THE CENTRAL CONTROL OF THE PULMONARY CHEMOREFLEX

BY

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Thesis submitted for the degree of Doctor of Philosophy to the Faculty of Science of the University of London

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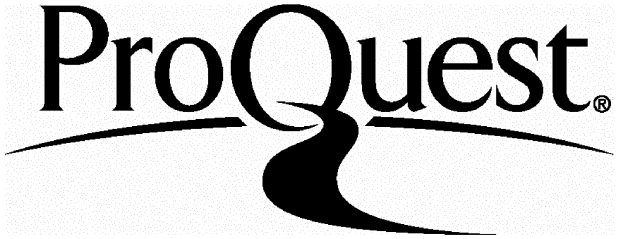

ABSTRACT

This thesis explores the central organization of the reflex associated with pulmonary C-fibre stimulation. In particular the circulatory and respiratory changes associated with the pulmonary chemoreflex are analyzed. Electrophysiological techniques are used to dissect the medullary networks involved in elaborating the cardiac vagal changes that occur in response to phenylbiguanide administered into the pulmonary circulation. The primary objective is to explain why the cardioinhibition of the pulmonary chemoreflex is unaffected by central respiratory activity. Results from experiments performed on cat, rat, rabbit and dogfish are described.

Two different populations of cardiac vagal preganglionic neurones are described in the cat. One population displays respiratory related activity and the other, tonic non-respiratory related activity. Both populations are implicated in the pulmonary chemoreflex. The results of vagal stimulation experiments, performed with anodal block, demonstrate that both populations of cardiac vagal preganglionic neurones have chronotropic action in the cat, rat and rabbit.

A hypothesis, based on the existence of two populations of cardioinhibitory vagal preganglionic neurones, is advanced to interpret certain features of the vagal control of the heart which hitherto, have eluded explanation. A philosophy is presented that endeavours to examine the possible evolutionary physiology of such a system.

DEDICATION

To Veronica, my beautiful future wife.

ACKNOWLEDGEMENTS

Special thanks are due to Prof.R.G. O'Regan for suggesting that I work with Dr. David Jordan, one of the best physiologists in Britain. David has been a patient and thoughtful supervisor, and has transformed my understanding of the autonomic nervous system.

Many enjoyable discussions concerning cardio-respiratory physiology were held with Prof.M. DeBurgh Daly, Prof. K.M. Spyer and Prof. E.W. Taylor; without this interaction this thesis would be impoverished.

I have had the good luck to work with Yun Wang and Michael Young, and received a great deal of support from Dr. Andrew Ramage.

The financial support of the MRC is gratefully acknowledged.

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Abbreviations

BCL	basic cardiac cycle length
BP	blood pressure
CGRP	calcitonin gene related peptide
CVLM	caudal ventrolateral medulla
DLH	D,L homocysteic acid
DVMN	dorsal vagal motor nucleus
ECG	electrocardiogram
EPSP	excitatory post synaptic potential
$ETCO_2$	end tidal carbon dioxide
FRC	functional residual capacity
GABA	gamma-aminobutyric acid
HDA	hypothalamic defence area
HRP	horseradish peroxidase
Hz	hertz
IS-SD	initial segment-somatodendritic
LAP	lower airway pressure
LHRH	luteinizing hormone releasing hormone
NA	nucleus ambiguus
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
PBG	phenylbiguanide
PDG	phenyldiguanide
PSTH	peri stimulus time histogram
RLN	recurrent laryngeal nerve
RVLM	rostroventrolateral medulla
sEPSP	slow excitatory post synaptic potential
TP	tracheal pressure
UAP	upper airway pressure
5-HT	serotonin

1. General Introduction

1.1 The pulmonary chemoreflex

Before the pioneering experiments of Brodie (1900), investigators believed that the respiratory expanse below the glottis was insensitive to chemical irritants. He described a vagal reflex "triad" of apnoea, bradycardia and systemic hypotension evoked in cats when blood serum was injected into a jugular vein. This is the first and probably the best description of what was latter to be called the pulmonary chemoreflex (Dawes & Comroe 1954). In some cases the excitation of the vagal fibres lasted so long as to cause the death of the animal. Brodie concluded that the active substance is produced during the process of clotting, that the corpuscles take an active part, and that the upper layer sediment of white corpuscles and platelets may be involved. This predates by eighty to ninety years the demonstration that serotonin release by platelets is involved in the pulmonary chemoreflex (Wiggins et al. 1985; Armstrong & Kay 1990). Brodie also made the following fascinating observation :

"occasionally animals have been met which show no effect on respiration although the cardiac effect is typical".

In a subsequent paper Brodie described a similar constellation of effects induced by inhalation of a small quantity of bromine vapour and abolished by section of the pulmonary vagal branches (Brodie & Russell 1900). A picture emerges therefore of a pulmonary vagal reflex which is triggered by stimuli in the bloodstream or the airstream and which powerfully slows the heart. It is of interest that Brodie made the analogy between his mammalian pulmonary chemoreflex and the work of McWilliam (1885), who had described that the stimulation of the gills of eel, perch, carp and rudd leads to cardiac arrest.

The pulmonary chemoreflex is occasionally confused with the reflex described by Von Bezold and Hirt in 1867. The latter is primarily concerned with cardiac afferents and can be triggered by injecting veratrum alkaloids into the coronary artery. This reflex is also referred to as the Bejold-Jarisch reflex, the Bejold effect and the Bezold reflex.

These are unhelpful terms and could be replaced with the term coronary chemoreflex (Dawes & Comroe 1954). Brodie proved that his reflex was not another manifestation of the coronary chemoreflex through careful selective cardiac denervation.

After Brodie's observations, progress in this field was slow until the receptor involved in the pulmonary chemoreflex was identified. Paintal (1955), was the first to record the low voltage action potentials from vagal filaments (pulmonary C-fibres) in cats when phenyldiguanide (a serotonin agonist) and other chemicals were injected into the right atrium. Paintal called the afferent endings "type-J" receptors, J representing "juxta-pulmonary capillary" (Paintal 1969).

Many of the facts concerning pulmonary C-fibre physiology have been established by the long and careful scientific enquiry of the Coleridges. There is now considerable evidence that the tonic activity in afferent C-fibres from the lungs powerfully influences respiratory frequency, tidal volume, bronchomotor tone and systemic vasomotor tone (Coleridge & Coleridge 1984). However, much less is known concerning the ongoing pulmonary C-fibre activity and its relationship to vagal cardiomotor tone.

1.2 Anatomical details of pulmonary C-fibres

The vagal pulmonary branches are primarily composed of unmyelinated C-fibres. A degeneration study of the adult feline vagal pulmonary branch revealed that of the 5000 afferent fibres distributed to the lungs and intrapulmonary airways, about 80% are unmyelinated (Agostoni et al. 1957). A recent duplication of that study, but using electron microscopy, found 92% of these fibres are unmyelinated (Jammes et al. 1982). The sensory terminals of unmyelinated afferents have been most extensively surveyed in the mouse lung (Hung et al. 1972). C-fibres are regularly present in the mouse alveolar walls and ducts, areas supplied by the pulmonary circulation and overlapping the predicted distribution zone of Paintal's J receptors. However, in studies on both rat (Meyrick & Reid 1971) and human lung (Fox et al. 1980), investigators revealed a scant number of C-fibres in the alveolar wall, which they ascribed to species variation. This may be an incorrect assumption as the appropriate studies based on allomorphosis or

allometric calculations based on ontogeny have not been performed. In other words are pulmonary C-fibres obvious in a mouse because a mouse is small ? It would be of interest to determine the scaling effect upon C-fibre firing rate, C-fibre number and arborization in lung parenchyma, as animals change in size and shape. However there is no information concerning more basic C-fibre details such as sensory field density, number of terminal branches per axon and size of terminal arborization. Also no direct observations on C-fibre activity in the newborn are available to make the calculation.

1.3 General sensory properties

Pulmonary C-fibres have a sparse (<1Hz) irregular tonic discharge which is easily missed in multifibre vagal slips from animals with open pneumothorax (Coleridge & Coleridge 1984). The C-fibre receptor is polymodal, it is both chemosensitive and mechanosensitive. The inflation threshold in open chest dogs is about three times the threshold for myelinated slowly adapting receptors (Kaufman at al. 1982), and almost equal to that of rapidly adapting receptors. Bronchial C-fibres have the highest inflation threshold. This would suggest that the pulmonary C-fibre should be silent during normal tidal breathing in a closed chest preparation. Surprisingly, it assumes an inspiratory rhythmicity during spontaneous breathing (Coleridge & Coleridge 1977a). Obviously changes in lung mechanics must be considered when interpreting data from lung afferents in animals with thoracotomies.

Pulmonary C-fibres are stimulated by a variety of foreign irritants, but they appear to be insensitive to lung autocoids. However the pulmonary circulation is richly supplied with the enzymatic machinery to destroy most autocoids (Junod 1977). Therefore the apparent insensitivity to autocoids may be a consequence of this special environment. Paintal (1969) has hypothesized that one important stimulus to J-receptors (i.e., pulmonary C-fibres) might be the increase in interstitial fluid volume during pulmonary oedema. The observation, that pulmonary congestion evoked a vago-vagal response closely resembling the pulmonary chemoreflex, had been reported forty years earlier by Churchill and Cope (1929). However there have been a number of difficulties with the experimental validation of this hypothesis. First, pulmonary congestion stimulates all four types of pulmonary vagal afferents: slowly adapting receptors, rapidly adapting receptors,

bronchial C-fibres and pulmonary C-fibres. Second, the phenomena that accompany the oedema (such as hypercapnia and hypoxia), and the methods used to produce the oedema, (such as clamping the aorta or the pulmonary veins or injecting caustic alloxan) may have been the cause of the reflex rather than the oedema per se. Recently however, Roberts et al. (1986) have obtained convincing evidence that pulmonary C-fibres are recruited during lung oedema. Their approach involved the infusion of large volumes of Krebs-Henseleit solution and then the withdrawal of blood to restore pulmonary pressure to control levels but leaving interstitial oedema which was quantified by measuring extravascular lung water and examining lung tissue microscopically.

On the balance of evidence obtained to date, the physiology of the "J-receptor" is still confounding. It is not known what element is sensed by the receptor in a resting breathing subject. Since it is situated at a blood-air interface, is it an interoceptor or an exteroceptor ? Is the receptor primarily nociceptive or is it actually monitoring the gel hydration level of the pulmonary interstitium ? Is it indirectly rheoreceptive ? Are the receptors influenced by physiological mixed venous or alveolar gases or both ? Whatever the origin of the afferent traffic along C-fibres, the central nervous system does not filter it out, as certain cardiorespiratory variables are influenced tonically by pulmonary C-fibres.

1.4 Components of the pulmonary chemoreflex

The pulmonary chemoreflex is classically described as a triad of apnoea, bradycardia and systemic hypotension (Dawes & Comroe 1954). In addition to this triad the reflex also causes inhibition of somatic motor function (Ginzel & Eldred 1969) tracheo-bronchial constriction (Coleridge et al. 1982; Russell & Lai-Fook 1979) laryngospasm (Haxhiu et al. 1988), tracheal mucosecretion (Schultz et al. 1991), cough (Jain 1972), bronchial vasodilation (Coleridge et al. 1992), coronary vasodilation (Clozel et al. 1985; Ordway et al. 1986), negative dromotropy (Brender & Webb-Peploe 1969), and negative inotropy (Cassidy 1979). The striking increase in vagal tone to the heart may have a protective action since it is well established that this greatly diminishes reperfusion arrhythmias (Zuanetti et al. 1987). There is a pattern to the rest of this diverse list of reflex responses which results from the sudden surge of pulmonary C-fibre activity in response to inhaled

irritants or intravenously injected boli of noxious chemicals. The pattern is defensive in nature, and the structure defended may be the respiratory exchange surface. If this hypothesis is correct, then it is possible that a similar chemoreflex may be described in different zoological groups, but pertaining to the appropriate gas exchanger. This is presumably what Brodie was considering when he drew a connection between the reflexes emanating from the mammalian lung and the fish gill. Unfortunately there is a paucity of data on nonmammalian species with gill respiration and none on species with integumentary respiration. Only Satchell (1977) has investigated this possible evolutionary connection. He described a "J-reflex" in the spiny dogfish (*Squalus acanthias*) when phenyldiguanide was injected into the central veins or the inspired waterstream. The receptors involved were localized to the branchial circulation. As Satchell points out, the evolving pattern resembles the mammalian response closely and essentially involves the inhibition of three pumps: the respiratory pump, the cardiac pump and the muscle pump. The net effect is a tremendous drop in venous return, cardiac output and consequently gaseous exchange. This has the protective action of minimizing the flux of irritant into the bloodstream, an essential reflex action since the mixed venous blood content of irritant is low which would potentially drive circulatory convection in an unreactive biological system. In mammals, the sympathoinhibition appears to be an unpatterned global inhibition of all vascular beds (Brender & Webb-Peploe 1969), this facet of the pulmonary chemoreflex makes it a particularly attractive reflex to study. Quite apart from this defensive action in a pathological circumstance it is becoming increasingly clear that pulmonary C-fibres have considerable functional significance in the control of cardio-respiratory parameters at rest.

1.5 Physiological role of pulmonary C-fibres

At rest there is a continuous low frequency stream of impulses travelling up large numbers of pulmonary C-fibres (Coleridge & Coleridge 1984). Paintal suggests that a sustained input of even less than 1 impulse per second can be of considerable reflex significance (Paintal 1970, 1973; Anand & Paintal 1980). Folkow et al. (1976) have referred to vagal unmyelinated C-fibres as the "submerged part of the iceberg", due to the large reflex changes that accompany very small electrical stimulation frequencies. In

a series of vagal cooling experiments on the rabbit and dog, Hammouda & Wilson (1939) developed the hypothesis that resting breathing rate is determined by a balance between an inhibitory input from the lower respiratory tract, blocked at a vagal temperature of 8°C, and an excitatory input blocked only at 2-3°C. These observations which suggest that pulmonary C-fibres tonically influence breathing rate through their ability to shorten both expiratory and inspiratory time, have since been confirmed by many investigators (Karczewski & Widdicombe 1969a,b; Philipson et al. 1973; Fishman et al. 1973; Miserocchi et al. 1978). The tonic control that pulmonary C-fibres exert over the respiratory network is also exerted over the sympathetic vasomotor outflow. Receptors in the cardiopulmonary region subserved by vagal afferents exert a tonic inhibition on the central vasomotor nerves controlling the sympathetic outflow to the resistance and capacitance vessels (Mancia et al. 1973). When the influence of the arterial baroreceptors is eliminated, cooling of the cervical vagus in the anaesthetized cat, rabbit and dog results in constriction of the resistance vessels of the skeletal muscle, intestine and kidney and of the splanchnic capacitance vessels (Guazzi et al. 1962; Pillsbury et al. 1969; Oberg & White 1970; Mancia et al. 1973). In addition the liver volume clearly decreases (Carneiro & Donald 1977). Mancia and Donald (1975) examined the important question whether the tonic inhibition arises from the atria, the ventricles or the lungs. In their experiments with an extracorporeal oxygenator selective extirpation of these reflexogenic zones established that receptors in the lungs, the atria, and the ventricles each were responsible for the tonic inhibition of what they termed the "vasomotor centre". Experiments with anodal block and vagal cooling have established that the tonic vagal inhibition from the cardiopulmonary area is principally or even totally mediated via nonmedullated fibres (Thoren 1977a,b;Thoren et al. 1975). It would appear from the detailed studies of Mancia et al. (1973; 1975a,b), that cardiopulmonary receptors are unable to affect the cardiovascular system when the carotid arterial baroreceptors are strongly activated suggesting that perhaps pulmonary C-fibres and carotid sinus nerve afferents converge on common motor pools. There is a quantitative difference however between these reflex systems: the muscle vasomotor fibres are less engaged by the cardiopulmonary receptors than by the arterial baroreceptors but only in quadrupeds (Thoren 1979). When pulmonary C-fibre activity is powerfully and synchronously increased to provide a defensive pattern sympathetic activity is globally decreased

(Brender & Webb-Peploe 1969). In the resting unperturbed animal the low background discharge of pulmonary C-fibres is harnessed for tonic vasomotor inhibition for specified vascular beds. Which vascular beds appears to depend on whether most of the blood volume is at heart level (quadrupeds) or below heart level (bipedals), (Thoren 1979). It is unknown how much of tonic vasomotor inhibition is indirect through inhibition of inspiration synchronous sympathetic activity i.e. is the ongoing stream of pulmonary C-fibre primarily directed to the respiratory pattern generator?

There is evidence that vagal bronchomotor tone in cats is entirely dependent upon pulmonary C-fibre input, for Jammes and Mei (1979) found that selective section of afferent vagal fibres at the level of the nodose ganglion abolished all bronchomotor tone. Application of procaine to block the C-wave of the evoked compound action potential also abolished bronchomotor tone. Jammes and Mei postulated that vagal bronchoconstrictor tone is maintained largely by background activity in C-fibres from the lung. This is incomplete evidence since procaine blocks both C-fibre motor and sensory pathways. However, the experiments of Roberts et al. (1982) in dogs confirm the fact that pulmonary C-fibre input (but not solely C-fibre input) tonically drives tracheal tone, here the motor innervation (superior laryngeal nerve) is anatomically separate from the sensory input (pulmonary vagus). Vagal cooling to below 2°C decreases tension in a tracheal segment.

1.6 Central neural pathways of the pulmonary chemoreflex

1.6a Nucleus tractus solitarius

The medullary projections of afferent pulmonary C-fibres and bronchial C-fibres were antidromically mapped for the first time by Kubin et al. (1991). The two receptor subtypes did not differ in their central projection pattern. Rostral to the obex, the central branches were localized to the medial portions of the nucleus tractus solitarii (NTS) and area postrema. Caudal to the obex the densest branching was found in the dorsal portion of the commissural subnuclei. Pulmonary C-fibres have scant or no input to the lateral, ventrolateral or ventral subnuclei unlike their myelinated counterparts, which have some degree of projection to these subnuclei (Kalia & Richter 1985). The technically difficult

study of Kubin et al. (1991) successfully tracked a small sample of 12 fibres out of an estimated 10,000 bronchopulmonary C-fibre population in the cat. However there is reason to believe that this sample is representative. Neurones in commissural NTS are required for full expression of the pulmonary chemoreflex in rat. Injections of cobalt abolish the reflex if applied focally to this site (Bonham & Joad 1991). The anatomical location of this site agrees strikingly with the caudal site described by Kubin et al. (1991); however whether a rostral projection site is present in the rat is unknown. Verberne and Guyenet (1992) have demonstrated that a bilateral block of excitatory amino acid transmission in the lateral commissural nuclei of the rat attenuates the hypotension of the pulmonary chemoreflex. Vardhan et al. (1993) claim to be able to differentially block the carotid body chemoreflex from the pulmonary chemoreflex by very discrete chemical lesions in the commissural nucleus.

Bennett et al. (1985) recorded ipsilateral unmyelinated pulmonary input to neurones in the medial NTS whereas myelinated pulmonary input was distributed to both medial and lateral regions of the NTS. The electrophysiological findings of Bennett et al., provided the fascinating result that individual NTS neurones may receive myelinated input or unmyelinated input from the lungs, but never convergent excitatory input from both. This is perhaps an indication that the medulla processes information on the basis of timing, with parallel and independent channels for fast high frequency information and the relatively slow transmission of low frequency C-fibre data. Of particular note is the possibility that this mirrors the parallel vagal preganglionic output of myelinated and unmyelinated efferents to heart and lungs. The question arises as to what possible interconnections exist between this selective processing of rapid and slow input and the emergent fast and slow fibre preganglionic output.

The study of airway and pulmonary afferent organization in the nucleus tractus solitarius is inordinately biased in favour of slowly adapting pulmonary stretch receptors (SARs). This is somewhat surprising in view of their meagre numbers and late phylogenetic appearance. In contrast the sensory feedback from the vast surface of the gas exchanger and richly innervated branchiomeric driven structures is poorly understood. For example there are no studies correlating upper airway ventilation and NTS firing patterns, (partly

because of the routine practice of tracheostomy), and there are no studies on NTS firing patterns to pulmonary C-fibres. The SAR feedback system has been so overstudied in contrast, that three different terminologies have arisen, all describing the same phenomenon: Ralpha, Rbeta cells (Baumgarten & Kanzow 1957), Ialpha, Ibeta cells (Berger 1977); I+,I-,I0 cells (Cohen and Feldman 1984). The classification is based on variations of SAR and central inspiratory input. Following the original demonstration of the Ralpha and Rbeta inspiratory neurones in the NTS by Von Baumgarten, numerous investigators have confirmed their findings;in addition a group of neurones termed P-cells (or pump cells) were identified as probable second order neurones for SARs. (Long & Duffin 1986). It is unknown how these neurones behave during the pulmonary chemoreflex, but since Rbeta and P-cells receive monosynaptic input from SARs (Backman et al. 1984) they might be expected not to receive input from unmyelinated fibres (Bennett et al. 1985), although this has not been specifically investigated.

1.6b Respiratory neurone activity

There are only a few studies on central respiratory activity during the pulmonary chemoreflex (Katz & Horres 1972; Koepchen et al. 1977; Daly et al. 1992). The first two studies concern respiratory neurones which unfortunately are not characterized according to axonal projection or location and lack categorization with reference to the phrenic nerve. Nevertheless it is possible to apply a modern classification of respiratory cells (Richter et al. 1986) to at least to Koepchen's paper. It is quite clear that pulmonary C-fibres inhibit inspiratory cells, stage II expiratory cells but powerfully excite post-inspiratory cells (stage I expiration). More recently Daly et al. (1992) have recorded from multiple respiratory outputs: phrenic nerve, recurrent laryngeal nerve, intercostal nerves and from ventral respiratory group neurones in the anaesthetized cat. Pulmonary C-fibre activation inhibited both inspiratory and expiratory neuronal activity and excited post-inspiratory neurone activity. Prabhakar et al. (1986) have analyzed post-inspiratory activity recorded from single fibres of a phrenic rootlet in the anaesthetized cat. During hyperventilation (hyperoxic hypocapnia) activity of post-inspiratory fibres continued in a tonic fashion although mass phrenic displayed no rhythmic activity. Prabbhakar et al. (1986) report that after bilateral vagotomy the majority of units displaying post-

inspiratory activity no longer displayed sustained discharge during hyperventilation, suggesting the importance of vagal afferents in producing tonic drive. During phasic breathing stretch and irritant receptor activation inhibit the length of post-inspiratory activity and only pulmonary C-fibres seemed to prolong it (Prabhakar et al. 1986; Holmes and Remmers 1989; St John & Zhou 1989). Holmes & Remmers (1989) clearly show that activation of pulmonary C-fibres in the decerebrate cat produces tonic discharge in the thyroarytenoid, posterior cricoarytenoid and diaphragm. The most dramatic increase occurs in the laryngeal constrictor muscle: the thyroarytenoid. Haxhiu et al. (1988) also found tonic activation of thyroarytenoid in anaesthetized dogs during the apnoeic spell of the pulmonary chemoreflex. In addition this tonic activity appeared in other upper airway constricting muscles: the lateral cricoarytenoid, the upper and middle pharyngeal constrictor and in chest wall muscles: internal intercostals and triangularis sterni. Upper airway dilating muscles, diaphragm and external intercostals exhibited decreased activity in agreement with the findings of Holmes and Remmers (1989) in the cat. The external oblique in the anaesthetized dog is excited by myelinated pulmonary fibres and inhibited by pulmonary C-fibres (Hollstein et al. 1991). Thus all these studies are in agreement that the apnoea of the pulmonary chemoreflex is a post-inspiratory apnoea, and in this regard resembles the reflex apnoea obtained by laryngeal stimulation (Remmers et al. 1986). The entire reflex is certainly facilitated through the post-inspiratory apnoeic state of the central oscillator i.e. through the post-inspiratory linking of vagal preganglionic neurones (leading to bradycardia, bronchoconstriction, laryngospasm and pharyngeal constriction), and post-inspiratory inhibition of sympathetic preganglionic neurones (leading to vasodilatation and bradycardia). Respiratory modulation of autonomic outflow would appear to be a very economical method of orchestrating disparate target organs during the progression of a reflex pattern.

1.6c Somatic motor function

The central pathways for the reflex depression of spinal reflex arcs (Paintal's J-reflex) have been analyzed by Kalia (1973). The supra bulbar projections appear to involve the caudate nucleus and cingulate gyrus, and ablation of these areas abolishes the response, according to Kalia. It is known that vagal C-fibre afferents have multiple projections to the anterior insular cortex in rats (Ito 1992). It is strange therefore to learn that the

original description was obtained in decerebrate cats whose rigidity lessened when PDG was injected into the right atrium (Ginzel & Eldred 1969). In the spinal cord, inhibitory pathways onto monosynaptic reflexes descend adjacent to the central canal and involve interneurones in the ventral pericanalicular region of the upper lumbar segment. The pulmonary chemoreflex is associated with a most powerful bulbo-spinal descending inhibition of both flexor and extensor α-motoneurones and all associated γ-motoneurones, sympathetic preganglionic neurones and spinoreticular neurones of almost all laminae (Deshpande & Devanandan 1970; Ahluwalia et al. 1977; Rao & Devanandan 1977; Thies & Foreman 1983). The pulmonary chemoreflex is pre-eminent in its ability to produce such global unpatterned descending inhibition. What is of interest is that this reflex is hardly ever engaged in modern vertebrates (with the exception of such unusual events such as the Bhopal gas tragedy). The chemoreflex induced by injecting PBG into the pulmonary circulation is a pharmacological curiosity, yet the circuitry exists to engender it. It is known that a similar depression of monosynaptic reflexes, bradycardia and hypotension occurs during the carotid baroreflex (Schulte et al. 1959), splanchnic and pelvic afferent stimulation (Evans & McPherson 1958) and REM sleep (Parmeggiani & Morrison 1990) and stimulation of the ventrolateral periaqueductal grey (Lovick 1991). Perhaps the circuitry persists in vertebrates because it is now readapted for other purposes.

1.6d The depressor response

Sun & Guyenet (1987) have recorded from barosensitive rostroventrolateral medullary (RVLM) neurones in the rat inhibited by vagal C-fibre afferents and utilizing GABA receptors as the means of inhibition. The source of the GABAergic neurones may be the caudal periambiguual area also referred to as the depressor area, A1 Area, or external formation of nucleus ambiguus at obex level, but usually referred to as the caudal ventrolateral medullary area (CVLM) (Bieger & Hopkins 1987; Willette et al. 1983). Ablation or inhibition of the CVLM causes a pressor response and inhibition of both the baroreflex and the pulmonary chemoreflex, whereas excitation of the CVLM produces a depressor response and bradycardia. (Willette et al. 1983; Gordon 1987; Agarwal et al. 1990). Guyenet (1990) has proposed a simple scheme to account for these results: second order pulmonary C-fibre neurones in the nucleus tractus solitarius project to the

CVLM and release a glutamate-like substance which activates GABAergic like medullary interneurones, which then project to the rostral ventrolateral medulla. The latter would then be responsible for the inhibition of rostral ventrolateral medullary neurones. The hypothetical scheme appears compatible with existing anatomical data (Ross et al. 1985; Sun & Guyenet 1986). Verberne and Guyenet (1992) have postulated that the sympatho-inhibitory component of the Bezold-Jarisch reflex may use a central pathway similar to that of the arterial baroreflex. The possible caveat to this hypothesis is that the RVLM studies are concentrating on a population of barosensitive neurones, as this is a common criterion for identification of a RVLM neurone. There may be pulmonary C-fibre sensitive cells which are not barosensitive. The "presympathetic" neurones of the RVLM may be grouped and organized according to target-organ location, (an "autonomic homonculus") or target-organ function of the appropriate sympathetic preganglionic neurone. Alternatively this region may actually be organized according to reflex pattern formation, and thus be shaped by the vagaries of reflex evolution, reflex cross-adaptation and maladaptation.

1.6e Hypothalamic control of the pulmonary chemoreflex

Several studies have analyzed the interaction between the hypothalamic defence area (HDA) of the cat and the pulmonary chemoreflex (Achari & Downman 1969, 1970; Achari et al. 1973). The response to right atrial PDG was abolished by HDA stimulation and this still occurred after cerebellectomy. Fastigial nuclei stimulation is capable of blocking the bradycardia of the pulmonary chemoreflex but not the apnoea and this effect still occurred after intercollicular decerebration (Achari & Downman 1970). According to Kidd (1987), the cerebellar neurones involved do not appear to utilize GABA to effect an inhibition of pulmonary C-fibre input. It is well established that the bradycardia of the baroreflex is blocked during the defence response (Humphreys et al. 1971; Humphreys & Joels 1972; Djojosugito et al. 1970) as is the bradycardia of the coronary chemoreflex (Wennergren et al. 1976). The CVLM is a possible locus for descending hypothalamic defence area projection since GABA agonists injected here raise blood pressure and gate the baroreflex and the pulmonary chemoreflex depressor responses. (Willette et al. 1983).

1.7 Reflex cardiac effects of pulmonary C-fibre activation

A major component of the pulmonary chemoreflex is a fall in right ventricular cardiac output that cannot be ascribed to a reduction in venous return or an increase in pulmonary vascular resistance (Bäer & Nusser 1958), it appears to be due primarily to the vagally mediated bradycardia. This powerful negative chronotropic effect appears within the pulmonary circulation time of the animal and often produces a period of asystole lasting several seconds (Coleridge et al. 1964). Restoration of sinus rhythm is usually accompanied by several cycles of atrio-ventricular block (Brender & Webb-Peploe 1969). In fact all possible cardiac vagal actions are manifest during this reflex: chronotropic , dromotropic, inotropic, and vasomotor. A cholinergic coronary vasodilatation occurs in dogs (Ordway et al. 1986; Clozel et al. 1985). Measurements of the transmural distribution of blood flow indicated that vasodilation occurred in all layers of the myocardium, (Clozel 1985). Analysis of vagal coronary control during this reflex has only been performed in canine preparations, which is unfortunate because there is now considerable evidence that cholinergic vasodilation is the exception rather than the rule in vagal coronary control (Kalsner 1985,1989). From a central control point of view, it might be predicted that all cardiac vagal preganglionic neurones, regardless of location or function are activated during this reflex, unless they are constrictor to the coronary arteries in which case these should be inhibited. But there are two caveats to this hypothesis: first, it assumes that the prime reflex response is coronary vasodilation, which teleologically makes sense since cardiac output plummets and systemic vascular resistance also falls (Brodie 1900), so the proportion of output to the heart would be drastically reduced if coronary vasoconstriction ensued. Second, it assumes that certain species have vagal cholinergic constrictor fibres, if this is correct it would mean extraordinary central rewiring has occurred in certain species for this reflex.

Slowly adapting pulmonary stretch receptor activity promotes tachycardia (Daly 1972;Angell-James & Daly 1978), this accounts for one of Anrep's (1936) two basic mechanisms which generate respiratory sinus arrhythmia. The other mechanism is central inspiratory inhibition of cardiac vagal tone. Coleridge & Coleridge (1984) suggested that

the vagally mediated bradycardia of the pulmonary chemoreflex may be more pronounced in spontaneously breathing animals, when it is accompanied by arrest of breathing, than in artificially ventilated ones when phasic lung inflation is uninterrupted. Surprisingly when this hypothesis was actually tested recently (Daly & Kirkman 1989) it was found that inflation of the lungs did not inhibit the bradycardia of the pulmonary chemoreflex although it did inhibit the bradycardia of the carotid body reflex, the coronary chemoreflex and the baroreflex. Moreover the pulmonary inflation actually tended to increase the degree of pulmonary C-fibre bradycardia (Daly & Kirkman 1989). This observation already existed in the literature albeit in an indirect form. Sutton (1981) showed that the bradycardia caused by injecting serotonin (5-HT) into the nodose ganglion of the cat was unaffected by ramp inflation of the lungs. Pulmonary and abdominal C-fibre afferents express 5-HT_3 receptors along their entire length but particularly at their terminals and cell bodies (Segu et al. 1981). Daly (1991) pursued the pulmonary C-fibre bradycardia further to demonstrate that central inspiratory activity also has no influence on the level of cardiac slowing across a wide range of respiratory drive. The lack of respiratory modulation might have been explained by postulating a direct action of PBG on postganglionic nerve terminals or the pacemaker, but the response has a latency of approximately 5 seconds and is abolished by vagotomy. Also there might have been a sympatho-inhibitory difference amongst the reflexes (Koizumi & Kollai 1992). However Daly performed the experiments with beta blockers and total sympathetic denervation of heart and lungs, and found no difference in the results. Therefore this strange phenomenon is of vagal origin but cannot be so readily assimilated into the body of knowledge accumulated since Anrep (1936) or current models of cardiorespiratory integration. According to Richter and Spyer (1990), early inspiratory and possibly also late inspiratory inhibition of vagal cardiomotor neurones is pronounced and totally suppresses what must be a relatively weak tonic excitatory input. Three other reflexes associated with bradycardia were analyzed by Daly: the carotid body chemoreflex, the baroreflex and the coronary chemoreflex, and in all of these clear evidence of respiratory modulation was obtained. A survey of the literature with regard to respiratory modulation of other cardioinhibitory reflexes reveals that the bronchial C-fibre reflex in the dog (Roberts 1981) the diving reflex in the seal (Daly & Angell-James 1980) the oculocardiac reflex in humans and nasopharyngeal reflex in dogs (Gandevia et al. 1978a,b) are also

distinctly modulated by central inspiratory activity. It would appear that this distinct unmodulated trait of the pulmonary chemoreflex has not been highlighted hitherto because of the usual association of apnoea with bradycardia during the pulmonary chemoreflex. It appears only Brodie (1900) and Daly (1994) have commented upon the fact that it is possible to observe a cat's response to pulmonary C-fibre stimulation in which there is little effect upon respiration, yet the cardiac effect is unaltered. It is unknown whether laryngeal reflex cardioinhibition is unmodulated as it is extremely difficult to dissociate the inhibition of breathing from the inhibition of heart rate. In fact the apnoeic threshold is lower than the threshold for producing bradycardia during electrical stimulation of the superior laryngeal nerve (SLN)(Daly & Kirkman 1989). In order to account for the peculiarity of the pulmonary chemoreflex, Daly has posited the existence of two distinct populations of cardiac vagal preganglionic neurones, one of which is coupled to the respiratory network and responsible for the reflexes associated with respiratory modulation. The other group however, is independent of the respiratory network and receives no inhibitory input from pulmonary stretch receptors. This group is dedicated to the pulmonary chemoreflex. It is interesting that although all peripheral cardiac efferent work has been biased to exclude all C-fibre efferents, evidence for multiple populations still surfaces. Kunze (1972) recorded from single units in the right cardiac vagal branch of the cat, she found 5 neurones responsive to baroreceptive and chemoreceptive input, and 13 neurones responsive to baroreceptive input only. Iriuchijima (1972) postulated separate populations for SLN induced bradycardia compared with carotid sinus nerve evoked bradycardia. Davidson et al. (1976) reported that some cardiac vagal preganglionic neurones might be very responsive to one stimulus and not responsive to the other. However this was never formally quantified or pursued in further investigations. Unfortunately, there has never been a study dedicated to analysing baroreceptive, chemoreceptive and SLN input onto cardiac vagal preganglionic neurones. In order to place the multiple population hypothesis in context, it is necessary to briefly review current knowledge pertaining to the distribution and physiological properties of cardiac vagal preganglionic neurones.

