}H] noradrenaline or [^{3}H] dopamine. Although sympathectomy appears to have no effect on the [^{3}H] dopamine content of the carotid body tissue, the synthesis of [^{3}H] noradrenaline falls by approximately 18% when compared to control values.

3.4 DISCUSSION

In the present study, it was of particular interest to determine the relative concentrations of endogenous noradrenaline and dopamine in the rat carotid body since this has been a point of considerable controversy in the literature. The present study was unable to identify a so-called 'dominant' amine and both were present at much lower concentrations than have been previously reported using HPLC (see Mir et al., 1982). The results in this study (3.6 pMol and 4.2 pMol/carotid body for noradrenaline and dopamine respectively), most closely resemble those obtained by Chiocchio et al. (1981) who reported that the rat carotid body contained 4.5 and 5.1 pMole of noradrenaline and dopamine respectively, using a radioenzymatic assay. In addition, Hanbauer and Hellstrom (1978) obtained values of 2.5 pMole for noradrenaline and 8.5 pMole for dopamine. As a result of the low concentrations of the amines and the large variability in the present study, HPLC was not subsequently used for quantitative estimates of amine content.

Preliminary experiments were designed to determine the conditions that were necessary to ensure that the incorporation of [^{3}H] tyrosine into carotid body catecholamines was maximal. In order to do this, experiments were performed to determine the optimum precursor concentration and the time course of catecholamine synthesis.

The synthesis of [^{3}H] noradrenaline reached a maximum

level at a tyrosine concentration of 40μM while [^{3}H] dopamine synthesis was maximal in the presence of 60μM tyrosine. From these values, a K_m for [^{3}H] catecholamine synthesis can be estimated to be approximately 25μM and this is in good agreement with the value of 17μM obtained in the rabbit carotid body (Fidone and Gonzalez, 1982). The estimated value is also similar to that obtained by Levitt et al. (1965) for noradrenaline synthesis in the perfused guinea-pig heart (20μM). On the basis of these results, all subsequent experiments contained [^{3}H] tyrosine at a final concentration of 50μM in the incubation medium.

As shown in Figure 3.2, incorporation of [^{3}H] tyrosine into [^{3}H] noradrenaline was maximal after a 60 minute incubation in 50μM tyrosine, while [^{3}H] dopamine synthesis reached its optimal level after a 90 minute incubation period. These results differ from those of Fidone and Gonzalez (1982) and Rigual et al. (1986) who reported that [^{3}H] catecholamine synthesis was linear for periods of up to 4 hours in the rabbit and cat carotid body, respectively. This discrepancy may indicate a significant difference in the activities of tyrosine hydroxylase between species since this enzyme represents a rate-limiting point in the synthesis of the catecholamines. Although a 90 minute incubation period would seem appropriate in order to maximally synthesize both amines, in subsequent experiments the incubation period was extended to 120 minutes in order to ensure that all of the newly-synthesized catecholamines mixed freely in all pools of endogenous amine.

Addition of 6-MPH_4, a synthetic analogue of the cofactor for tyrosine hydroxylase, was found to have no effect on the synthesis of either catecholamine. This finding disagreed with that of Fidone and Gonzalez (1982) who reported an 84% and 350% increase in dopamine and noradrenaline synthesis respectively after administration of 100μM cofactor to incubation media containing rabbit carotid body tissue. The results in the present study suggest that there are adequate endogenous stores of the natural cofactor for tyrosine hydroxylase in the carotid body of the rat. In subsequent experiments, exogenous cofactor was omitted from the incubation medium.

In the second series of experiments, the effects of sympathectomy and carotid sinus nerve-denervation on catecholamine synthesis were determined. In all cases the [^{3}H] dopamine content was unaltered and these results are in agreement with studies performed on rabbit (Fidone and Gonzalez, 1982) and rat carotid bodies (Hanbauer and Hellstrom, 1978). These results suggest that dopamine is synthesized and stored within the Type I cells of the carotid body. The [^{3}H] noradrenaline content was unaltered following denervation but was significantly reduced following sympathectomy. These observations are quantitatively dissimilar to other reports but are in general overall agreement with a number of workers, eg. Fidone and Gonzalez (1982) on the rabbit, Hanbauer and Hellstrom (1978) on the rat and Mir *et al*. (1982) on the rat carotid body, who all suggested a noradrenaline store

outside the carotid body which may contribute towards the overall noradrenaline content in the intact preparation. In view of these findings, carotid bodies were routinely sympathectomized during isolation.

In the present study, the conditions required to ensure that the rat carotid body catecholamine pools were optimally prelabelled, were fully established. These results formed the basis for subsequent experiments concerned with the release of the amines in response to a variety of physiological and pharmacological agents.

CHAPTER 4

STUDIES ON THE RELEASE OF CATECHOLAMINES FROM THE RAT CAROTID BODY IN VITRO

4.1 INTRODUCTION

An important criterion in defining the action of neurotransmitter-like substances is the demonstration of release in response to physiological stimuli. Much of the evidence for stimulus-related release of catecholamines from the carotid body has been based on morphological studies of alterations in the intensity of catecholamine fluorescence of the Type I cells or in the numbers of dense-cored vesicles in these cells. There have been relatively few biochemical studies and these have focused on changes in amine content in the carotid body rather than directly on release. Many attempts have been made to correlate changes in amine content or amine release with alterations in chemosensory discharge in afferent fibres during stimulation (eg. Rigual *et al.*, 1986), in the hope that the role of the catecholamines in the chemoreception process could be fully determined.

Sensory discharges of the carotid sinus nerve have been shown to increase in frequency following a fall in arterial O_2 tension, pH, or an increase in CO_2 tension (Eyzaguirre *et al.*, 1975; McDonald, 1977b). In addition, increases in temperature, tonicity or stimulation using chemical substances such as cyanide or K^+ ions also increase the firing rate (see Eyzaguirre and Fidone, 1980). In the carotid bodies of rabbits and cats, hypoxia has been shown to evoke dopamine release and an associated chemosensory discharge *in vitro*. Evidence obtained from other tissues

such as the rat striatum have implicated the involvement of membrane cholinergic receptors in the regulation of endogenous dopamine release (Westfall et al., 1976). Moreover, several studies have demonstrated that afferent fibres from the carotid body alter their discharge frequency following application of exogenous acetylcholine to the in vitro preparation. In addition, it has been shown that acetylcholine can alter the membrane potential of Type I cells in the cat carotid body. Type I cell membranes are thought to possess both nicotinic and muscarinic receptor sites (Eyzaguirre and Monti-Bloch, 1982) since both cat and rat carotid bodies have been shown to bind [^{125}I] alpha-bungarotoxin, a nicotinic marker (Chen et al., 1981; Dinger et al., 1981) while application of muscarinic agents are known to depolarize the Type I cells of cats, rabbits and mice. Moreover, it has been postulated that cholinergic agents may elicit catecholamine release from the Type I cells since the dopamine content of the rat carotid body has been shown to be reduced by methacholine administration (but not nicotine) and the effect can be blocked by methylatropine (Hellstrom and Hanbauer, 1981).

In the studies presented in the present chapter, the release of newly synthesized catecholamines in response to a variety of natural and chemical stimuli were investigated directly using an in vitro superfusion method as introduced by Eyzaguirre and Lewin (1961). The superfused carotid body preparation was developed in order to simplify the interpretation of electrophysiological experiments by

excluding the effects of alterations in blood flow through the carotid body as a possible cause of the recorded changes in chemoafferent activity. This method has since been used to successfully study the release of newly synthesized catecholamines from the carotid body.

It was hoped that the results in this study would provide an indication as to the possible intracellular mechanisms involved in the secretory process of the rat carotid body.

4.2 METHODS

The release of newly-synthesized catecholamines from the rat carotid body was studied using an *in vitro* superfusion system as described in detail in Chapter 2.

The concentrations of the various reagents used in the stimulus phase are shown in Table 4.1. In some experiments, calcium was omitted from the superfusion medium and 100μM EGTA was added in order to chelate any remaining Ca^{2+} ions that may have been present. In experiments involving atropine, this was added to the superfusion medium 10 minutes prior to the stimulus phase. Nitrendipine was prepared as a 1mM stock solution in ethanol and diluted as required with Tyrodes medium. Sodium cyanide was prepared as a 20mM stock solution in Tyrodes medium and added to the superfusion reservoir just prior to the stimulus phase in order to minimize HCN evolution (which is maximal within the pH range 7.4-7.5).

4.3 RESULTS

The time course of the release of [^{3}H] noradrenaline and [^{3}H] dopamine from superfused rat carotid bodies using unsupplemented Tyrodes medium is shown in Figure 4.1.

Superfusion of carotid body tissue with unsupplemented Tyrodes medium resulted in an initial rapid release of both [^{3}H] noradrenaline and [^{3}H] dopamine which reached a steady basal release phase after 40 minutes.

The effects of various agents on the release of newly-synthesized [^{3}H] noradrenaline and [^{3}H] dopamine from carotid body tissue prelabelled with 50μM [^{3}H] tyrosine for 2 hours are shown in Table 4.1.

Following the introduction of a hypoxic stimulus after 40 minutes, the amount of radioactivity associated with [^{3}H] noradrenaline and [^{3}H] dopamine had significantly increased when compared to their corresponding basal release phases.

The response to hypoxia or carbachol was reduced by the addition of nitrendipine or atropine to the superfusion medium and in addition, the hypoxia-related response was reduced in the absence of extracellular calcium. The release of [^{3}H] dopamine was unaffected in the presence of high extracellular K^+ but there was a significant increase in the release of [^{3}H] noradrenaline. Sodium cyanide was able to evoke the release of both amines. The 46% increase in radioactivity associated with dopamine was comparable to the values obtained during stimulation with either hypoxia (52%) or carbachol (39%).

Figure 4.1: Release of [^{3}H] catecholamines from the superfused rat carotid body

Groups of eight carotid bodies prelabelled with [^{3}H] tyrosine were superfused for an initial period of 40 minutes to establish the basal release phase as described in Chapter 2. The tissue was superfused for a further 10 minutes with medium supplemented as shown in Table 4.1 and this was followed by a 10 minute recovery phase. Catecholamines released into the superfusate were separated on columns of Dowex-50 cation exchange resin as described in Chapter 2 and quantified by determination of their radioactivity.

The point at which a potential stimulus was introduced into the superfusion medium is shown by the arrow.