1.8 Cardiac vagal preganglionic neurones

The anatomical distribution of vagal preganglionic neurones projecting to the heart, and the location of the "cardioinhibitory centre", have been a source of controversy for over a hundred years. Whilst a number of workers working on a wide range of vertebrates claimed that cardiac vagal preganglionic neurones originated in the dorsal vagal nucleus:

(Molhant 1910; Miller & Bowman 1916; Getz & Sirnes 1949; Mohiuddin 1953; Mitchell & Warick 1955; Calaresu & Cottle 1965; Cohen et al. 1970; Cohen & Schnall 1970; Schwaber & Schneiderman 1975; Todo 1977; Todo et al. 1977; Smolen & Truex 1977; Schwaber & Cohen 1978; Kaufman et al. 1979)

an almost equal number claimed that they originated in the nucleus ambiguus:

(Kosaka 1909; Szentagothai 1952; Urabe & Tsubakawa 1960; Calaresu & Pearse 1965a,b; Gunn et al. 1968; Kerr 1969; Borison 1970; Lee et al. 1972; McAllen 1973; Thomas & Calaresu 1974; McAllen & Spyer 1976; Chen & Chai 1976; Dugin 1978).

There are also papers that claim that cardiac preganglionic neurones originate in both nuclei:

(Gunn 1968; Bennett et al. 1979; Nosaka et al. 1979a,b,1982; Sugimoto et al. 1979; Hopkins & Armour 1979; Garcia et al. 1978; Geis & Wurster 1980; Geis et al. 1981; Kalia & Mesulam 1980; Bennett et al. 1981; Ciriello & Calaresu 1980, 1982; Steusse 1982; Jordan et al. 1986; Ford et al. 1990; Cabot et al. 1991).

It is of interest to analyze how such confusion arose, and attempt to isolate the factors involved. One important factor appears to involve certain inconvenient anatomical features of this motor system. The motor fibres of the nucleus ambiguus run close by the fibres of the dorsal vagal nucleus. This is beautifully illustrated by Ramon Y Cajal (1909, fig 311). Therefore, the search for "a cardioinhibitory centre" using stimulation or

ablation (eg Lee et al. 1972) will yield equivocal results. In certain studies the central stimulation parameters are incompatible with known electrophysiological properties of C-fibres or the criterion for a positive cardiomotor site required the discrete stimulation to produce immediate slowing of the heart (Gunn et al. 1968). The somata of cardiac vagal preganglionic neurones are dispersed along a considerable area of brainstem in relationship to the very small numbers involved. This may account for the frequent failure of studies which electrically stimulate foci in the DVMN to elicit bradycardia (eg Laughton & Powley 1987). In one study DVMN stimulation failed to evoke a compound action potential in the cervical vagus ipsilaterally (Calaresu & Pearce 1965). The fact that vagal visceromotor function is preserved following destruction of the dorsal motor nucleus led Kerr (1969) to conclude that the DVMN is not the source of cardioinhibitory fibres. Kerr should have concluded that the DVMN is not the only site of cardioinhibitory fibres, if it is a site of cardioinhibitory fibres at all. Borison and Domjan (1970) made precisely the same illogical conclusion in their DVMN ablation studies.

The cardiac ganglia are located in the fat pads surrounding the heart, mainly on the posterior surface, and limited by the auriculoventricular sulcus (Woollard 1930). However horseradish peroxidase (HRP) is commonly injected into the cardiac ventricles a site where not only are there no ganglia (except in snakes) but there are very few postganglionic vagal fibres (except in avian species)(Woollard 1930).

The most reliable data concerning cardiac vagal preganglionic neurones comes from central recordings of these cells, antidromically identified on electrical stimulation of the intrathoracic vagal branches to the heart. The use of DLH (McAllen & Spyer 1976) in order to distinguish axons from somata, was a crucial step in overcoming the problems associated with axonal crowding of the vagal outflow. It also bypassed the deleterious effect that anaesthesia has on the neuronal excitability of vagal preganglionic cells. This technique established that cardiac preganglionic in the cat are located in the ventrolateral nucleus ambiguus and possess B-fibre axons. These neurones are powerfully excited by the arterial baroreceptors and when active exhibit pulse rhythm in their discharge that depends in large part on this input (McAllen & Spyer 1978). Intracellular recording of these neurones in vivo, shows that they greatly resemble post-inspiratory respiratory neurones (Gilbey et al. 1984). This provided elegant proof of one of Anrep's mechanisms behind sinus arrhythmia.

The electrophysiological investigation of the dorsal population of cardiac preganglionic neurones in the cat was undertaken by Ford et al. (1990). These workers provided convincing evidence that the dorsal vagal motor nucleus contains C-fibre cardiac preganglionic neurones. In contrast to the ventral group these neurones are apparently not influenced by the arterial baroreceptors (Ford et al. 1990) and their function is obscure This distribution of two populations of cardiac vagal preganglionic neurones in the cat has also been described in the rat (Nosaka et al. 1982).

It would appear from the great inconsistencies of the HRP retrograde tracing technique, that this method is incapable of establishing the ratio of neurones located in the two motor nuclei of the vagus. The amount of terminal axoplasm, the thickness of axon and size of the parent cell determine the density of retrograde labelling (Reynolds 1977). Increments in fixation of as little as one hour significantly decreases labelled cells (Rosene & Mesulam 1978). This effect of fixation is greatest when horseradish peroxidase (HRP) concentration is low, in small lightly labelled cells (ie the dorsal C-fibre population of cardiac vagal preganglionic neurones). In this regard the central and peripheral counts of C-fibre cardiac vagal preganglionic neurones in the cat differ extraordinarily. According to Sugimoto et al. (1979) only 10% of cardiac vagal preganglionic neurones in the medulla of the cat are C-fibres but according to Agostoni et al (1957), using a peripheral degeneration technique in the cat, 90% of cardiac vagal preganglionic neurones are C-fibres. Certain branches of the cardiac vagus appeared to have more preganglionic backlabelling in the DVMN than others e.g. the recurrent cardiac nerves of the dog (Hopkins & Armour 1979a,b,1982). Therefore the central counts obtained may depend on which branch is chosen to label.

Another striking factor that emerges from the literature is the widespread use of the misnomer "cardiac vagal motoneuron" when applied to a preganglionic neuron. There are no cardiac vagal motoneurones in the medulla oblongata, the vagus contains the axons of cardiac ultimate interneurones, cardiac vagal motoneurones are located more peripherally in the ganglia near the heart. Therefore the search for a "cardioinhibitory centre" or indeed cardioinhibitory neurones is suspect since preganglionic neurones do not represent the final common path.

"The terminal path may, to distinguish it from internuncial common paths, be called the

final common path."

Sherrington (1947)

The medullary cardiac preganglionic neurone is an internuncial common path. This distinction is not a question of pedantry. There are several important consequences resulting from this arrangement. If two preganglionic neurones have a postganglionic neurone in common, the final common path must be capable of responding with different rhythms which different preganglionic neurones impress upon it. Sherrington realised the importance of the concept, and has this to say about the final path:

"It must be to a certain degree aperiodic. If its discharge be a rhythmic process, as from many considerations it appears to be, the frequency of its own rhythm must be capable of being at least as high as that of the highest frequency of any of the afferent arcs that play upon it; and it must be able also to reproduce the characters of the slowest."

If reflex cardioinhibition involves a coordinated pattern of activity from more than one locus of the medulla descending upon the ganglia, then it would be a fundamental error to search in the medulla for cardioinhibitory neurones. In this regard the findings of McAllen and Spyer (1978) that a single cardiac vagal preganglionic neurone when stimulated by excitatory amino acids leads to cardiac slowing, may simply reflect a large neural unit size (the product of convergence and divergence) rather than the sole physiological cardioinhibitory pathway. With regard to terminology, "cardiac vagal motoneurones" will from here on be referred to as cardiac vagal preganglionic neurones if they are antecedent to the final common path or cardiac vagal postganglionic neurones if they are the final path.

1.8a Ontogeny

Windle (1933) bore witness to a most interesting phenomenon concerning the ontogenesis of vagal preganglionic neurones. The brain stem of embryos were analysed during early feline gestation, at a stage when vagal preganglionic neurones form a compact single nucleus immediately below the neural tube, with increasing gestational age the somata were then observed to migrate ventrolaterally. Early fetal development and maturation of rat vagal preganglionic neurones has recently been reexamined by Rinaman and Levitt

(1992). At the thirteenth day (E13) of gestation a few labelled neurones are seen midway between dorsal and ventral surfaces trailing neurites that reach medially towards the fourth ventricle, whilst others exhibit proximal dendritic arbors. At E14 a new group of labelled cells near the germinal zone of the fourth ventricle begin to form an indistinct DVMN, some dendrites enter the nucleus tractus solitarius.

There is evidence that the vagal bradycardia of the pulmonary chemoreflex matures earlier than the cardioinhibition of the baroreflex, and the coronary chemoreflex (Downing 1960; Gootman et al. 1981). Schleman et al. (1979) found a J-reflex functioning at adult levels in 2 day old piglets (sus scrofa). The J-reflex of kittens on the other hand has been reported to be only functional by the 10th postnatal day (Kalia 1976). The possibility that this could be due to the known maturational reactions to barbiturates or ontogenesis of $5HT_3$ receptors (as PDG was used) was not tested. There is a dearth of information on cardiac vagal control in the kitten.

1.8b Phylogeny

Certain design features of cardiac vagal control, with two populations of preganglionic neurones and distally located final common pathway embedded near the cardiac pacemaker, appear to be stable across vertebrates. The evolutionary physiology of the vagus may hold clues to the possible function of the dorsal group of cardiac vagal preganglionic neurones, the group of neurones which is does not migrate and about whose physiological role, so little is known. HRP studies have shown that all mammalian vertebrates display a ventrolateral scattering of vagal preganglionic neurones across the medulla (Withington-Wray et al. 1987). Whilst there are two main vagal motor nuclei, somata may be observed in an intermediate zone between the two nuclei. Cabot et al. (1991) have postulated that the brainstem organization of cardiac parasympathetic efferents is stable across avians and mammals, and that the dual cardiac representation within the mammalian medulla oblongata might embody a phylogenetically stable template applicable to vertebrates more generally. In the goldfish *Carassius auratus* (Morita & Finger 1987) and catfish *Ictalurus punctatus* (Kanwal & Caprio 1987), oro-branchial exteroceptive information is organized in a special "vagal lobe" (below the facial lobe) and interoceptive visceral branches do not enter the vagal lobe but project to the general visceral nucleus:the commissural nucleus of Cajal, which is more caudal. Cell

bodies of efferents within the interoceptive visceral branch are distributed throughout the caudal half of the vagal motor column. In the goldfish the efferent projection of ramus cardiacus forms the most medial group with no circumscribed nucleation, this is considered part of the general visceral column by Morita and Fink (1987). The "vagal lobe" is more lateral and exhibits branchiomeric segregation, this is the special visceral column, the probable NA homologue of amniotes. Ihmied & Taylor (1992) have suggested that the migration of vagal preganglionic neurones to a more ventrolateral position coincides with evolution\ development of air breathing in vertebrates. The axolotl, *Ambystoma mexicanum*, which is remarkably neotenous, has an increased number of ventrolaterally placed vagal preganglionic neurones once fully metamorphosed from an aquatic gill breather to a terrestrial lung breather (Ihmied & Taylor 1992). The quantitative analysis of HRP labelled somata within vertebrate species or across vertebrate classes poses formidable technical difficulties. The true meaning behind this vagal preganglionic topography however, is on the balance of evidence, quite uncertain. A recent study on a very ancient fish: the hagfish, *Eptatretus burgeri* (Matsuda et al. 1991) reveals a ventrally placed vagal branchiomotor column, whose neurones have hairpin axon loops (horizontal axis) projecting dorsally, before exiting ventrolaterally. Thus the ontogenetic migration (Windle 1933) may not be a recapitulation of a phylogenetic sequence but rather, represents a basic blueprint for development that vertebrates share. This is essentially a reformulation of a very old controversy in the zoological sciences between Von Baer and Haeckel. According to Von Baer recapitulation is impossible; young embryos are undifferentiated general forms, not previous adult ancestors (Gould 1977). The "undifferentiated general form" may resemble that found in *Amphioxus*, where the ventral roots innervate only the myotomes and all the visceromotor fibres leave by the dorsal roots (Burnstock 1969). There is difficulty in constructing an effective taxonomy for the vagal motor outflow because both branchiomeric myotomes and viscera are supplied by the tenth cranial nerve. There is also difficulty on the afferent side because the nodose ganglion is of placodal origin whereas the jugular ganglion is of neural crest origin.

The heart of the hagfish is aneural, but a closely related cyclostome the lamprey (*Lampetra fluviatilis*) exhibits the first rudimentary vagal innervation, Taylor (1993).

Little is known about this species cardiac vagal control, which is regrettable since this vertebrate marks the very beginning of the coupling of the rhombencephalon to the cardiac pacemaker through the vagus. The elasmobranch *Scyliorhinus canicula*, or dogfish has an interesting pattern of discharge to its cardiac vagus. Two populations of cardiac vagal preganglionic neurones have been reported in this species (Barrett & Taylor 1985a,b). One group fires with a powerful respiratory rhythm and the other fires tonically, with a non-respiratory related rhythm. These populations have different morphologies, different locations in the medulla and different reflex involvements (Barrett & Taylor 1985b). Lazar et al. (1992) consider the medial respiratory modulated branchiomotor group of neurones in fish (the weakly electric elephant nose fish, *Gnathonemus petersis* was the fish actually studied) to be the homologue of the nucleus ambiguus, because NA motoneurones show dendritic bundling and extensive dendritic arborization in the cranial and ventrolateral direction as do the neurones of the medial group of fish. In lower mammals the NA extends in front of the level of the cephalic pole of the dorsal efferent nucleus. Sensory visceral afferents terminate among dendrites of motoneurones of caudal nucleus of fish and DVMN of mammals. Efferent axons form a hairpin loop or arch (horizontal or frontal axis) in the medial motor cell group of fish and NA of birds and mammals. The axonal loop is really a spiral when considered in three dimensions and has a chiral opposite on the other side of the medulla. Although the cardiac ganglion of the dogfish has not been studied, it has been postulated that the respiratory related discharge evolved to ensure synchrony between the sinoatrial oscillator and the respiratory oscillator because water flows through gill lamella as a kind of counter current to the direction of flow of blood from the afferent branchial vessels. Maximal O_2 flux therefore requires critical timing between two potentially independent pumps. A gill chemoreflex has been described by Satchell (1977) to injected PDG in the elasmobranch *Squalus acanthias*. This resembles the mammalian pulmonary chemoreflex, and involves bradycardia and respiratory inhibition. However it is unknown how the two populations of cardiac vagal preganglionic neurones behave during this reflex. Mott (1951) has recorded from afferents emanating from the branchial circulation of the common eel *Anguila anguila*, which were sensitive to raised intravascular pressure. Although Mott intended to study possible baroreceptor reflexes of the branchial vessels and construed all her results in this light, it is possible that this interpretation is incorrect.

First,the latency to the cardioinhibitory reflex upon raising afferent branchial arterial pressure was not immediate, but in all the records presented, actually takes many heart beats. Second, afferents with baroreceptor related neural traffic were not found. The question arises: was Mott actually studying gill afferents sensitive to oedema and capable of reflexly evoking bradycardia?

The gill chemoreflex is a primitive and highly conserved response which appeared (on the basis of Satchell's work and the fossil record) around the Ordovician about four hundred million years ago (Hardisty 1979; Dejours 1975). Early palaeozoic aquatic creatures lived in hypoxic conditions. At this time life was switching from low-efficiency fermentation to aerobic energy production when photosynthetic organisms evolved oxygen. The pressure of oxygen is estimated to have been 7-14 torr.(Symposium on the evolution of the earth's atmosphere, 1965). Efficient gas exchangers must have been important in the evolutionary struggle for survival. At this period vertebrates evolve a cardiac vagal innervation, and the hindbrain shows evidence of simple reflex wiring involving visceral input/visceral output relationships.

In summary, analysis of the pulmonary chemoreflex should shed light on the hindbrain cardiorespiratory network, and perhaps indicate the physiological function of those cardiac vagal preganglionic neurones that remain fixed in the dorsal medulla of most vertebrates.

2. Central recording of respiratory cells and vagal preganglionic neurones in the anaesthetized cat and rat.

2.1 Introduction

The pulmonary chemoreflex is a primitive stereotyped response which occurs when phenylbiguanide (PBG) or capsaicin is injected into the pulmonary circulation. The reflex is highly conserved and has been elicited in every animal tested from elasmobranchs (Satchell 1977) to homo sapiens (Jain et al. 1972). This facet of the reflex make it an ideal archetype for the analysis of hindbrain control of breathing and the heart. The paucity of studies on central recording of respiratory cells and vagal motoneurones during the pulmonary chemoreflex provides added impetus to study both dorsal and ventral medullary respiratory groups in a variety of species. Also the hypothesis (Daly 1991) that cardiac vagal preganglionic neurones other than the classical B-fibre preganglionic neurones in the cat may be involved in the pulmonary chemoreflex is specifically addressed. This first chapter is concerned with the response of the anaesthetized cat and rat to the right atrial injection of phenylbiguanide.

2.1 Methods

2.1a General

Experiments were performed on adult cats of either sex (2-4.3kg) and on male Sprague Dawley rats (250-570g). The surgery was performed on a table incorporating a stereotactic holder and supported with pneumatic cushions. The following physiological variables were monitored in all animals: temperature (maintained at 37°C through a rectal probe and Havard homoiothermic apparatus),end tidal CO_2 ($ETCO_2$)(ADC fast response analyzer),tracheal pressure (Validyne transducer), arterial blood pressure, arterial blood gases (Corning 158 pH/blood gas analyzer), right phrenic nerve activity (Neurolog system), ECG lead II (Neurolog system). Urinary output was monitored in the cats.

2.1b Anaesthesia

In the cats anaesthesia was induced and maintained with i.p. alpha-chloralose (Aldrich)(50mg/kg) and urethane (Sigma) (0.5g/kg) or pentobarbitone sodium (Sagatal) (60mg/kg) i.p. The rats were anaesthetized with pentobarbitone sodium (Sagatal; 60 mg/kg) i.p. initially and then supplemented, when necessary, by Sagatal (6mg) via a cannula in the femoral vein.

2.1c Operative procedures

In all experiments a femoral vein was cannulated with polyethylene tubing for administration of drugs and anaesthetic agents, and the trachea was intubated with polythene tubing low in the neck to maintain a clear airway two sidearms were fitted to the tracheostomy tube to monitor tracheal pressure and carbon dioxide. The right femoral artery was cannulated. The right external jugular vein was cannulated and the catheter advanced into the right atrium. This was determined by pressure measurement and postmortem confirmation.

2.1d Preparation of the cats

A paediatric swan ganz 4fr was introduced into the femoral artery and advanced 20 cm. The bladder was catheterized transurethrally or through a suprapubic cystostomy to monitor urinary output. After the commencement of artificial ventilation, with air enriched with O_2 and positive end expiratory pressure (1cm H_2O), a right thoracotomy was performed. The ribs were retracted and a right upper lobectomy performed to expose the azygos vein. The azygos was tied and divided to gain access to the cardiac and pulmonary branches of the right thoracic vagus. Small insulated bipolar stimulating electrodes were placed upon the intact cardiac and pulmonary branches, and the electrodes were secured to the surrounding tissues by cyanoacrylate glue. In a few cats the intact right recurrent laryngeal nerve was hooked up to an electrode assembly which was secured to the tracheostomy tube. The right phrenic nerve was dissected from a dorsolateral approach with the animal in the stereotactic headframe, then cut peripherally and desheathed. The vertebral spine of C7 and the spine of L2 or L3 were utilized to elevate and stabilize the animal, thereby minimizing pulsation artefact of ventilation and

heartbeat. After removing the overlying muscle, the occipital bone was cut away for about 5mm on each side of the midline, from the atlanto-occipital membrane to just rostral to the occipital ridge. The exposed edges of bone were plugged with bone wax and then the dura covering the cerebellum was cut and reflected laterally. To expose the dorsal surface of the brainstem the cerebellum was displaced with a small retractor. The animal was then paralysed with gallamine trietiodide (Flaxedil, M&B Ltd.; 5mg/kg) or vecuronium (Norcuron;200μg/kg). The level of anaesthesia under neuromuscular block was monitored by noting the regularity of the phrenic neurogram and the lack of cardiovascular change to paw pinch.

2.1e Preparation of the rats

Preparation of the rat was essentially similar to the cat, except the whole cervical vagus was utilized for stimulation, there was no thoracotomy and no Swan-Ganz catheter introduced into the femoral artery. In the rats in which there was synchronous ventilation of the upper and lower airways, the technique of Jones et al. (1993) was used. This involved passing two cannulae into the trachea, one faced cranially below the cricoid cartilage, and the other faced caudally towards the lower airways. A solenoid valve controlled the flow of air from a positive and negative pressure source. At times specified by the digital output ports of a CED 1401 interface, negative pressure was applied to the upper airways and positive pressure to the lower airways, thus simulating inspiration. To simulate expiration the lungs were allowed to return to a functional residual capacity set by a positive end expiratory pressure, and positive pressure was simultaneously applied to the upper airways. The applied pressures were controlled by passing the air through underwater seals. The length of inspiration and expiration could be controlled by CED spike2 software, and various experimental manoeuvres assigned to certain keyboard characters.

2.1f Electrode details

i) Single barrel electrodes were pulled from Clarke borosilicate glass microelectrodes (GC150F-10) to a tip diameter of 1-2μm. These were filled with pontamine sky blue (20mg/ml in 0.5M sodium acetate) for recording and staining. The extracellular recording preamplifier used was a Dagan (gain 1000) and further amplification and filtering was

accomplished through a Tektronix 5103N oscilloscope (second gain 5). Bandpass filtering for the neural signals was 3-1000 Hz.

ii) 5 barrel electrodes were constructed from Clarke glass microelectrodes cleaned in concentrated nitric acid and acetone prior to use. Small brass collars bound the five glass segments together. The electrode was pulled on a Narishige electrode puller and bumped to a final tip diameter of 3-5μm. These electrodes were filled with saline (4.0M), DLH (0.2M pH8.5), and pontamine sky blue. Microelectrophoresis and automatic current balancing was performed with a Neurophore (Medical System Corp.). The balancing current passed through the pontamine electrode and a retaining current of +10nA passed through the DLH barrel. Recording was through the saline barrel (details are as single electrodes).

2.1g Experimental arrangement and procedure.

The experimental protocol was essentially the same for all experiments. The peripheral nerves were stimulated at 1Hz, 1ms duration and at a voltage supramaximal to that which caused either direct or reflex bradycardia. A master programmable pulse generator (master 8, A.M.P.I.) was linked to four isolated stimulators (Digitimer). The brainstem was explored for antidromic and orthodromic activity. The typical protocol for analysis of a response to vagal stimulation involved determining which branch(es) elicited the response, the threshold, refractory period analysed with double pulse stimulation, and presence of collision. If the neurone fired spontaneously a free run was taped for subsequent PSTH analysis of possible relationship to ECG, phrenic nerve activity or tracheal pressure. Various reflex manoeuvres were tested: pulmonary chemoreflex, baroreflex (inflation of aortic balloon), HDA stimulation, raising the end expiratory pressure, pulmonary deflation and pulmonary inflation to various transpulmonary pressures (the latter was accomplished by having a 50 ml syringe attached to the outlet pipe of the ventilator)(since there was a thoracotomy the tracheal pressure equals the transpulmonary pressure). If the neurone was silent DLH was applied to evoke activity. It proved to be impossible to apply all these tests to a single neurone.

2.1h Recording and analysis

All data and commentary were captured on video tape through an Instrutech digital data

recorder (eight analogue channels and eight timing channels). In eight channel mode the frequency response is DC-4.5kHZ. The inputs were sent to a 1401 interface (CED) which was linked to a Viglen 486 computer system. On line data display utilized CED chart program and CED Sigavg program. Off line analysis was accomplished with CED spike2 which is a flexible software system that permits some degree of custom analysis programming from a small library of commands.

2.1i Histological localization of sites of recording

50 μA-minutes of pontamine sky blue iontophoresis was sufficient to deposit the dye. After the experiment the animals medullae were stored in 10% formal saline for fixation purposes (>3 days). Serial frozen sections (80μm thick) were cut and stained with neutral red. The rat medullary sections were analysed with reference to the atlas of Paxinos & Watson (1986) and the cat recording sites displayed on standard sections of medulla taken from Berman (1968).

2.2 Results

2.2a The pulmonary chemoreflex

The drug phenylbiguanide (PBG) excites pulmonary C-fibres at short latency when injected into the right atrium. The archetypal triad of apnoea bradycardia and hypotension is readily observed (see fig 2.1). Whereas the cardioinhibition of the pulmonary chemoreflex lacks respiratory modulation (fig 2.1),the bradycardia of the baroreflex is respiratory phase dependent (fig 2.2).

2.2b Respiratory cells

Central recording from the medulla was performed to ascertain whether differences in the activity patterns of the various classes of respiratory cell could account for the lack of respiratory modulation of the pulmonary chemoreflex. In other words, are post-inspiratory neurones homogeneous in their response patterns? are inspiratory cells affected to the same degree? Since post-inspiratory laryngeal motoneurones greatly resemble cardiac vagal preganglionic neurones (Richter & Spyer 1990), special attention

was given to their response to PBG.

165 respiratory cells were recorded in total (n=97 in rat, n=68 in cat). The behaviour of the respiratory cells were identical in the two species and the results will be considered together. Of the total, 74 cells were unclassified with respect to the phrenic nerve and will not be considered further. Of the remaining 91 neurones, 37 were successfully tested with phenylbiguanide (5-50 μg/kg). Only stable recordings and responses within the pulmonary circulation time of the species were considered for analysis. In the two species PBG evoked an inhibition of inspiratory neurones (n=22) located in both the dorsal and ventral respiratory groups (fig 2.3). Also PBG evoked an inhibition of expiratory (n=9) neurones (stage II expiration) located in both the dorsal and ventral respiratory groups (fig 2.4) In contrast, post-inspiratory cells (n=6) were powerfully excited by PBG.

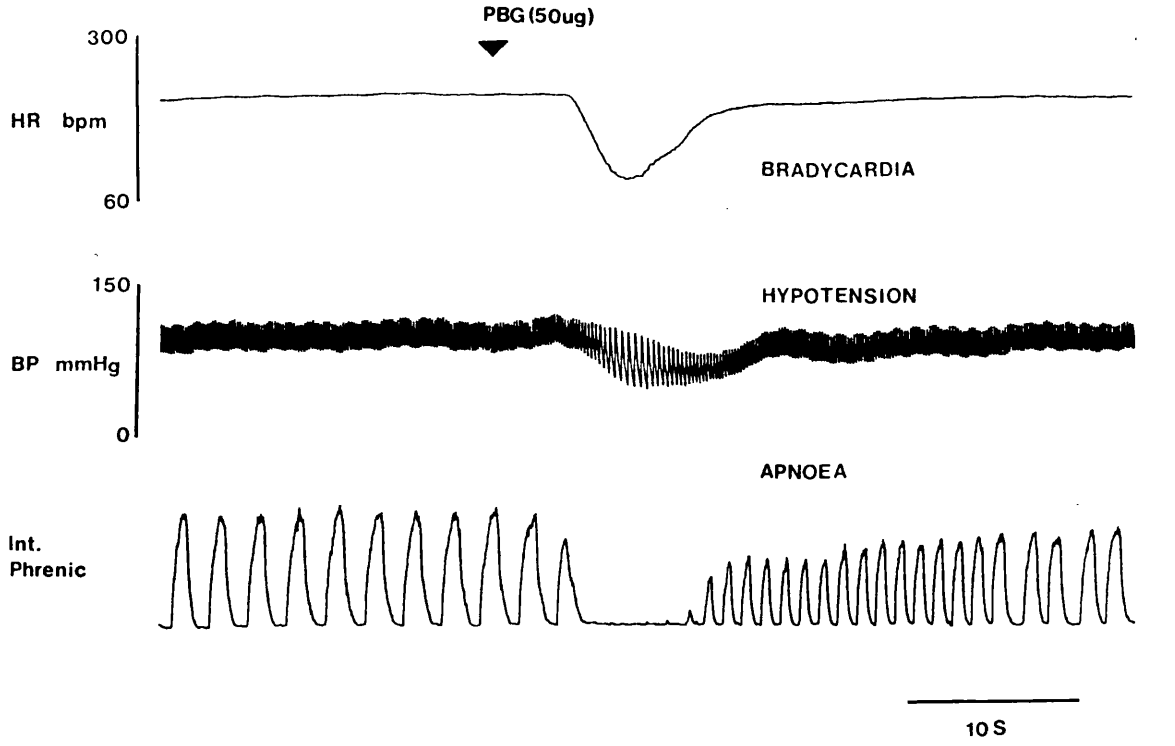
In the cat 8 laryngeal motoneurones were identified by antidromically stimulating the right recurrent laryngeal nerve. 2/2 inspiratory laryngeal motoneurones were inhibited and 3/3 post-inspiratory laryngeal motoneurones were excited by PBG (fig 2.5). The remaining 3 were unclassified and untested. Also in the rat 4 laryngeal motoneurones were identified by antidromically stimulating the cervical vagus below the pharyngeal branch. 2/2 post-inspiratory neurones were excited by PBG (fig 2.6). The remaining 2 were unclassified with respect to the phrenic nerve and untested.

The results show that not only is the response of the various classes of respiratory neurones homogeneous, but that there also exist striking inter-species similarities. The results do not support the hypothesis that differential responses of the various classes of respiratory neurones account for the lack of respiratory modulation of the pulmonary chemoreflex bradycardia. This confirms the findings of Daly et al. (1992).

FIGURE 2.1

Top figure illustrates the typical pulmonary chemoreflex of the anaesthetized cat. Note the expiratory apnoea followed by acceleration of breathing. This is not due to arterial blood gas changes because the animal is oxygenated and artificially respired. The reflex develops rapidly with a latency less than five seconds. This shows that the receptors involved are supplied by the pulmonary circulation. After 5 seconds the drug gains access to the coronary and bronchial circulations. Careful attention was paid to the reflex latency in all experiments with PBG.

Bottom figure illustrates the response of another cat to the same dose of PBG. The effect upon respiration is minimal although the cardiovascular response is powerful. The $ETCO_2$ is 4.0%. Only a minority of cats display this pattern (about 10%, n=40 animals) while possessing a normal arterial blood gas profile. However most cats will display this pattern if the apnoea of the pulmonary chemoreflex is prevented by stimulating the chemoreceptors, achieved by raising the $ETCO_2$. This same pattern may be obtained after atenolol (1mg/kg), indicating that cardiac vagal preganglionic neurones are involved. Observe the third phrenic burst and the complete lack of respiratory modulation of the cardioinhibition during inspiration. The cardiac vagal preganglionic neurones involved in this reflex, appeared to be unaffected by central inspiratory activity, and this was the case across a wide range of respiratory drive. This confirms the findings of Daly (1991).


PBG(50ug)
300
HR bpm
60
BRADYCARDIA
150
HYPOTENSION
BP mmHg
0
APNOEA
Int.
Phrenic
10 S

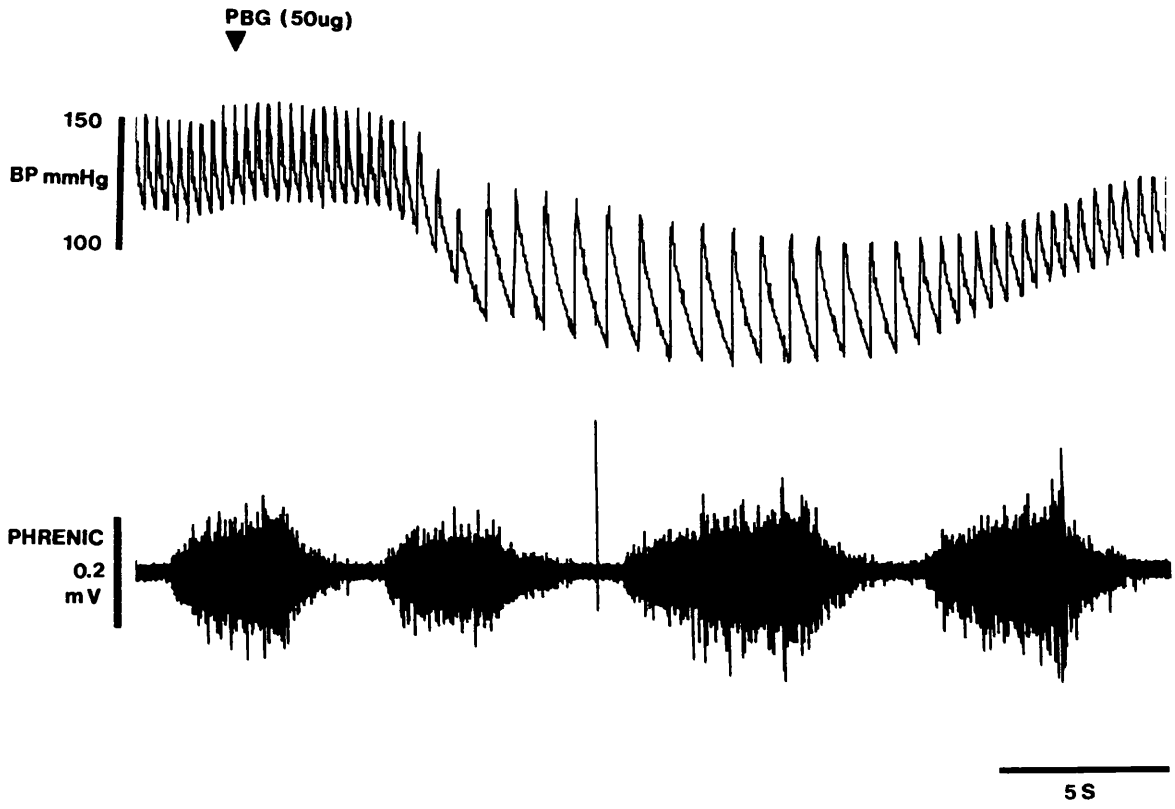
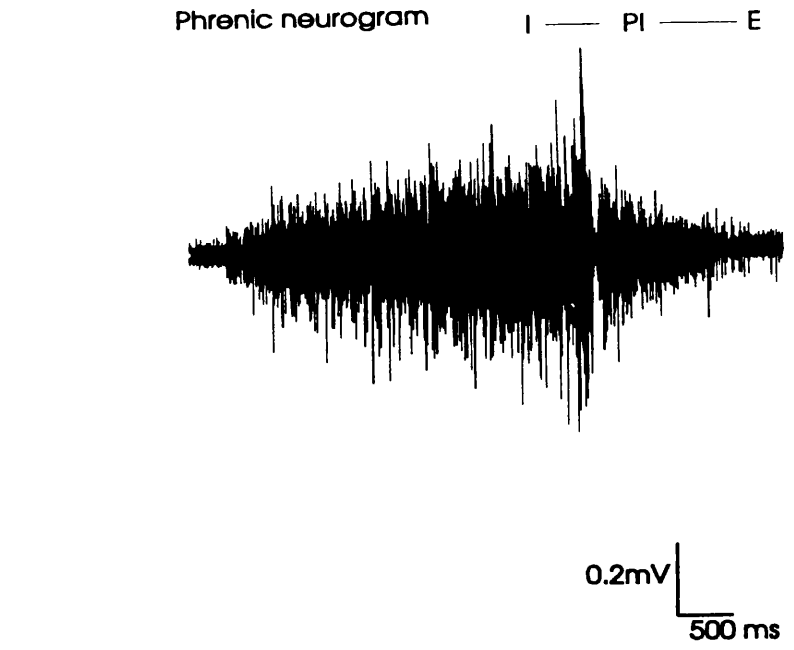

PBG (50ug)
150
BP mmHg
100
PHRENIC
0.2
mV
5 S

FIGURE 2.2

Top figure illustrates multi-unit phrenic recording delineating the three phases of respiration in the anaesthetized paralysed ventilated cat. I=inspiration, PI=post-inspiration, E= stage II expiration. There is late inspiratory drive in this phrenic burst, followed by 50ms of electrical silence,(the late inspiratory inhibition) and decrementing post-inspiratory activity.

Bottom figure illustrates the gating of brief baroreceptor stimuli applied at various stages of the respiratory cycle in the cat. Note powerful cardiac slowing is present in late expiration and early expiration, these are the preferred firing phases of the cycle for post-inspiratory neurones. In contrast to the pulmonary chemoreflex, the cardiac vagal preganglionic neurones mediating the baroreflex are markedly respiratory modulated. This confirms the findings of Neil & Palmer (1972) and Potter (1981).


Phrenic neurogram
I
PI
E
0.2mV
500 ms

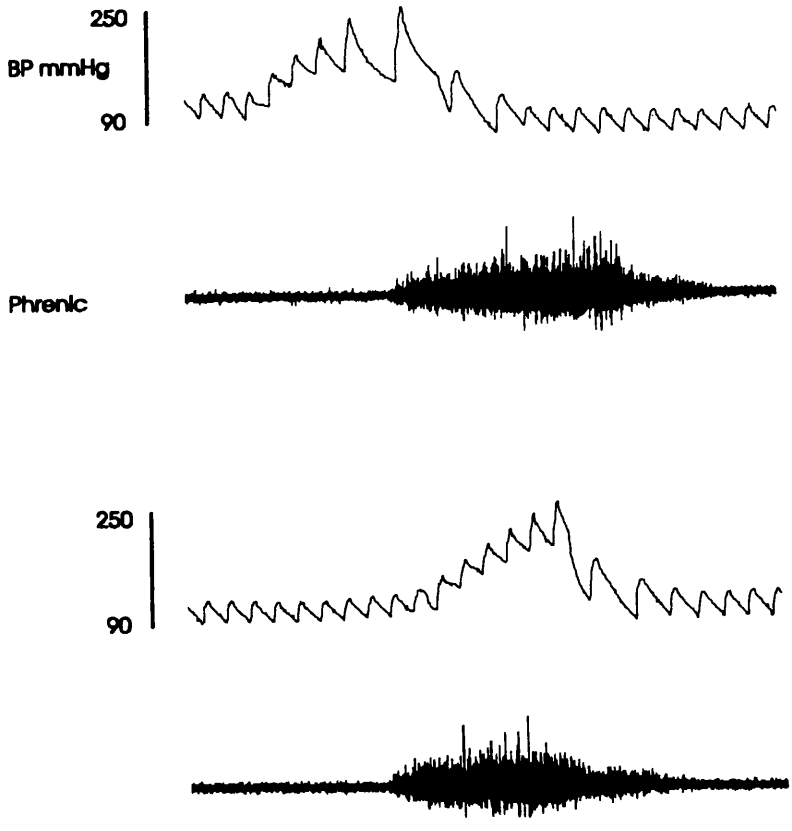

250
BP mmHg
90
Phrenic
250
90
1 sec

FIGURE 2.3

Four examples of inspiratory cell inhibition during the pulmonary chemoreflex in the cat. The traces from above downwards are: raw phrenic nerve activity, discriminated spikes of the respiratory cell and blood pressure. The arrows indicate the time of the injection of 50μg of PBG into the right atrium.

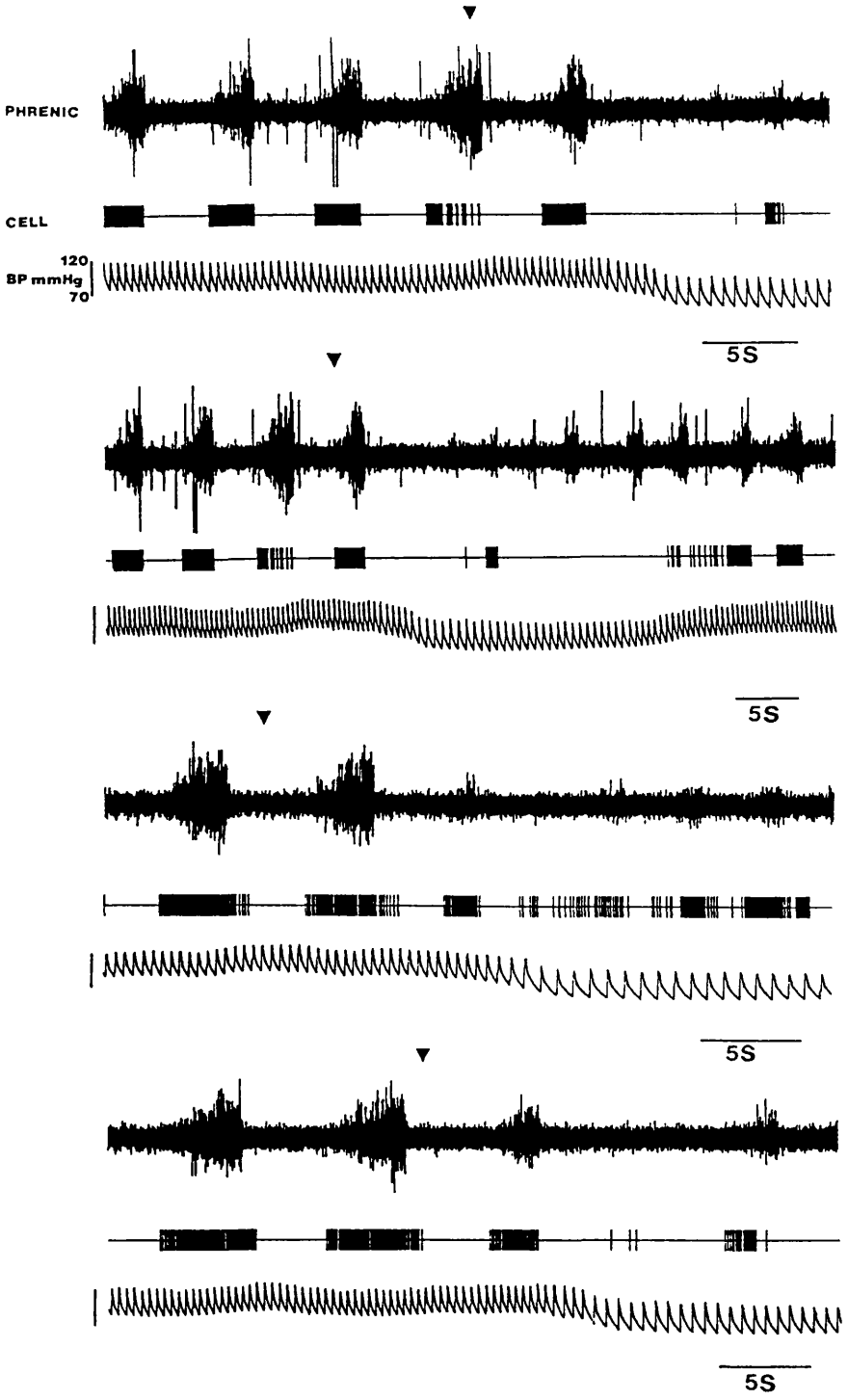

PHRENIC
CELL
120
BP mmHg
70
5S
5S
5S
5S

FIGURE 2.4

Upper panel: example of inhibition of a stage II expiratory cell (classified with respect to the phrenic nerve) during the pulmonary chemoreflex in the rat. This animal has synchronous ventilation of upper and lower airways. (UAP= upper airway pressure; LAP= lower airway pressure).

Lower panel: Rapid ventilation of the rat entrains the expiratory cell. This highlights the fact that the pulmonary chemoreflex in the spontaneously breathing animal must be an amalgam of reflex interactions. The rapid shallow breathing obtained during pulmonary C-fibre stimulation in the ventilated animal corresponds to a true acceleration of the "respiratory clock", in the spontaneously breathing animal interesting closed loop feedback cycles must occur between stretch receptors and pulmonary C-fibres.

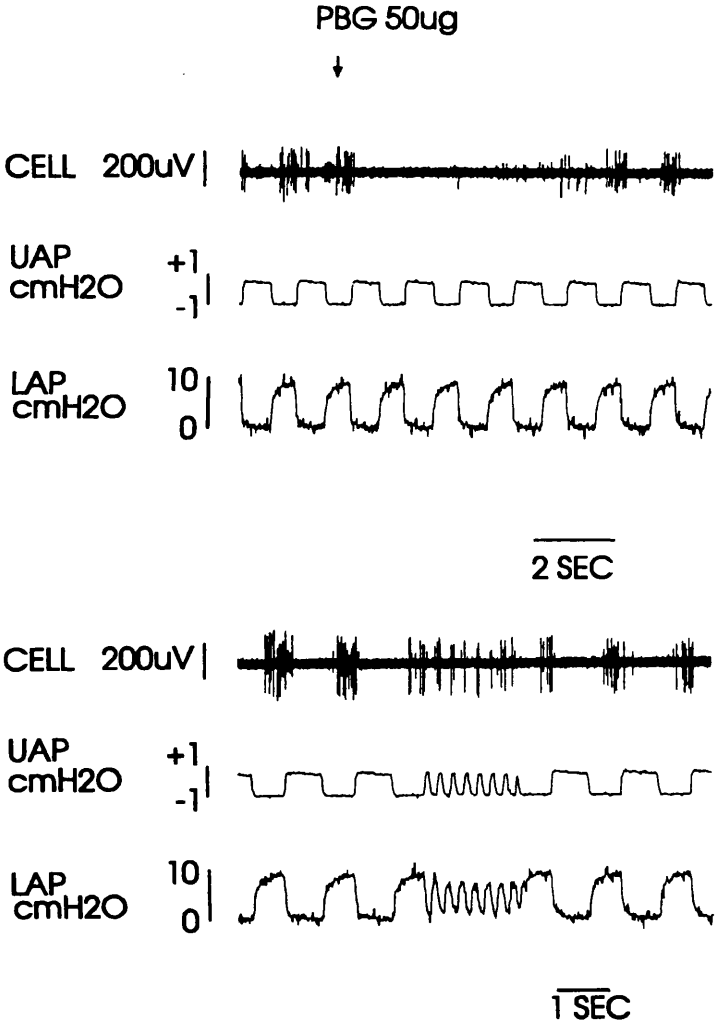

PBG 50ug
CELL 200uV
UAP
cmH2O
+1
-1
LAP
cmH2O
10
0
2 SEC
CELL 200uV
UAP
cmH2O
+1
-1
LAP
cmH2O
10
0
1 SEC

FIGURE 2.5

Multibarrel electrode recording of post-inspiratory laryngeal motoneurone of the cat (background DLH 20nA). Upper panel shows excitatory response to PBG at short latency. Of particular note is the continued rhythmical output despite "phrenic apnoea".

Lower panel,right hand side: shows antidromic response (labelled with star) to RLN stimulation and in the middle: cancellation with spontaneous action potential. (The tall spike is the stimulus artefact).

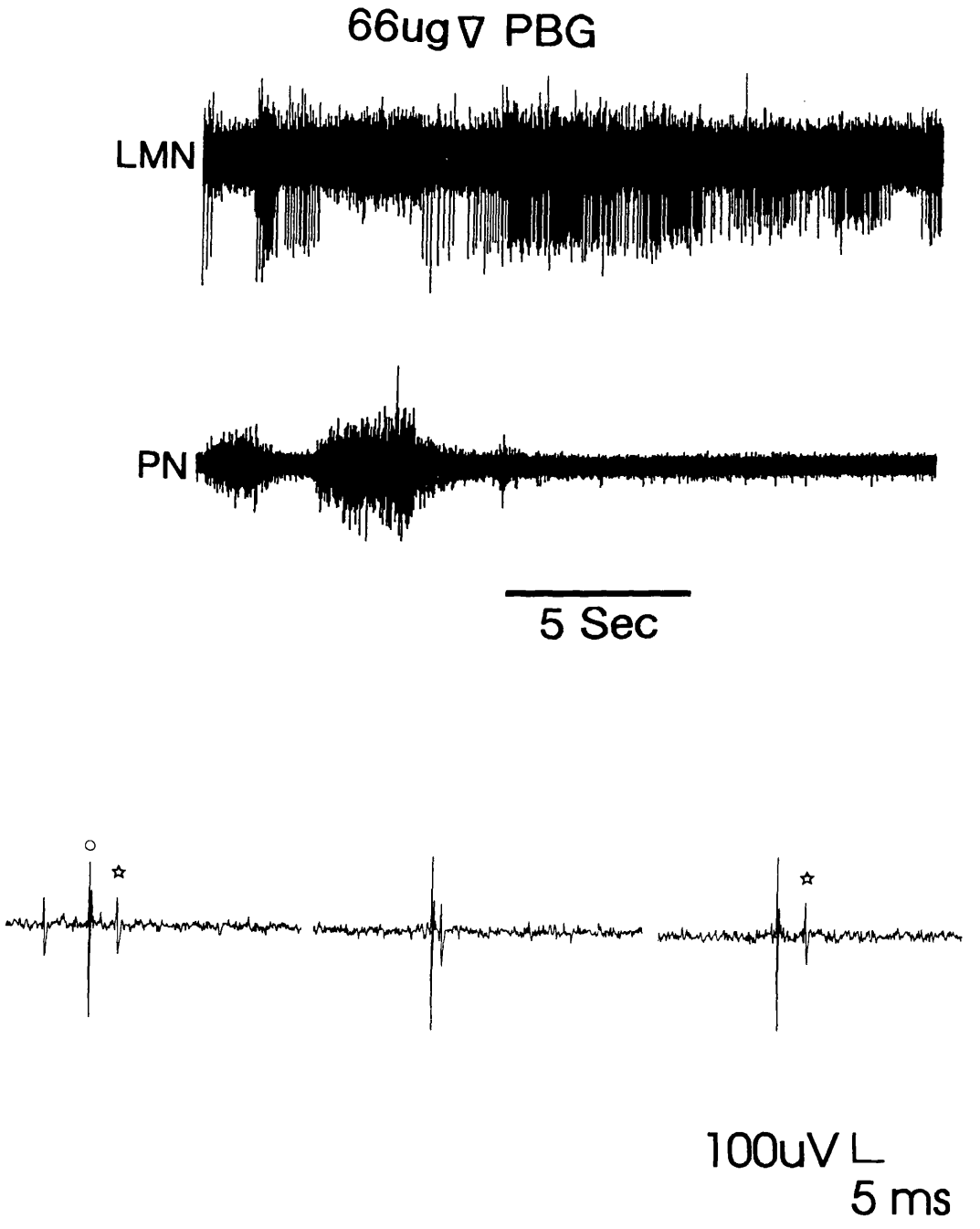
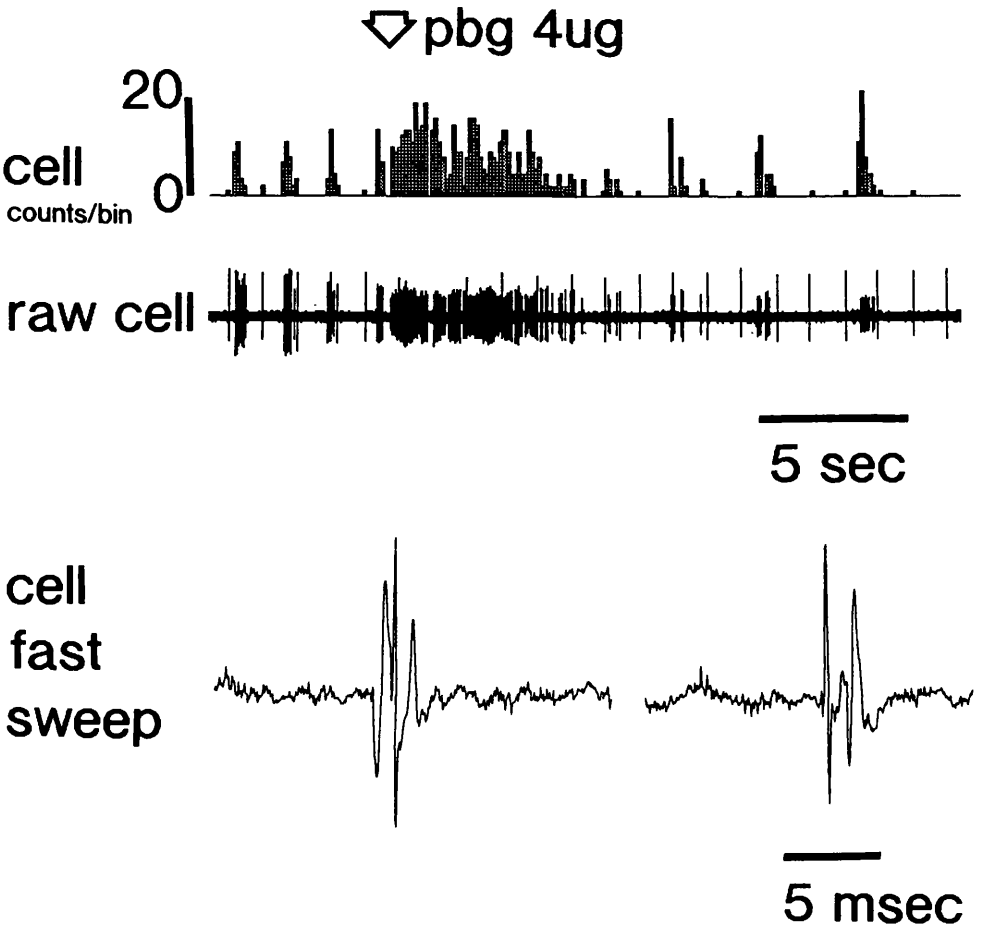

66ug ▽ PBG
LMN
PN
5 Sec
100uV
5 ms

FIGURE 2.6

Post-inspiratory laryngeal motoneurone in the rat (classified with respect to the phrenic nerve, not shown).

Upper panel: The top trace is a ratemeter record of the discriminated spikes of the cell (100ms bins), and the raw record of the neurone. Note the shorter latency of the response compared to the cat and the increase in activity of the cell during a period of phrenic apnoea. The RLN stimulus (1 Hz) was left running throughout the experiment.

Lower panel: The antidromic response to vagal stimulation is illustrated on the lower right panel. The lower left hand side illustrates cancellation with a spontaneous spike that precedes the stimulus artefact (the tall potential).


pbg 4ug
20
cell
counts/bin
0
raw cell
5 sec
cell
fast
sweep
5 msec

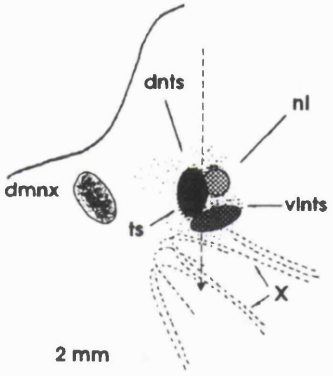
2.2c NTS neurones

The projection of pulmonary C-fibres was analysed to ascertain whether the organization of the NTS could help explain why the bradycardia of the pulmonary chemoreflex is unaffected by lung inflation or central inspiratory activity.

54 orthodromic responses were recorded in response to stimulation of the cardiac and pulmonary vagal branches of the anaesthetized cat. 4/54 were considered to be afferent axonal recordings. This decision was based on an analysis of spike shape,firing pattern, absence of collision, frequency following, latency and histological location. 50 neurones were deemed to be synaptically driven on the basis of latency jitter, spike shape, the output spike number being greater than the input, and the absence of collision.

FIGURE 2.7

Result of a single penetration into the medulla oblongata of the cat. The recordings obtained are laid out sequentially from top (dorsal) to bottom (ventral). One pulse is delivered to the cardiac (C) or pulmonary (P) branch as indicated by the circle. The interpretation of these results is as follows: the first cell receives only cardiac B-fibre afferent information, the second cell receives pulmonary A-fibre and pulmonary B-fibre information, the third multiunit recording is the tractus solitarius and contains activity resembling the A,B and C waves of a vagal compound action potential. Finally the fourth recording is from a respiratory neurone receiving excitatory information from myelinated pulmonary afferents. This could correspond to the vlNTS. Whilst the schema above is speculative, it is quite clear that these cells receiving myelinated information do not receive unmyelinated information. The vertical dashed line in the coronal section of medulla on the left, indicates the possible electrode track.


dnts
nl
dmnx
vlnts
ts
X
2 mm

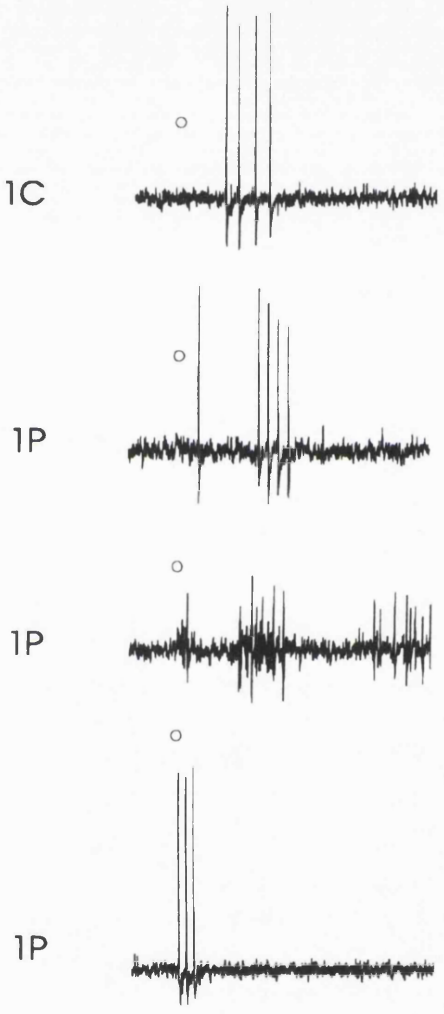
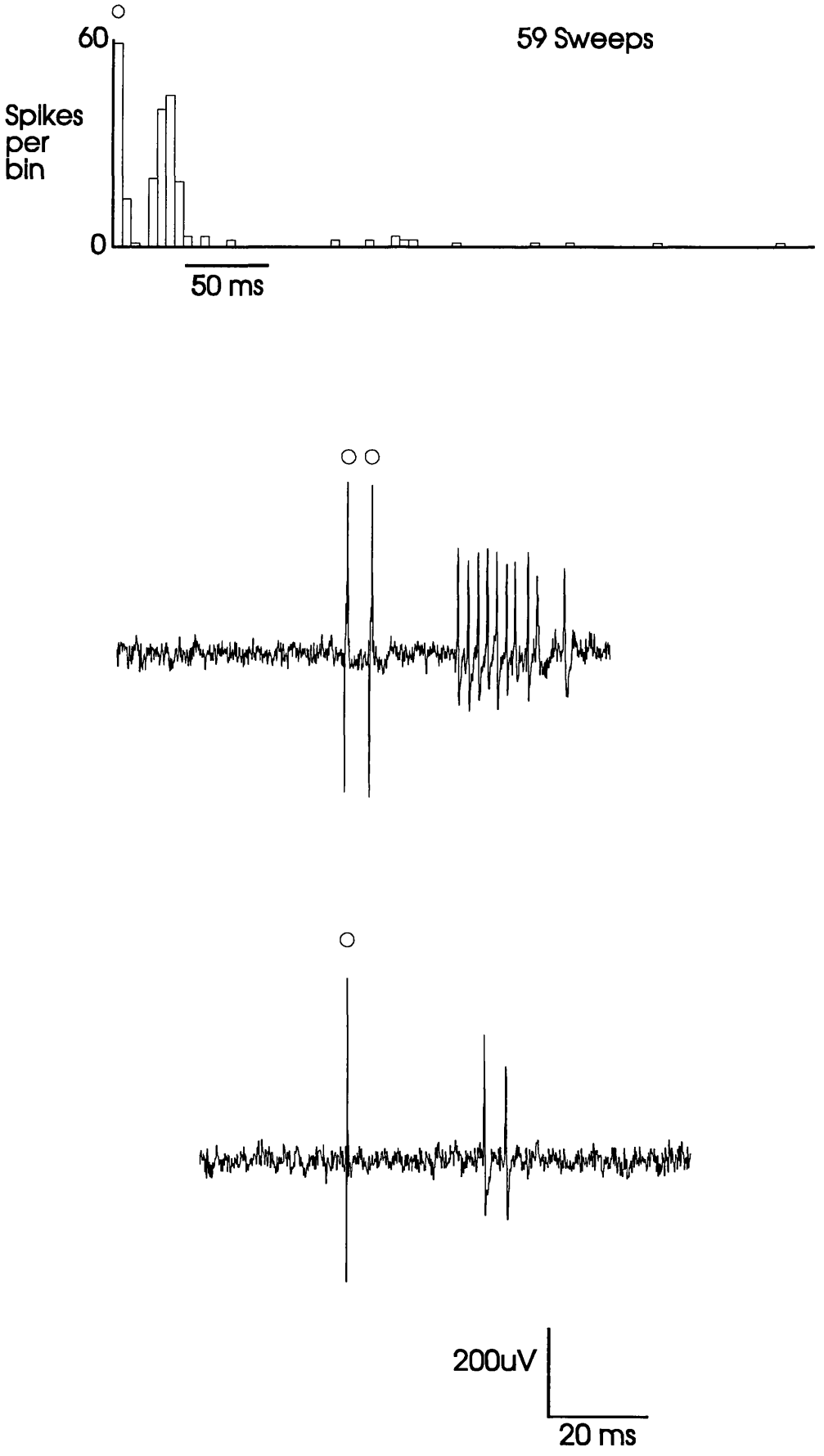

1C
1P
1P
1P
200uV
50 ms

FIGURE 2.8

Example of a neurone receiving short latency cardiac input only. All these records refer to the same cell. Upper panel is a post stimulus time histogram (5 ms bins), stimulus artefact at time zero, indicated by circle. Middle panel shows raw record of the neurones response to a twin pulse stimulus to the cardiac branch. Lower panel shows the response to a single stimulus delivered to the cardiac branch.


60
59 Sweeps
Spikes
per
bin
0
50 ms
200uV
20 ms

The categorization of these synaptic events fell naturally into three divisions. Fast synaptic input 5-15ms (A), medium 20-75ms (B), and long latency 100-250ms(C). In the following cells tabulated there was no convergence of myelinated and unmyelinated information onto a common cell. Overall the commonest neurone encountered possessing afferent synaptic input, was one in which fast myelinated input converged from heart and lungs.

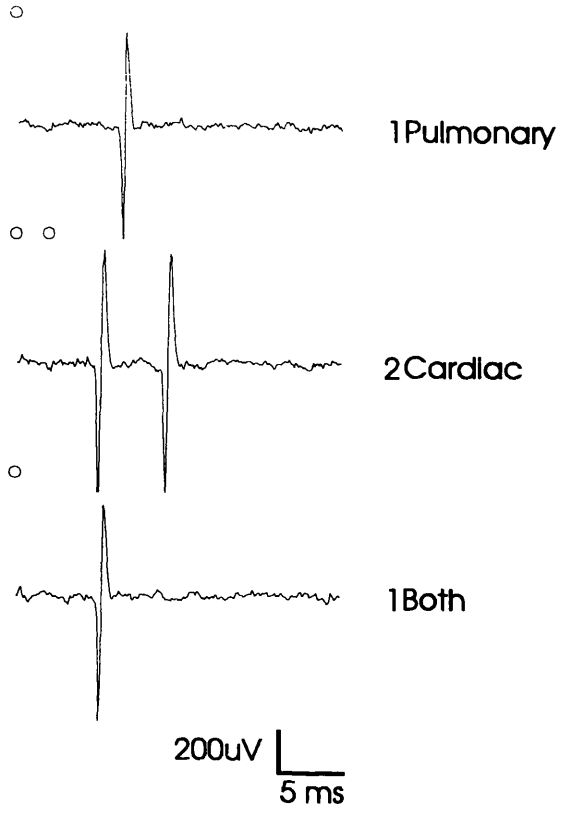
	A	B	C
Cardiac alone	8-15 ms n=7	20-75 ms n=9	100-300 ms n=7
Pulmonary alone	5-10 ms n=4	20 ms n=1	180-250 ms n=5
Cardiac and Pulmonary myelinated	8-15 ms n=8	20-25 ms n=2	
Cardiac and Pulmonary unmyelinated			100-170 ms n=1

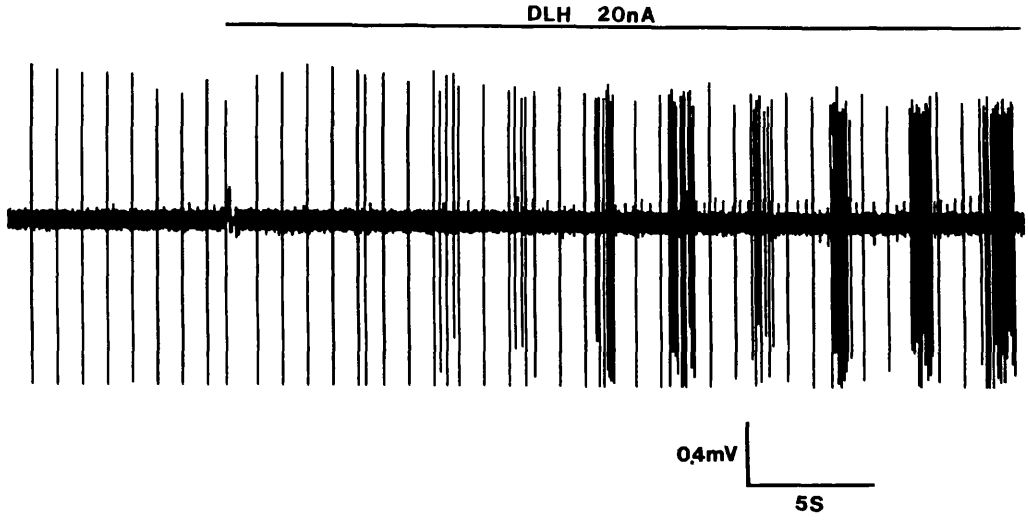
TABLE 2.1

FIGURE 2.9

Multibarrel recording of a neurone receiving myelinated input from heart and lungs. Upper panel shows raw record of orthodromic responses to pulmonary and cardiac stimulation illustrated by circles. Although both branches appear to drive the neurone when stimulated separately, when they are stimulated together only one spike is triggered. Lower panel shows effect of iontophoresing DLH onto the neurone, whilst the stimulus to the pulmonary branch is on (1Hz). When the excitability of the neurone was raised it fired with a tracheal pressure related rhythm (and not a phrenic related rhythm). This demonstrated that the neurone was a pump cell. Even when the excitability of the neurone was raised there was no evidence of unmyelinated input.

This was one of 5 pump cells recorded which all had myelinated pulmonary input (5-12 ms) and no evidence of pulmonary C-fibre input. These cells were not included in table 2.1.


1Pulmonary
2Cardiac
1Both
200uV
5 ms


DLH 20nA
0.4mV
5S

The results of the orthodromic recordings confirm the findings of Bennett et al. (1985), with regard to the specific projection pattern of unmyelinated input onto NTS cells which lack convergence of myelinated input of vagal afferents from both heart and lungs (Table 2.1). This provides support for the hypothesis that the neuronal networks that mediate the reflex tachycardia that arises from the stimulation of slowly adapting receptors are separate from the networks that mediate the reflex bradycardia that arises from the stimulation of lung C-fibres.

FIGURE 2.10

Interesting example of a cell which has short and long latency input. This cell is not an NTS neurone but is in fact a vagal preganglionic neurone. This is based on the histological location of the recording site (see plate 1. overleaf). Of course since there is early synaptic input there is no antidromic spike, and it is impossible to say whether the neurone is cardiac or pulmonary.

In total four neurones (in four different animals) with properties very similar to this one were recorded. All these neurones possessed powerful pulmonary C-fibre input. These cells were not included in table 2.1. The existence of such neurones highlights the limitations of the extracellular recording technique.

P= pulmonary branch stimulation (1 pulse 1ms 15V).
C= cardiac branch stimulation (1 pulse 1ms 15V).

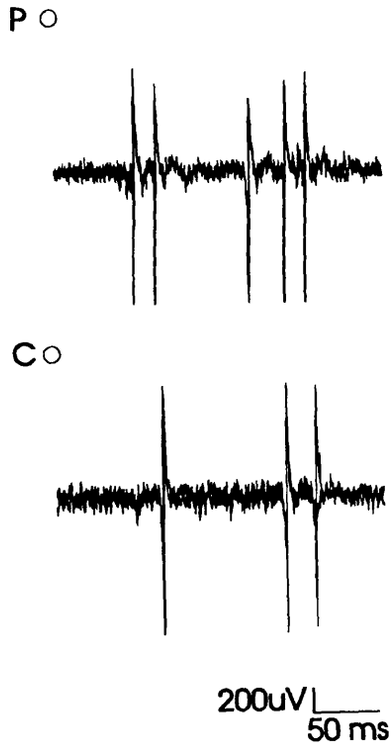
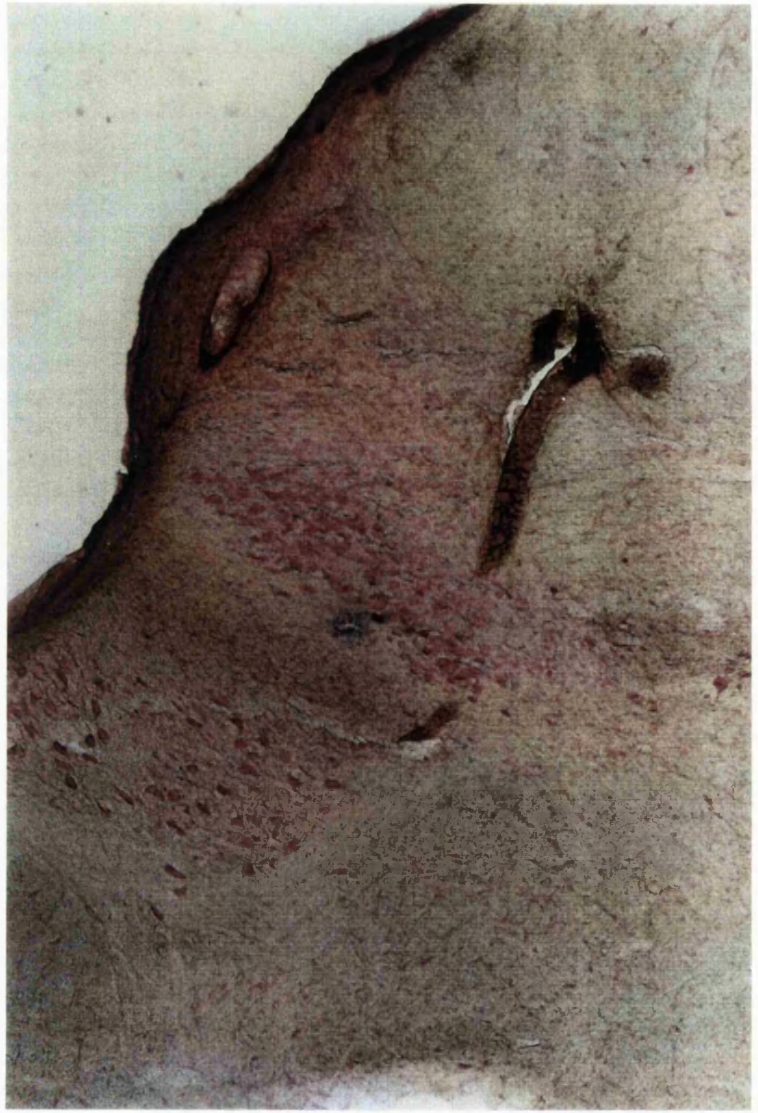
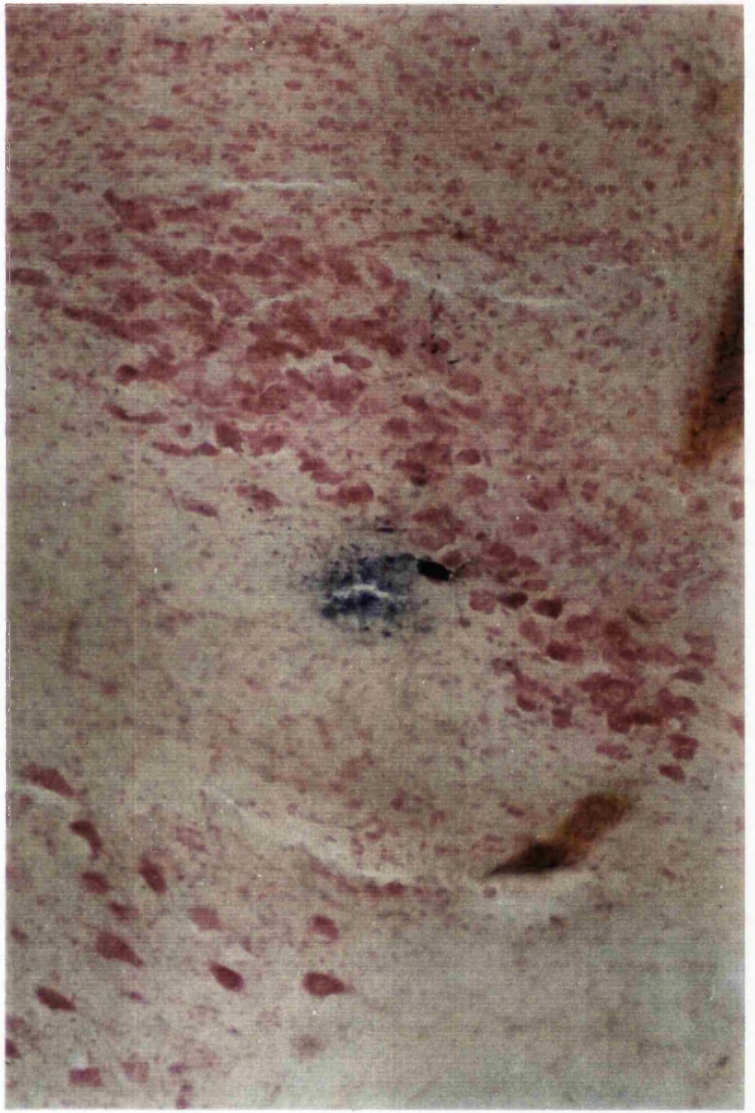

P
C
200uV
50 ms

PLATE 1.