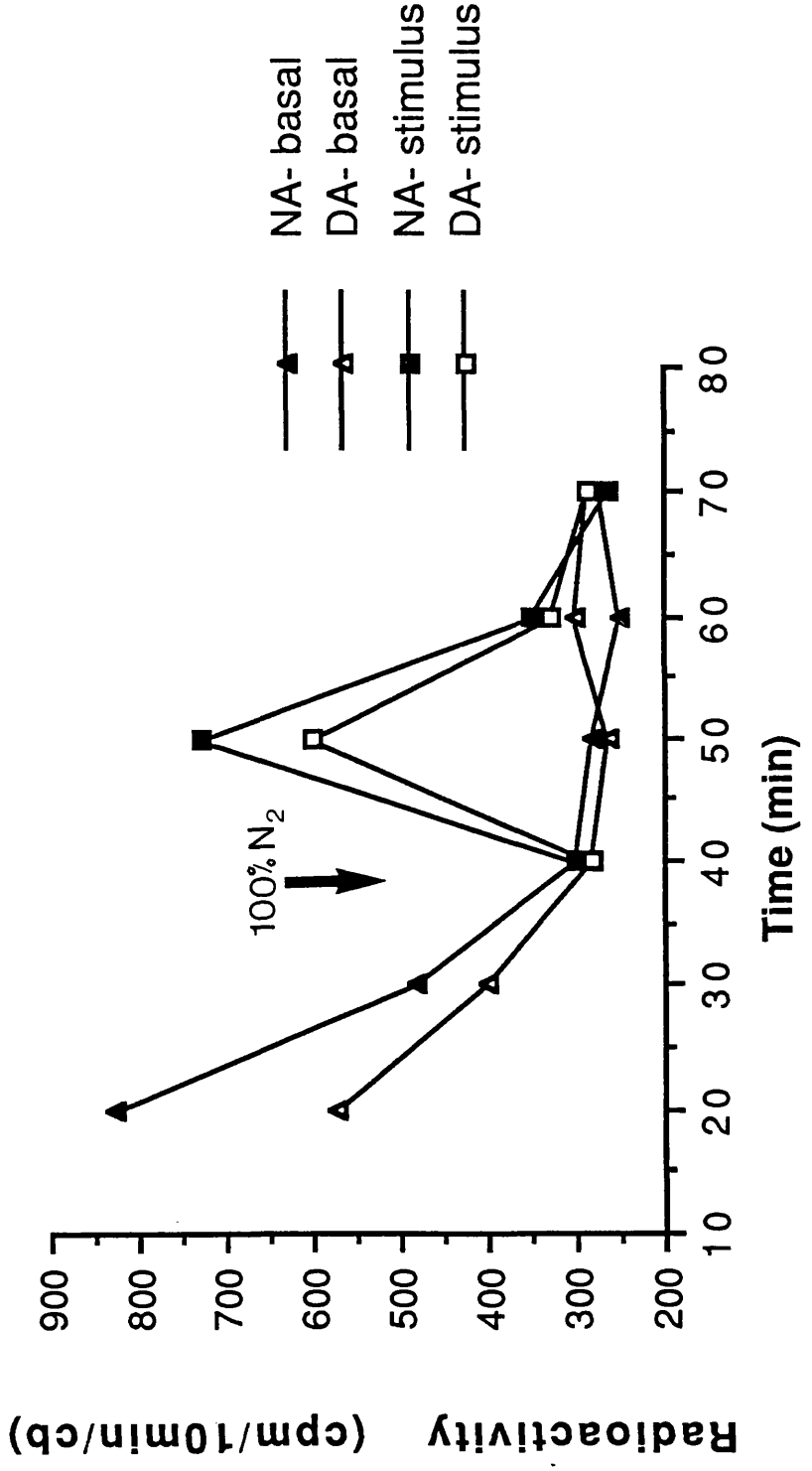

NA- basal
DA- basal
NA- stimulus
DA- stimulus
100% N_2
Radioactivity (cpm/10min/cb)
Time (min)
900
800
700
600
500
400
300
200
10
20
30
40
50
60
70
80

FIG 4·1

Table 4.1

Carotid body tissue prelabelled with [^{3}H] tyrosine was superfused as described in Figure 4.1. Catecholamines released into the superfusate were separated by a method described in Chapter 2 and quantified by determination of their radioactivity.

Each value represents the mean ± SEM of the increase in radioactivity during the 10 minute stimulation period compared to the radioactivity associated with the average of the two 10 minute periods immediately before and after the stimulation period.

'n' = numbers of observations in each case.

* indicates values significantly different from the corresponding basal release phase ($p < 0.01$).

** indicates values significantly different from a hypoxic stimulus alone ($p < 0.01$).

*** indicates values significantly different from a carbachol stimulus alone ($p < 0.01$).

Table 4.1: Effect of hypoxia, muscarinic stimulation, depolarization and metabolic inhibition on the release of [^{3}H] catecholamines from the rat carotid body

Additions to the superfusion medium	Increase in radioactivity %		
	Noradrenaline	Dopamine	'n'
Hypoxia	42.1 ± 8.9*	52.4 ± 3.7*	10
Hypoxia - calcium	31.3 ± 5.2	15.9 ± 3.3**	10
Hypoxia + nitrendipine (1μM)	18.2 ± 4.5**	20.2 ± 3.6**	10
Hypoxia + atropine (100μM)	11.3 ± 2.5**	11.5 ± 1.5**	10
Carbachol (100μM)	23.9 ± 4.4*	39.3 ± 3.2*	8
Carbachol (100μM) + nitrendipine (1μM)	16.7 ± 2.8***	10.7 ± 1.8***	8
Carbachol (100μM) + atropine (100μM)	0 ± 1.5***	0 ± 0.3***	8
KCl (55mM)	7.3 ± 1.2	0 ± 1.8	6
NaCN (2mM)	7.5 ± 2.1	46.5 ± 4.3*	6

4.4 DISCUSSION

The release of [^{3}H] noradrenaline and [^{3}H] dopamine from the unstimulated, superfused carotid body, decreased rapidly during the first 40 minutes of superfusion after which time a second, slower release phase (basal release) became apparent. The change in slope (Figure 4.1) may be attributed to the emptying of two different catecholamine pools with the initial phase representing the release of a more readily releasable pool (Bevington and Radda, 1985).

The results in Table 4.1 show that hypoxia stimulates the release of both amines, a finding which has not previously been reported in the rat. Fidone *et al*. (1982) studied the effects of various oxygen concentrations on [^{3}H] dopamine release from the prelabelled rabbit carotid body and reported that the rate of [^{3}H] dopamine release was inversely related to the oxygen tension of the medium. Fidone *et al*. (1982) were able to stimulate secretion using 30% O_2 in N_2 as a 'hypoxic' stimulus while in the present study [^{3}H] dopamine release could only be evoked in the presence of 100% N_2 (pO_2 = 40mmHg). The rat carotid body would therefore seem to be less sensitive to moderate levels of hypoxia than the rabbit carotid body. The reasons for this difference in sensitivity are unknown but may be a result of differences in tyrosine hydroxylase activity between species and also a different effect of hypoxia on enzyme activity during catecholamine synthesis, since in many systems, agents which are known to be potent

secretagogues also modify synthesis.

In the present study, the release of the two amines was affected to different extents during stimulation by a variety of agents and this would suggest that they are released from separate Type I cells. This suggestion is in agreement with a number of others and may reflect the distribution of dopamine-β-hydroxylase between Type I cells. The release of [^{3}H] dopamine was significantly reduced in the absence of extracellular calcium and this finding was in agreement with that of Fidone et al. (1982) who demonstrated that hypoxia-induced [^{3}H] dopamine release from the rabbit carotid body required the presence of extracellular calcium. In addition, the results from the present study have shown that the L-type calcium channel antagonist, nitrendipine, was able to significantly reduce the hypoxia-induced release of dopamine to a similar extent as the removal of extracellular calcium. These results suggest that the release of dopamine from the rat Type I cells is largely dependent on the entry of extracellular calcium into the cells via plasma membrane calcium channels which are gated by membrane potential (voltage-dependent). The presence of these channels in Type I cell membranes had been previously suggested by Gual and Stensaas (1985). Although there clearly is a requirement of extracellular calcium for a full secretory response, additional mechanisms of control may operate to regulate the secretory process since catecholamine release in response to hypoxia is not completely inhibited in the absence of extracellular calcium.

The muscarinic cholinergic agonist carbachol was found to stimulate the release of both catecholamines suggesting the presence of cholinergic receptors on rat Type I cell membranes. The response to carbachol could be blocked by atropine which suggests that carbachol operates via a muscarinic receptor-mediated event. Muscarinic receptor activation may lead to a number of intracellular events which begin with the activation of membrane-bound enzymes resulting in the generation of second messenger systems (Chapter 1) and in some cases, the mobilization of intracellular calcium from organelle-bound stores. Since the carbachol-induced release of the catecholamines could not be totally abolished by nitrendipine, which would be expected to prevent the entry of extracellular calcium via the voltage-sensitive calcium channels, it is possible that secretion is maintained at a lower level by intracellular calcium stores.

In terms of catecholamine release, the rat carotid body showed a remarkable insensitivity to superfusion with a high extracellular potassium concentration. This result was surprising since high (> 30mM) concentrations of potassium have been shown to trigger catecholamine release from a number of secretory systems including the adrenal chromaffin cells (Schneider et al., 1981; Sasakawa et al., 1984; Burgoyne and Cheek, 1985; Cheek et al., 1989), rat striatal slices (Westfall et al., 1976) and the caudate nucleus (Nieollon et al., 1977). High levels of extracellular potassium have also been found to stimulate dopamine release

following membrane depolarization of cat carotid body Type I cells (Almaraz et al., 1986). Membrane depolarization is thought to result in the opening of voltage-dependent calcium channels thus causing an increase in the cytosolic calcium concentration (Chapter 5). It may well be that in the rat carotid body, this calcium increase is insufficient to evoke release of the catecholamines but may be sufficient to induce the secretion of other endogenous putative neurotransmitters such as acetylcholine.

In contrast to potassium, sodium cyanide was able to stimulate dopamine release and this was quantitatively similar to the response evoked by hypoxia. A similar effect has been reported in the cat carotid body (Obeso et al., 1985). Cyanide is known to be a potent chemoreceptor stimulant in vivo (Joels and Neil, 1968; McQueen, 1980) and an uncoupler of oxidative respiratory metabolism. In the carotid body, it is thought to operate by altering cellular ATP levels and promoting cellular calcium fluxes which may trigger a secretory response. It has been suggested that hypoxia may exert its effects on the carotid body in a similar way to that of cyanide, but experimental evidence for alterations in cellular ATP has been contradictory (Obeso et al., 1985; Talib et al., 1988).

The results in this chapter have provided an indication of the agents which are capable of inducing catecholamine secretion from the rat carotid body. In the following chapters, the actual intracellular mechanisms which may contribute to the overall response are studied in some detail.

CHAPTER 5

THE ROLE OF CALCIUM IN THE SECRETORY RESPONSE OF THE CAROTID BODY

5.1 INTRODUCTION

Many hormones, neurotransmitters and other intercellular communicators exert their effects by altering the concentration of free ionized calcium in the cytosol of their target cells. Agents that are able to elevate the cytosolic calcium concentration do so mainly by opening voltage-dependent calcium channels in the plasma membrane, thereby permitting the entry of extracellular calcium or by mobilizing calcium from intracellular stores (Chapter 1). Calcium fluxes may be studied in a number of ways. Some workers have used Ca^{2+}-sensitive electrodes (eg. Streb et al., 1983) or fluorescent intracellular probes, for example, Quin 2 (Joseph et al., 1984).

The results in Chapter 4 demonstrated that catecholamine release in response to hypoxia was not completely inhibited in the absence of extracellular calcium or in the presence of the calcium channel antagonist, nitrendipine. The results suggested that although extracellular calcium plays an important role in the secretory response to hypoxia, additional control mechanisms must operate in the Type I cells in order to 'fine' regulate the response.

In view of these results, the involvement of changes in the cytosolic calcium concentration in the secretory response was investigated further. This was achieved by following the effects of the various agents, on the efflux of ^{45}Ca from the pre-loaded carotid body, since alterations in ^{45}Ca efflux provides a sensitive index of changes in

intracellular calcium concentration. The agents were categorized as muscarinic agonists, depolarizing stimuli or agents which may have some direct metabolic effect on the Type I cells. The results are discussed in relation to the ability of the various types of agents to stimulate catecholamine secretion release from the isolated carotid body.

5.2 METHODS

^{45}Calcium efflux measurements from superfused carotid bodies which had been pre-loaded with ^{45}Ca were made using a modification of the method of Frankel *et al*. (1978), as described in detail in Chapter 2.

The concentrations of the various reagents used in the stimulus phase are shown in Table 5.1. In some experiments, atropine was added to the superfusion medium 10 minutes prior to the stimulus phase in order to atropinize the cells.

Nitrendipine was prepared as a 1mM stock solution in ethanol and diluted as required with Tyrodes medium. In experiments involving cyanide, this was prepared as a concentrated stock solution in Tyrodes medium and added to the superfusion medium just prior to the stimulus phase.

5.3 RESULTS

A time course of ^{45}Calcium efflux from pre-loaded rat carotid body tissue, superfused with unsupplemented Tyrodes medium, is shown in Figure 5.1.

There was a rapid efflux of ^{45}Calcium from the tissue during the first 30 minutes of superfusion. A slower, more steady efflux phase was established after 40 minutes and this was maintained throughout the 70 minute superfusion regime.

After the initial 40 minutes of superfusion with unsupplemented Tyrodes medium, it was possible to determine the effects of various agents on ^{45}Calcium efflux from the tissue. The results are shown in Table 5.1.

When carotid bodies were superfused with hypoxic superfusion medium after 40 minutes, there was a 53% increase in ^{45}Ca efflux within 4 minutes of introduction of the stimulus when compared to the basal release phase. Addition of the calcium channel antagonist nitrendipine resulted in a significant reduction (approximately 77%) of the response to hypoxia.