The location of the neurone illustrated in fig 2.10. The photograph on the left is a low power micrograph (×4) of the dorsal medulla of the cat. The section on the right is a high power (×10) micrograph of the same area. The scale bar refers to the high power micrograph on the right.





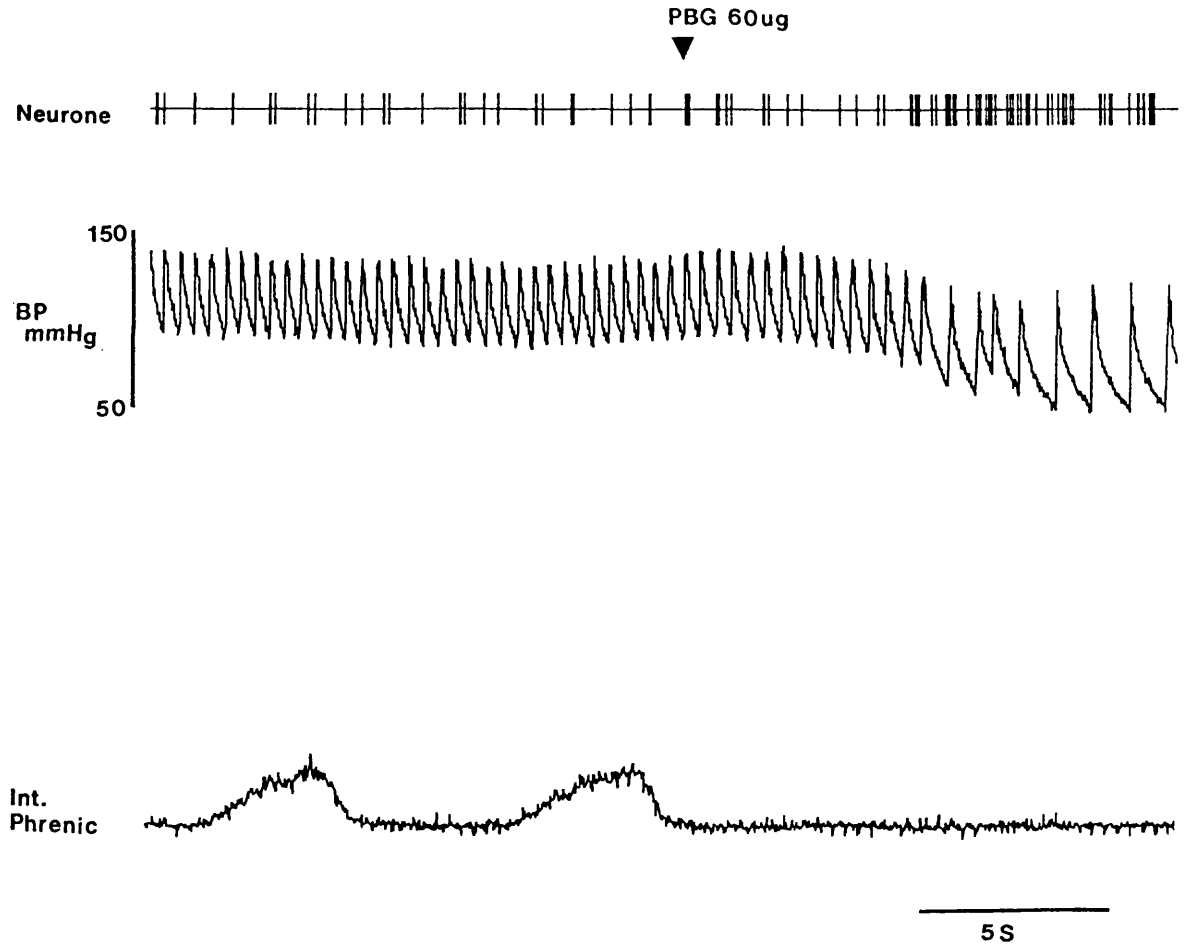
100 um

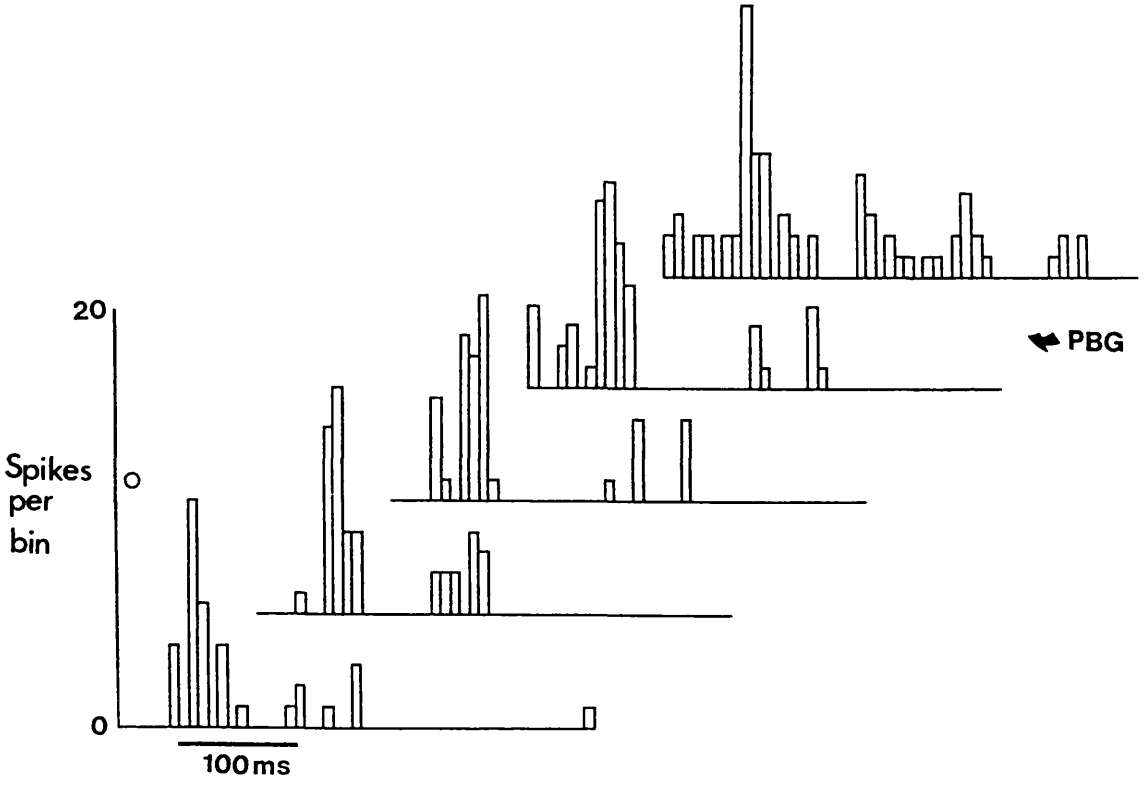
FIGURE 2.11

Upper panel shows discriminated spikes of a neurone with cardiac input excited by PBG (60μg) administered into the right atrium The stimulus at 1Hz was left running throughout.

Lower panel shows peri-stimulus time histogram to cardiac branch stimulation at time zero (circle), there is no stimulus artefact; (9 second time slices with 1 second between slices,8 ms bins). The fifth slice is taken during the PBG test all the other slices are prior to PBG.

The histogram is constructed by sequentially analysing 10 seconds of data at a time (9 sweeps of data per histogram). There is a bimodal distribution of intervals and an increase of firing after PBG (fifth histogram).


PBG 60ug
Neurone
150
BP
mmHg
50
Int.
Phrenic
5S


20
PBG
Spikes
per
bin
0
100ms

2.2d Dorsal vagal preganglionic neurones in the rat

Only a limited study of the rat's DVMN was performed. Vagal preganglionic neurones were identified by conventional criteria based on latency, spike shape, collision test, and histology. Only those neurones which pass all these criteria are discussed. The rat study was deliberately limited because of inevitable uncertainty concerning the projection of neurones that are identified through whole cervical vagal stimulation. However a group of neurones (n=6),5/6 of which were located on the lateral edge of the DVMN shared certain properties which are of some interest (antidromic latency 27-40ms). Unfortunately all these neurones are from just two animals.

The results indicate that the rat pulmonary chemoreflex has C-fibre to C-fibre reflex components, 5/6 C-fibre vagal preganglionic neurones located near the DVMN showed increased activity to PBG (fig 2.13); and these neurones lacked respiratory related activity (fig 2.12). The function of these neurones is unknown.

FIGURE 2.12

Upper panel: collision test of vagal preganglionic neurone. Stimulus artefact is the large spike, the stimulus is triggered from DLH evoked spikes. Collision occurs at 27 ms in this example. This is a vagal C-fibre efferent.

Lower panel: spontaneous activity during asphyxic stimulus (ventilator off). Note the complete lack of respiratory modulation under circumstances where respiratory drive was powerful. All the neurones recorded had ongoing or DLH-induced discharge patterns which were unrelated to central inspiratory drive,blood pressure or tracheal pressure. The question arises: do these nonrespiratory modulated C-fibre vagal preganglionic neurones participate in the pulmonary chemoreflex?

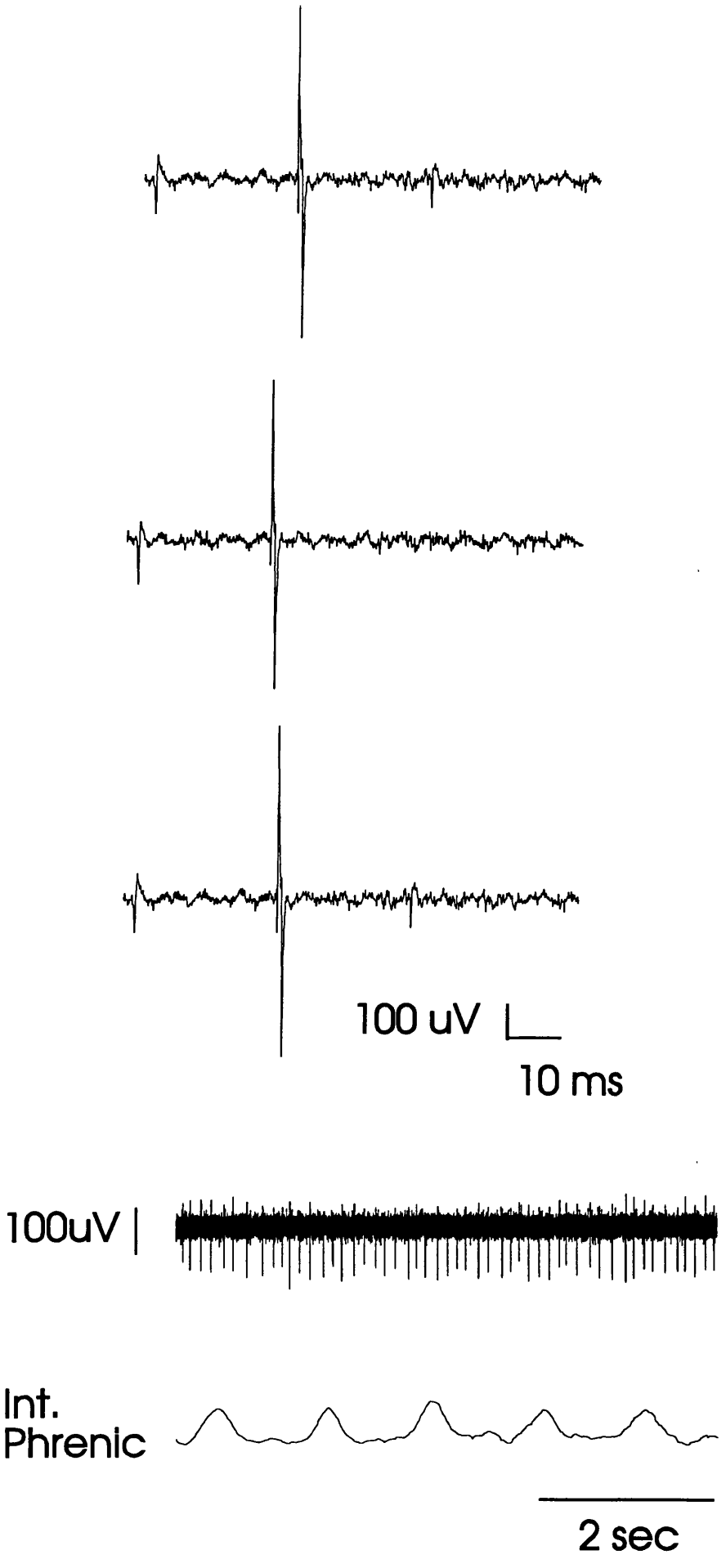

100 uV
10 ms
100uV
Int.
Phrenic
2 sec

FIGURE 2.13

C-fibre vagal efferent excitatory response in the rat to C-fibre input evoked both by chemical and mechanical stimuli.

Upper panel: Multibarrel recording of a vagal preganglionic neurone's response to PBG (background DLH 20nA throughout). Note the initial intense burst of activity that occurs at short latency to PBG injected into the right atrium. This precedes the hypotension of the pulmonary chemoreflex.

Lower panel: The response to large lung inflation. The large inflation threshold and associated hypotension indicates possible pulmonary C-fibre involvement. Heart rate was not measured because the paralysing agent gallamine (an M_2 muscarinic antagonist) blocks reflex bradycardias.

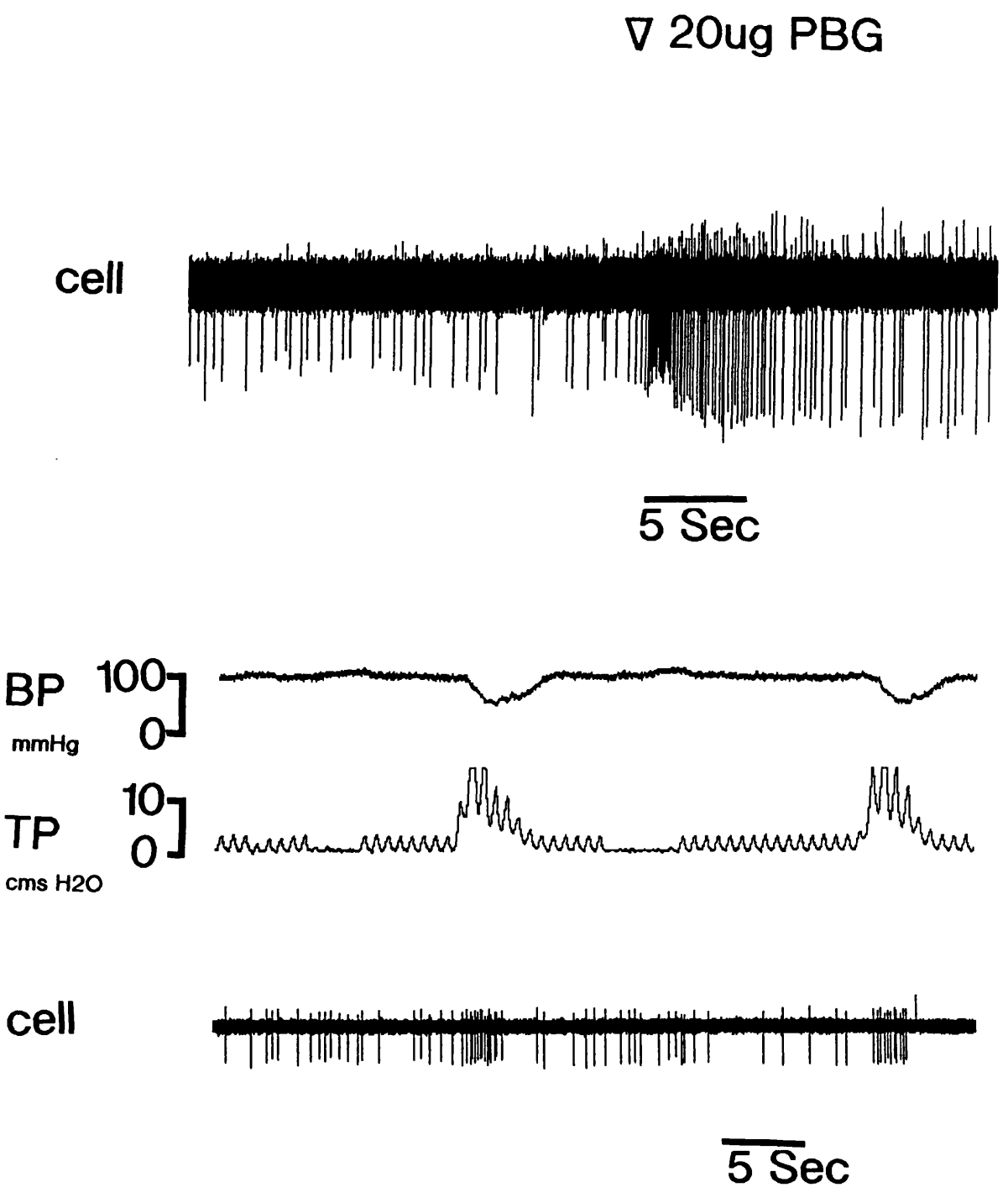

∇ 20ug PBG
cell
5 Sec
BP
100
0
mmHg
TP
10
0
cms H2O
cell
5 Sec

FIGURE 2.14

Upper panel: paired pulse stimulation of vagus (circles), note the IS-SD failure.

Lower panel: the same neurone. The activity is plotted as a post stimulus time histogram (5ms bins),the stimulus artefact is indicated by a circle,the next peak is the antidromic which is not constantly present due to spontaneous cancellations. Note the long latency vago-vagal inhibitory input.

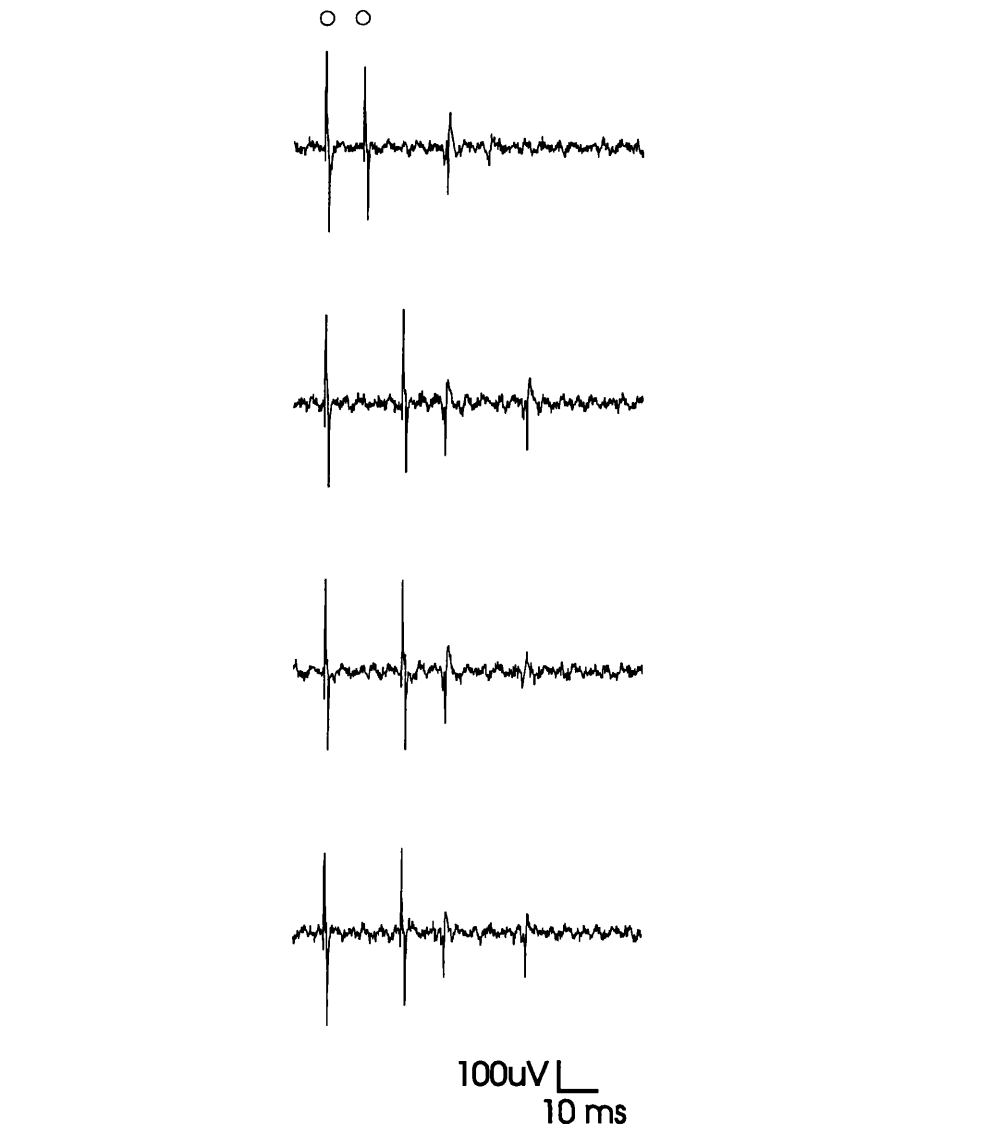
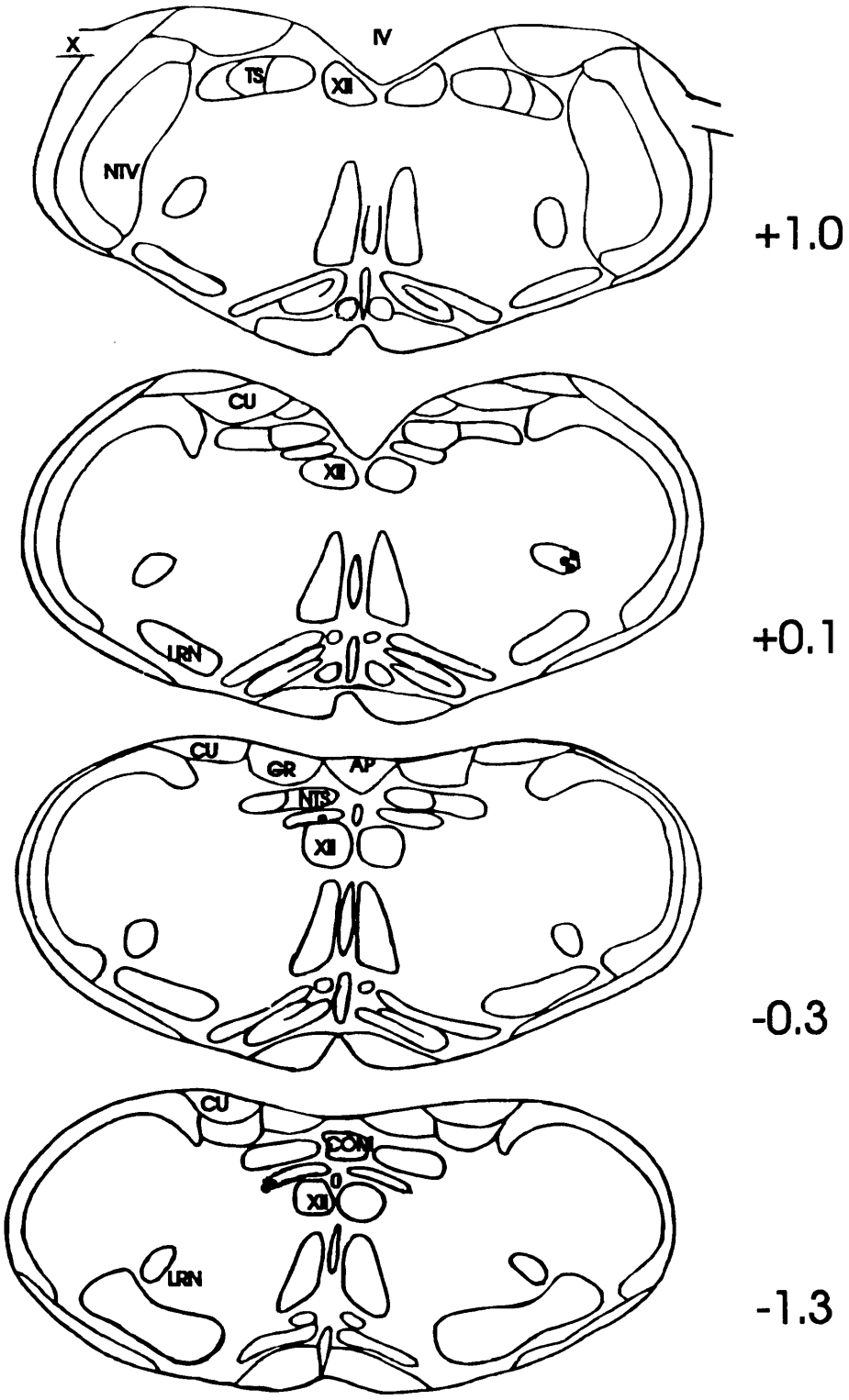

100uV
10 ms
20
22 sweeps
Spikes
per
bin
0
100 ms

FIGURE 2.15

Histology of rat medulla showing location of vagal preganglionic neurones in the DVMN and laryngeal motoneurones in the nucleus ambiguus.

Abbreviations:

XII= hypoglossal nucleus
TS= tractus solitarius
IV= fourth ventricle
NTV= trigeminal nucleus
CU= cuneate nucleus
LRN= lateral reticular nucleus
GR= gracile nucleus
AP= area postrema
COM= commissural nucleus
X= vagus


X
IV
TS
XII
NTV
+1.0
CU
XII
SP5
LRN
+0.1
CU
GR
AP
NTS
XII
-0.3
CU
CON
XII
LRN
-1.3

2.2e Cardiac vagal preganglionic neurones in the cat.

The accessibility of the cardiac branch in the cat affords the investigator the opportunity of positively identifying preganglionic neurones projecting to the heart. Therefore a more intensive investigation than that performed in the rat was conducted. It was possible to obtain high quality single and multibarrel recordings of cardiac preganglionic neurones from the region near the nucleus ambiguus (n=5) the intermediate area (n=1) and near the DVMN (n=27). The majority of the evoked responses were considered to arise from cell bodies rather than axons since many showed a fractionation of the spike into IS-SD components and durations greater than 2 ms. DLH iontophoresis produced an increased firing and this is considered to affect cell bodies not axons. Criteria for identification of cardiac efferents included: constant response latency, histological location and collision. Only neurones that fulfilled at least 2/3 of these criteria were deemed to be preganglionic neurones.

FIGURE 2.16

Histology of the cats medulla. The dots indicate the location of cardiac efferents. The dorsal group are located just on the ventral and ventrolateral surface of the DVMN and the ventral group are located just ventral and ventrolateral to the NA.

★= Pulmonary efferents (all C-fibres).

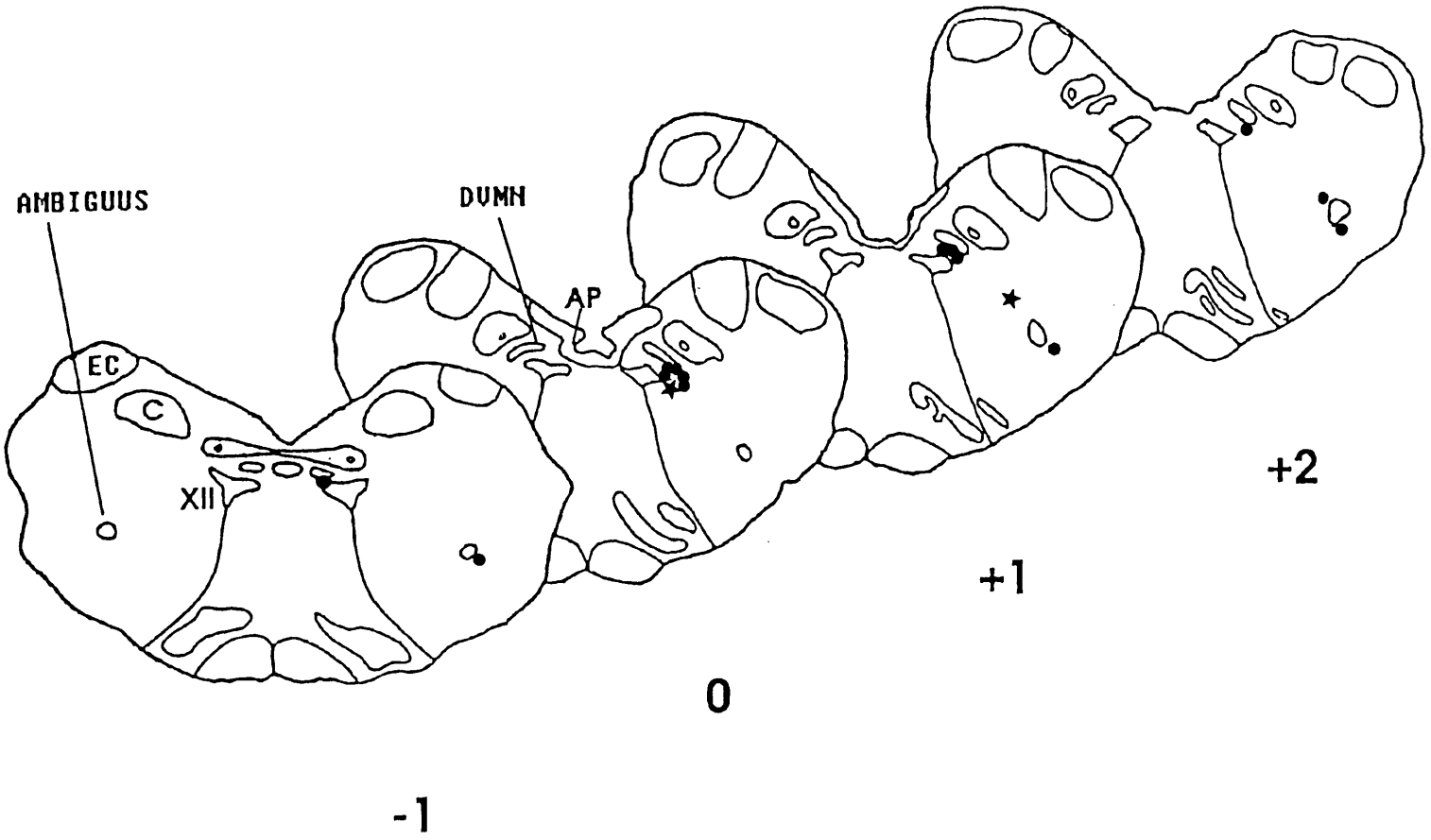

AMBIGUUS
DVMN
AP
EC
C
XII
+2
+1
0
-1

FIGURE 2.17

Long latency response to cardiac branch stimulation (at dot), the cardiac branch was stimulated with a pulse of 8V and 1ms duration.

The C-fibre cardiac vagal efferent shows spontaneous cancellation.

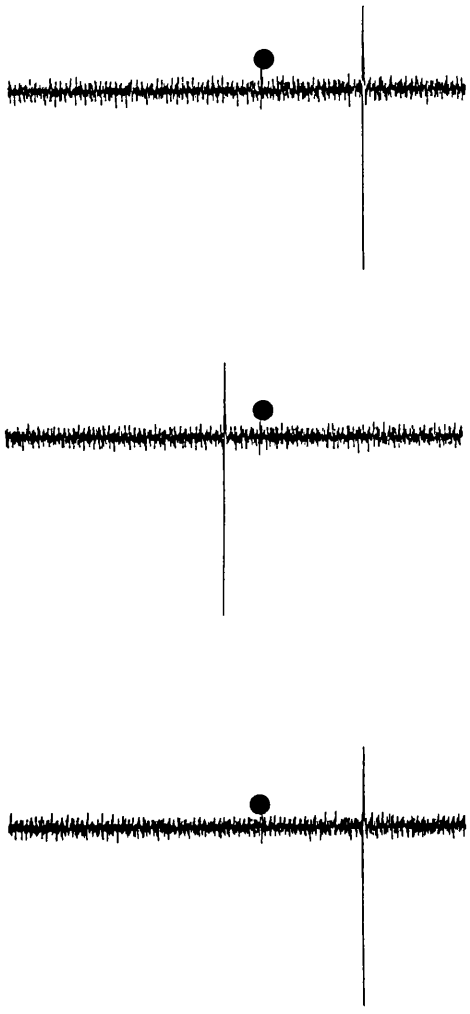
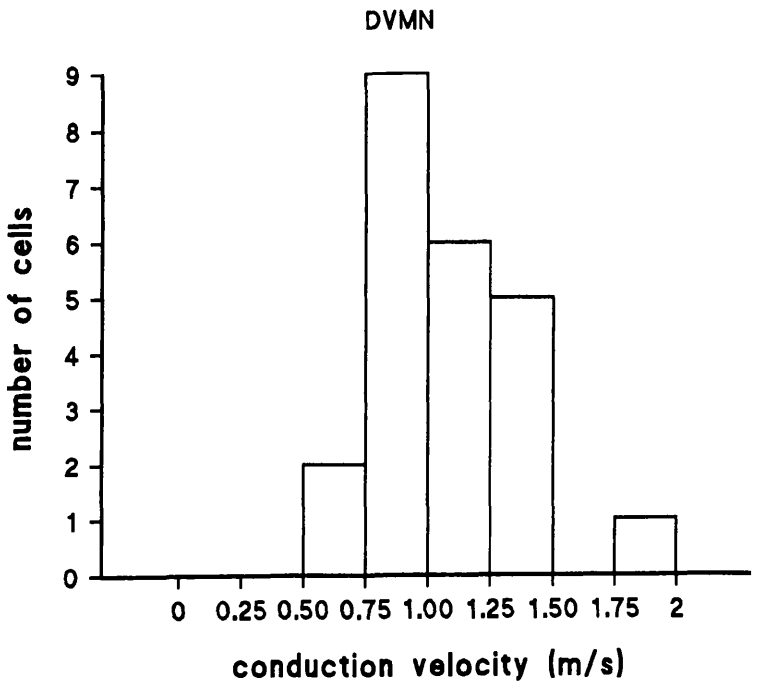

200 ms

FIGURE 2.18

From the antidromic latency and a consideration of the evoked potentials in the tractus solitarius it is possible to estimate the conduction distance and velocity of the cardiac efferents. The conduction distance was calculated to be 160mm.

All responses 2 metres/sec or less are considered to involve unmyelinated axons. On this basis then, cardiac preganglionic neurones in the dorsal medulla are all C-fibres whereas the ventral group near the NA are all B-fibres. The one cardiac vagal preganglionic neurone recorded in the intermediate area had a C-fibre axon (plate 2).