The introduction of carbachol into the superfusion medium induced a 45% increase in ^{45}Ca efflux when compared to the basal efflux phase. Nitrendipine was able to block the carbachol effect by approximately 50%. In addition, the muscarinic antagonist atropine completely blocked the response to carbachol. However, when atropine was incorporated into the superfusion medium during hypoxic

Figure 5.1: ^{45}Calcium efflux from the superfused rat carotid body

Carotid body tissue pre-loaded with ^{45}Ca was superfused with unsupplemented Tyrodes medium over a period of 70 minutes as described in Chapter 2. The superfusate was collected over 2 minute periods and each fraction was quantified by determination of its radioactivity.

The point at which a potential stimulus was introduced into the superfusion medium is shown by the arrow.

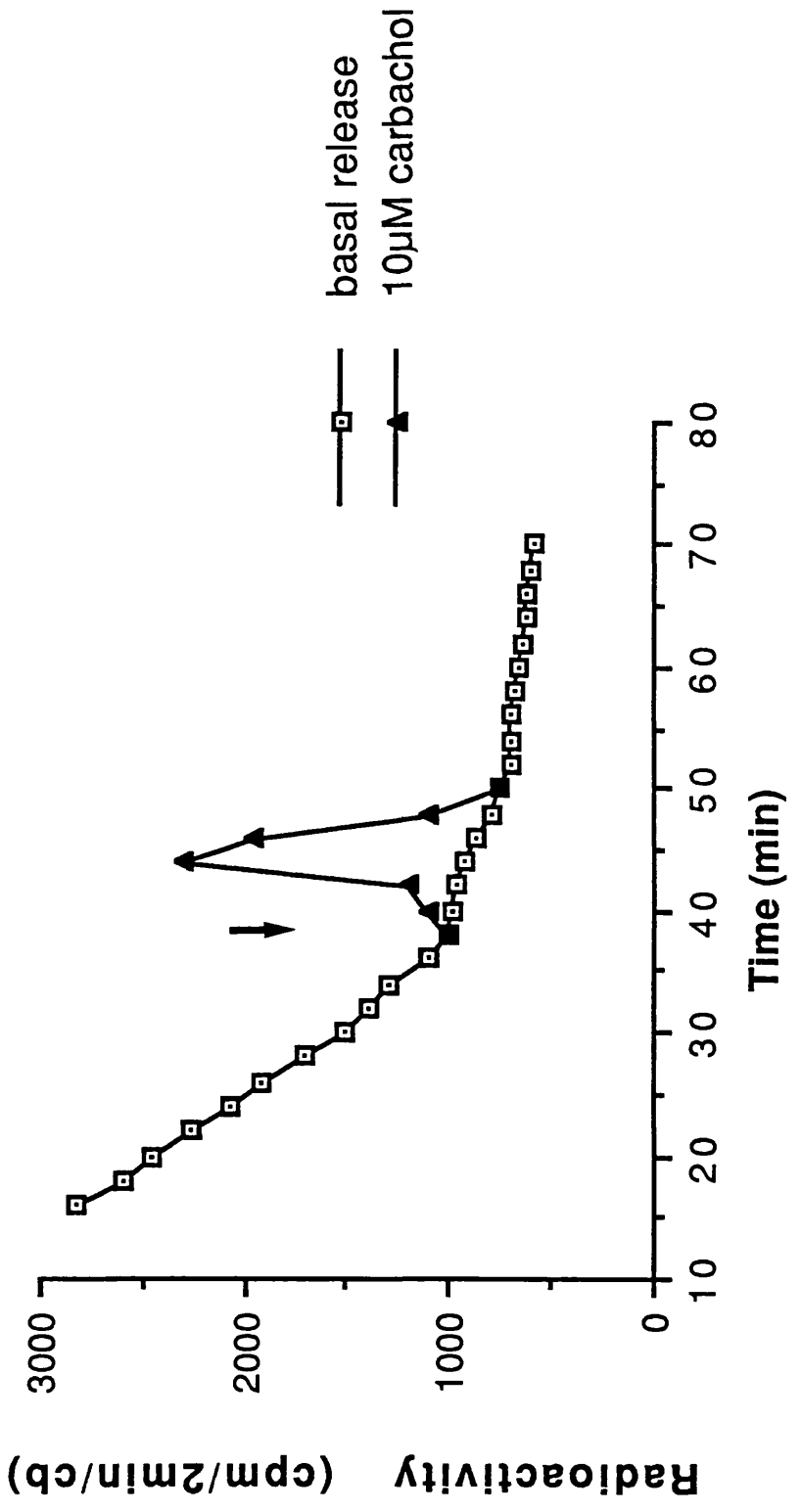

basal release
10µM carbachol
Radioactivity (cpm/2min/cb)
3000
2000
1000
0
10
20
30
40
50
60
70
80
Time (min)

FIG 5·1

Table 5.1

Groups of 4 carotid bodies preloaded with ^{45}Ca were superfused with Tyrodes medium for an initial period of 40 minutes to establish the basal release phase as described in Chapter 2. The tissue was superfused for a further 10 minutes with medium supplemented as shown in Table 5.1 and this was followed by a 10 minute recovery phase. ^{45}Ca released into the superfusate was determined.

Each value represents the mean ± SEM of the increase in radioactivity during the 10 minute stimulation period compared to the radioactivity associated with the average of the two 10 minute periods immediately before and after the stimulation period or as compared to an appropriate control experiment.

The number of observations in each case are shown in parentheses.

* indicates values significantly different from the corresponding basal release phase ($p < 0.001$).

** indicates values significantly different from hypoxia alone ($p < 0.01$).

*** indicates values significantly different from carbachol alone ($p < 0.01$).

Table 5.1: Effect of hypoxia, muscarinic stimulation, depolarizing stimuli and metabolic inhibition on ^{45}Ca efflux from the carotid body

Additions to the superfusion medium	Increase in radioactivity %
Hypoxia	52.9 ± 5.9* [8]
Hypoxia + Nitrendipine (1μM)	11.9 ± 2.3** [8]
Hypoxia + Atropine (10μM)	26.2 ± 4.3** [8]
Carbachol (10μM)	45.3 ± 7.8* [8]
Carbachol (10μM) + Nitrendipine (1μM)	22.5 ± 5.0*** [8]
Carbachol (10μM) + Atropine (10μM)	0 ± 1.8*** [8]
KCl (55mM)	29.3 ± 7.7* [6]
NaCN (2mM)	39.3 ± 7.4* [6]

stimulation, the efflux of ^{45}Ca was only reduced by approximately 50% (Table 5.1). Both 55mM K^+ and 2mM NaCN were able to induce significant increases in the release of labelled Ca^{2+} (approximately 30% and 40% respectively) when compared to their corresponding basal release phases.

5.4 DISCUSSION

When carotid bodies pre-loaded with ^{45}Ca were superfused with Tyrodes medium containing the normal physiological concentration of ^{40}Ca (2.2mM), there was an initial rapid release of ^{45}Ca from the tissue. This rapid release phase can in part be attributed to the displacement of extracellular label and the exchange of extracellular ^{40}Ca and intracellular ^{45}Ca. A steady basal release phase was reached as the exchange of ^{40}Ca and ^{45}Ca approached equilibrium after approximately 30 minutes of superfusion.

Hypoxia was found to evoke ^{45}Ca efflux from the carotid body and this effect could be partially blocked by nitrendipine which prevents the entry of extracellular calcium via the plasma membrane calcium channels. Electrophysiological studies performed on a variety of mammalian carotid bodies have confirmed the presence of voltage-dependent calcium channels although no pharmacological characterization of the channels has been undertaken (eg. see Acker, 1981).

The results from the present study suggest that hypoxia clearly does induce calcium conductance through these gated channels. However, as calcium continues to be extruded from the carotid body in the presence of nitrendipine, it is possible that this calcium may also be released from intracellular storage sites. The endoplasmic reticulum, mitochondria and calciosomes have been implicated as Ca^{2+} sequestering organelles in a wide variety of tissue types

and they may be the source of this calcium (Chapter 1). A significant increase in ^{45}Ca efflux was induced by stimulation with carbachol and this response could be blocked by atropine providing evidence for the presence of muscarinic-type cholinergic receptors on the Type I cells which may be linked to calcium movement. Both muscarinic and nicotinic cholinergic receptors have been demonstrated on the Type I cell membranes of both cat and rabbit carotid bodies (Eyzaguirre and Monti-Bloch, 1982). In a variety of other tissues, ^{45}Ca efflux following stimulation with carbachol results from the mobilization of intracellular calcium stores (eg. Masters et al., 1984; Pozzan et al., 1986; Mouillac et al., 1989). In the present study, however, the response to carbachol could be partially reduced with nitrendipine, suggesting that voltage-dependent calcium channels may be involved in the movement of calcium from the extracellular environment to the intracellular compartment.

Since atropine was able to block the calcium efflux during hypoxic stimulation, the possible involvement of a muscarinic-type mechanism in the response to hypoxia cannot be ruled out.

It is well established that release of neurotransmitter substances and hormones is triggered by cell depolarization that causes the opening of voltage-sensitive calcium channels and the entry of calcium into the cytosolic compartment (see Exton, 1988). An increase in the extracellular potassium concentration is routinely used in vitro to induce cell depolarization in excitable cells

and thereby to increase cell permeability to various ions including calcium.

In the present study, 55mM K^+ was found to induce ^{45}Ca efflux from pre-loaded carotid bodies, suggesting that depolarization of the cell membranes is associated with calcium movement in the Type I cells. Elevations in cytoplasmic calcium as a result of potassium depolarization may not be exclusively due to entry via the plasma membrane calcium channels since there are a small but increasing number of examples of cells whereby manipulations of the extracellular K+ environment may influence phosphoinositide metabolism and thus the potential mobilization of intracellular calcium (Best and Bolton, 1986; Novotny et al., 1983).

Superfusion with high extracellular potassium was unable to evoke catecholamine secretion (Chapter 4). These results may suggest that cellular depolarization alone results in a calcium flux which is of insufficient magnitude to induce secretion. It is possible that membrane depolarization is the initial trigger for the secretory response but in view of the fact that hypoxia was able to elicit the release of the catecholamines and high extracellular potassium was not, there must be a further regulatory mechanism involved in the hypoxic response in addition to the initial depolarization event.

Addition of 2mM NaCN to the superfusion medium resulted in a significant increase in ^{45}Ca efflux (Table 5.1). In addition, the results in Chapter 4 demonstrated that NaCN

was also able to evoke catecholamine secretion. These results suggest that the secretory response to cyanide may be related to elevated cytosolic calcium levels, although the mechanism by which cyanide induces an increase in the intracellular calcium concentration can only be postulated from the data available.

Cyanide is a known uncoupler of mitochondrial oxidative phosphorylation. It is able to inhibit the activity of the terminal oxidase enzyme in the electron-tranfer chain (Slater, 1967). The arrest of oxidative metabolism normally results in a decrease in the concentration of adenosine triphosphate (ATP), the energy source required for a variety of cellular functions, including the movement of secretory vesicles during exocytosis (Knight and Baker, 1987). A decrease in the production of ATP by oxidative phosphorylation could potentially result in an elevation of the cytosolic calcium concentration as a result of a reduction in calcium extrusion by way of the Ca-ATPase pump at the plasma membrane. Under these conditions, extracellular calcium may still enter the cell through the plasma membrane down the steep concentration gradient.

The intracellular calcium concentration may also be elevated as a result of the mobilization of intracellular calcium stores. Inhibition of oxidative phosphorylation would prevent the electrophoretic uptake of calcium by the mitochondria since proton extrusion by the respiratory chain is required to exchange calcium ions (Brand, 1988; McCormack and Denton, 1986). The resultant elevation in the

cytoplasmic calcium concentration may produce an efflux of ^{45}Ca due to Ca^{2+}-Na^{+} exchange at the plasma membrane (see Meldolesi and Pozzan, 1987).

Clearly, even in the presence of NaCN, cellular ATP concentration is sufficient for exocytosis and it may be maintained in the short term by anaerobic metabolism using glucose as a substrate.

In view of the similarities between hypoxia and NaCN in terms of their abilities to induce both ^{45}Ca efflux and catecholamine secretion, it is possible that hypoxia may operate via an effect on cellular oxidative metabolism, although as previously discussed, other regulatory mechanisms are likely to be involved in the response to hypoxia. In Chapter 6, an example of a way in which cytosolic calcium may be elevated in the stimulated carotid body is investigated.

CHAPTER 6

THE ROLE OF POLYPHOSPHOINOSITIDE METABOLISM IN THE CAROTID BODY

6.1 INTRODUCTION

The data presented in Chapters 4 and 5 demonstrated that the interaction of various putative secretagogues with the carotid body elicited marked changes in both the efflux of ^{45}calcium and catecholamine secretion from the Type I cells. Agonists that elevate cytosolic calcium do so either by mobilizing calcium from intracellular stores or by opening plasma membrane calcium channels (see Exton, 1987). The mobilization of intracellular calcium is often the result of the generation of myo-inositol 1,4,5-trisphosphate (IP_3) following hydrolysis of the plasma membrane phospholipid (phosphatidylinositol 4,5-bisphosphate or PIP_2) by the enzyme phospholipase C (Chapter 1). In the present study, it was suggested that the response to hypoxia could be mediated at least in part by the activation of muscarinic cholinergic receptors on the Type I cell membranes. Stimulation of muscarinic receptors particularly of the M_2 type (Birdsall and Hulme, 1983), have been shown to induce a rapid hydrolysis of inositol phospholipids in many tissues, eg. Fisher and Bartus (1985).

As a result of a widespread interest in membrane lipid signalling systems, [^{3}H] inositol has proved to be a valuable tool in the attempts to understand the functions of the inositol phospholipids and their water soluble inositol phosphate metabolites. Uptake of the isotope from the extracellular medium into cells can occur by way of a low affinity passive carrier or by a Na^+-dependent pump (Downes,

1989). A fraction of the [^{3}H] inositol which enters the cytoplasmic pool is readily incorporated into the inositol lipids. Radioautographic studies in the pancreas (Hokin and Huebner, 1967) have shown that the incorporation of [^{3}H] myo-inositol into phosphatidylinositol (PI) occurs mainly in the rough endoplasmic reticulum (RER; Gerber et al., 1973) with a smaller fraction in the plasma membrane. Label incorporated into phosphatidylinositol 4-phosphate (PIP) and PIP_2 is almost exclusively in the plasma membrane (see Hokin, 1985).

There have been no previous attempts to assess the possible involvement of the phospholipid signalling system in the secretory mechanism of the carotid body. In the present chapter, the effects of hypoxia and carbachol on membrane lipid turnover have been investigated. In particular, their ability to induce hydrolysis of the inositol phospholipids was assessed to test the hypothesis that hypoxia may operate via a muscarinic effect which may involve a membrane lipid transduction mechanism. The results presented in this chapter are discussed in relation to those obtained from other tissue types.

Since the Dowex method for the separation of the inositol phosphates (Chapter 2) is unable to resolve the various isomers, positional locants are only referred to when they are necessary for clarity.

6.2 AGONIST-INDUCED [^{3}H] INOSITOL EFFLUX

It is possible to monitor polyphosphoinositide metabolism in tissues by following the release of free myo-inositol from the stimulated tissue. During stimulation, an agonist operating via the phospholipid transduction system would be expected to alter the rate of hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PIP_2) and hence the rate of production of IP_3 and diacylglycerol (DAG). The subsequent dephosphorylation of the inositol phosphates would lead to the production of free myo-inositol which may be re-incorporated into the membrane inositol lipids, or released from the cell.

6.2.1 METHODS

In these studies, the effect of various agents on the efflux of [^{3}H] inositol was determined using the methods as described in Chapter 2.

The muscarinic cholinergic agonist carbachol was prepared as a stock solution in Tyrodes medium (10mM) and added to the superfusion medium at a final concentration of 100μM, just prior to the stimulus phase. The muscarinic cholinergic antagonist atropine was prepared as a 1mM stock solution in Tyrodes medium. In all experiments involving this antagonist, the tissue was atropinized prior to the stimulus phase, by adding atropine at a final concentration of 100μM to the medium 10 minutes before the introduction of the

agonist stimulus.

In some experiments, lithium chloride was used at a final concentration of 10mM in order to inhibit the enzyme inositol monophosphatase. Lithium was added to the superfusion medium at the start of the superfusion regime.

6.2.2 RESULTS

The effect of superfusing carotid bodies prelabelled with [^{3}H] inositol under control release conditions with unsupplemented Tyrodes medium is shown in Figure 6.1. In these control carotid bodies, the rate of [^{3}H] inositol efflux declined rapidly for the first 20 minutes of the superfusion and more slowly for a further 20 minutes, reaching a steady basal release phase after 40 minutes. At this time, it was possible to introduce various agents into the superfusion medium. The effects of these various additions on the efflux of [^{3}H] inositol are shown in Table 6.1.

When carotid bodies were superfused with Tyrodes medium that had been equilibrated with 100% N_2 (pO_2 <40mmHg), there was no significant increase in the [^{3}H] inositol efflux compared to the control basal release phase. In comparison, carbachol induced a 38% increase in [^{3}H] inositol efflux within approximately 4 minutes of introduction of the stimulus (Figure 6.1). The muscarinic receptor antagonist atropine significantly blocked the response to carbachol ($p< 0.001$) as shown in Table 6.1. In experiments involving

Figure 6.1: Release of [^{3}H] myo-inositol from the superfused rat carotid body

Groups of four carotid bodies prelabelled with [^{3}H] inositol were superfused with unsupplemented Tyrodes medium over a period of 70 minutes as described in Chapter 2. The superfusate was collected over 2 minute periods and each fraction was quantified by determination of its radioactivity.

The point at which a potential stimulus was introduced into the superfusion medium is shown by the arrow.

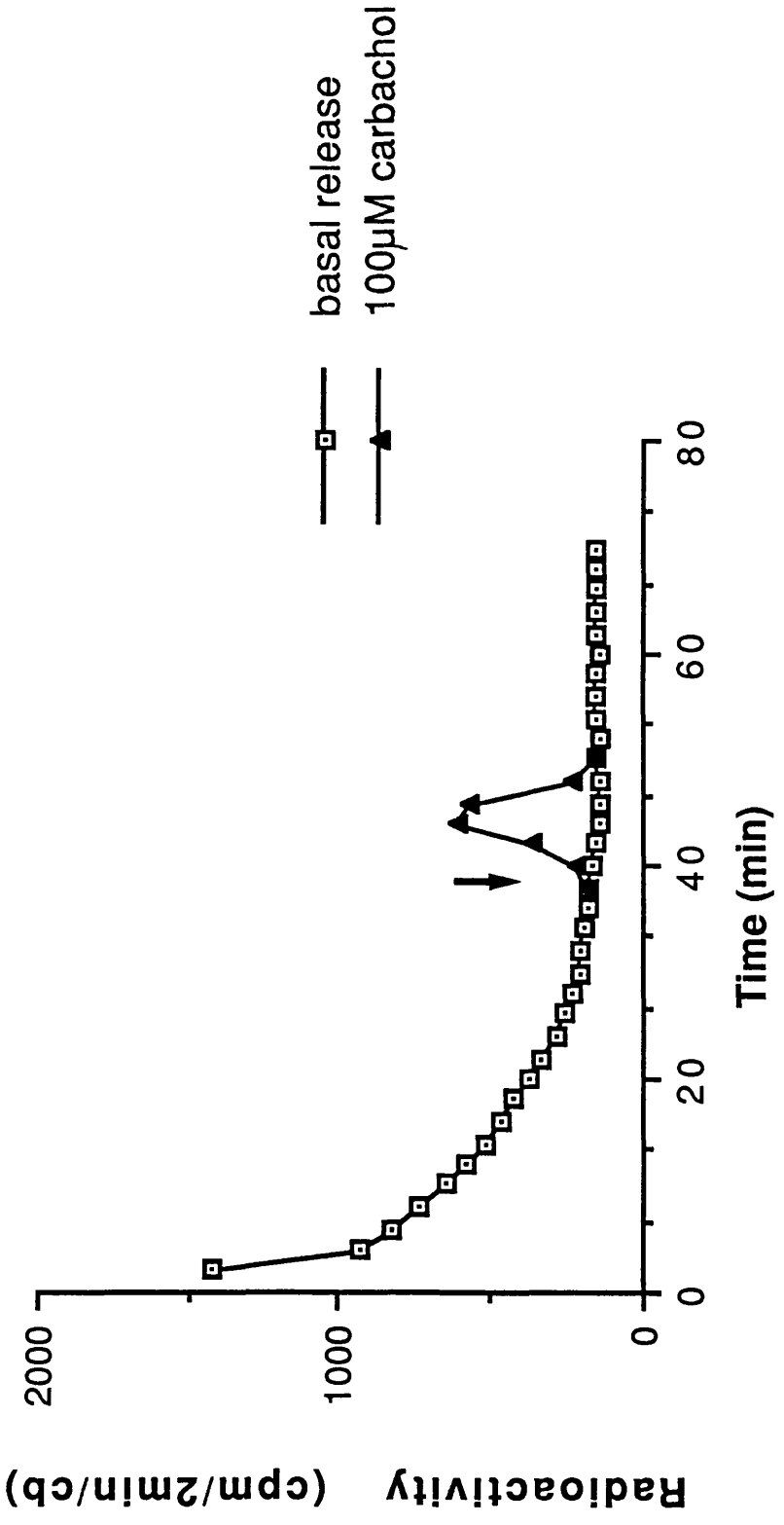

basal release
100μM carbachol
Radioactivity (cpm/2min/cb)
2000
1000
0
0
20
40
60
80
Time (min)

FIG 6·1

Table 6.1

Groups of 4 carotid bodies pre-labelled with [^{3}H] inositol were superfused with Tyrodes medium for an initial period of 40 minutes to establish the basal release phase. The tissue was superfused for a further 10 minutes with medium supplemented as shown in Table 6.1 and this was followed by a 10 minute recovery phase. [^{3}H] inositol released into the superfusate was determined as described in detail in Chapter 2.

Each value represents the mean ± SEM of the percentage increase in radioactivity during the stimulation period compared to the two 10 minute control periods immediately before and after the stimulus phase.

The number of observations in each case are shown in parentheses.

** indicates values significantly different from the corresponding basal release phase ($p<0.001$).

*** indicates values significantly different from a carbachol stimulus alone ($p<0.001$).

Table 6.1: Effects of hypoxia and carbachol on [^{3}H] inositol efflux from the carotid body

Additions to the superfusion medium	Increase in radioactivity %
Hypoxia (pO_2 <40mmHg)	4.70 ± 0.81 [8]
Carbachol (100µM)	38.83 ± 5.95 [6] **
Carbachol (100µM) + Atropine (100µM)	6.64 ± 0.33 [6] ***
Carbachol (100µM) + Lithium (10mM)	4.17 ± 0.68 [6] ***

lithium, the [^{3}H] inositol efflux in response to carbachol stimulation was also inhibited ($p < 0.001$; Table 6.1).

6.3 AGONIST-INDUCED POLYPHOSPHOINOSITIDE HYDROLYSIS

The accumulation of [^{3}H] inositol phosphates following polyphosphoinositide hydrolysis was determined in carotid bodies which had been prelabelled with [^{3}H] inositol, as described in Chapter 2.

The final step in the phosphoinositide re-cycling system is the conversion of inositol monophosphate to free myo-inositol by the enzyme inositol monophosphatase. Lithium is able to inhibit inositol monophosphatase in a variety of tissue types, resulting in a decrease in free inositol and a corresponding increase in one or more of the water-soluble inositol phosphates. The inhibitory action of lithium amplifies the agonist-induced response and can thus be employed as a method to detect receptor-mediated breakdown of the inositol lipids.

6.3.1 METHODS

In these studies, the effects of various treatments on the accumulation of the inositol phosphates was determined following recovery of the phosphates as described in detail in Chapter 2.

Additions to the incubation medium were made in a final volume of 50µl just prior to the stimulus phase, using solutions at 10x their final concentration.

6.3.2 RESULTS

The effects of the various additions to the incubation medium on the accumulation of the inositol phosphates are shown in Table 6.2.