DVMN
number of cells
9
8
7
6
5
4
3
2
1
0
0 0.25 0.50 0.75 1.00 1.25 1.50 1.75 2
conduction velocity (m/s)

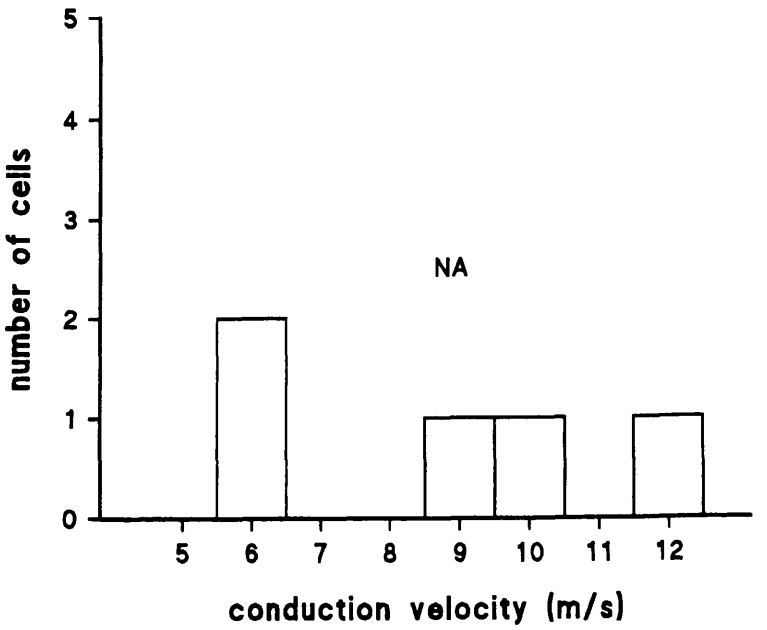
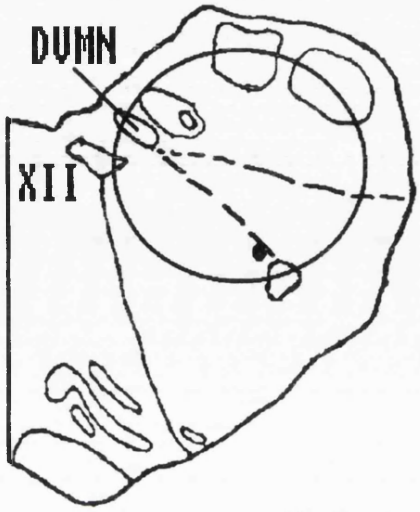

number of cells
5
4
3
2
1
0
NA
5 6 7 8 9 10 11 12
conduction velocity (m/s)

PLATE 2.

The cardiac vagal preganglionic neurone located in the intermediate area between NA and DVMN. Note the vagal efferent loop. The pontamine stain lies on the path taken by migrating embryonic vagal preganglionic neurones. This neurone had a latency to cardiac branch stimulation of 150ms (dark field microscopy).



+2.0 mm

0.5мм

FIGURE 2.19

The firing pattern of the two populations of cardiac preganglionic neurones compared. The top panel shows integrated phrenic nerve activity and the spontaneous activity of a C-fibre cardiac efferent. Note the lack of respiratory modulation and the low firing frequency.

The lower panel shows a post-inspiratory B-fibre cardiac preganglionic neurone and a neighbouring inspiratory cell (which has the smallest spike amplitude). The very tall spikes represent stimulus artefact. Note the high firing frequency and total inhibition of activity during inspiration.

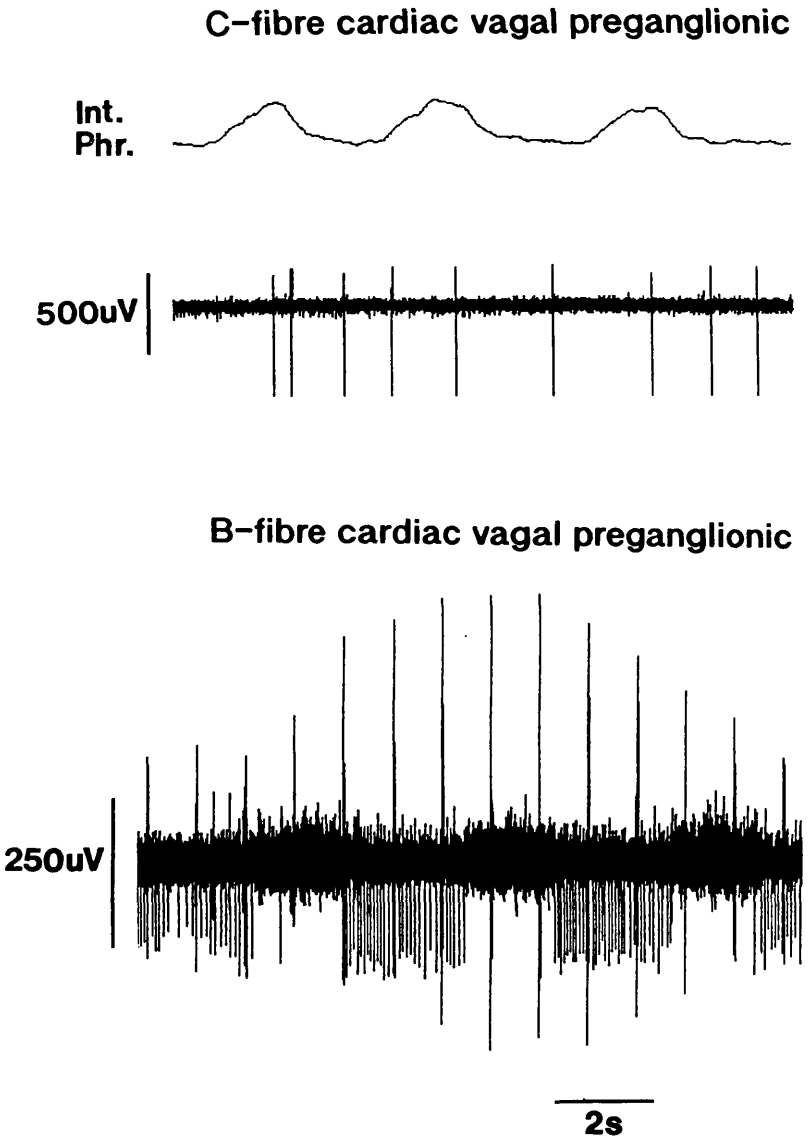

C-fibre cardiac vagal preganglionic
Int.
Phr.
500uV
B-fibre cardiac vagal preganglionic
250uV
2s

FIGURE 2.20

Upper panel: Raw record of a C-fibre cardiac vagal preganglionic (the same cell as fig 2.19) in relation to the integrated phrenic nerve.

Lower panel:
The activity of this C-fibre efferent averaged over 58 respiratory cycles with respect to the integrated phrenic. (bin width=25ms)

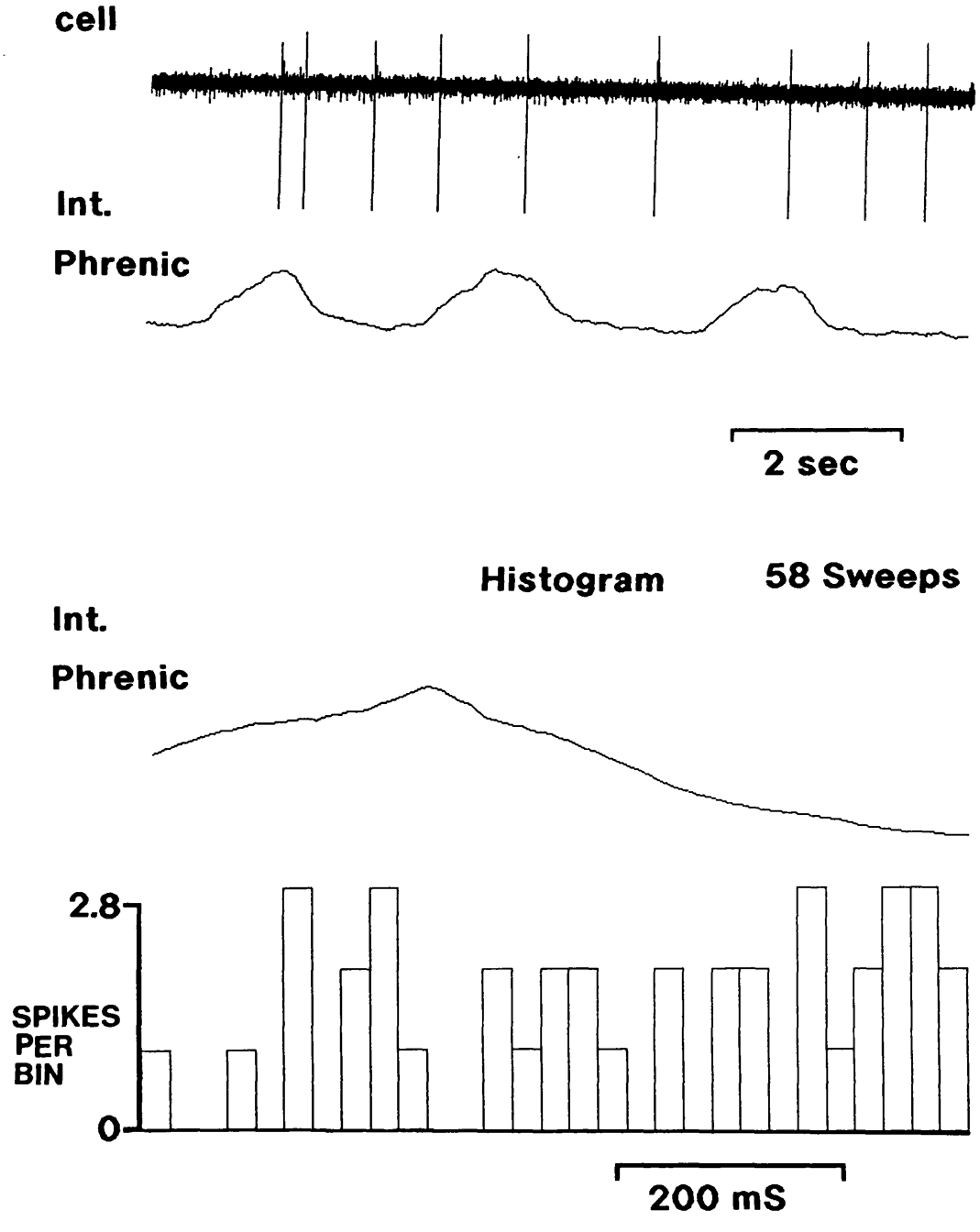

cell
Int.
Phrenic
2 sec
Histogram
58 Sweeps
Int.
Phrenic
2.8
SPIKES
PER
BIN
0
200 mS

FIGURE 2.21

PSTH constructed for a C-fibre efferent with respect to tracheal pressure and ECG. There is no obvious relationship between the firing of the neurone and either of these parameters. All the spontaneously firing neurones with C-fibre axons (n=9) exhibited these same discharge patterns.

Since these cardiac vagal efferents have no ECG related activity or respiratory related activity and have C-fibre axons, they would not conventionally be thought of as cardioinhibitory neurones. So the question arises: are these type of neurones involved in the pulmonary chemoreflex?

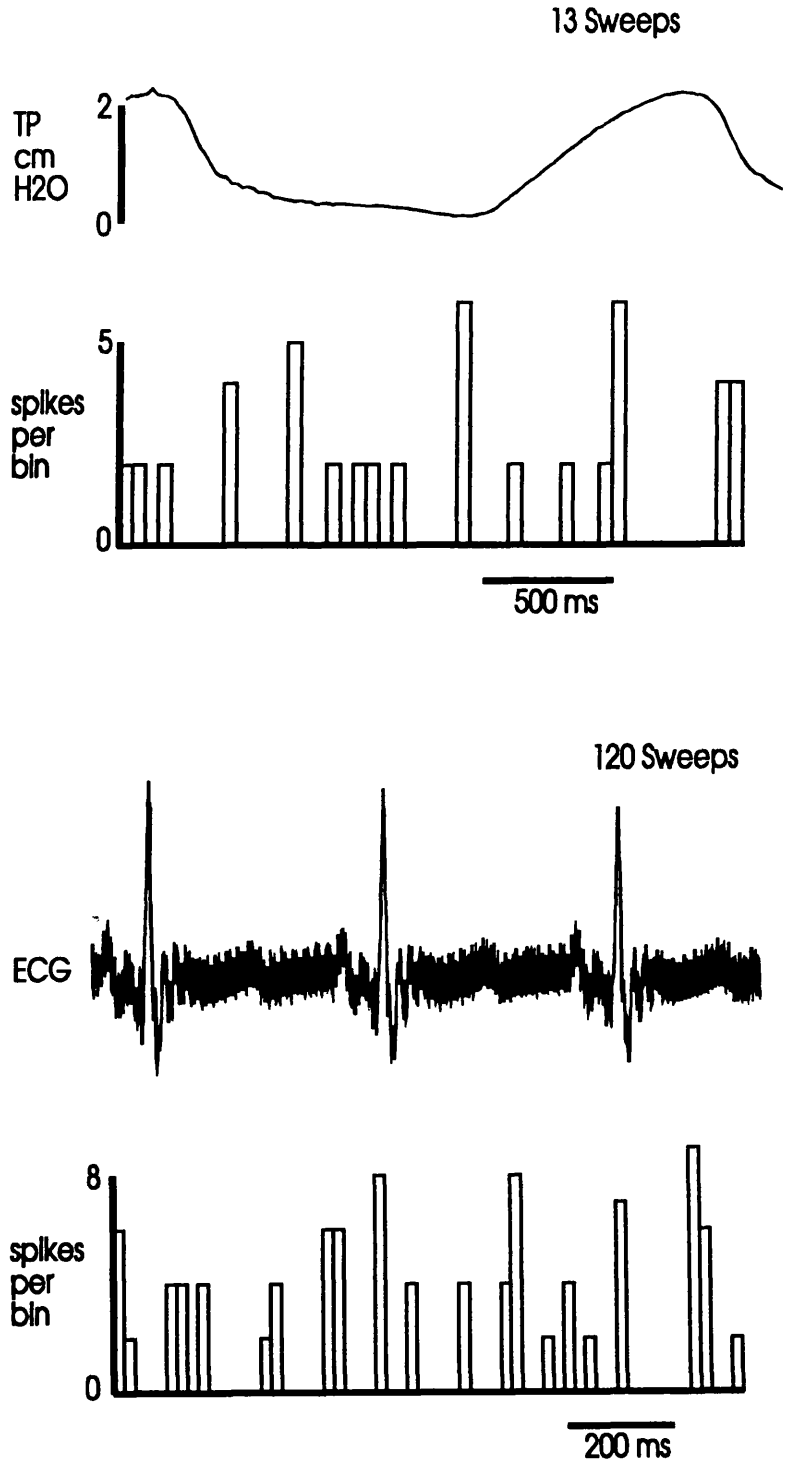

13 Sweeps
TP
cm
H2O
2
0
5
spikes
per
bin
0
500 ms
120 Sweeps
ECG
8
spikes
per
bin
0
200 ms

FIGURE 2.22

Excitation of a cardiac vagal C-fibre efferent (discriminated spikes) during the pulmonary chemoreflex. In order to highlight the spontaneous activity of the neurone the stimulus artefact and the antidromic spike have been edited out. This is performed by placing the event times into a buffer as an array and programming a subtraction routine. By constructing a time window only the spike which most closely fits the expected antidromic latency is removed. Note the neurone fires infrequently in the control run, but greatly increases its discharge at short latency to PBG. The excitation precedes the hypotension and the bradycardia. There was no question of movement artefact during the response. After the burst like response the neurone is lost. This is the advantage of leaving the cardiac stimulation (1Hz) running during the experiment.

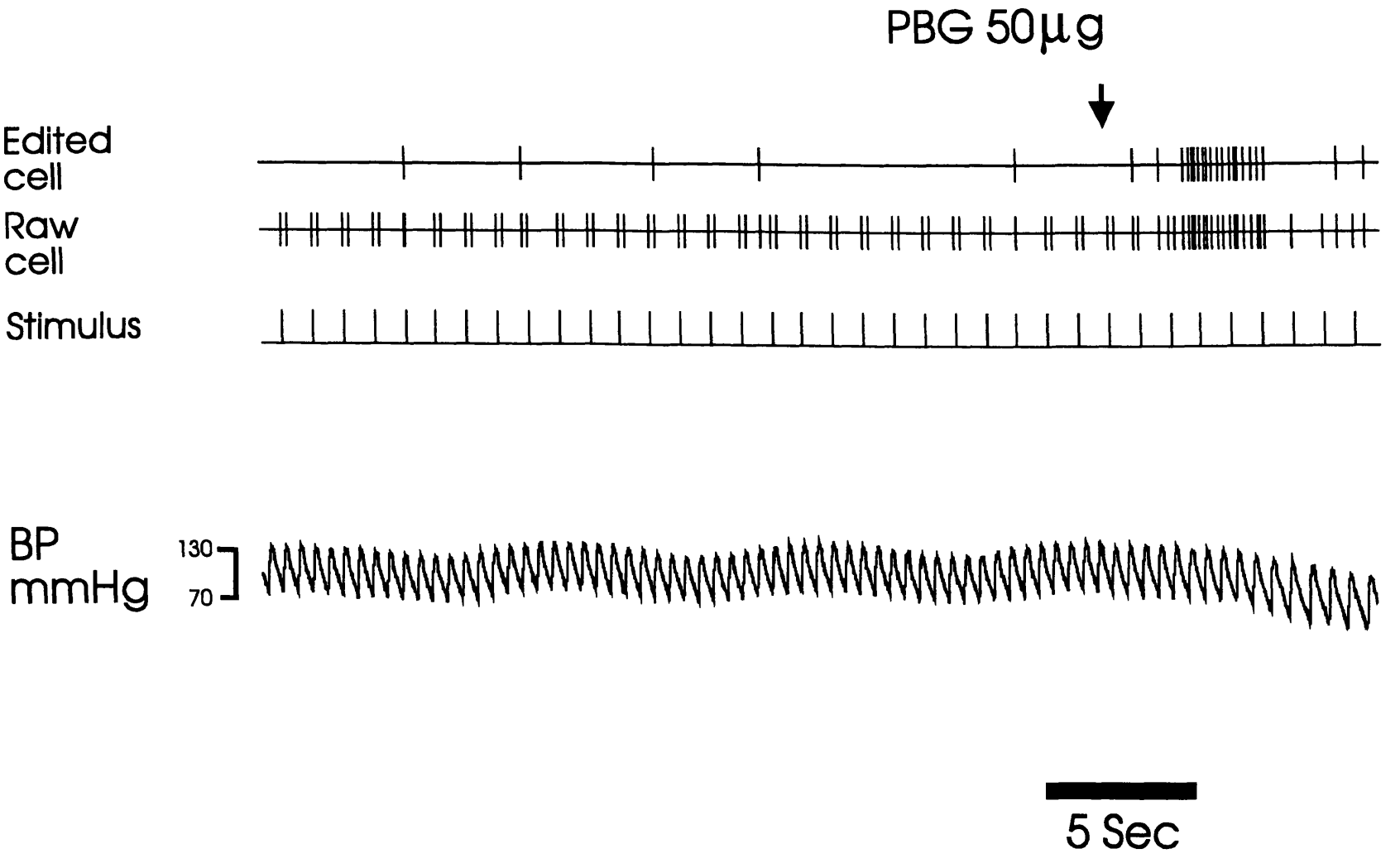

PBG 50µg
Edited cell
Raw cell
Stimulus
BP mmHg
130
70
5 Sec

FIGURE 2.23

This illustrates the disadvantage of stimulating during the recording of cardiac C-fibre efferents. During the "on" phase the cardiac branch is stimulated at 1 Hz. Like the rat's dorsal vagal preganglionic neurones some of these cells exhibit vago-vagal inhibitory inputs. The cell is edited as in the previous figure 2.22. There is an excitatory response to PBG when the branch stimulation is off.

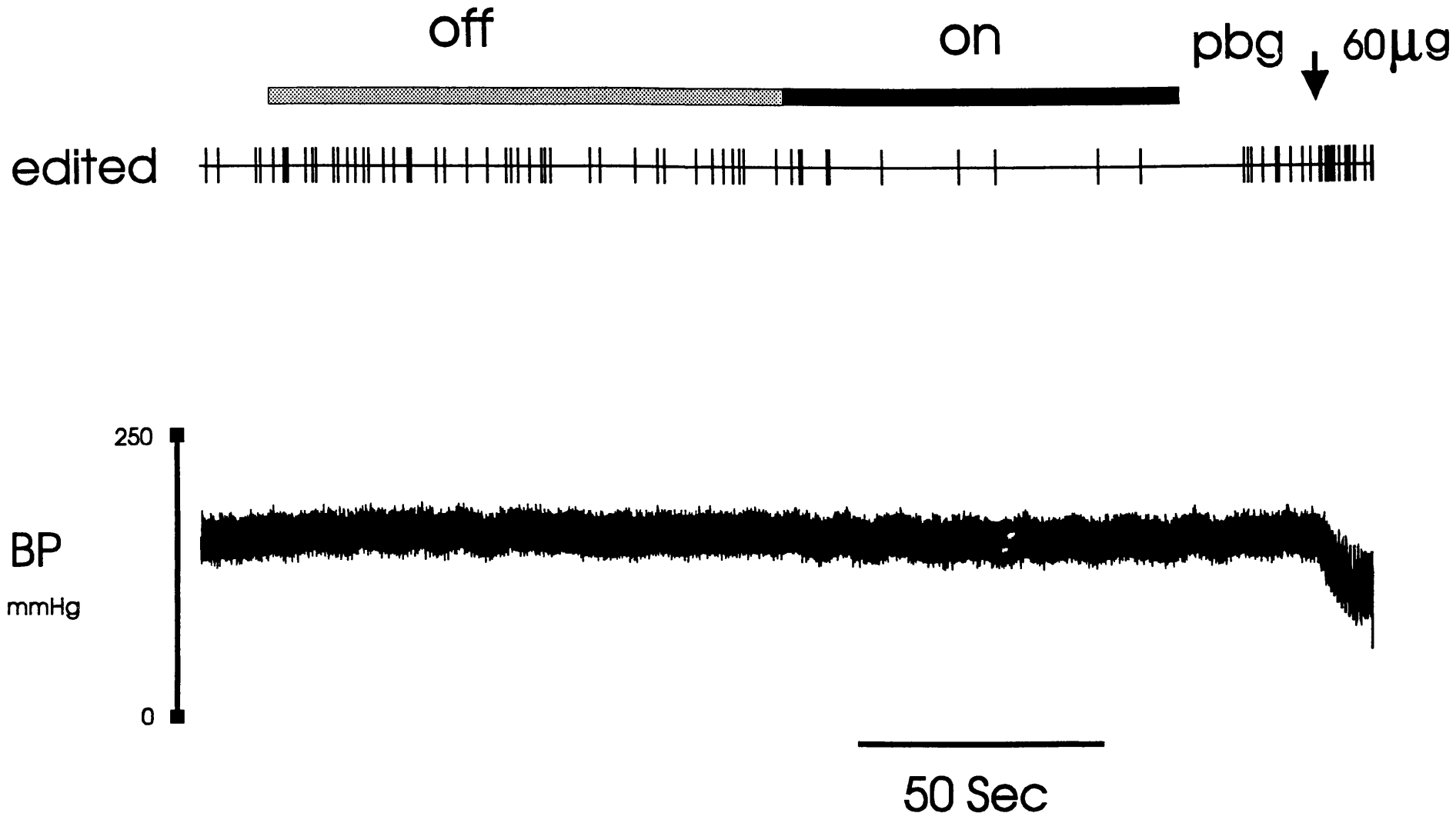
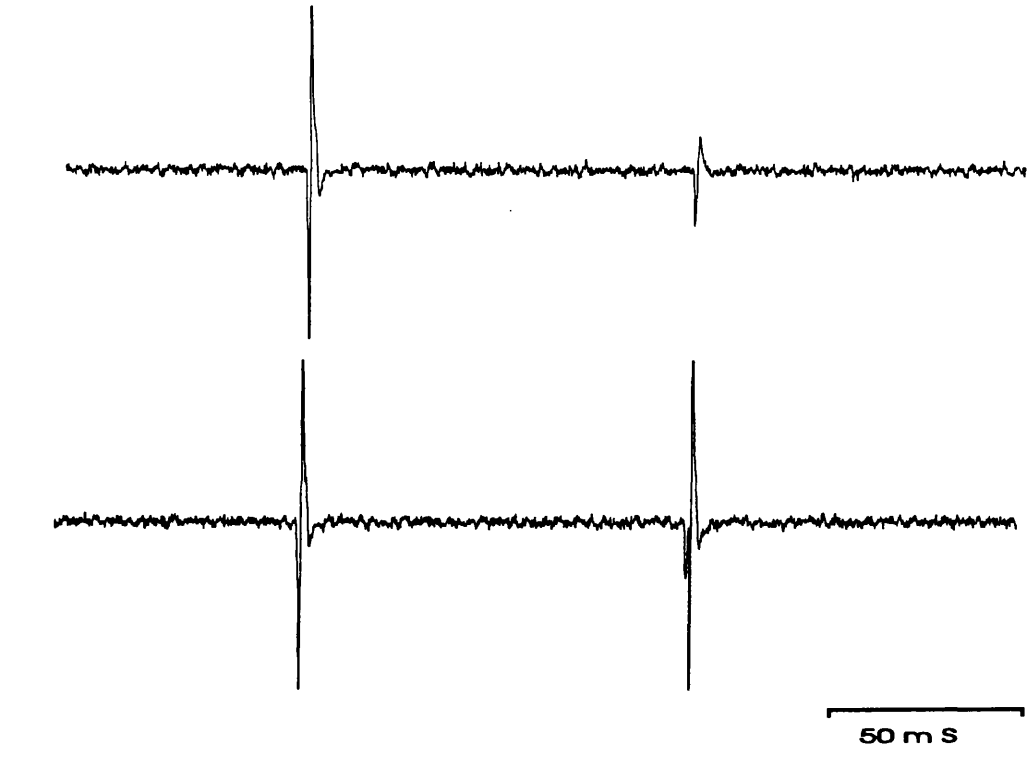
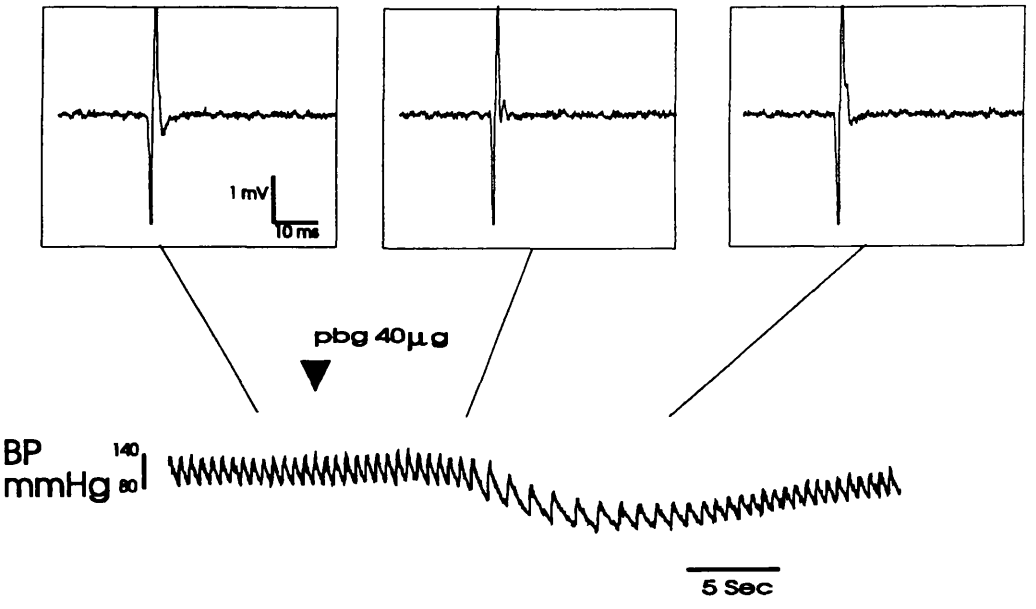

off
on
pbg
60µg
edited
250
BP
mmHg
0
50 Sec

FIGURE 2.24

Upper panel shows IS-SD failure of a C-fibre efferent. Two pulses (not shown) are applied at time zero, and 200 ms later two antidromic responses are recorded. The stimuli are separated by 100ms. At stimulation intervals above 100ms the neurone faithfully follows the stimuli. At 100ms or less there is clear IS-SD failure.

Lower panel shows a different C-fibre efferent which was only occasionally active. This neurone was not excited by PBG, although the reflex was potent. The possibility exists that this neurone was not activated because of inhibitory input from the cardiac branch that occurs during continuous stimulation. Alternatively the dorsal population of cardiac vagal preganglionic neurones may be more heterogeneous in their response patterns to PBG.


50 m S


1 mV
10 ms
pbg 40µg
BP
mmHg
140
80
5 Sec

There was considerable difficulty in obtaining stable recordings, working reflexes and spontaneously firing neurones all in the same experiment. Nine cells of the C-fibre group exhibited good spontaneous activity whereas the other 18 were silent or fired infrequently. 2/3 spontaneously active C-fibre group (fig 2.22) and none of the 8 silent neurones recorded during a PBG evoked bradycardia showed an increase in activity. 1/2 spontaneously active neurones of the B-fibre group were excited during a PBG evoked bradycardia. Whereas the value of a negative result is dubious when the deleterious effects of anaesthesia upon vagal tone are considered, there is little doubt upon the positive excitatory effect of PBG upon at least some C and B cardiac vagal preganglionic neurones. In addition a considerable number of cardiac preganglionic neurones may have escaped attention because of early synaptic input masking the antidromic spike (as in fig 2.10). All the neurones that possessed long (100-180ms) and short (40-75ms) latency input from the intrathoracic branches, possessed powerful pulmonary C-fibre input. If the findings of Bennett et al. (1985) are correct then these are less likely to be NTS cells. These was proved in one case only (plate 1). In summary, the findings both in the rat and the cat provide sufficient evidence to postulate that non-respiratory cardiac vagal preganglionic neurones are involved in the pulmonary chemoreflex. The possible role these C-fibre to C-fibre reflexes play in the pulmonary chemoreflex, is addressed in the next chapter.

3. Selective electrical stimulation of cardiac vagal C-fibres in the anaesthetized cat, rat and rabbit.

3.1 Introduction

The action of cardiac vagal preganglionic C-fibre efferents was not determined from central recordings (chapter two). In order to study the possible chronotropic action of C-fibre efferents, direct selective stimulation of this group is necessary.

Previous studies attempting a central electrical DVMN stimulation have either failed or afforded unconvincing evidence to support the hypothesis that this is a "cardioinhibitory centre". This approach is to be avoided for three reasons: first the axons of B-fibre cardiac vagal preganglionic neurones lie close to the axons of the C-fibre efferents, therefore electrical stimulation will yield a false positive result, second the C-fibre efferents are scattered along a considerable longitudinal stretch of neuraxis, therefore focal stimulation may yield a false negative result. Third, since the DVMN is so close to NTS neurites , current spread and electrical stimulation of NTS pathways involved in reflex cardioinhibition may account for a false positive result.

Early studies on the effect of vagal stimulation employed the use of induction coils and this was subsequently combined with recordings of evoked compound action potentials displayed on cathode ray oscilloscopes. To assess the contribution of unmyelinated preganglionic axons, a stimulation intensity was chosen which activated only myelinated axons and the effect on heart rate noted. By increasing the stimulus intensity the unmyelinated fibres were recruited and any additional effect on heart rate was then ascribed to their action on the ganglion. This approach therefore, aimed to compare the effects of B-fibres versus B+C fibres.

This approach is to be avoided for four reasons: First, increasing stimulus intensity increases B-fibre recruitment therefore B+C should be greater than B alone and this has nothing to do with C-fibre recruitment, (a false positive). Second, the stimulus intensity

which activates B-fibres may produce a near maximal response and the effect of C-fibre recruitment may be masked (a false negative). Third heart rate rather than cardiac cycle length (R-R interval) has been recorded in most vagal stimulation studies. It is possible to produce enormous R-R interval changes and minuscule heart rate changes on the appropriate part of the heart rate/R-R interval curve (false negative). This part of the curve is reached during the high intensity stimulation of B+C fibres. Finally and perhaps most importantly, nothing is known concerning the projection patterns of B and C fibres onto their postganglionic targets, the possibility of convergence offers the possibility of occlusion. B and C-fibre preganglionic convergence has been demonstrated in sympathetic ganglia (Janig et al. 1983).

Selective study of unmyelinated fibres has been accomplished with the techniques of: cooling (Ritchie & Straub 1956; Paintal 1965; Patberg et al. 1984), anodal block (Sasen & Zimmerman 1980), heating (Klump & Zimmerman 1980), local anaesthetics (Heavner & De Jong 1974), pressure block (Clark et al. 1935), capsaicin application (Jansco & Such 1983).

Local anaesthetic block does not discriminate adequately between non-myelinated and small myelinated fibres (Nathan & Sears 1961). Differential cooling might offer a simple test of the hypothesis that the bradycardia of the pulmonary chemoreflex is a C-fibre to C-fibre reflex (Daly 1991). It is possible that there is already evidence of this in the literature. In cats the bradycardia induced by PBG has been reported not to be abolished until the vagi are cooled to 3°C (Dawes et al. 1951). It is unknown whether this bradycardia is due to cardiac vagal C-fibre efferents or sympathoinhibition or both. The great advantage of anodal block over differential cooling is that the limitation of C-fibre discharge when A-fibres are blocked is not so marked. This becomes more important if C-fibres have weak chronotropic action. Continuous anodal block or prolonged high frequency stimulation is to be avoided as this leads to nerve deterioration quite rapidly (Whitwam & Kidd 1975;Thoren et al. 1977).

A modified anodal block technique has been described (Accornero et al. 1977) and successfully used to demonstrate the cardioinhibitory actions of vagal C-fibre efferents in the rat (Nosaka et al. 1979) and rabbit (Wooley et al. 1987). The modification in the technique involves the use of triangular shaped pulses to avoid anodal break excitation.

The possibility of asynchronous firing of myelinated axons during the anodal block was eliminated by Accornerro et al. (1977) through the use of the collision test of Douglas and Ritchie (1957).

In cats, it is generally accepted that cardiac slowing is mediated entirely (Middleton et al. 1950; Kidd & McWilliam 1982) or mainly (Heinbecker & Bishop 1935) by myelinated B-fibres and these myelinated axons also seem to mediate most of the reductions in atrial contraction and all of the slowing of A-V conduction evoked by vagal stimulation (Kidd & McWilliam 1982). However, a chronotropic action for C-fibre cardiac vagal efferents has been purported in other species, in the turtle heart (Heinbecker 1931), the rat (Nosaka et al. 1979) rabbit (Heinbecker & Bishop 1935; Wooley et al. 1987) and guinea pig (McWilliam & Wooley 1987). The cardiac division of the fourth branchial nerve of the elasmobranch *Scyliorhinus canicula* is completely myelinated (Barrett & Taylor 1985b). The comparison of amphibian and fish myelination to that in occurring in the nerves of mammalian species must be made with caution. This is simply because elasmobranchs for instance, inhabit a world of 10 degrees centigrade. This can create the illusion of species differences and effectively mask underlying templates which all vertebrates may share. It is surprising that amongst mammals, there are striking species differences reported, and this concerns such a basic motor system: the vagal control of the heartbeat.