When carotid bodies were subjected to a 10 minute hypoxic stimulus, there were significant increases in the amounts of radioactivity associated with each of the measured inositol phosphates. The most marked increases occurred in the inositol monophosphate and inositol bisphosphate (IP and IP_2) fractions. In the case of the carbachol stimulus, there were also significant increases in all of the inositol phosphate levels but particularly in the IP and IP_4 fractions. Atropine significantly inhibited the effects of carbachol on inositol phosphate accumulation.

Table 6.2

After an initial 2 hour pre-labelling period with [^{3}H] myo-inositol, groups of 4 carotid bodies were re-incubated for 10 minutes in 0.5ml Tyrodes medium containing 1mM unlabelled myo-inositol and 10mM LiCl and supplemented as shown. At the end of this period, 0.5ml TCA was added and the tissue homogenized. Following centrifugation, the deproteinized supernatants were neutralized and the inositol phosphates separated by anion-exchange chromatography.

Each value represents the mean ± SEM of the percentage increase in radioactivity associated with the stimulation period compared to the appropriate control period.

The number of observations in each case are shown in parentheses.

* $p<0.05$ relative to a control experiment

** $p<0.01$ relative to a control experiment or a carbachol stimulus alone

*** $p<0.001$ relative to a control experiment or a carbachol stimulus alone

Table 6.2: Effects of hypoxia and carbachol on the accumulation of [^{3}H] inositol phosphates in the carotid body

Additions to the incubation medium	Increase in radioactivity (%)			
	IP	IP_2	IP_3	IP_4
Hypoxia (pO_2< 40mmHg) [6]	25.38 ± 3.10 **	26.15 ± 4.26 **	18.90 ± 2.08 *	11.18 ± 2.91 *
Carbachol (1mM) [7]	70.19 ± 3.41 ***	31.42 ± 5.10 ***	35.43 ± 4.92 ***	54.02 ± 5.20 ***
Carbachol (1mM)/ Atropine (1mM) [7]	9.09 ± 1.51 ***	10.61 ± 1.88 **	0 ± 1.14 ***	11.90 ± 2.26 ***

6.4 DISCUSSION

Superfusion of the carotid body with unsupplemented Tyrodes medium resulted in an initial rapid release of [^{3}H] inositol from the tissue which reached a steady basal release phase after 30 minutes. The rapid release phase may be attributed to the displacement of extracellular label from the tissue.

Exposure of the carotid body to hypoxic superfusion medium appeared to have no significant effect on the efflux of [^{3}H] inositol (Table 6.1). However, the results in Table 6.2 indicated that hypoxia was able to induce small but significant increases in the accumulations of IP_3 and IP_4. As explained in Chapter 1, increases in intracellular IP_2, IP and free myo-inositol are mainly derived from successive dephosphorylations of IP_3. Therefore, from the results in this study, it would appear that the actual increase in the IP_3 concentration following hypoxic stimulation, was insufficient to generate an excess of free myo-inositol. This excess would normally manifest as an efflux of [^{3}H] inositol.

When carbachol was introduced as a stimulus into the superfusion medium, there was a significant release of [^{3}H] inositol 2 minutes from the introduction of the stimulus. This time lag has been reported in other systems, such as the pancreatic islets (Zawalich et al., 1989) and is thought to occur as a result of the time taken for the hydrolysis of

PIP_2 and the subsequent dephosphorylation cascade to produce free myo-inositol. The release of [^{3}H] inositol in response to carbachol was almost 10-fold that observed with hypoxia and the effect was almost completely blocked with an equimolar concentration of atropine. These results would suggest that the efflux of [^{3}H] inositol results from a specific muscarinic receptor-mediated mechanism. Atropine clearly blocks the activation of phospholipase C which hydrolyses PIP_2. The effect of carbachol was also abolished in the presence of lithium, and this is known to inhibit the enzyme inositol monophosphatase which is responsible for the conversion of IP to free myo-inositol. This would prevent the efflux of the labelled inositol.

In addition to promoting inositol efflux, carbachol was also found to stimulate large accumulations of the inositol phosphates. In particular, the increases in IP_3 and IP_4 were considerably greater than those produced as a result of hypoxic stimulation. Clearly, the increase in IP_3 is sufficient to ensure a substantial inositol efflux and this was demonstrated in the results in Table 6.1. These results are in general agreement with previous studies of the effect of carbachol on a variety of tissues including brain and parotid gland (Berridge et al., 1983), exocrine pancreas (Rubin et al., 1984), ileum smooth muscle (Sekar and Roufogalis, 1984) and iris smooth muscle (Akhtar and Abdel-Latif, 1984). All of these authors reported carbachol-induced increases in IP_3, IP_2 and IP.

The results in Table 6.2 revealed that both hypoxia and

carbachol were able to induce a significant increase in the formation of IP_4. Phosphorylation of IP_3 to IP_4 is thought to follow from the activation of a 3-kinase in the presence of an adequate source of IP_3 substrate. IP_4 is thought to influence the distribution of calcium between the internal and external environment. Irvine and Moor (1986) have suggested that IP_4 formation occurs after the mobilization of intracellular calcium by IP_3. It is thought that IP_4 so formed may control the opening of voltage-dependent calcium channels in the plasma membrane.

Moreover, the production of IP_4 is thought to be an effective way of terminating the IP_3 signal and of generating the more highly phosphorylated derivatives, IP_5 and IP_6 (Jackson et al., 1987; Carpenter et al., 1989). These substances may potentially represent other cellular signals, but their exact physiological roles have yet to be determined.

Atropine was found to significantly inhibit the accumulation of the inositol phosphates in response to stimulation with carbachol. This effect has been reported in other tissues (eg. Akhtar and Abdel-Latif, 1984) and suggests that carbachol clearly operates via a specific muscarinic receptor-mediated event. Both M_1 and M_2 muscarinic receptor subtypes have been shown to be linked to phosphoinositide hydrolysis (Gil and Wolfe, 1985; Brown and Goldstein, 1987) in different tissues.

The results in the present chapter indicate that in the rat carotid body, carbachol may exert its stimulatory effect

via a mechanism involving the hydrolysis of PIP_2 and the formation of IP_3. The production of both IP_3 and IP_4 may lead to the elevation of cytosolic calcium. In comparison, modest increases in the concentrations of these inositol phosphates following hypoxic stimulation would seem to suggest that hypoxia is unlikely to exert its effects on the Type I cells via inositol lipid hydrolysis. Alternative mechanisms by which hypoxia may operate in the rat carotid body are discussed in Chapter 7.

CHAPTER 7

GENERAL DISCUSSION

The mammalian carotid body is a peripheral chemoreceptor that is thought to play a role in monitoring and responding to changes in the partial pressures of arterial blood gases. It is generally accepted that the carotid body may respond to these changes by depolarizing the afferent nerve endings which are in synaptic contact with the Type I cells. In this way, the carotid body is able to send neural messages to the respiratory centres located in the medulla of the hindbrain. The actual mechanism whereby the carotid body recognizes changes in arterial gas tensions and converts these into neural messages is less well understood.

Although the identity of the actual chemosensor has yet to be firmly established, it has been demonstrated in the present study that the rat carotid body is able to release the catecholamines dopamine and noradrenaline in response to stimulation, suggesting a definite role for the Type I cells in the sensing mechanism. During the course of this study, a number of *in vitro* techniques were employed in an attempt to define some of the cellular events that link stimulus recognition by the Type I cells to the release of catecholamines.

In vitro studies concerned with elucidating secretory mechanisms of hormones and neurotransmitters often involve the use of classical pharmacological secretagogues. These studies generally provide an insight into the functional activity of a particular tissue *in vivo*. The carotid body is extremely sensitive to blood flow, and therefore comparisons between the effect of a pharmacological agent *in vitro* and

in vivo may be difficult since the results from *in vivo* experiments may be attributed to agents acting on the carotid body vasculature. Simulated hypoxia *in vitro* is likely to be one of the few stimuli whose effects may be comparable to the *in vivo* situation. In the present study, the rat Type I cells were found to respond to hypoxia by releasing the catecholamines noradrenaline and dopamine. This effect has not previously been observed in the rat carotid body although other workers have shown that hypoxia stimulates the release of dopamine from rabbit and cat carotid bodies (see Chapter 4).

Hypoxia is clearly a major physiological stimulus for the carotid body since it has also been shown to elicit discharges in the afferent nerve endings (for example, see Rigual *et al*., 1986; Delpiano and Acker, 1980). However, the effect of hypoxia in releasing the catecholamines is not unique to the carotid body. Chronic post-natal hypoxia has been shown to cause long-lasting changes in dopamine release from the rat striatum which are thought to manifest as morphological and functional alterations in development of the brain (Odarjuk *et al*., 1987).

There is some evidence to suggest that calcium fluxes are an important part of the response to hypoxia in the carotid body as well as in other tissues such as the ventricular muscle of the heart (Allen and Orchard, 1983). In the present study, the release of dopamine in response to hypoxia was significantly reduced in the absence of

extracellular calcium. Similar effects have been reported in the rabbit (Fidone et al., 1982) and cat (Obeso et al., 1986) using in vitro carotid body preparations. In addition, the present study demonstrated that the calcium channel antagonist nitrendipine was able to reduce the hypoxia-stimulated release of dopamine to a similar extent. These results suggest that the release of dopamine shows classical calcium dependency which is a criterion used to define true secretory mechanisms. The results also provide indirect evidence for the presence of L-type voltage-dependent calcium channels and suggest that the entry of calcium via these channels is an important part of the secretory process. The observation that the release of dopamine is not completely inhibited in the presence of nitrendipine suggests that additional mechanisms apart from the entry of extracellular calcium may operate to regulate the secretory process, and these were explored later.

Carbachol was found to stimulate the release of both amines from the rat carotid body and the blocking effect of atropine would indicate that carbachol operates by a specific interaction with membrane muscarinic receptors. Muscarinic receptor activation may be coupled to the generation of second and higher order messenger systems as explained in detail in Chapter 1. A large variety of external stimuli have been shown to raise the intracellular concentration of inositol trisphosphate and diacylglycerol following polyphosphoinositide hydrolysis in a wide range of tissues. In the present study, the contribution of polyphos-

phoinositide metabolism during the response to both hypoxia and carbachol was assessed by measuring intracellular accumulations of the inositol phosphates. It was hoped that the results might provide a further insight into the mechanisms involved in the secretory response. The intracellular concentrations of both inositol tris- and tetrakisphosphates were elevated following a carbachol stimulus. In addition, results from this study also indicated that carbachol could induce ^{45}calcium efflux. In previous studies using permeabilized cells, receptor sites for inositol trisphosphate have been shown to be present on the endoplasmic reticulum (Streb et al., 1984). Binding of IP_3 may induce the release of calcium which is known to be sequestered in these organelles. Results from the present study would suggest that this is not the only mechanism by which carbachol operates, since its effect on both secretion and calcium efflux is reduced by nitrendipine, which seems to implicate the involvement of the voltage-dependent calcium channels. It may well be that the mobilization of intracellular calcium may initiate secretion, but that maintenance of the response requires the entry of extracellular calcium which would also serve to replenish the intracellular compartments (Rasmussen and Barrett, 1984). The mechanism by which the voltage-dependent calcium channels are activated can only be postulated.

The generation of IP_4 may facilitate entry of calcium by this route, since this metabolite has been shown to allow movement of calcium from the extracellular to the

intracellular compartment. In addition, this study does not rule out a potentially important role for diacylglycerol, the other cleavage product of PIP_2 hydrolysis. Diacylglycerol may activate cytoplasmic protein kinase C by increasing its affinity for Ca^{2+} (Nishizuka, 1983). This enzyme is thought to phosphorylate proteins specifically involved in the secretory response. Lahiri et al. (1988) performed studies using phorbol 12, 13-dibutyrate (PDB) to promote activation of protein kinase C in the carotid body. PDB elicited a prompt chemosensory discharge in afferent fibres. These authors suggested that a network of protein phosphorylation and dephosphorylation may control chemosensory activity. Calcium-dependent protein kinases have also been implicated in the regulation of tyrosine hydroxylase activity in other catecholaminergic tissues such as the adrenal chromaffin cells (Pocotte et al., 1986). This aspect obviously requires further investigation in the carotid body.

Hypoxia-induced catecholamine release and calcium efflux were both significantly reduced in the presence of atropine, which suggested the possible involvement of cholinergic receptor activation in the response process. The results from the present study showed that hypoxia elicited a relatively modest increase in the intracellular IP_3 concentration. If hypoxia operates, in part, via a muscarinic-type mechanism, it would seem unlikely that this involves the direct activation of phospholipase C. Indeed, the partial blocking effect of atropine would seem to

suggest that there are additional mechanisms involved in regulating the hypoxia-induced increase in cytosolic calcium.

In recent years, the 'metabolic hypothesis' of chemoreception has received much attention (see Belmonte and Gonzalez, 1983). This hypothesis is based on the assumption that hypoxia may exert its effects on the Type I cell in a way which likens it to a metabolic poison which acts by inhibiting the respiratory chain (Mulligan and Lahiri, 1981). Metabolic poisons such as cyanide are thought to be powerful chemostimulants, since applications of these agents to the carotid body have been found to result in afferent nerve depolarization (eg. Monti-Bloch and Eyzaguirre, 1980). In the present study, cyanide was found to evoke both calcium efflux and catecholamine secretion to an extent similar to hypoxia, suggesting that they may operate via a common mechanism. The metabolic hypothesis proposes that these chemostimulants may decrease the ATP content of the Type I cells, but it has not been suggested how this may depolarize the Type I cell membranes and initiate secretion. In other tissues such as ventricular myocytes, cytosolic ATP levels have been found to decrease in response to low O_2 or cyanide (Allshire et al., 1984; Cobbold and Bourne, 1984).

In the rat carotid body, it has been established that part of the response to hypoxia involves a depolarization of the Type I cell membranes, thereby initiating calcium influx via the voltage-dependent calcium channels. A decrease in cytosolic ATP may be indirectly responsible for this

depolarization, since ATP-dependent mechanisms (for example, the Na^+-K^+ plasma membrane pump) may contribute towards maintenance of the membrane potential. Perturbations in membrane potential may open the gated calcium channels. Similar mechanisms have been proposed in central neurons (see de Weer, 1975) and smooth muscle (Fleming, 1980).

This mechanism would account for an increase in the cytosolic calcium concentration, since a decrease in ATP would impair the action of the membrane pumps responsible for calcium uptake across intracellular membranes. In addition, there would be a reduction in ATP-dependent calcium pumping across the plasma membrane. The hypoxia-induced calcium efflux demonstrated in the present study may therefore represent Ca^{2+}/Na^+ exchange. Calcium may also be released from the secretory granules or from specific binding sites on the granules and these have been demonstrated in the carotid body of the rat and rabbit using electron microscopy and electron probe x-ray microanalysis (Hansen and Smith, 1979; Hess, 1977b).

Studies of the cat carotid body have provided evidence for (Obeso et al., 1985) and against (Acker and Starlinger, 1984) decreases in the intracellular ATP content following hypoxia. In addition, the metabolic hypothesis has not been verified in the rabbit carotid body, since metabolic inhibitors have been shown to decrease the ATP content while no change has been reported using hypoxia (Talib et al., 1988). ATP levels have yet to be monitored in the rat carotid body. Clearly, the suggested decrease in the

cytosolic concentration of ATP following hypoxia would be expected to have some effect on the movement of the catecholamine-containing granules. It may be that this transient event is sustained in the short term by anaerobic metabolism. Stimulus-induced alterations in cellular metabolism, with the eventual release of neurotransmitters or hormones, are not unusual. In the pancreatic β cells for example, it has been suggested that glucose-stimulated insulin release may be triggered by a product of glucose metabolism (see Grodsky et al., 1963).

Various theories of chemoreception by the carotid body have been proposed and have postulated the location of the hypoxic transduction machinery. The progressive increase in afferent discharge which develops as pO_2 is lowered has been attributed to a decrease in oxidative metabolism which is thought to exert an effect on one or more cytochromes of the mitochondrial respiratory chain. Mills and Jobsis (1972) and Nair et al. (1986) have suggested the involvement of two forms of cytochrome a_3 which differ in their affinity for oxygen. Mills and Jobsis (1972) have suggested that they are located in different components of the carotid body complex and that the lower affinity cytochrome is more important in the sensory mechanism. These authors suggest that the lower affinity cytochrome is likely to be associated with the Type II cells rather than the Type I cells, although this has not been confirmed by experimental evidence.

The 'chromophore sensor' hypothesis (Lloyd et al, 1968; Lahiri, 1981) implicates the involvement of a plasma

membrane protein in the detection of arterial oxygen tensions and has the distinct advantage of locating the sensory machinery within the membrane. The findings in the present study demonstrated that plasma membrane-mediated events may play a role in the response mechanism, since carbachol was shown to evoke amine secretion and the muscarinic receptor antagonist, atropine, was able to partially inhibit the hypoxia-induced response. These findings may implicate the plasma membrane in the sensory process.

The results obtained in the present study refute the overall theory of chemoreception, as proposed by Osborne and Butler (1975). As explained in Chapter 1, these authors postulate a positive feedback loop in the carotid body, whereby catecholamine release is depressed during hypoxia, in order to remove the inhibitory effect of dopamine on afferent nerve endings. McDonald and Mitchell (1975) suggest that the Type I cells are interneurones responding to the effects of an excitatory transmitter released from nerve endings during hypoxic stimulation. In the present study, the effect of atropine on hypoxia-induced catecholamine secretion would seem to indicate a more direct role for hypoxia on the Type I cells. A more extensive investigation of the inter-relationships between hypoxia and carbachol should provide a better understanding of the physiological role of muscarinic agents in the secretory response.

This study has made a significant contribution to the understanding of events involved in the stimulus-secretion coupling mechanism in the rat carotid body, since changes in the intracellular free calcium concentration within the Type I cells have been shown to link stimulus recognition to the secretion of catecholamines. Further research into hypoxia-induced alterations in cellular respiratory metabolism in the rat Type I cells may fully elucidate the chemosensory mechanism, and provide a more complete understanding of the physiological functions of the carotid body.

APPENDICES

APPENDIX I

APPENDIX I

Conversion of Dowex AG 50W-X H^+ form to Na^+ form

In order to separate the catecholamines by cation exchange chromatography, the commercially available Dowex AG 50W-X cation exchange resin of the hydrogen form was initially converted to the sodium form by the following method:

(i) The resin was initially rinsed with 3x 500ml distilled water in order to remove any loose contaminants. The resin bed was allowed to drain between each rinse.

(ii) The bed was washed with 3x 500ml 1M NaOH and the resin was stirred to ensure thorough conversion. The bed was allowed to drain completely between each application of NaOH.

(iii) The resin in its sodium form was finally rinsed with 6x 500ml distilled water before use.

APPENDIX II

APPENDIX II

Composition of Solvent System for Catecholamine Analysis by HPLC-ED

A citrate-acetate solvent system at pH 5.2 was employed for separation of the catecholamines on reversed-phase HPLC columns.

Components	g/2 litres
Citric acid	11.5
Sodium Acetate	13.6
Sodium Hydroxide	4.8
EDTA	0.74
Sodium Octyl Sulphonate (SOS)	0.20

The above components were dissolved in 1.5 litres of distilled water and the solution was brought to pH 5.2 with glacial acetic acid. A further 300ml distilled water was added and the solution was made up to a final volume of 2 litres with 200ml HPLC grade methanol. The solution was vacuum filtered through a 0.2µM cellulose-acetate filter (Sartorius) and further degassed with helium.

The ratio of SOS to methanol could be altered to vary the elution times of the amines.

APPENDIX III

APPENDIX III

Publications

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Biochemical studies on the release of catecholamines from the rat carotid body in vitro

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The effects of hypoxia and carbachol on the release of newly synthesized catecholamines from superfused rat carotid bodies have been examined. Hypoxic superfusion medium was found to evoke catecholamine release which was dependent on the extracellular calcium concentration and was reduced by nitrendipine and atropine. Superfusion with the muscarinic agonist, carbachol, stimulated catecholamine release independently of the oxygen tension of the medium. The effect of carbachol on catecholamine release was abolished by atropine, suggesting that it was mediated by activation of cholinergic receptors of the muscarinic type. Both hypoxia and carbachol stimulated the release of ^{45}Ca from carotid bodies prelabelled with ^{45}Ca. The release of ^{45}Ca with either stimulus was reduced by atropine and nitrendipine. These results suggest that although extracellular calcium plays an important role in the exocytotic secretory process of the carotid body, the mobilization of intracellular calcium pools may also contribute to the secretory response.

Introduction

The carotid bodies are a pair of peripheral arterial chemoreceptors located in most vertebrates in the area of the carotid bifurcation [1]. They are able to respond to the level of oxygenation of the circulating arterial blood by contributing to the reflexes which maintain blood oxygen at normoxic levels [2,3].

The organ contains two major cell types; Type I or glomus cells, which exist in clusters, and Type II or sustentacular cells, which have a supportive role similar to glial cells, and envelope clusters of Type I cells. The cells lie within a connective tissue stroma containing groups of myelinated and unmyelinated nerve fibres along with a rich capillary network.

Histochemical studies have established the presence of dense-core chromaffin-like vesicles in the cytoplasm of the Type I cells [4,5]. These vesicles are known to contain putative neurotransmitters such as the catecholamines dopamine and noradrenaline. The relative amounts of these two catecholamines vary between species and in the rat dopamine is thought to be the predominant catecholamine [6]. Part of the response of the organ to hypoxia is thought to be the release of dopamine, which may act to modulate chemoreceptor discharge via its own excitatory or inhibitory actions [7].

The pharmacological response of the chemoreceptors to cholinergic agonists varies greatly among the mammalian species studied to date. Nicotinic mechanisms are thought to predominate in the cat, whereas in the rabbit, muscarinic actions largely contribute to the inhibitory response [8]. To date, little is known about those mechanisms operating in the rat carotid body.

In contrast to the wealth of information that is available concerning the structure and physiological function of the carotid body, relatively little is known about the secretory process itself or the mechanism whereby changes in oxygen tension are linked to the secretory response. It was the aim of this study to undertake a biochemical investigation into the secretory process of the rat carotid body.

Materials and Methods

All the reagents used were of analytical grade. L-[2,6-^{3}H]Tyrosine (spec. act., 1.78 TBq/mmol; radioactive concn., 37 MBq/ml) and $^{45}CaCl_2$ (spec. act. 1 GBq/mg Ca; radioactive concn. 37 MBq/ml) were obtained from Amersham, U.K. Carbamylcholine chloride (carbachol), atropine sulphate, EGTA and ethyl carbamate (urethane) were obtained from Sigma Poole, U.K.

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itrendipine was a gift from Dr R.I. Norman, Depart-ent of Medicine, University of Leicester. Polypro-ylene Econo-columns and Dowex AG 50W-X4 ation-exchange resin were purchased from Bio-Rad, ichmond, CA, U.S.A.

issue preparation and incubation

Male Wistar rats (180–200 g body weight) were aesthetized with Urethane and the carotid bifurcation as exposed. Carotid bodies were removed from both des of the neck and placed into ice-cold physiological edium where they were quickly freed of surrounding nnective tissue before being placed into the incuba-on medium. The tissue was incubated at 37 °C for 2 h 1 ml modified Tyrode's medium [9] containing: NaCl 12 mM), KCl (4.7 mM), $CaCl_2$ 2.2 mM), $MgCl_2$ (1.1 M), sodium glutamate (42 mM), Hepes (5 mM) and ucose (5.6 mM) at pH 7.42. The medium also con-ined the catecholamine precursor, L-tyrosine at a con-ntration of 50 μM and 14.8 MBq L-[2,6-^{3}H]tyrosine. e length of the incubation period and precursor con-ntration were determined in preliminary experiments. roughout the incubation the medium was continually s-equilibrated with 100% O_2. At the end of the in-bation period, the medium was aspirated and the sue was washed with 3×1 ml ice-cold Tyrode's edium ready for transfer to the superfusion apparatus.

perfusion

Following incubation, the tissue was mounted in a all polypropylene chamber which was positioned up-eam of a peristaltic pump set at a flow rate of 1 l/min. The chamber was connected to reservoirs of perfusion media via lengths of iso-versinic tubing in der to minimize oxygen exchange. The pO_2 of the dium in the reservoirs and tubing was monitored ing an oxygen electrode. A two-way tap in the line stream from the tissue chamber enabled the tissue to exposed to superfusion media containing the various tative secretagogues of interest.