3.1a Pharmacology of the C-fibre cardiac vagal efferent response in the rabbit

The bradycardia mediated by non-myelinated axons in the rabbit is resistant to the nicotinic ganglion blocker hexamethonium (Woolley et al. 1987; Ford & McWilliam 1986; McWilliam & Woolley 1990). The bradycardia elicited both by B and C fibres is sensitive to atropine. The slow return to baseline heart rate after C-fibre stimulation and the hexamethonium resistance suggested to McWilliam et al. (1990), that at the cardiac ganglion a transmitter other than acetylcholine is involved. The inability of nicotinic cholinergic antagonists to abolish ganglionic events is commonly interpreted as evidence of non cholinergic pathways. Seabrook et al. (1990) reported that responses resistant to mecamylamine (cholinergic nicotinic antagonist) in a neonatal rat cardiac ganglion utilize noncholinergic transmission. However it must be emphasised that nicotinic blockers block nicotinic transmission not cholinergic transmission in toto. In fact it turns out that the

sEPSPs in the rat cardiac ganglia recorded by Seabrook et al. (1990) do involve cholinergic receptors but that these are muscarinic not nicotinic. (Selanyko & Skok 1992a,b,c). In fact this nicotinic/muscarinic co-transmission should have been no surprise: many parasympathetic and sympathetic ganglia possess this mechanism. This is the most important and basic aspect of autonomic neurotransmission. Blumberg and Janig (1983) have a described B-fibre sympathetic preganglionic pathways utilizing cholinergic nicotinic transmission to postganglionic sympathetic vasoconstrictor neurones whilst C-fibre preganglionic input is via cholinergic muscarinic transmission. The question arises: are cardiac ganglia organized in a similar fashion? Recent experiments on mammalian cardiac ganglion neurones, cultured or acutely dissociated in vitro, have showed sEPSP synaptic events which are mediated through M_1 and M_2 receptors. (Allen & Burnstock 1990; Xi-Moy et al. 1993; Selyanko & Skok 1992b). This phenomenon was first reported by Kuffler's group in the mudpuppy (*Necturus maculosus*) (Hartzell et al. 1977). The recent in vitro mammalian work has also revealed interesting facts concerning the organization of the cardiac vagal postganglionic neurones. Xi et al. (1991) have described two populations of principal cardiac ganglion neurones, with different morphologies and electrophysiological properties (these elegant experiments utilized intracellular recording and labelling). One population fires tonically to a step depolarization stimulus, and the other fires in a phasic fashion. It is becoming increasingly clear that most parasympathetic and sympathetic ganglia are organized in a very similar fashion (Saffrey et al. 1992). However, the parallels in the central organization of cranial and sacral parasympathetic preganglionic outflow and the peripheral construction of the ganglia has not featured in the literature;this is perhaps due to the increasing division in the disciplines of in vitro and in vivo electrophysiology.

In 1914, Dale demonstrated that acetylcholine produced two responses that were mimicked by nicotine and by muscarine and that the muscarinic action was blocked by atropine. It is only since 1980 that muscarinic receptor behaviour was better defined through the use of the drug pirenzepine (Hammer & Giachetti 1982). The phrase "muscarinic receptor behaviour" is used, because in 1980 it was unknown whether there were different receptor subtypes or different coupling stratagems used by one receptor and/or different binding sites for antagonists. The techniques of molecular biology have

now confirmed the existence of as many as five different receptor subtypes, and these have been sequenced, cloned and expressed (Hammer & Giachetti 1982). All cardiac ganglion neurones dissociated in cell cultures express muscarinic receptors (Saffrey et al. 1992). In situ hybridization indicates that most of the muscarinic receptor genes are expressed in vitro and in situ. This has been repeatedly demonstrated by autoradiography and electrophysiological analysis (Saffrey et al. 1992).

Receptor sensitivity to the blocking actions of pirenzepine is present in the cardiac ganglion of the guinea pig (Allen et al.1990), dog (Xi-Moy et al. 1993) and chicken (Jeck et al. 1988). Block of the nicotinic ganglionic transmission in the chicken heart by ±tubocurarine unmasked an excitatory muscarinic transmission, which was mediated through M_1-receptors stimulating a low and prolonged release of acetylcholine (Jeck et al. 1988). There is evidence for M_1 transmission in airway parasympathetic ganglia (Barnes 1993;Bloom et al. 1988), superior cervical ganglion (Ashe & Yarosh 1984; Brown & Constanti 1980),and enteric nervous system (North et al. 1985).

The neuropeptides; galanin, substance P, CGRP, LHRH and NPY have been localized in the cardiac ganglia of a number of species (Knoppe et al. 1992) but best described in the transparent cardiac ganglion of the mudpuppy (*Necturus maculosus*). No physiological role has been ascribed to any peptide in cardiac ganglia with the possible exception of NPY which mediates vago-sympathetic antagonism (Potter 1987). Peptidergic transmission is characterized by long latency and long duration signalling (Horn 1992;Knoppe 1992). Nicotinic receptors operate on the millisecond timescale (fast EPSP), and muscarinic receptor operation lasts seconds (sEPSP), in a physiological system this means the difference between beat by beat data and breath by breath data. Peptidergic transmission lasts many minutes (late sEPSP), therefore it is unlikely that peptides are of any importance in dynamic cardiovascular control. Also peptidergic transmission cannot account for the lack of respiratory modulation of the pulmonary chemoreflex bradycardia in the cat, since the phenomenon develops rapidly (the first breath) and lasts only for a few seconds before respiratory sinus arrhythmia resumes (personal observation). It may be theorized that the most likely candidate to explain this phenomenon is a muscarinic mechanism, therefore in the following experiments pirenzepine(an M_1 antagonist) was used to test this theoretical prediction.

Although cardiac labelling with HRP delineates DVMN and NA as the source of preganglionic neurones in most species studied (Withington-Wray et al. 1987) there are reported species differences in the chronotropic action of vagal stimulation. It is clear from the literature that there are many problems with the experiments that have yielded this result. It is the object of the present study to investigate the chronotropic action of C-fibre stimulation in anaesthetized rat, cat and rabbit using the modified anodal block technique (Accornero et al. 1977). This technique has been previously applied to the rat (Nosaka et al. 1979) and the rabbit (Woolley et al. 1987) but never to the cat. In addition the hexamethonium resistance of C-fibre provoked bradycardia in rabbits (Woolley et al. 1987) is tested for pirenzepine resistance. These experiments are conducted to test the hypothesis that C-fibre cardiac vagal preganglionic neurones in the cat, which have already been shown to lack respiratory rhythm (Chapter 2), are involved in the cardioinhibition of the pulmonary chemoreflex.

3.2 Methods

3.2a General

For rats and cats, this is essentially the same as that described in chapter two. Female rabbits (New Zealand Whites) weighing 1.8-2.4kg were anaesthetized with urethane (Sigma)(1.4g/kg) administered via a marginal ear vein or pentobarbitone sodium (Sagatal) i.p. The animals were pretreated with atenolol (1mg/kg). This selective $beta_1$-antagonist was added to outrule sympatho-inhibition as a contributing mechanism in the evoked bradycardias.

3.2b Preparation of the cats

High cervical bilateral vagotomy was performed and the intact right cranial cardiac branch was placed upon recording electrodes. Stimulating bipolar electrodes were placed upon the cut peripheral end of the right cervical vagus. The inter-electrode distance was 2-3 mm the electrode was made of silver (diam.: 0.5mm).

3.2c Preparation of the rats

The preparation of the rat was identical to the cat. It was possible to locate the cranial

cardiac branch in the rat and this branch when stimulated could arrest the heart and was traced to cardiac ganglia in the fat pads surrounding the heart. The cardiac branch was found to be readily accessible without resort to lobectomy. The fat pads were dissected in vitro after the experiment. The ganglia were made visible by applying 1% neutral red. The ganglia were stored in 10% formal saline and then paraffin sectioned 3 days later. The sections were stained for Nissl substance.

3.2d Preparation of the rabbits

It was difficult to maintain rabbits in an acceptable state post thoracotomy. This approach was abandoned, and the recording electrodes were placed not on the cardiac branch but on the lower cervical vagus. In other respects the preparation of the rabbits was the same as that for the cats and rats.

3.3 Results

The modified anodal block technique of Acornerro et al. (1977) was found to be a simple, convenient method for selectively stimulating C-fibres. Care was taken to ensure good quality unambiguous action potential recordings (fig 3.1, fig 3.2). Signal averaging techniques were used to accentuate possible myelinated break-through and subtract movement artefact from the cardiac branch records. This was considered insufficient however, since the main concern is for a single or multiple asynchronous volley breakthrough which the signal averaging process will actually mask. This is an important issue since B-fibre preganglionic efferents are extremely potent even in small numbers. Therefore every sweep was individually scrutinized (200 sweeps per stimulation protocol i.e. 10Hz for 20 seconds). The results dramatically illustrate the species similarities (fig 3.3) (fig 3.5). Greater certainty of the potency of the anodal block can be obtained through single volley stimuli. The experiments of Brown & Eccles (1934) were revisited (fig 3.6 & 3.7). The state of modern computing means that hundreds of thousands of cardiac cycle lengths can be calculated in seconds, a feat that would have taken the early experimenters months to perform manually.

FIGURE 3.1

Experimental arrangement for anodal block technique. Atenolol (1mg/kg) treated animals had bilateral vagotomy performed. Stimulating electrodes were placed on the cut end of the vagus. In order to stimulate all the fibres the cathode faced the heart, the evoked compound action potential (this is from a rabbit) displays A,B and C waves. When the polarity is switched and a triangular shaped pulse is applied, the A and B waves are completely blocked and the amplitude of the C wave is partially reduced. The dot indicates the point of stimulation.

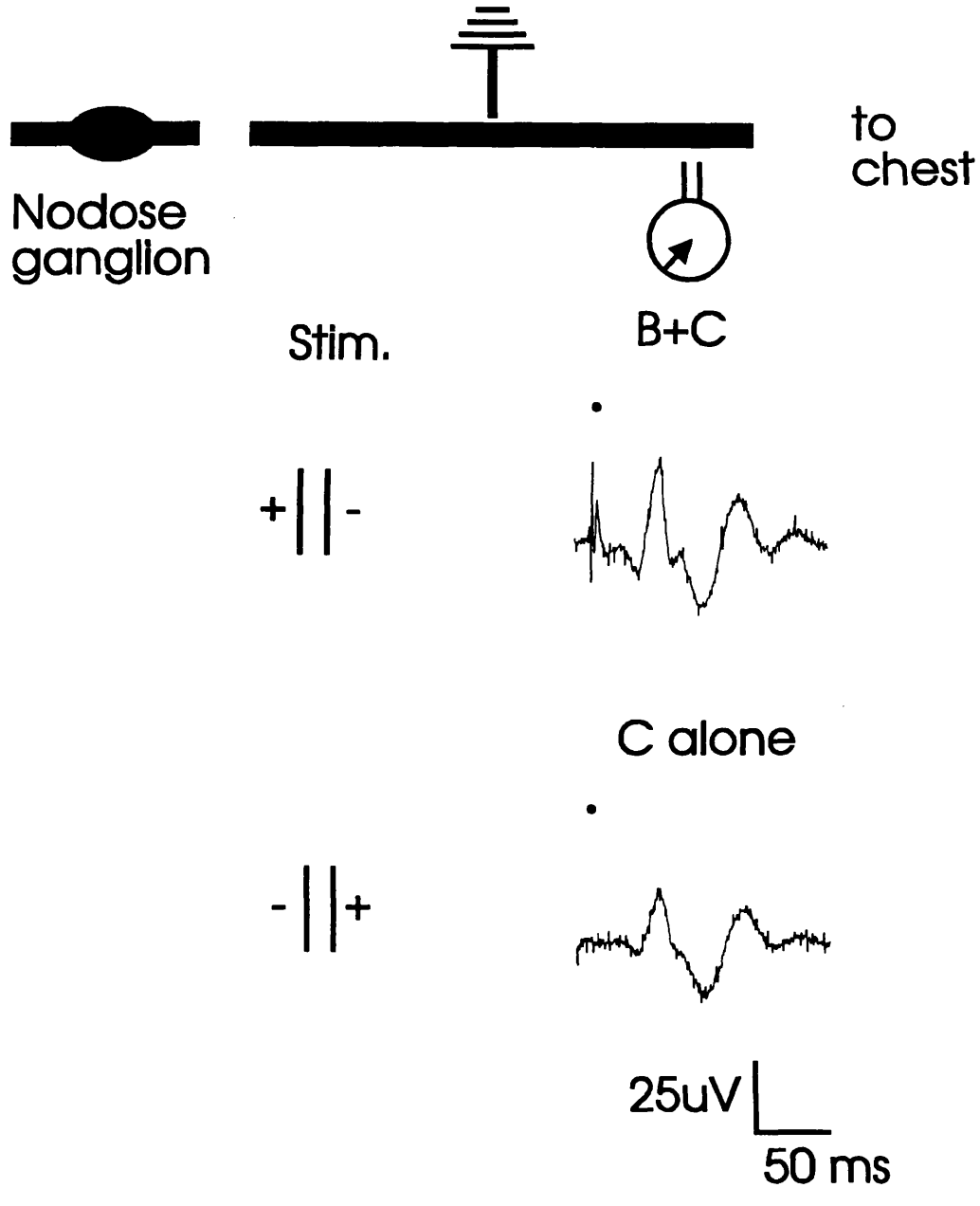
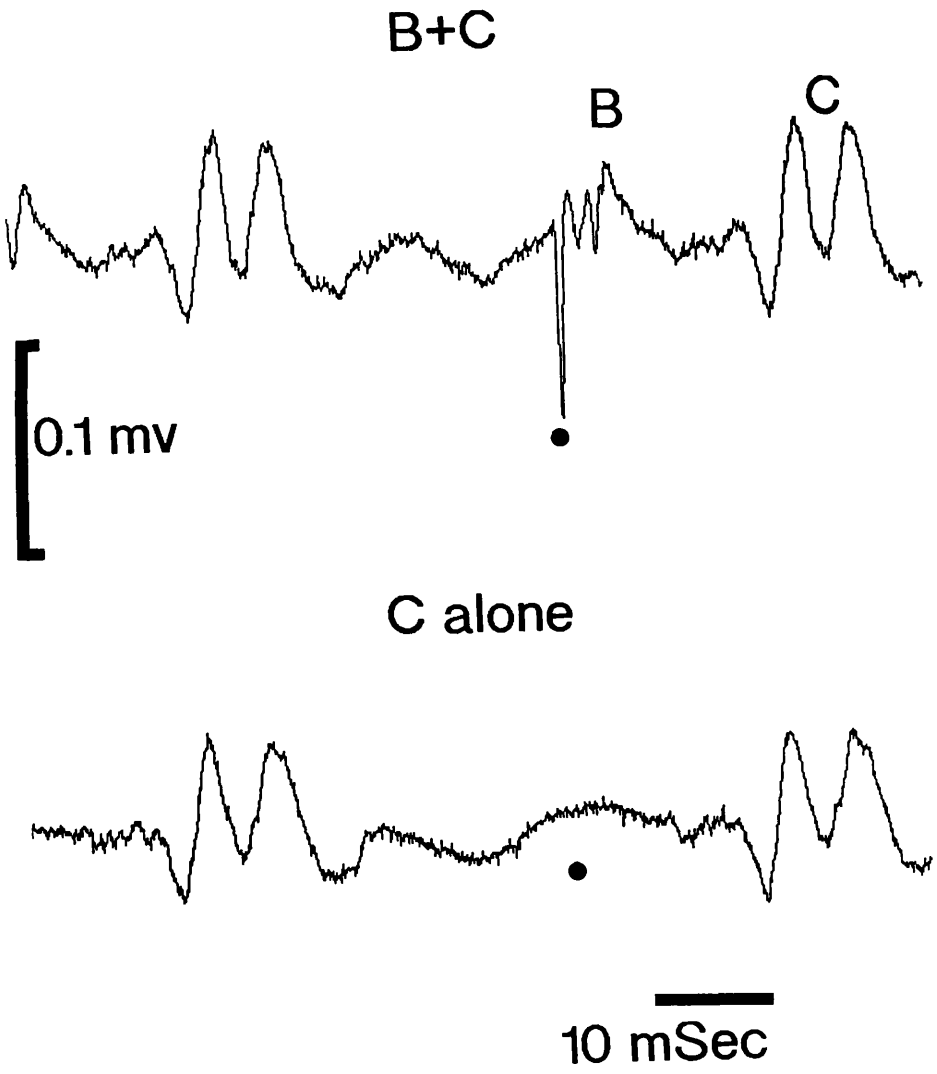

to
chest
Nodose
ganglion
Stim.
B+C
+
-
C alone
-
+
25uV
50 ms

FIGURE 3.2

A recording from the right cranial cardiac branch of the rat. 10 Hz stimulation (dots indicate stimulation times), note the complete abolition of the A and B waves. There is good frequency following by the C-fibres. Compare the latency of the C-wave to the previously recorded C-fibre preganglionic neurones of the rat (27-40 ms).



RAT
Cardiac Branch

FIGURE 3.3

Cardiac C-fibre efferent stimulation compared in three species. Stimulation protocol was 10Hz 1ms 10V for 20 seconds for the cat and rabbit. The rat heart rate change illustrated was obtained by stimulating at 5Hz 1ms 9V. Note the species similarity.

Instantaneous frequency, calculated by taking the reciprocal of the intervals between R wave events of the ECG, was used as an indication of the heart rate. However this only yields accurate values at each R-wave event. Values between events were obtained by comparing the interval between the current time and the last event with the interval between the previous two events. If the interval between the current time and the last event is less than the interval between the previous two events, the current value is taken as the reciprocal of the interval between these two events. However, if it is greater, then the current value is taken as this reciprocal of the interval between the current time and the last event. This results in an instantaneous frequency which decays as 1/t if there are no events for a while (t= interval between the last two events).

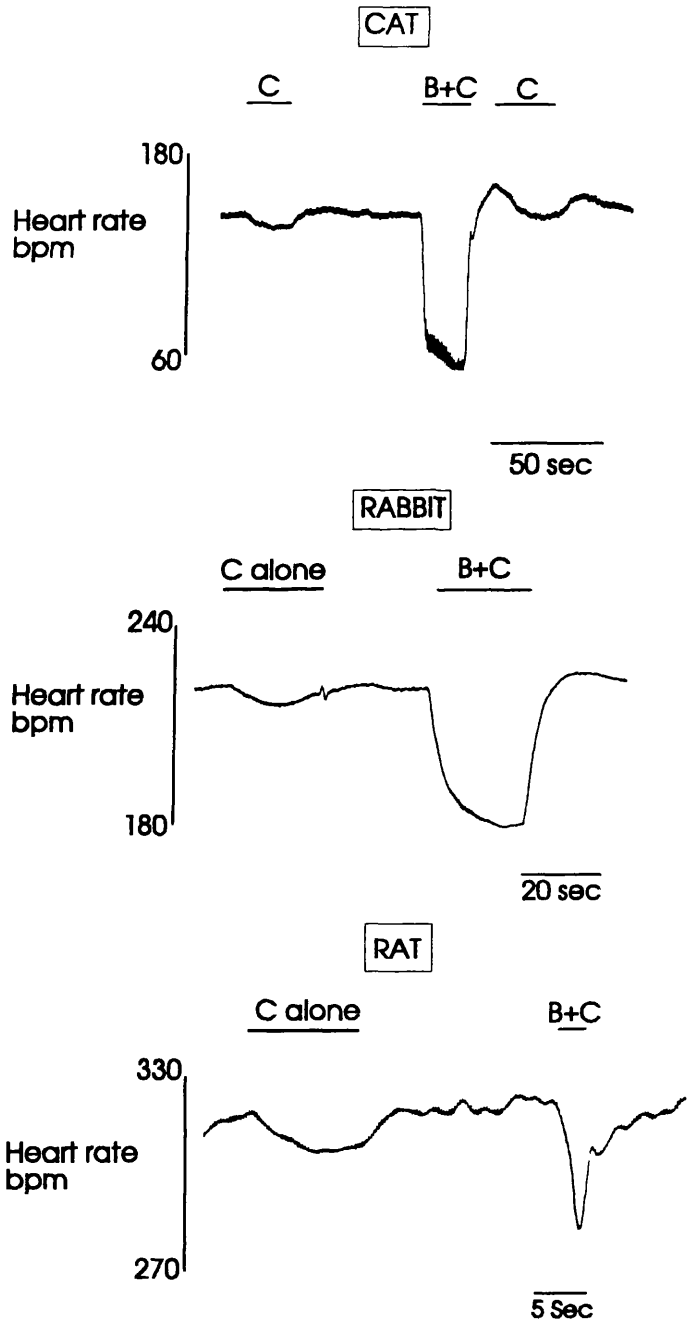

CAT
C
B+C
C
180
Heart rate
bpm
60
50 sec
RABBIT
C alone
B+C
240
Heart rate
bpm
180
20 sec
RAT
C alone
B+C
330
Heart rate
bpm
270
5 Sec

FIGURE 3.4

This illustrates the heart rate changes for a rabbit. Note the C-fibre bradycardia is resistant to high dose hexamethonium.

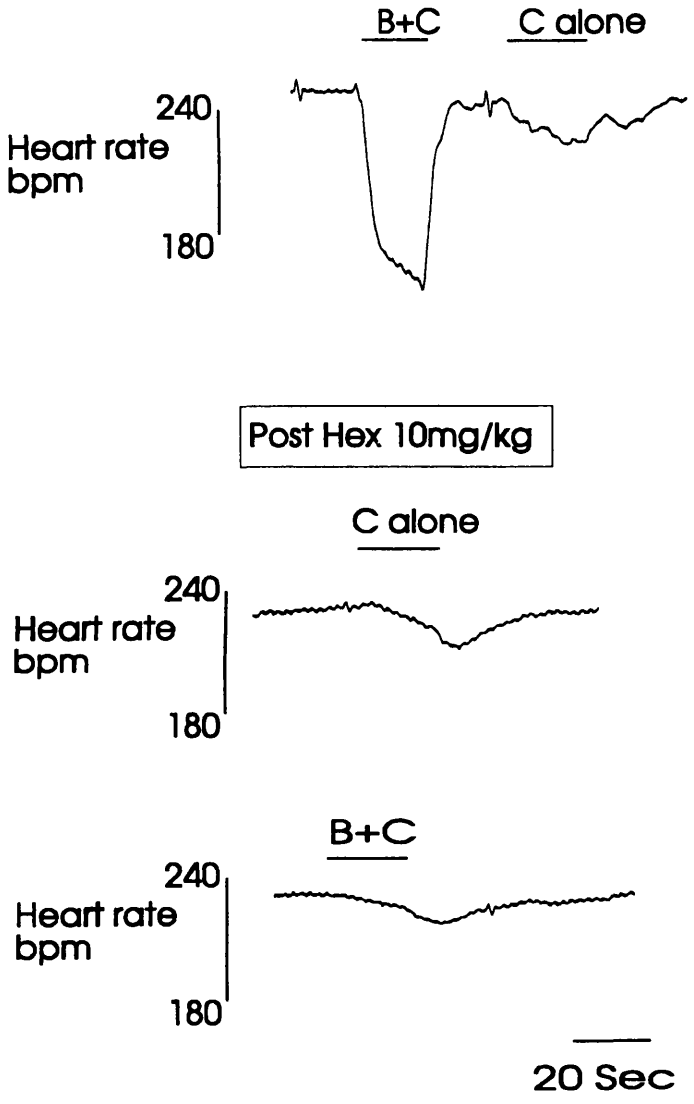

B+C
C alone
240
Heart rate
bpm
180
Post Hex 10mg/kg
C alone
240
Heart rate
bpm
180
B+C
240
Heart rate
bpm
180
20 Sec

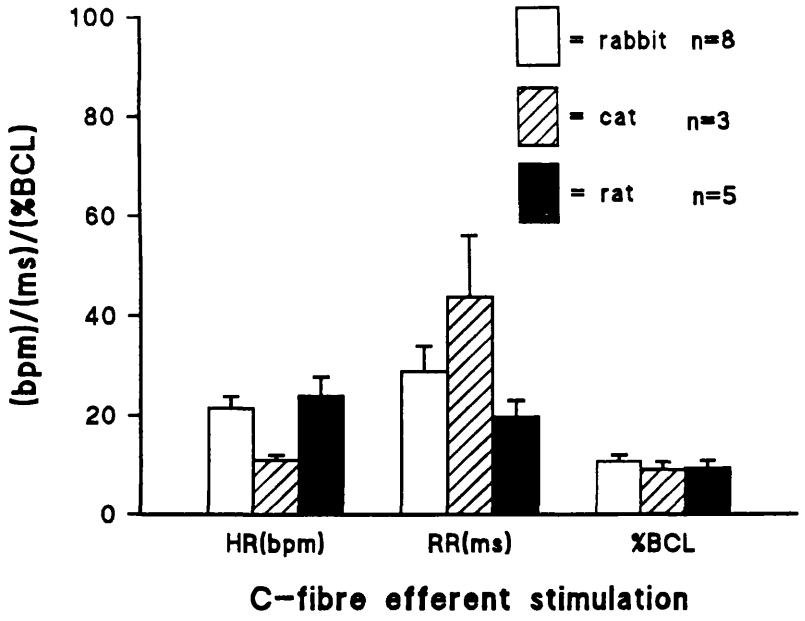
FIGURE 3.5

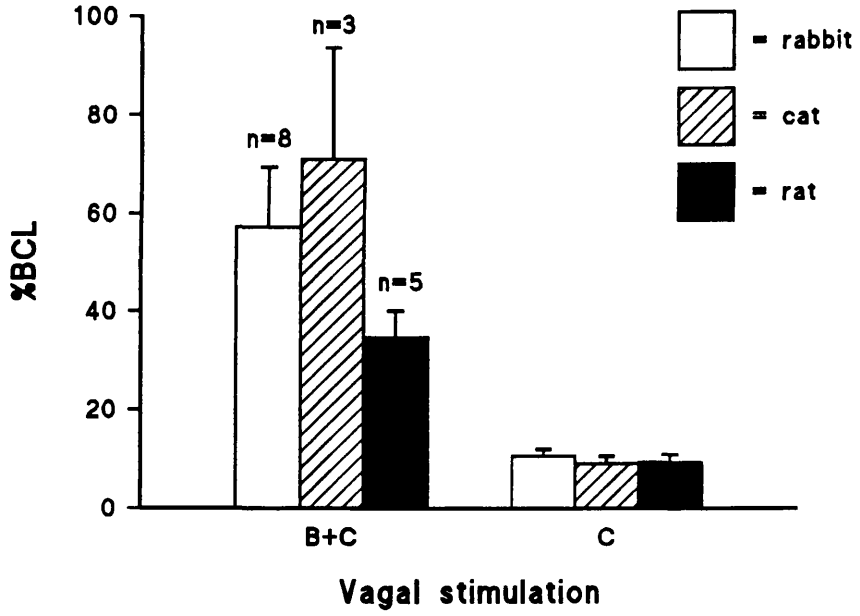
Upper graph: This shows the effect of pure cardiac C-fibre stimulation (10Hz, 1ms, 5-15V for 20 seconds) across the species (n= number of animals,mean±SEM). The stimulation protocol was the same for each species, but the heart rate change was greatest in the rat and the RR interval change was greatest in the cat. Neither of these measures are appropriate for comparing species with differing intrinsic pacemaker rates. When the lengthening of the cardiac cycle is expressed as a percentage of the basic cycle length (%BCL) for that species (atenolol treated vagotomized) then it is clear that there is no species difference.

Middle graph: The constancy of the C-fibre effect is in contrast to the B-fibre effect. 10 Hz stimulation is a submaximal frequency for B-fibres (see central recordings), however it may represent an optimal frequency for C-fibre efferents. Maximal B-fibre stimulation will cause cardiac arrest.

Lower graph: The effect of hexamethonium in the rabbit. The interesting feature here is the equalization of the effect of B+C to C alone post hexamethonium. The more potent nicotinic antagonist chlorisondamine was also tested (n=3) at high dose (2.5-5mg/kg) and this also failed to block the C-fibre bradycardia.

R-R intervals were constructed by plotting the time difference between successive R-wave events against time; the individual points were then joined together without smoothing.


100
80
60
40
20
0
(bpm)/(ms)/(%BCL)
= rabbit n=8
= cat n=3
= rat n=5
HR(bpm)
RR(ms)
%BCL
C-fibre efferent stimulation


100
80
60
40
20
0
%BCL
n=8
n=3
n=5
= rabbit
= cat
= rat
B+C
C
Vagal stimulation

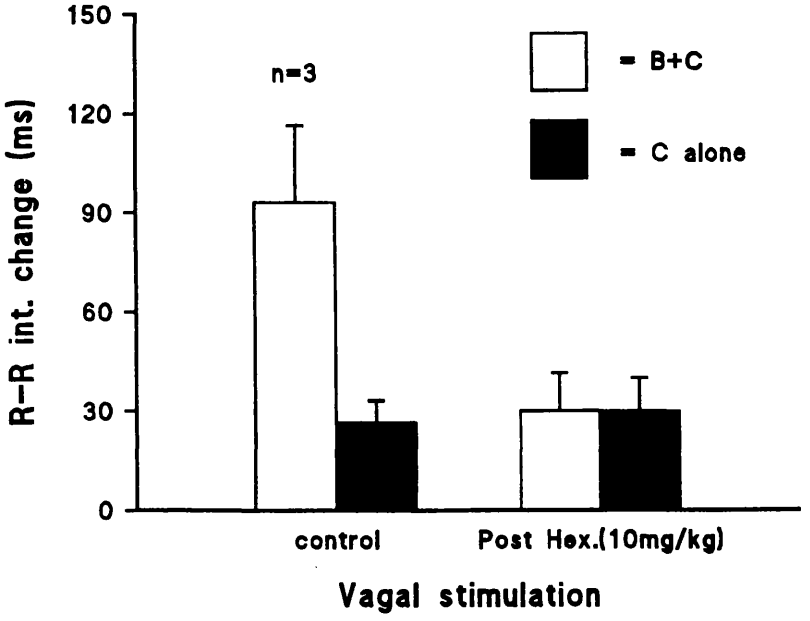
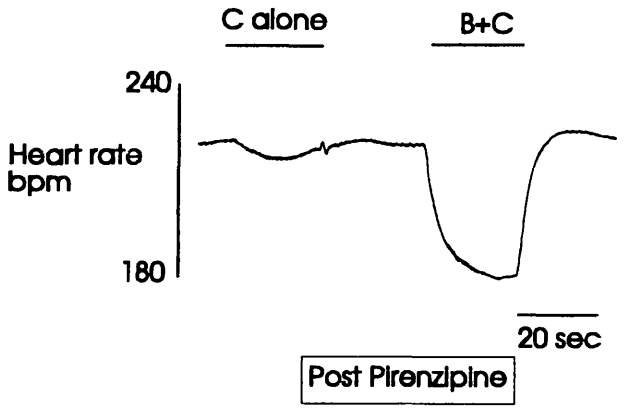

150
120
90
60
30
0
R-R int. change (ms)
n=3
= B+C
= C alone
control
Post Hex.(10mg/kg)
Vagal stimulation

FIGURE 3.6

Effect of pirenzepine (400nmol/kg) on the rabbit bradycardia.

There is complete block of C-fibre efferents and partial block of B+C efferents. The use of pirenzepine as an M_1-muscarinic antagonist is complicated by the fact that it will also block M_2 receptors at high dose. Second pirenzepine was noted to have a very short duration of action which was dose dependent. This makes cumulative dose response curves unreliable if this time dependency is ignored. In the rabbit the effect of pirenzepine was tested at 60 seconds post i.v. injection. In the dose range 50-400nmol/kg the blocking action was observed to last between 5-15 minutes. Since these experiments compare B fibres with C-fibres the experiment contains an inbuilt control for M_2 receptors. Pirenzepine consistently produced complete block of C-fibre efferents at a dose which only partially blocked B+C fibre efferents (n=5 animals). The results may be explained by postulating M_1 ganglionic transmission for C-fibre preganglionic neurones and M_2 transmission for B-fibre postganglionic neurones. Alternatively the dose difference may reflect a preganglionic fibre number difference. It is not known what the B:C cardiac preganglionic ratio is.


C alone
B+C
240
Heart rate
bpm
180
20 sec
Post Pirenzipine

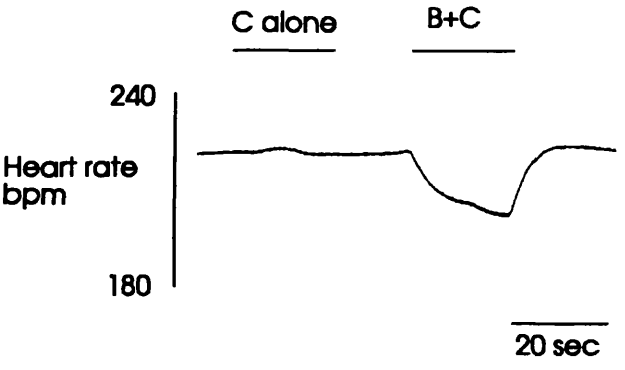
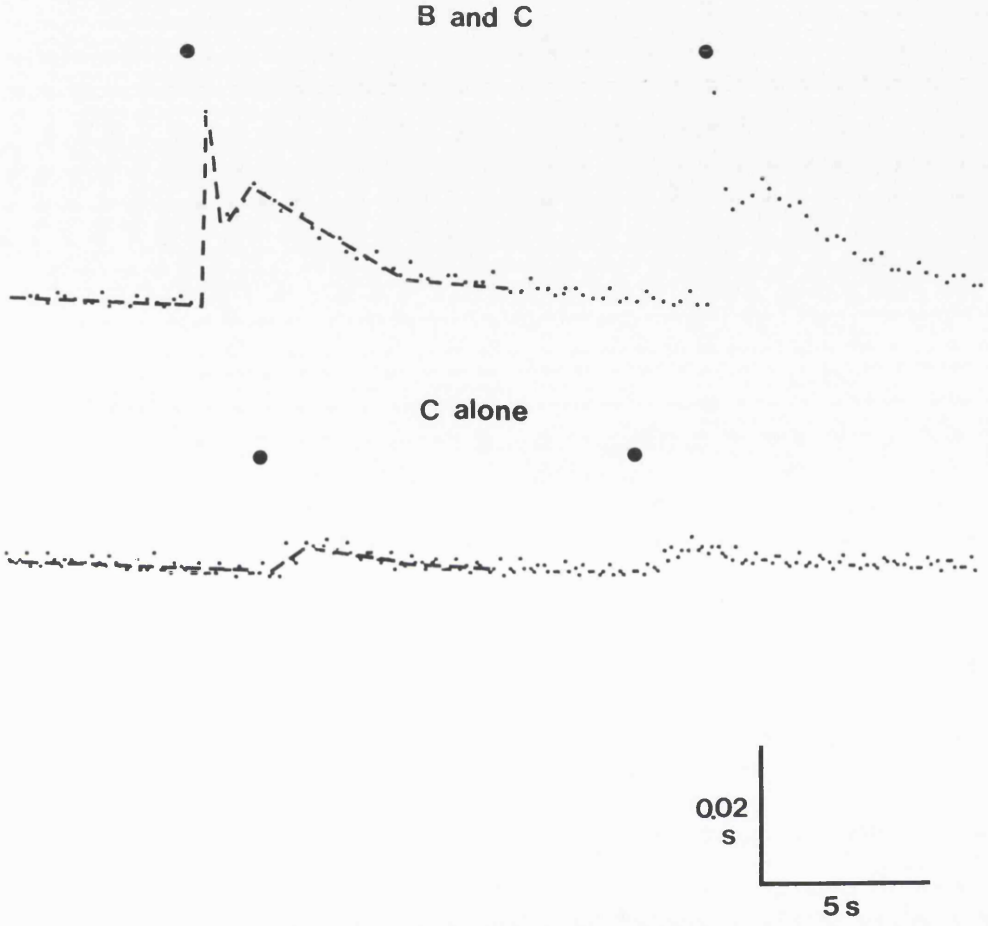

C alone
B+C
240
Heart rate
bpm
180
20 sec

FIGURE 3.7

Upper panel: Single shock delivered to the vagus of a rabbit (RR interval plot;the xaxis is time, the yaxis is RRinterval). This compares B+C to C alone.

Lower panel: Taken from Brown & Eccles (1934), a single shock (B+C) to the right vagus of the cat. The data is plotted according to Donders (1868), on the yaxis is plotted the lengthening of each cycle expressed as a percent of the control or basic cycle length (%BCL) and on the xaxis: the interval that lies between the vagal stimulus and the end of that cycle. This permits a calculation of the latency of the pacemaker's response to vagal stimulation.

Note the double inhibitory waveform and the species similarities, and note that a single shock to C-fibre efferents alone resembles the second inhibitory wave of Brown & Eccles (1934).


B and C
C alone
0.02
s
5 s

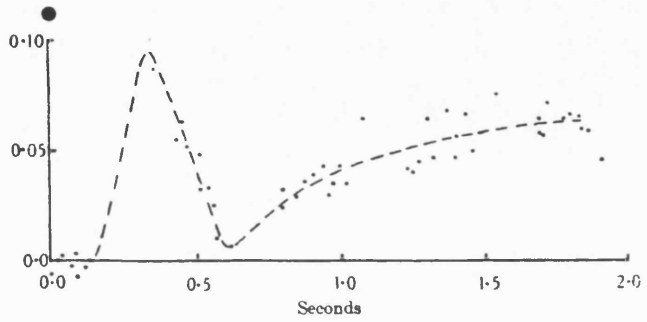

0·10
0·05
0·0
0·0
0·5
1·0
1·5
2·0
Seconds

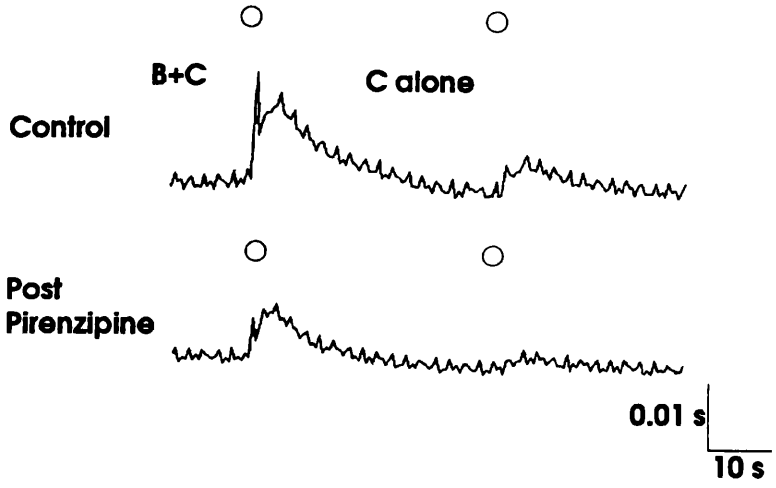
FIGURE 3.8

Comparison of a single shock delivered to the decentralized vagus of the rabbit. The stimuli are triggered to land in a similar phase of the cardiac cycle (100ms post R-wave). Note that a pure B stimulus evokes both primary and secondary waves. Note the effect of recruiting C-fibres by increasing the voltage actually recruits more B-fibres (the primary wave is bigger). These waveforms are consistent with the hypothesis that the temporal summation of fast nicotinic EPSPs and slow muscarinic EPSPs shape the response of the pacemaker to vagal stimulation;and that C-fibre efferents utilize muscarinic transmission at the ganglion.

The second inhibitory wave was found to be resistant to hexamethonium and sensitive to pirenzepine in the rabbit.

100nMol/kg pirenzepine administered and ECG triggered single shocks administered every 40 seconds B+C alternating with C alone. This proved to be a useful means of studying the pharmacology of pirenzepine, and in addition the single shocks with anodal block (0.025 Hz) had minimal deleterious effect upon the nerve. These pirenzepine experiments suggest that C-fibre efferents utilize M_1-muscarinic transmission at the ganglion.


C alone
B alone
B+C
0.01 s
10 s


B+C
C alone
Control
Post
Pirenzipine
0.01 s
10 s

4 Activity of medullary respiratory cells during the pulmonary chemoreflex in the anaesthetized rabbit

4.1 Introduction

"Supposing we succeeded by one means or another in maintaining an animal in a state of steady respiratory chemistry, without at the same time having the volume of the lung affected by breathing movements. In that situation the usual stimuli which arise through volume changes would not be acting on the medulla. If in these circumstances section of the vagi still reduced the rate of breathing, this would indicate that before vagal section some other stimulus must have been transmitted in the vagi, accelerating respiration".

Breuer (1868)(Transl. E.Ullman 1970)

Dr. Breuer's logically conceived experiment was performed with unidirectional ventilation in rabbits. This is the first description and proof of a pulmonary vagal excitatory drive to respiration. In 1889 Henry Head described a "paradoxical reflex" in rabbits, which consisted of a reversal of the usual Hering-Breuer inhibitory response to lung inflation, when the vagi were rewarming after being placed in Gad's cooling thermode. "Head's paradoxical reflex" has been a source of considerable confusion for over a century (Coleridge & Coleridge 1984). This appears to be due to the fact that subsequent investigators have either disregarded or not read Breuer's and Head's original papers. The "gasp reflex",a brief and powerful inspiratory burst, is often equated with Heads paradoxical reflex (Widdicombe 1954b; Reynolds 1962). It is quite clear however, both from Head's description and the accompanying plates, that the vigour of diaphragmatic contraction during the paradoxical inspiration in rabbits was the same as that of a normal inspiration. It is highly probable that pulmonary C-fibres mediate both Breuer's tonic excitatory drive and Head's paradoxical reflex. The latter survives vagal cooling to as low as 3°C (Whitteridge & Bulbring 1944). It is reasonable to propose that pulmonary C-fibres in rabbits are activated by lung inflation as they are in cats (Coleridge et al. 1968; Armstrong & Luck 1974) and dogs (Coleridge et al. 1965; Coleridge & Coleridge 1977b., Kaufman et al. 1982). Widdicombe (1964) has demonstrated Head's reflex in rabbits when the inflation reflex and the deflation reflex are blocked by vagal

cooling to 5°C. Widdicombe (1964) has ascribed Head's reflex to the action of myelinated "irritant receptors" which produce maintained inspiratory effort when partially blocked by cooling and a gasp reflex when fully warmed. However at 3°C the impulse activity along myelinated fibres is abolished as is the deflation reflex (mediated by Widdicombe's "irritant receptors") (and ironically, proved by Widdicombe himself, 1964). The blocking temperature for myelinated fibres is 6-8°C (Paintal 1965;Franz & Iggo 1968) but many C-fibres will conduct isolated impulses at 0°C (Abbott et al. 1965; Franz & Iggo 1968; Coleridge et al. 1982a).

The pulmonary chemoreflex (to injected PDG or PBG) in the rabbit is unusual because the apnoea is inspiratory, not expiratory like the rat and the cat.(Dawes 1951; Karczewski & Widdicombe 1969; Guz & Trenchard 1971; Miserocchi et al. 1978). This difference prompted Dawes and Comroe (1954) to exclaim "species differences are quite extraordinary" with regard to the pulmonary chemoreflex. The source of this difference may be peripheral or central. Is PBG activating the same receptor in the lung of the rabbit?,or is the brainstem of the rabbit uniquely organized with respect to this reflex? Direct recordings obtained from pulmonary C-fibres in the rabbit clearly demonstrate that these receptors are sensitive to PBG and respond with short latency to right atrial injections of PBG (Russell & Trenchard 1979; Matsumoto et al. 1992). The pulmonary chemoreflex in rabbits is mediated by pulmonary C-fibres; this has been demonstrated by procaine application to the vagus (Matsumoto et al. 1992). Therefore the source of the unique inspiratory apnoea of the rabbit is not peripheral. Whatever the actual mechanism, the pulmonary chemoreflex of rabbits is decidedly odd because a powerful bradycardia is maintained in the face of an "inspiratory apneusis" and concomitant chest wall expansion. This is entirely at odds with Anrep's hypothesis (Anrep et al. 1936) of respiratory sinus arrhythmia. However the recent findings of Daly (1991) in cats, concerning the lack of respiratory modulation of pulmonary C-fibre evoked bradycardia, point to remarkable species similarity. It seems reasonable therefore to record the respiratory neurones of the rabbits medulla during the pulmonary chemoreflex and then compare this pattern to that previously recorded in the cat and rat (chapter 2).

4.2 Methods

4.2a Anaesthesia

Female New Zealand White rabbits (weighing between 1.5-2.2kg) were anaesthetized with Urethane (1.4g/kg) administered into a marginal ear vein.

4.2b Preparation of the rabbits

The right femoral artery and vein were cannulated for BP recording and administration of drugs. The trachea was cannulated and the animals artificially respired. A cannula was advanced towards the right atrium via the right external jugular vein. The side port and occluded end of this cannula ensured minimal projection of PBG into the inferior vena cava. Correct positioning at the cavo-atrial junction was confirmed at post-mortem. Monitoring of physiological variables was conducted as described in chapter two. Two small holes were drilled near the infra-orbital ridge of the maxillae bilaterally. This allowed placement of the skull in the stereotactic frame. When the animal was prone and secured in the frame, a midline incision was made from bregma to cervical spine 7. The phrenic nerve was dissected, cut peripherally and desheathed using a dorsolateral approach. The occipital craniotomy and preparation of the medulla was the same as that described for the cat in chapter 2. Single barrel electrodes filled with pontamine sky blue were used for the recording and histological staining of successful recordings sites (again histological protocol was the same as that described in chapter 2). The medullary sections were analysed with reference to the histological atlas of Meesen & Olszewski (1949).

4.2c Experimental protocol

Medullary neurones were classified as respiratory by their phase relationship to the phrenic nerve. If the recording was stable 0.1- 0.25 ml of 200μg/ml PBG was injected into the right atrium.

RESULTS 4.3

Rabbits (n=14) demonstrated a powerful and distinctive cardiorespiratory pattern during the pulmonary chemoreflex (fig 4.1). It closely resembles Head's paradoxical reflex (fig 4.2). The responses of neurones to bolus injections of PBG into the right atrium (10-25μg/kg, 200μg/ml) were all of short latency but long duration 5-60 seconds. Stage II expiratory cells (10/11) were inhibited and usually this involved complete inhibition, respiratory rhythm then cycled between inspiration and post-inspiration (fig 4.4). All seven post-inspiratory cells were excited by the stimulus (fig 4.5), the only cell type to actually increase its firing rate in response to PBG. 9/13 inspiratory cells were inhibited (fig 4.6), showing decreases in both firing frequency and inspiratory time.

FIGURE 4.1

The pulmonary chemoreflex of three mammalian species. The top trace is from cat,the middle trace from rat, and the bottom trace from rabbit. Note the "inspiratory apnoea" of rabbits and the expiratory apnoea of rat and cat.

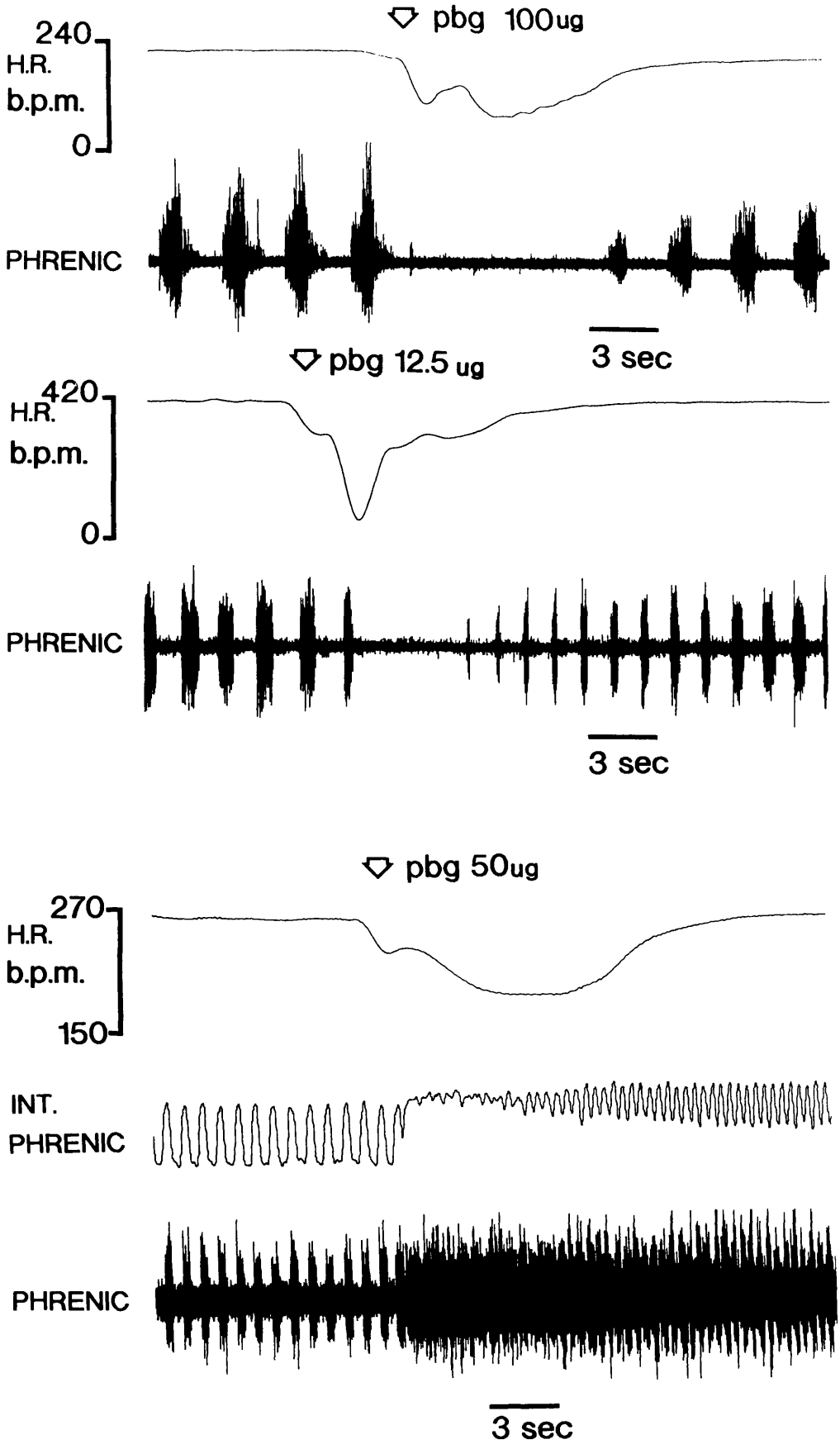

pbg 100ug
240
H.R.
b.p.m.
0
PHRENIC
3 sec
pbg 12.5 ug
420
H.R.
b.p.m.
0
PHRENIC
3 sec
pbg 50ug
270
H.R.
b.p.m.
150
INT.
PHRENIC
PHRENIC
3 sec

FIGURE 4.2

The original plate from Henry Head's paper of 1889;the top three traces of Head are combined with an integrated phrenic nerve recording from a PBG experiment (records have been aligned with similar time frames). At the top arrow Head inflated the lungs of a rabbit whose vagi had been cooled. Note the slow deep breathing and the "paradoxical" inspiratory response followed by tachypnoea. Observe the close similarity to the pulmonary chemoreflex in a rabbit with fully functioning vagi.

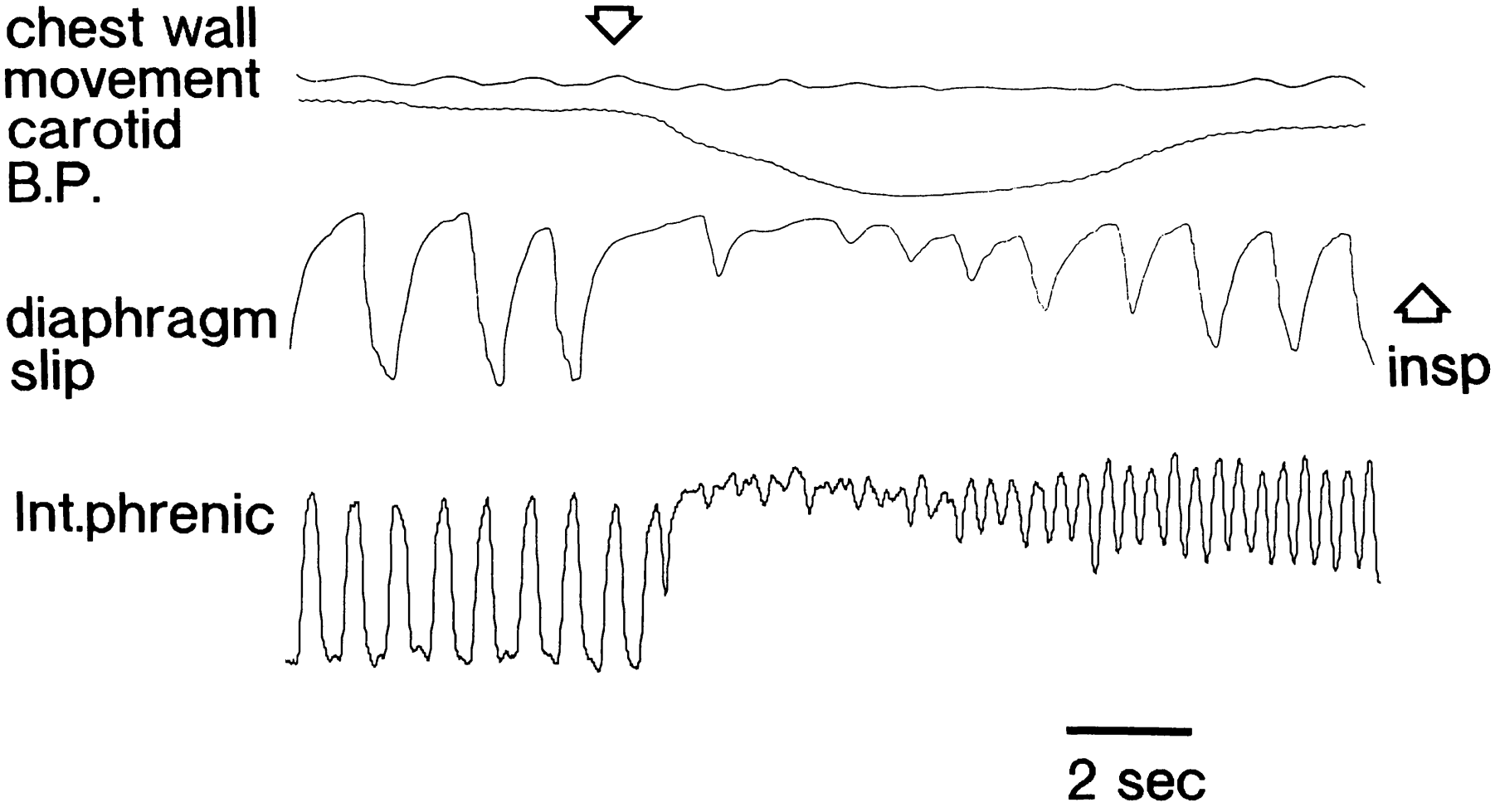
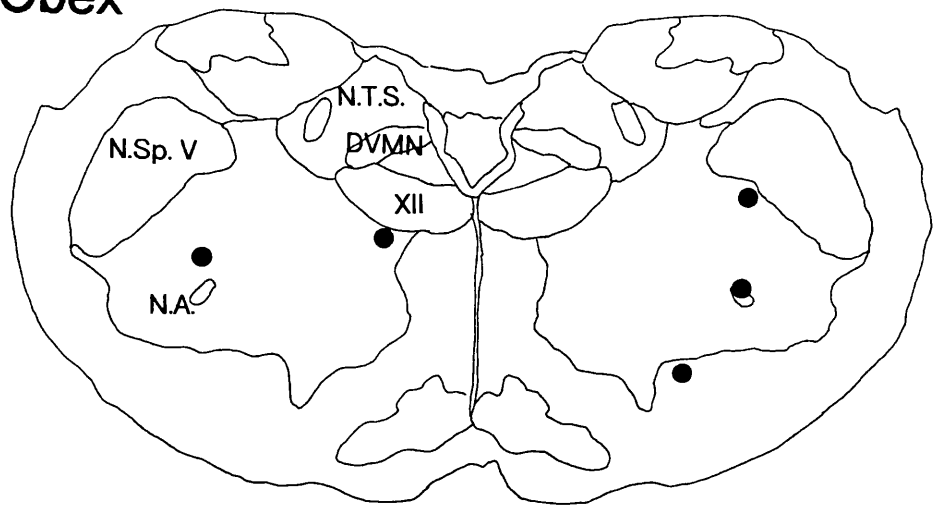

chest wall
movement
carotid
B.P.
diaphragm
slip
insp
Int.phrenic
2 sec

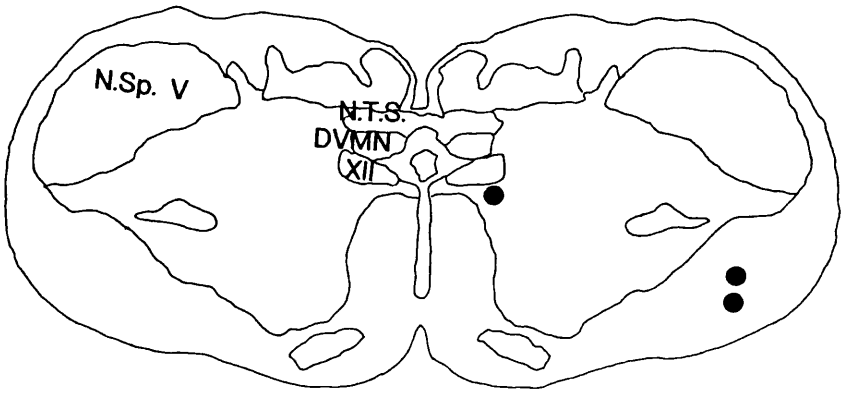
FIGURE 4.3

The medulla of a rabbit demonstrating the location of some of the recording sites of the respiratory neurones. Note one recording was obtained from the compact nucleus ambiguus.

Obex



-1.6



-2.2

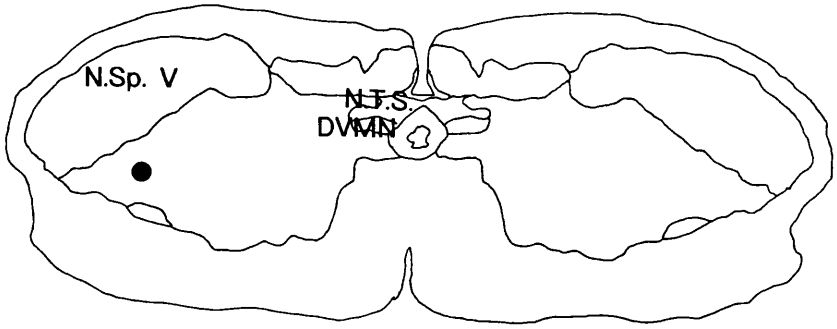


FIGURE 4.4

Example of a stage II expiratory cell inhibited by PBG (50μg) at short latency. Note there appears to be dramatic post-inspiratory drive to the diaphragm.

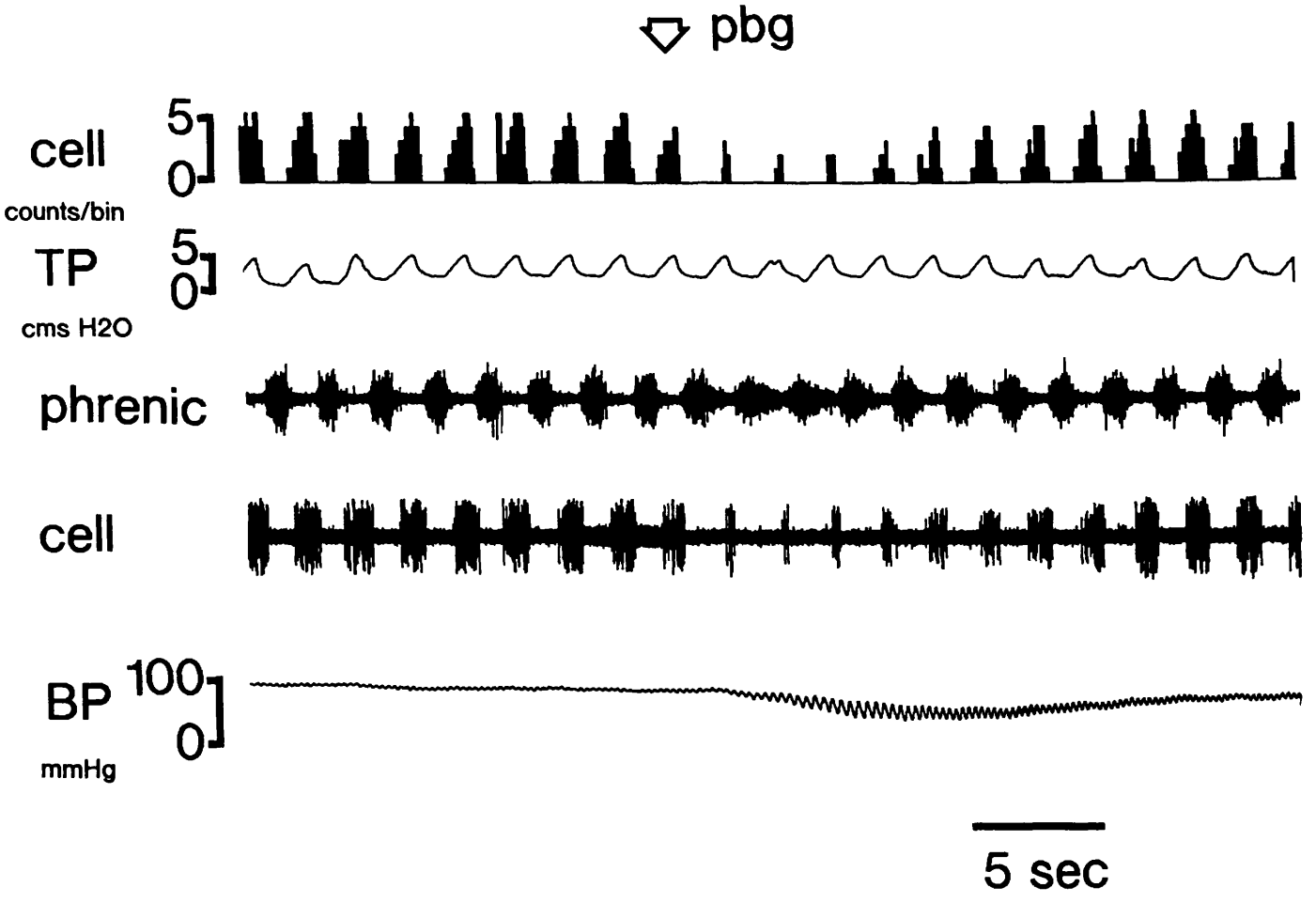

pbg
cell
5
0
counts/bin
TP
5
0
cms H2O
phrenic
cell
BP
100
0
mmHg
5 sec

FIGURE 4.5

The excitation of a post-inspiratory cell during the pulmonary chemoreflex of the rabbit. 50μg of PBG was injected into the right atrium at the time indicated by the arrow.

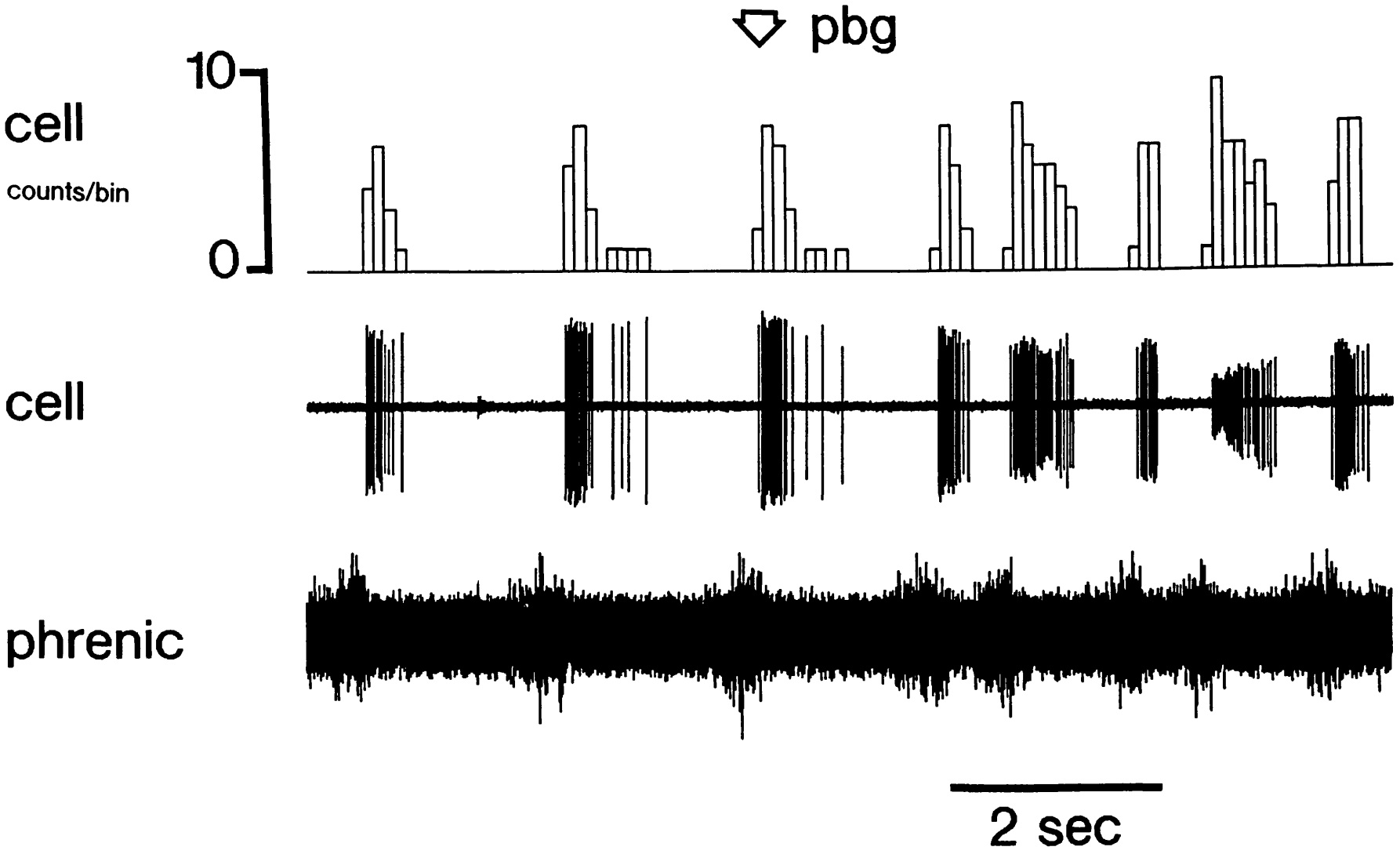

pbg
10
cell
counts/bin
0
cell
phrenic
2 sec

FIGURE 4.6

Inspiratory cell inhibition (50μg PBG injected at arrow) during the chemoreflex of the rabbit.