The superfusion media were maintained at 37 °C in a ter-bath and the remainder of the apparatus was used in a temperature-controlled chamber maintained 37 °C. The superfusate after passage over the tissue s allowed to drip directly onto pre-cooled Dowex AG W-X4 cation exchange resin contained in Econo-col-ns. Collections were made for 10 min periods and re continued for a total of 70 min from the start of superfusion. The basal release phase was reached er 40 min and this was followed by a 10 min stimulus se and a 20 min recovery phase.

paration of catecholamines

Cation-exchange chromatography was used to sep-te the catecholamines noradrenaline and dopamine m each other and from any unmetabolized tyrosine [10]. After passage of the superfusate through the columns they were washed with 10 ml H_2O followed by 4 ml 1 M HCl in order to remove any unmetabolized precursor. The columns were then washed with 10 ml 1 M HCl to elute the noradrenaline fraction followed by 10 ml 2 M HCl to elute the dopamine fraction. Samples of the noradrenaline and dopamine fractions were taken for liquid scintillation analysis.

The release of newly synthesized catecholamines under basal and stimulated and control conditions was expressed as ^{3}H cpm/carotid body per 10 min. The results were expressed as the percentage increase in radioactivity associated with the stimulus period when compared to the corresponding control period or when compared to the corresponding basal period. Normally the corresponding basal period was taken as the average of the catecholamine-associated radioactivity release during the two 10 min periods immediately before and after the stimulation period.

^{45}Ca efflux studies

The carotid body tissue was incubated at 37 °C for 30 min in 100 μl Tyrodes medium under an atmosphere of 100% O_2. All other components of the medium were at the concentrations described above with the exception of calcium chloride, which was replaced by 0.37 MBq $^{45}CaCl_2$. Following the incubation period, the medium was removed from the tissue which was then washed with 3×100 μl ice-cold, calcium-free Tyrode's medium and loaded into the superfusion apparatus described above. The superfusate was collected in 2 ml fractions using a fraction collector fed directly by the peristaltic pump set at a flow rate of 1 ml/min. The ^{45}Ca-loaded tissue was stimulated for a 10 min period after an initial 40 min basal release phase (as in the catecholamine release experiments). A 20 min recovery phase followed. The ^{45}Ca content of each fraction was determined by scintillation counting. ^{45}Ca efflux under basal and stimulated conditions was expressed as ^{45}Ca cpm/carotid body per 2 min.

Statistical analysis of the data

Where appropriate, the data were evaluated using Student's *t*-test.

Results

The time-course of the release of noradrenaline and dopamine from superfused rat carotid bodies under control and hypoxic conditions is shown in Fig. 1. It can be seen that the release of catecholamines declined to reach a constant basal rate after about 40 min of superfusion and that a hypoxic stimulus at this time produced a marked but transient stimulation of release of both noradrenaline and dopamine. The results in Table I show that both hypoxia and carbachol stimu-

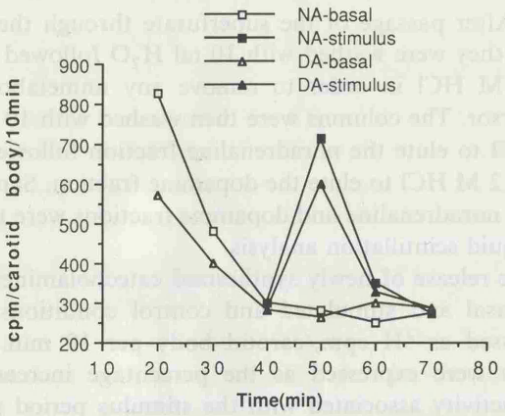

Fig. 1. Time-course of the release of [^{3}H]catecholamines from prelabelled rat carotid bodies superfused in vitro. Groups of eight carotid bodies prelabelled with [^{3}H]tyrosine were superfused as described in the text. The superfusion medium during the initial 40 min of superfusion was equilibrated with 100% O_2 and this was then continued (basal) or changed to medium equilibrated with 100% N_2 (stimulus). Superfusion under these conditions was continued for a further 10 min and the medium was then changed back to the original basal medium where appropriate. Noradrenaline (NA) and dopamine (DA) released into the superfusate were separated on Dowex resin as described in the text and quantified by determination of their radioactivity. The results are expressed as cpm/carotid body per 10 min and are representative of a typical experiment.

lated catecholamine release and that atropine and nitrendipine significantly reduced these responses. In addition, the effect of hypoxia was reduced in the absence of calcium from the superfusion medium.

TABLE I

Effect of hypoxia and carbachol on [^{3}H]catecholamine release from rat carotid bodies

Groups of eight rat carotid bodies prelabelled with [^{3}H]tyrosine were superfused as described in Fig. 1. The medium used during the stimulation period was either equilibrated with 100% N_2 (hypoxic stimulus) or contained 10 μM carbachol. In some experiments, nitrendipine (1 μM) or atropine (0.1 mM) were included in the stimulation medium and in further experiments calcium-depleted medium was used. Catecholamines released into the superfusate were separated on columns of Dowex resin, as described in the text, and quantified by determination of their radioactivity. The results are expressed as the percentage increase in radioactivity during the 10 min stimulation period compared to the radioactivity associated with the average of the two 10 min periods immediately before and after the stimulation period. Each value is the mean ± S.E. of eight observations. Values significantly different from: *, basal values ($P < 0.001$); ** hypoxic stimulus alone ($P < 0.01$); *** carbachol stimulus alone ($P < 0.001$).

Superfusion conditions	Percentage increase in radioactivity	
	noradrenaline	dopamine
Hypoxia	42.1 ± 8.9 *	52.4 ± 3.7 *
Hypoxia − calcium	31.3 ± 5.2	15.9 ± 3.3 **
Hypoxia + nitrendipine	18.2 ± 4.5 **	20.2 ± 3.6 **
Hypoxia + atropine	11.3 ± 5.0 **	11.5 ± 1.5 **
Carbachol	23.9 ± 4.4 *	39.3 ± 6.6 *
Carbachol + nitrendipine	16.7 ± 8.8	10.7 ± 1.8 ***
Carbachol + atropine	0 ***	0 ***

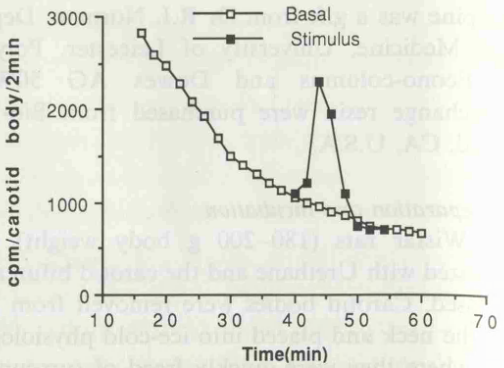

Fig. 2. Time-course of the efflux of ^{45}Ca from prelabelled rat carotid bodies superfused in vitro. Groups of four carotid bodies prelabelled with ^{45}Ca were superfused as described in the text. The medium for the initial 40 min of superfusion was equilibrated with 100% O_2 and this was then continued (basal) or changed to medium containing 10 μM carbachol (stimulus). Superfusion under these conditions was continued for a further 10 min and the medium was then changed back to the original basal medium where appropriate. ^{45}Ca released into the superfusate was determined as described in the text. The results are expressed as cpm/carotid body per 2 min and are representative of a typical experiment.

The time-course for the release of ^{45}Ca from superfused rat carotid bodies under control and carbachol stimulation is shown in Fig. 2. It can be seen that the the release release of ^{45}Ca decreased with time and that the addition of carbachol to the superfusion medium at 40 min produced a marked but transient increase in the rate of calcium efflux. The results in Table II show that both hypoxia and carbachol stimulated the efflux of ^{45}Ca from the carotid body and that these effects were reduced by atropine and nitrendipine.

TABLE II

Effect of hypoxia and carbachol on ^{45}Ca efflux from prelabelled rat carotid bodies

Groups of four carotid bodies prelabelled with ^{45}Ca were superfused as described in Fig. 2. The medium for the stimulation period was either equilibrated with 100% N_2 (hypoxic stimulus) or contained 10 μM carbachol (carbachol stimulus). In some experiments nitrendipine (1 μM) or atropine (10 μM) were included in the stimulation medium. ^{45}Ca released into the superfusate was determined as described in the text. The results are expressed as the percentage increase in radioactivity during the 10 min stimulation period compared to the radioactivity associated with the average of the two 10 min periods immediately before and after the stimulation period. Each value is the mean ± S.E. of eight observations. Values significantly different from: * basal values ($P < 0.001$); ** hypoxic stimulus alone ($P < 0.01$); *** carbachol stimulus alone ($P < 0.01$).

Superfusion conditions	Percentage increase in radioactivity
Hypoxia	52.9 ± 5.9 *
Hypoxia + nitrendipine	11.9 ± 2.3 **
Hypoxia + atropine	26.2 ± 4.3 **
Carbachol	45.3 ± 7.8 *
Carbachol + nitrendipine	22.5 ± 5.0 **
Carbachol + atropine	0 ***

iscussion

The mammalian carotid body is a chemoreceptor at is thought to play a role in monitoring and re-onding to changes in the partial pressures of oxygen d carbon dioxide in the arterial blood [2,3]. In the rat, rt of the response of the organ to hypoxia is the lease of dopamine which may act to modulate chem-eceptor discharge during hypoxic stimulation [7]. In is study we have used an in vitro superfusion tech-que in an attempt to define some of the cellular events at link stimulus recognition to the release process.

We have shown (Table I) that hypoxia stimulates the lease of both dopamine and noradrenaline from the carotid body. This result has not, to our knowledge, eviously been observed in the rat carotid body, al-ough other workers have shown that hypoxia stimu-es the release of dopamine from rabbit and cat caro-bodies [7,11]. We do not know whether both cate-olamines are released together from the same Type I l or whether they are released separately from differ-t cells. However, as the release of the two catechol-ines are affected to different extents by a variety of perfusion conditions, it is likely that the catechol-ines are released separately from different Type I ls.

The release of dopamine in response to hypoxia was nificantly reduced in the absence of extracellular cium, results which are in agreement with those of done et al. [7], who demonstrated that catecholamine ease from the rabbit carotid body required the pres-e of extracellular calcium. In addition, we have wn that the calcium channel antagonist, nitrendipine 13], inhibited the hypoxia-stimulated release of pamine to the same extent. These results suggest that release of dopamine from the carotid body is via a e secretory process that is dependent in part on the ry of extracellular calcium into the cell via voltage-sitive calcium channels. However, the observation t the release of dopamine is not completely inhibited he absence of extracellular calcium entry suggests t additional control mechanisms may operate to ulate the secretory process.

The cholinergic agonist, carbachol, was found to nulate the release of both dopamine and noradrena-from the carotid body (Table I), results which gest the presence of cholinergic receptors on the e I cell membranes. Moreover, the effect of carbachol catecholamine release could be completely blocked atropine, suggesting that the effect is mediated via eraction with cholinergic receptors of the muscarinic e. In a variety of tissues, muscarinic receptor activa-is associated with the activation of intracellular ssenger systems which lead to a number of events, luding the activation of protein kinase C and an ease in the intracellular concentration of inositol trisphosphate [14,15]. Moreover, the increase in inositol trisphosphate is thought to lead to the release of calcium from intracellular storage sites [16]. Our results suggest that at least part of the effect of carbachol on the carotid body is mediated via the release of calcium from intracellular stores, since we have shown that carbachol stimulates the efflux of calcium from the carotid body (Table II). However, this cannot be the only mechanism by which carbachol operates in the carotid body, since its effect both on secretion and on calcium efflux is reduced by nitrendipine (Tables I and II). It is possible that not only does carbachol stimulate the release of intracellular calcium to initiate the secretory response but that maintenance of this response requires the entry of extracellular calcium via voltage-sensitive calcium channels. It may well be that the opening of these channels follows from the carbachol-induced activation of protein kinase C.

In this study we have not attempted to investigate directly the mechanism by which the carotid body senses the extracellular oxygen tension. However, we have shown that the effect of hypoxia on catecholamine release and calcium efflux were both significantly reduced by atropine, suggesting the involvement of cholinergic receptor activation in the response process. The presence of acetylcholine in the carotid body has been suggested from a number of studies [17,18]. The possibility exists, therefore, that part of the carotid bodies response to hypoxia may initially involve the release of acetylcholine which via an autocrine mechanism subsequently stimulates the release of catecholamines. However, as atropine was not able to completely abolish the responses to hypoxia, it is unlikley that hypoxia operates entirely through such a muscarinic-type mechanism.

The results of this study suggest that calcium plays an important role in the stimulus-response mechanism of the rat carotid body. It appears likely that the response of the tissue to hypoxia involves an increase in the concentration of free calcium in the cytoplasmic compartment of the Type I cells. This calcium comes both from the extracellular environment of the cell and from intracellular storage pools. Extracellular calcium enters the cell following the opening of voltage-sensitive calcium channels and intracellular stored calcium may be released via an inositol trisphosphate activated mechanism.

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surface is re-epithelialized following its removal with a damaging agent such as ethanol. In the present study we observed the reconstitution of the epithelium in the large bowel of rats following treatment with 0·25 mM sodium deoxycholate in Krebs buffer.

Male Wistar rats were fasted overnight, anaesthetised with Sagatal (i.p. 60 mg/kg body weight) and the bile salt solution introduced into an *in situ* loop preared from the terminal section of the large bowel. After 20 min the solution was allowed to drain via the anus and the loop washed with Krebs. At intervals up to 2 h after treatment groups of rats ($n = 6$) were killed with an i.p. overdose of Sagatal and the intestine fixed and processed for both light and electron microscopy.

After 20 minutes exposure to the bile salt solution the entire surface epithelium was lost; the basal lamina was intact and there was no disruption of the lamina propria, although some connective tissue cells showed evidence of damage. Thirty minutes following treatment squamous cells were present at the margins of the ostia to the glands. One hour following treatment some interglandular regions were re-epithelialized, and by 2 hours the greater part of the surface epithelium was reconstituted by squamous and cuboidal cells. The observations show that in the large bowel as in the stomach, epithelial reconstitution is rapid and is part of the defence mechanism of the gut following surface damage.

D. 3. Uptake of microspheres by rat hepatocytes in tissue culture. By T. J. SELF, S. A. JOHNSON, N. W. THOMAS, M. WARREN*, J. FRY* and C. G. WILSON*. *Departments of Human Morphology and * Physiology and Pharmacology, University of Nottingham*

In animal experiments microspheres have been investigated as carriers for drugs to be targeted to specific organs or systems (Illum *et al. J. Pharm. Sci.* **75**, 1986). Hepatocytes contain a spectrum of drug-metabolising enzymes and *in vitro* phagocytose microspheres (Johnson *et al. J. Pharm. Pharmacol.* **38**, 1986), and thus might provide a model system for investigating the kinetics of intracellular drug release from a particulate delivery system. As a first step we examined the response of primary cultures of hepatocytes (5×10^5 cells/culture well: 6 wells/treatment), prepared from rats killed by cervical dislocation (Warren *et al. Xenobiotica* **15**, 1985), to doses (200, 400 and 1000 μl) of 500 nm polybead carboxylated microspheres. Cultures were fixed in 2% glutaraldehyde in 0·1 M cacodylate buffer, and post fixed in 1% OsO_4. During dehydration the cultures were pelleted and then embedded in epoxy resin. Dried mounts of particles on piloform-coated grids were photographed in a Philips EM410 and 400 measured in order to confirm the manufacturer's specifications. Calibrated micrographs obtained systematically from random samples of the cultures were used for point counting and also cell nuclei were measured using a GIS Imagan system on a Hewlett Packard 86B. The measured mean microsphere diameter was 492 + 19 nm compared with the manufacturer's datum of 500 nm. The number of microspheres/μm^3 for each dose was as follows: 200 μl—0·061; 400 μl—0·08; 1000 μl—0·086: the data shows that uptake is not increased in a dose-related manner.

D. 4. Use of scanning electron microscopy in the study of trauma in human skeletal material from archaeological sites. By J. WAKELY and S. J. WENHAM (introduced by M. A. ENGLAND). *Department of Anatomy, University of Leicester*

Trauma, whether accidental or deliberately inflicted, is one of the most common findings on examining human skeletons from archaeological sites. Unhealed injuries caused by sharp weapons, e.g. swords, cause characteristically flat smooth bone surfaces. On these surfaces fine scratch marks can be discerned microscopically, which can yield valuable information about the nature of the injury and the weapon that caused it. The three-dimensional imaging and high resolution produced by the scanning electron microscope is particularly suitable for this study. A simple technique has been developed for producing resin casts of cut bone surfaces where the size, fragility or irreplaceability of the original specimen makes direct examination impractical. Examples of the marks on bone taken from a variety of different specimens obtained from British sites is illustrated.

D. 5. *In vitro* synthesis of catecholamines in the rat carotid body. By KAREN SHAW, D. J. PALLOT* and W. MONTAGUE. *Department of Biochemistry and * Anatomy, University of Leicester*

The mammalian carotid body stores large amounts of catecholamines. There has been some controversy over the relative amounts of different amines stored in different species; the rat carotid body appears to store mainly dopamine, with smaller, variable, amounts of noradrenaline. We have become interested in the mechanisms which control synthesis and release of amines from the carotid body and to this end have developed an *in vitro* system using rat carotid bodies.

Carotid bodies were removed from rats anaesthetized with urethane (2 g/kg i.p.) and placed in

ice-cold Tyrode medium previously gassed with 100% O_2. The carotid bifurcations were then carefully cleaned, the carotid bodies removed and incubated in Tyrode medium containing various concentrations of tyrosine spiked with 400 μCi tritiated tyrosine for varying times. At the end of the incubation period individual carotid bodies were homogenised in 1 ml 0·1 M hydrochloric acid, spun at 1200 *g* for 5 min and the supernatant divided into two aliquots: one was used to determine the concentrations of dopamine and noradrenaline using high-performance liquid chromatography allied to electrochemical detection whilst the other was placed on a Dowex 50-ion exchange column in order to separate the dopamine and noradrenaline fractions. Aliquots of the dopamine and noradrenaline fractions obtained from the Dowex-50 columns were then counted in a Hewlett Packard scintillation spectrometer. From such data the amount of radioactivity incorporated in noradrenaline and dopamine could be calculated and, as the overall concentration of each amine was known, the specific activity for each compound could also be determined.

The poster demonstrates (*a*) the effects of varying the concentration of tyrosine in the medium, (*b*) the time course in incorporation of [^{3}H]tyrosine in the rat carotid body and (*c*) the effects of denervation of the carotid body upon the synthesis of catecholamine in the rat carotid body.

D. 6. Release of catecholamines from the rat carotid body *in vitro*. By KAREN SHAW, D. J. PALLOT* and W. MONTAGUE. *Departments of Biochemistry and * Anatomy, University of Leicester*

Carotid bodies removed from rats anaesthetised with ethyl carbamate (urethane) were pre-incubated in Tyrode solution containing 50 μM tyrosine spiked with 400 μCi tritiated tyrosine for 2 hours. They were then placed in a Millipore filter holder and superfused with Tyrode solution at a rate of 1 ml/min. After passing over the carotid bodies, the superfusion medium was allowed to drip on to columns containing Dowex-50 ion exchange resin. By appropriate elution schedules it was possible to separate the noradrenaline and dopamine that was released during the collection period and to subsequently determine the amount of each amine by liquid scintillation spectrometry. Collection periods in all experiments were 10 min.

When the carotid bodies were superfused with Tyrode's equilibrated with 100% O_2 there was an initial rapid release of both tritiated noradrenaline and dopamine which gradually stabilised over a 40 min period. Thereafter the release of both noradrenaline and dopamine remained stable for a further one hour.

In some experiments we examined the effects of superfusion with media containing low partial pressures of O_2 (*c.* 40 Torr) for 10 min after basal conditions with 100% O_2 superfusion had been reached. Under these conditions there was a dramatic increase in the amount of radioactivity released that was associated with both noradrenaline and dopamine.

In order to check that the phenomena we had observed was due to secretion of amines rather than cell leakage, these release experiments were repeated using Ca^{2+}-free Tyrode's. Under such conditions the amount of radioactivity in the superfusate was much reduced and the hypoxia-stimulated release was abolished.

D. 7. An analysis of the volume ratio of the tissue components in human cricopharyngeus muscle, in comparison with vastus lateralis. By I. WHITMORE, H. BROWNLOW and P. L. T. WILLAN. *Human Anatomy, Department of Cell and Structural Biology, University of Manchester*

In previous work from this laboratory, Bonington (*J. Anat.* **146**, 1986) noted the high proportions of connective tissue and elastic fibres seen in samples from human cricopharyngeus muscles. The present study has endeavoured to estimate the proportion of muscle fibre in relation to other tissue components.

Cryostat sections (9–15 μm thick) obtained from post-mortem specimens of cricopharyngeus and vastus lateralis were stained with haematoxylin and eosin. Photomicrographs were taken at one of two magnifications and enlarged to give prints 240 × 190 mm with final magnification factors of 205 or 410. Magnifications were verified using a 1 mm graticule photographed at the beginning of every film.

A 10 mm square transparent lattice was laid at random across each print, which was assessed to ascertain whether the points of intersection of the grid lay over muscle fibre, other tissue components or an area outside the section. Counts of the first two groups were recorded and the proportions of the tissue sections occupied by muscle fibres ascertained.

In vastus lateralis the proportion of the muscle occupied by muscle fibres was 75·4–90·8 % (mean 82·2 %), similar to other published findings, whereas in cricopharyngeus muscle fibres occupied 49·6–70·5 % (mean 60·4 %) of the tissue. No difference was noted when proportions were assessed with respect to age or sex in either muscle.

In view of previous reports of the wide variability of muscle fibre size in cricopharyngeus, it

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STATEMENT

The accompanying thesis/dissertation* submitted for the degree of Ph.D entitled

STIMULUS-SECRETION COUPLING IN THE RAT CAROTID BODY

is based on work conducted by the author in the Department of BIOCHEMISTRY of the University of Leicester mainly during the period between OCTOBER 1986 and AUGUST 1989

All the work recorded in this thesis/~~dissertation~~* is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University/~~part of this work has been submitted for another degree indicated in the University of~~*

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A B S T R A C T

Stimulus-secretion coupling in the rat carotid body

Karen Shaw PhD (Leicester) 1989

The effects of a variety of agents known to stimulate chemoafferent activity were employed to define in greater detail the stimulus-secretion coupling mechanism in the rat carotid body superfused in vitro.

Hypoxia, carbachol and sodium cyanide were independently able to elicit amine release. Hypoxia-evoked release was calcium dependent and was reduced by nitrendipine suggesting the involvement of voltage-dependent calcium channels in the secretory response. The effect of carbachol on catecholamine release was abolished by atropine indicating the presence of muscarinic cholinergic receptors on the Type I cells. Hypoxia-induced catecholamine release was partially blocked by atropine suggesting a possible role for muscarinic receptors in the secretory response.

Hypoxia, carbachol and cyanide stimulated the release of ^{45}Ca from carotid bodies pre-loaded with ^{45}Ca, and the release of ^{45}Ca by hypoxia or carbachol could be reduced by atropine and nitrendipine. These results suggest that the mobilization of intracellular calcium pools may also contribute to the secretory response.

Carbachol was able to stimulate the efflux of [^{3}H] inositol from pre-loaded carotid bodies and the response was abolished in the presence of atropine or lithium. Cytoplasmic concentrations of IP_3 and IP_4 significantly increased following stimulation with carbachol and the effect was abolished with atropine. In comparison, hypoxia was unable to induce [^{3}H] inositol efflux and only had a moderate effect on inositol phosphate accumulation. These results suggest that carbachol may control cytosolic calcium via the formation of inositol phosphate second messengers.

It is suggested that carbachol and hypoxia stimulate catecholamine secretion by altering the intracellular free calcium concentration in the Type I cells.

The major effect of hypoxia was to stimulate the entry of extracellular calcium via the voltage-dependent calcium channels whereas mobilization of intracellular calcium stores was a more important event during carbachol-induced catecholamine secretion.