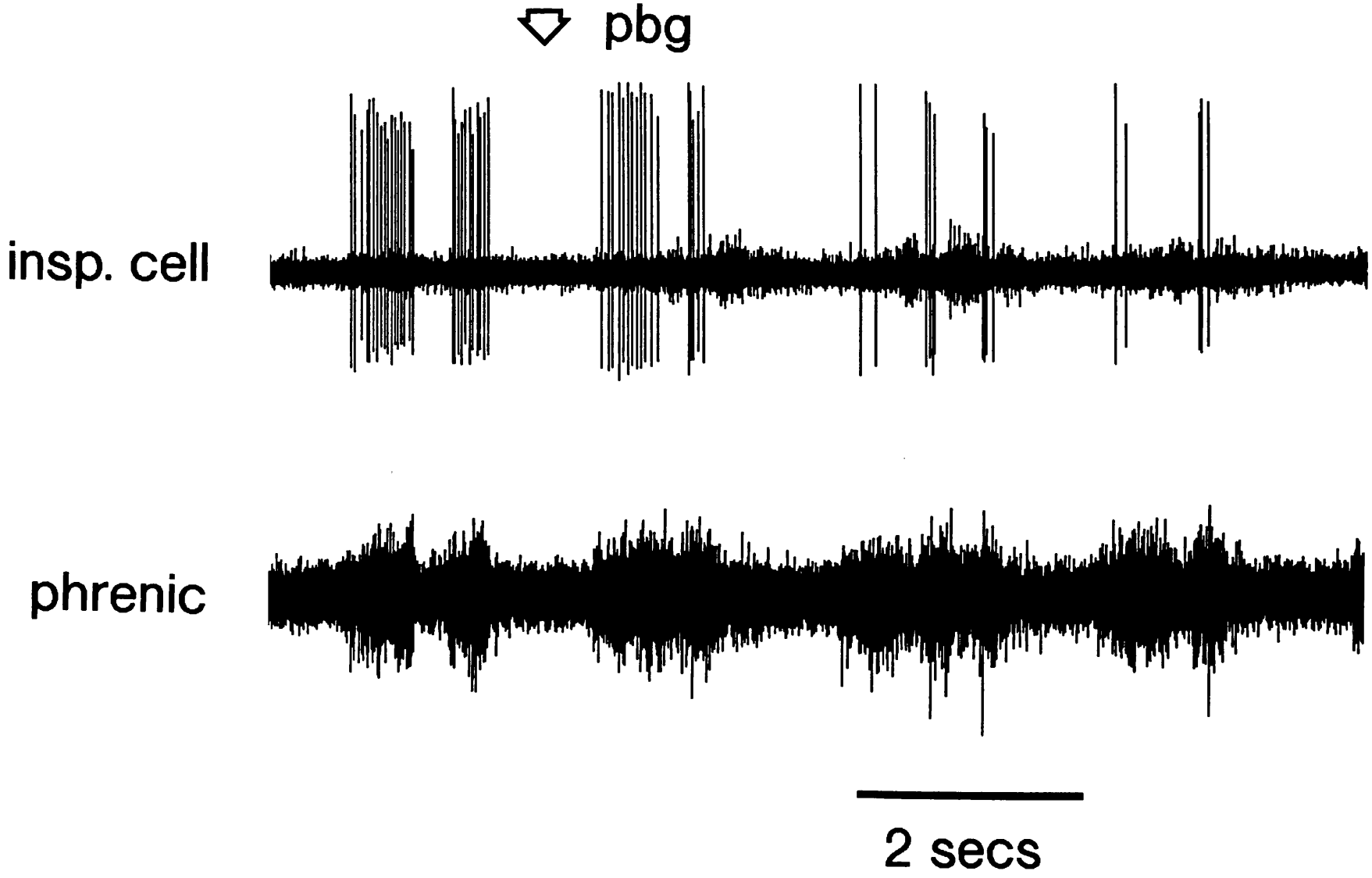
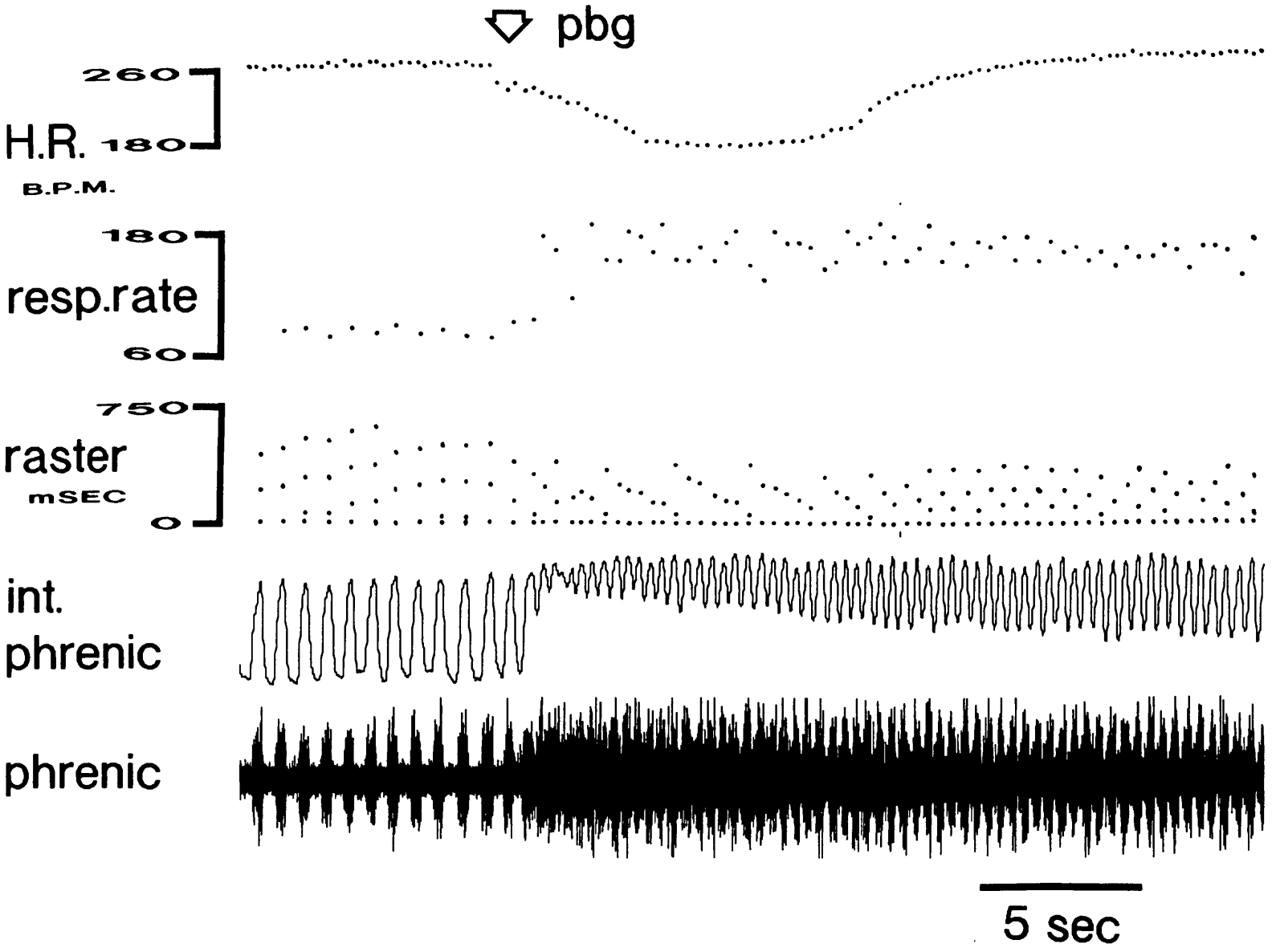

pbg
insp. cell
phrenic
2 secs

FIGURE 4.7

This spontaneously breathing rabbit was interesting because the heart rate and respiratory rate both approached 180/min after bolus injection of 50μg of PBG into the right atrium. A raster was constructed to check for the presence of cardiorespiratory synchrony, because if this is present the cardiac vagal preganglionic neurones involved must be respiratory modulated. The times of the peaks of the integrated phrenic were plotted along the xaxis of the raster and the delay to the next heartbeat plotted along the yaxis. Note that during the chemoreflex there is a 1:1 coupling of heartbeat and breathing. This is powerful evidence for B-fibre cardiac vagal preganglionic involvement in this reflex. If there was synchrony the raster would be a straight line. Instead a saw tooth pattern is observed with a turning point of 330ms (1/180). The presence of an additional tonic desynchronizing discharge neatly explains these findings. Is the C-fibre population the cause of this pattern?


pbg
260
H.R.
180
B.P.M.
180
resp.rate
60
750
raster
mSEC
0
int.
phrenic
phrenic
5 sec

4.4 DISCUSSION

The behaviour of respiratory neurones during the pulmonary chemoreflex in rabbits is in general accord with that previously described in rats and cats. This suggests that species differences are not as great as previously imagined. The great similarity between Head's paradoxical reflex and the pulmonary chemoreflex is explained by the strong post-inspiratory increase that these reflexes manifest. It is suggested that there is neither a peripheral nor a central difference between the reflexes in rabbits and cats. Indeed the only paradoxical feature of Head's paradoxical reflex is that there is no paradox at all (this statement of course, constitutes a failure in the logic of language). It is of particular interest that amongst the various theories of respiratory rhythmogenesis only the three phase theory (Richter et al. 1987) can account for the apparent peculiarity of the rabbits pulmonary chemoreflex. This theory satisfactorily explains the cardioinhibitory changes. The common practice of referring to the inspiratory apneusis of the rabbit's pulmonary chemoreflex is now shown to be incorrect. What appears as apneusis is in reality a fast inspiratory/post-inspiratory cycling of the respiratory rhythm generator with "fusion" of the phrenic impulses and tetany of the diaphragm. The upper limit on the speed of the respiratory rhythm generator or clock is unknown, however the rabbits diaphragm can respond to rapid signalling. Head (1889) noted that if a cut distal end of a phrenic nerve be placed on the beating heart the diaphragmatic slip contracts with the electrical activity of each heartbeat. The question arises: why does the rabbit choose to hold its chest at elevated functional residual capacity (FRC), when other species have expiratory apnoeas? The rise in venous return through what is essentially a Mueller's manoeuvre must be counter productive during this defensive response. Coleridge and Coleridge (1984) have speculated that the tonic diaphragmatic response constitutes a respiratory adaptation. Their argument runs as follows:the chest wall of a rabbit is frail, and there is small FRC at the position of rest; prolonged apnoea in this position might lead to collapse of alveoli, requiring very large inspiratory efforts to reinflate the lungs. This explanation is probably incorrect for two reasons: first, Crosfil and Widdicombe (1961) have compared various respiratory mechanical parameters across species (rat, cat and rabbit), although the rabbit has very compliant lungs there is little difference between the chest wall compliance of

a rat and a rabbit when expressed with respect to body weight. Second, rabbits do exhibit prolonged expiratory apnoea during upper airway stimulation with smoke (Dr. F. Kratschmer 1870). Kratschmer referred to this as an "expiratory tetanus". This yields quite a different pattern in the phrenic nerve of the rabbit to that of lower airway C-fibre stimulation (personal observation). Perhaps it is the classic mistake of a respiratory physiologist to assume respiratory modifications always serve a respiratory role. The tonic drive to diaphragm and chest wall may constitute an important element of the highly developed freezing response of rabbits. It is quite striking to observe how the rib cage and abdomen appear motionless during the pulmonary chemoreflex. This playing dead response may be confusing to predators who finally alight on their prey after a prolonged chase. The J-receptors of Paintal should certainly be stimulated during the pulmonary blood flow increases observed during vigorous exercise (Anand & Paintal 1980).

What is the central mechanism that permits this unique respiratory control in rabbits? The fact that there is a considerable post-inspiratory input to the rabbits diaphragm at rest and during the pulmonary chemoreflex makes the lagomorph invaluable in studies of respiratory rhythmogenesis and control. However the numerous studies in the past that have attempted to quantify respiratory cycle length (inspiratory time, expiratory time) with respect to the rabbits diaphragm or phrenic nerve must have yielded highly misleading data. It is of interest that while the dorsal respiratory group of the cat and dog contains predominantly inspiratory neurones and only 4-6% expiratory neurones (Cohen & Feldman 1984;Berger 1977) the dorsal respiratory group of the rabbit is reported to contain 44% expiratory neurones (Jiang et al. 1986;Jiang & Shen 1991). This is also the case when vagal afferents are eliminated (Wei et al. 1984). In addition the dorsal medulla of the rabbit contains post-inspiratory cardiac vagal preganglionic neurones which have B-fibre axons and are indistinguishable from their counterparts around the nucleus ambiguus (Jordan et al. 1982). It has been assumed that these represent neurones that failed to migrate ventrolaterally during embryogenesis. Is the dorsal medulla of the rabbit the source of the powerful post-inspiratory drive to the diaphragm? Have the neighbouring cardiac vagal preganglionic neurones assumed the firing characteristics of more ventrally placed neurones, not because they failed to migrate, but that their dendritic fields have become influenced by the powerful enveloping post-inspiratory

activity? In this regard the rabbits diaphragm more closely resembles a branchiomeric structure than the diaphragm of a cat. The degree of preceding speculation simply reflects an overwhelming ignorance concerning the medulla of a fascinating creature.

5. Role of the hypothalamic defence area during the pulmonary chemoreflex in the anaesthetized cat

5.1 Introduction

The interaction between the pulmonary chemoreflex and the hypothalamic defence area has been analysed previously (Achari & Downman 1970). Unfortunately the design of their experiments has led to possibility of a false positive. Using 20 second preconditioning HDA stimulation, PDG was injected during the fully developed cardiovascular response and the response to PDG was noted to be absent. However the response of pulmonary C-fibres to injected PBG depends mainly on its mean pulmonary concentration and the time it spends in the pulmonary capillary (Anand & Paintal 1980). Since HDA stimulation alters pulmonary transit time dramatically, is the abolition of the response to PDG a real interaction of reflexes or simply drug washout? Also if there is an interaction if reflexes where does the interaction take place?
The following experiments were designed to answer these simple questions.

5.2 Methods

5.2a Preparation of the cats

The same cats that were used in chapter 2 were also used to study the HDA ($n=40$). Details on preparation and anaesthesia can be found there. Access to the hypothalamic defence area was provided by extending the midline skin incision rostrally and after clearing the bone, using a dental drill to make a hole in the skull centred on a point 10mm rostral to the stereotactic zero. The edges of the bone were plugged with bone wax and the underlying dura incised.

5.2b Hypothalamic electrodes:

Bipolar concentric steel electrodes were used to stimulate the hypothalamus. These had bare tips of about 250μm.

5.2c Histology:

At the end of each experiment this area of brain was also removed and processed histologically in a manner similar to that described for the medulla (Chapter 2). After locating the sites of stimulation they were plotted onto standard sections of the cat hypothalamus taken from an atlas by Snider & Niemer (1961).

5.2d Experimental protocol

The hypothalamic defence area was explored until the typical defence pattern could be elicited by electrical stimulation (typically this was: 25V,70Hz;1mS). The pattern included a sustained rise in BP, tachycardia, pupillary dilatation, piloerection and phrenic nerve activity change. The locus was tested for baroreflex inhibition using an aortic balloon to raise pressure. The locus was then tested for pulmonary C-fibre interaction. A control injection of PBG was performed 0.2ml (200ug/kg). Repeated injections of PBG were never less than 300 sec apart, to avoid the complications of tachyphylaxis.The cardioinhibitory stimuli were matched to produce approximately equal R-R interval changes. When combined with HDA stimulation the PBG was delivered first then HDA 3 sec later for approximately 10 seconds . With this approach the pulmonary C-fibres were excited ahead of any cardiovascular changes elicited by HDA.

5.3 Results

First, the findings of Achari & Downman (1969) were confirmed see fig 5.2. This approach was then abandoned, in favour of the one already outlined i.e. one in which the HDA stimulation was started during a developing pulmonary chemoreflex (fig 5.3). It is not possible to group the results of these experiments since hypothalamic stimulation produced a wide array of different effects in different animals. This is to be expected in view of the crude nature of the experiment. It became of interest to compare a particular HDA's effect upon the cardioinhibition of the baroreflex and the pulmonary chemoreflex when it was observed that some sites could produce differential effects. The stimuli were chosen to produce an approximately equal sized bradycardia fig 5.1. The histology of some of the sites are displayed in fig 5.8. The HDA inhibition of NTS cells receiving

pulmonary C-fibre input is shown if fig 5.7. Evidence of clear inhibition was obtained in the two animals in which it was tested.

FIGURE 5.1

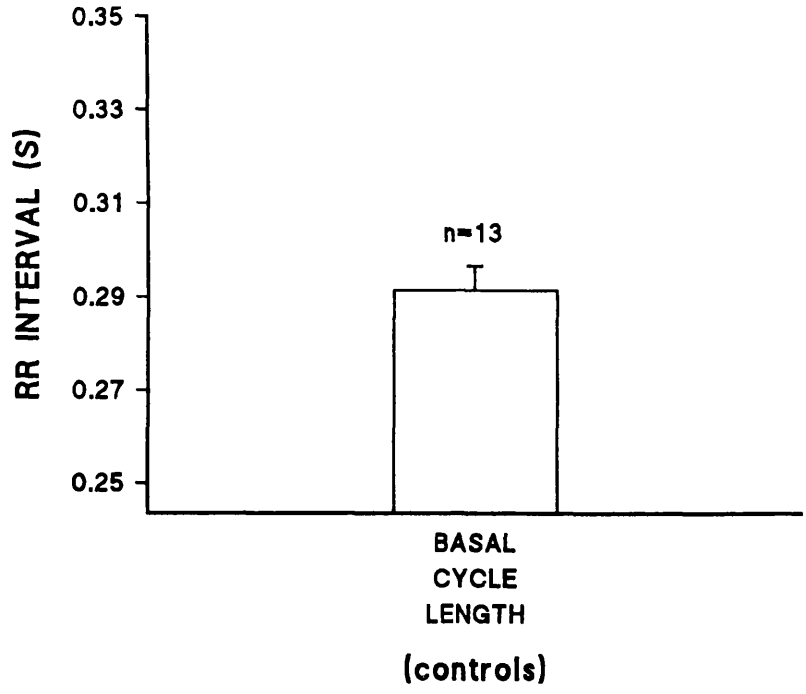
Upper graph

The resting cardiac cycle length (mean±SEM) for the group of animals studied. (n= number of animals)

Lower graph

The cardioinhibitory reflexes: both the baroreflex (baro) and the pulmonary chemoreflex (chemo) produced about a doubling of the cardiac cycle.

These graphs are presented to simply illustrate the constancy of the baseline and the similarity of the stimuli.


0.35
0.33
0.31
0.29
0.27
0.25
RR INTERVAL (S)
n=13
BASAL
CYCLE
LENGTH
(controls)

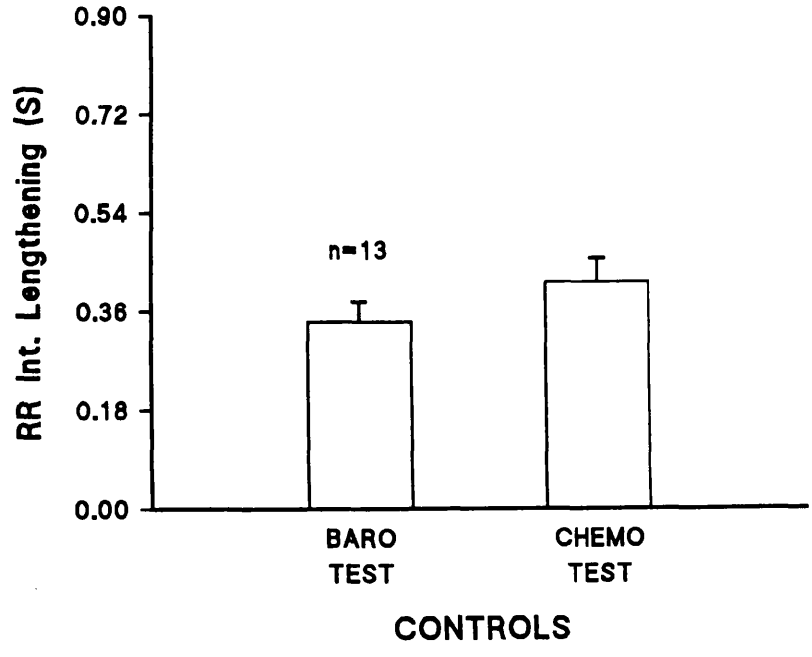

0.90
0.72
0.54
0.36
0.18
0.00
RR Int. Lengthening (S)
n=13
BARO
TEST
CHEMO
TEST
CONTROLS

FIGURE 5.2

Top panel: superimposed control PBG response, with PBG response during HDA stimulation. The lower traces are the control. The HDA is stimulated (70 Hz,1ms,28V) for about 10 seconds prior to PBG injection. Note the complete block of the pulmonary chemoreflex.

Lower panel: control HDA stimulation. Note the lack of block of the baroreflex and the post stimulation bradycardia which is respiratory modulated.

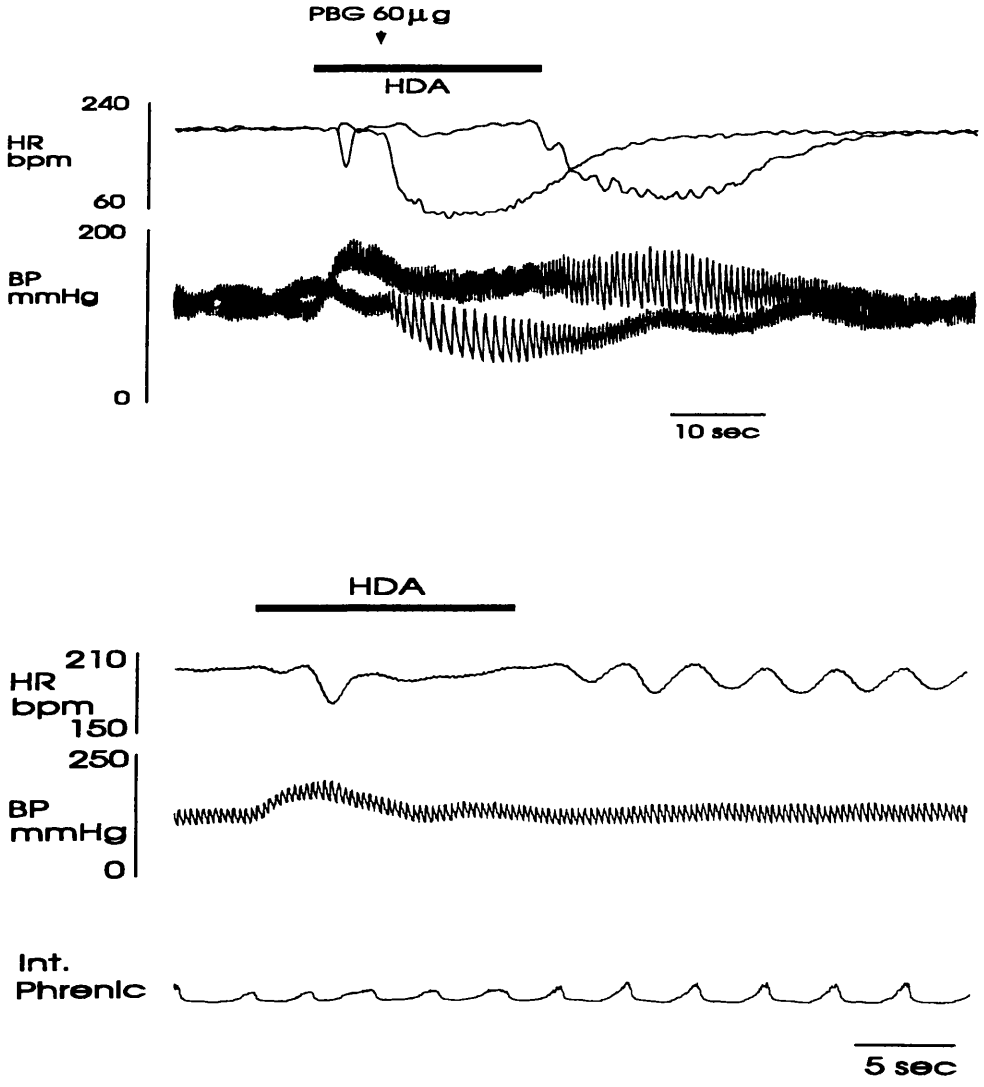

PBG 60µg
HDA
240
HR
bpm
60
200
BP
mmHg
0
10 sec
HDA
210
HR
bpm
150
250
BP
mmHg
0
Int.
Phrenic
5 sec

FIGURE 5.3

The stimulation of a HDA which does not block the baroreflex but completely blocks the pulmonary chemoreflex. Note that when the chemoreflex is blocked the cardiac vagal preganglionic neurones are not refractory to a baroreflex stimulus. The HDA effect could be acting at the NTS, since the afferents project to different regions; or there could be two separate populations of cardiac vagal preganglionic neurones dedicated to specific reflexes. Although it may be postulated that the combination of two excitatory inputs have outweighed the single inhibitory input from the HDA, this seemed less likely when this HDA was tested upon the baroreflex alone (next figure).

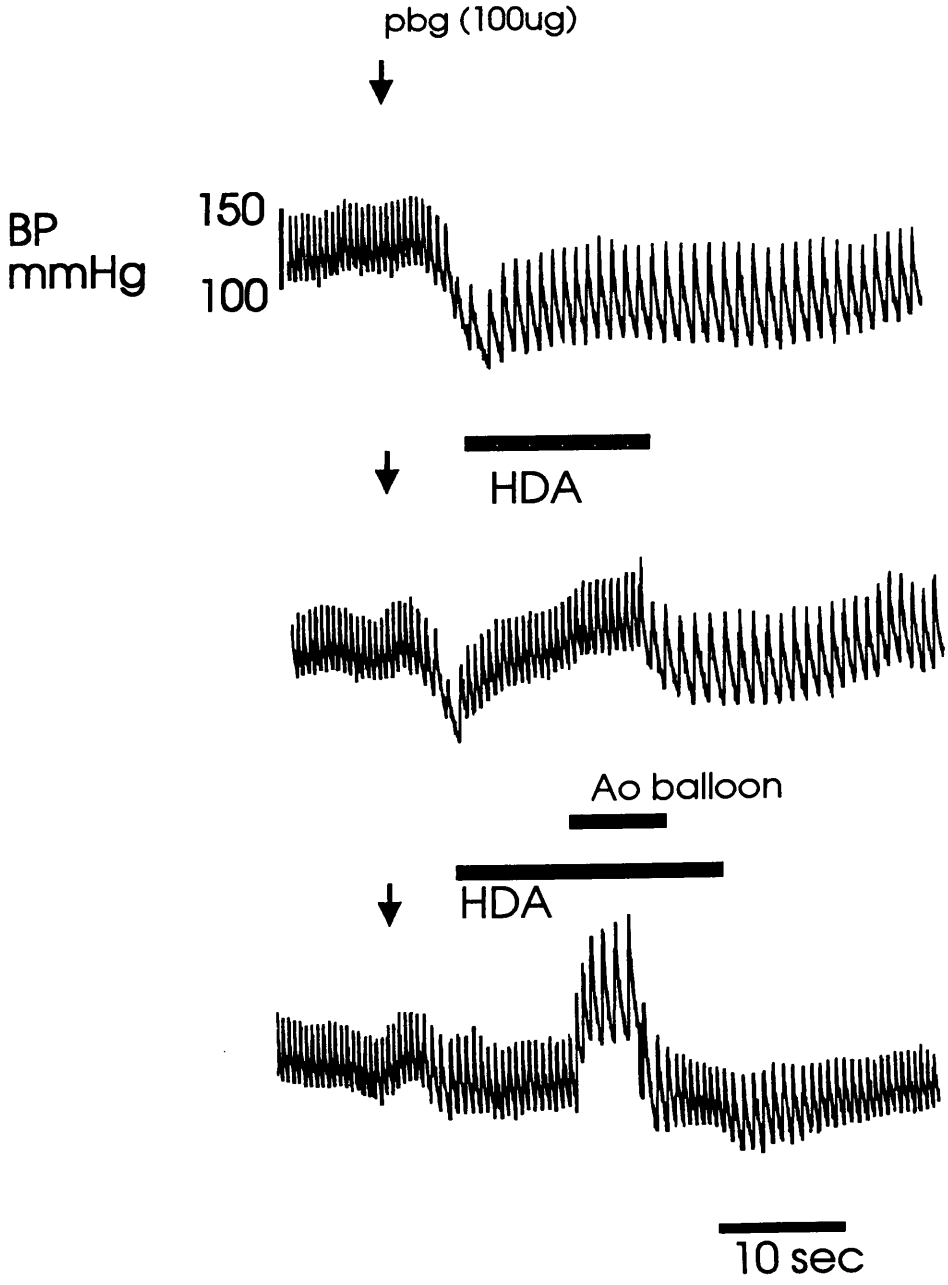

pbg (100ug)
BP
mmHg
150
100
HDA
Ao balloon
HDA
10 sec

FIGURE 5.4

The same HDA site as previously demonstrated in figure 5.3.
Note the lack of block of the baroreflex and the complete block of the PBG evoked bradycardia.

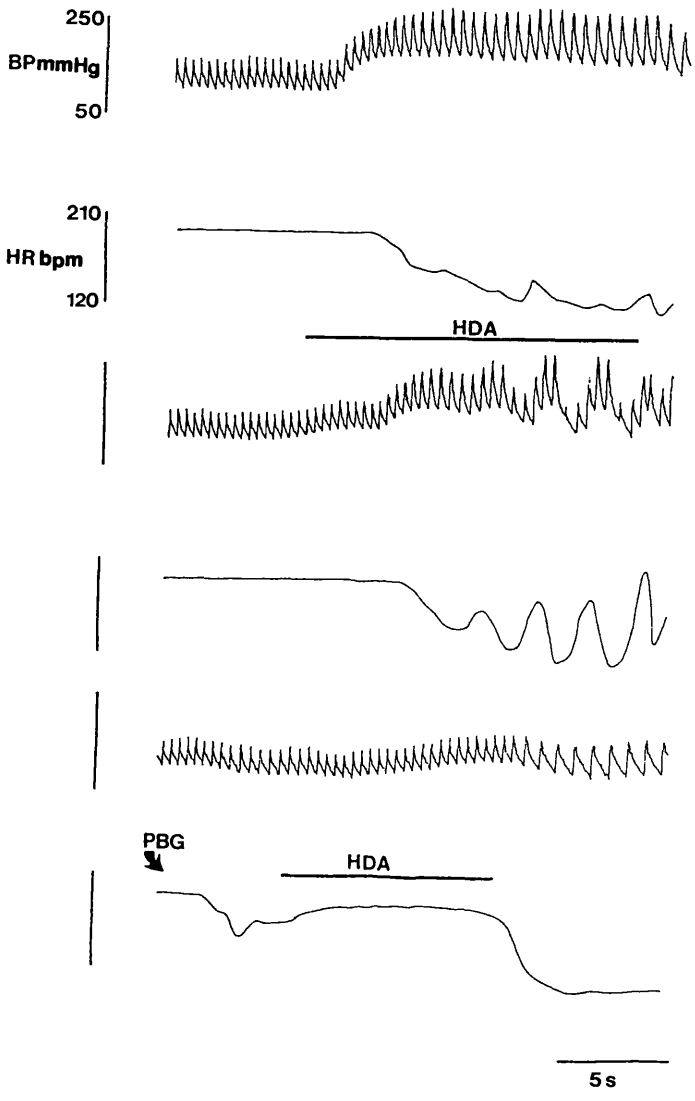
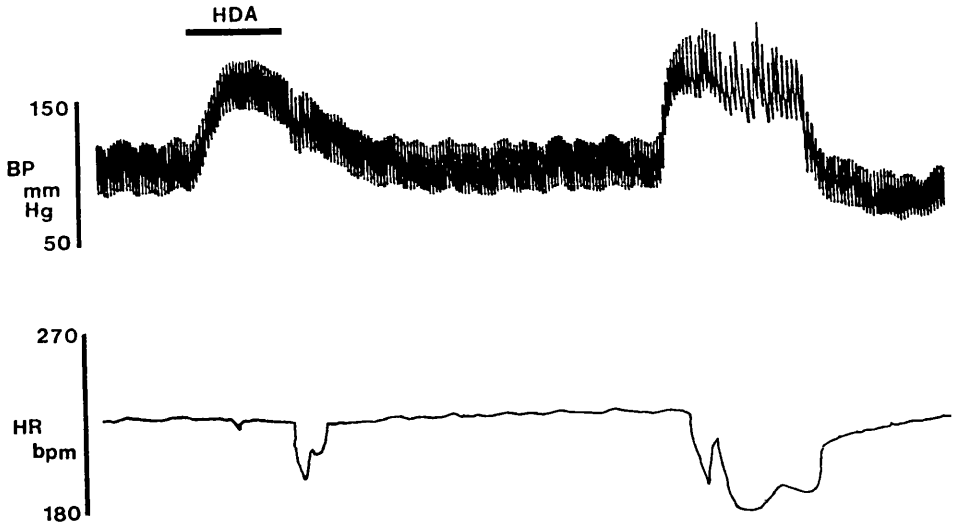

250
BPmmHg
50
210
HR bpm
120
HDA
PBG
HDA
5 s

FIGURE 5.5

This HDA site blocks the baroreflex but has no effect on the pulmonary chemoreflex.

Top panel: shows the control HDA stimulation (70Hz,1ms,30V)
Note the offset bradycardia. To the right is the control baroreflex. This area readily reversed the bradycardia of the baroreflex (not illustrated).

Lower panel: shows complete lack of effect of the HDA on the pulmonary chemoreflex.
Note the offset bradycardia illustrates the HDA is working but not gating this reflex.


HDA
150
BP
mm
Hg
50
270
HR
bpm
180

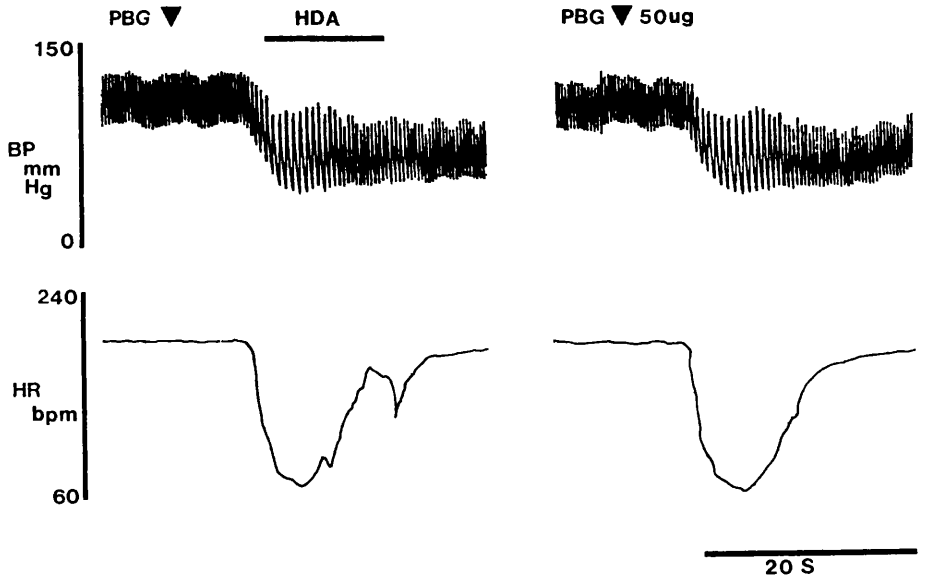
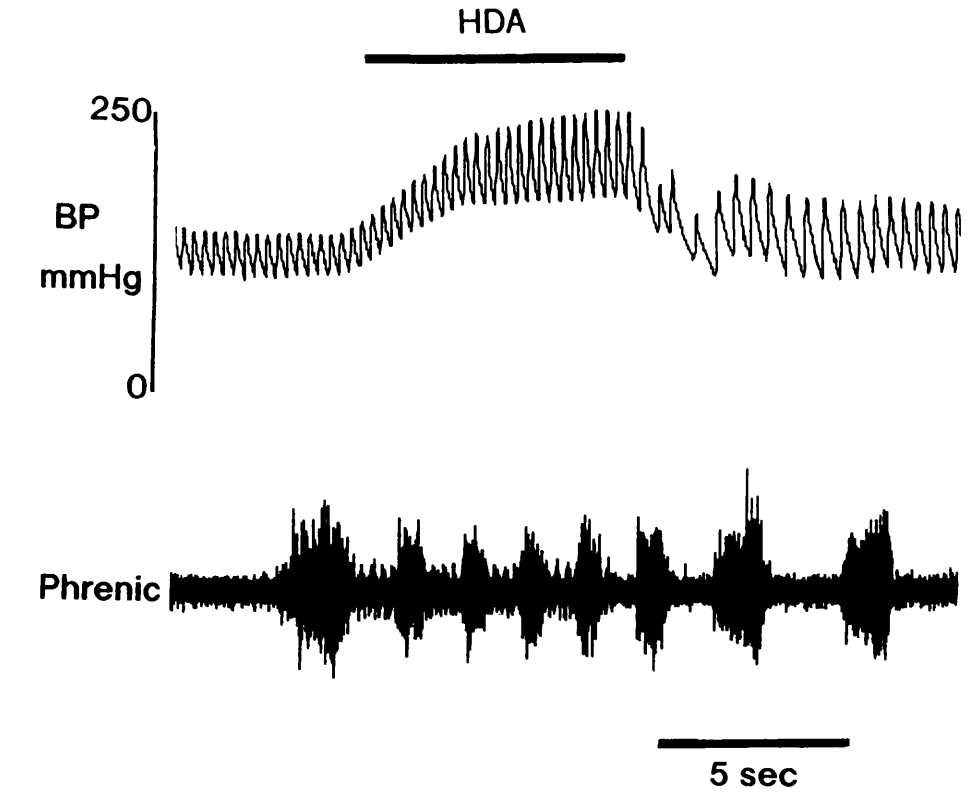

PBG
HDA
PBG 50ug
150
BP
mm
Hg
0
240
HR
bpm
60
20 S

FIGURE 5.6

This HDA site blocks both the baroreflex and the pulmonary chemoreflex

Upper panel: control HDA stimulation

Lower panel: block of the baroreflex


HDA
250
BP
mmHg
0
Phrenic
5 sec

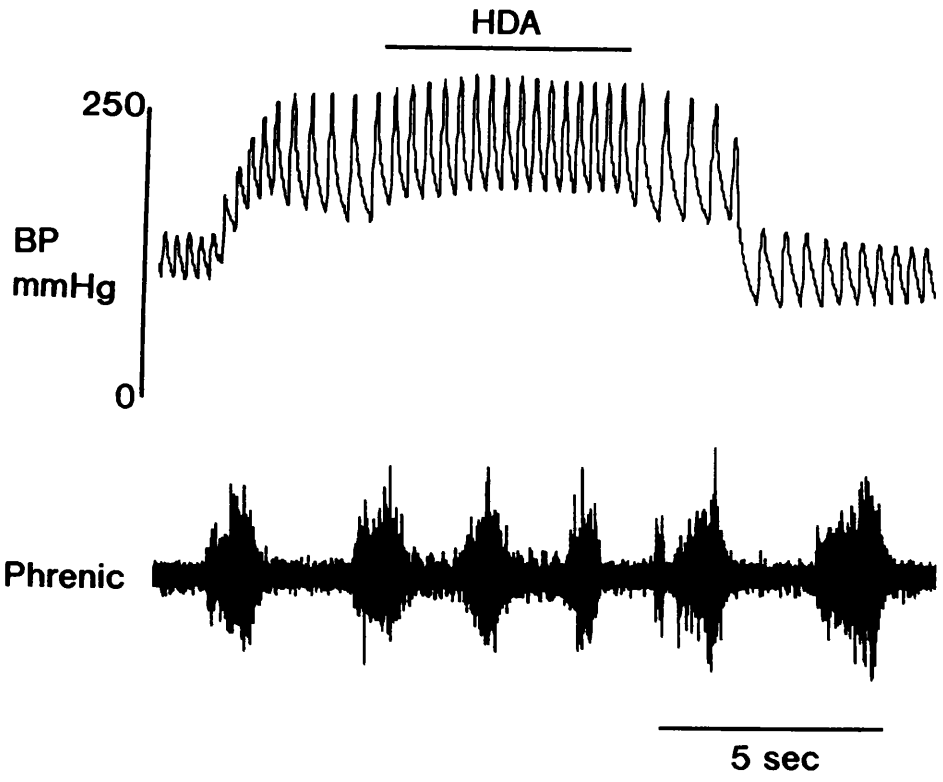

HDA
250
BP
mmHg
0
Phrenic
5 sec

FIGURE 5.7

The same site as previously described in figure 5.5.

Upper panel: control PBG reflex

Lower panel: block of the pulmonary chemoreflex triad.

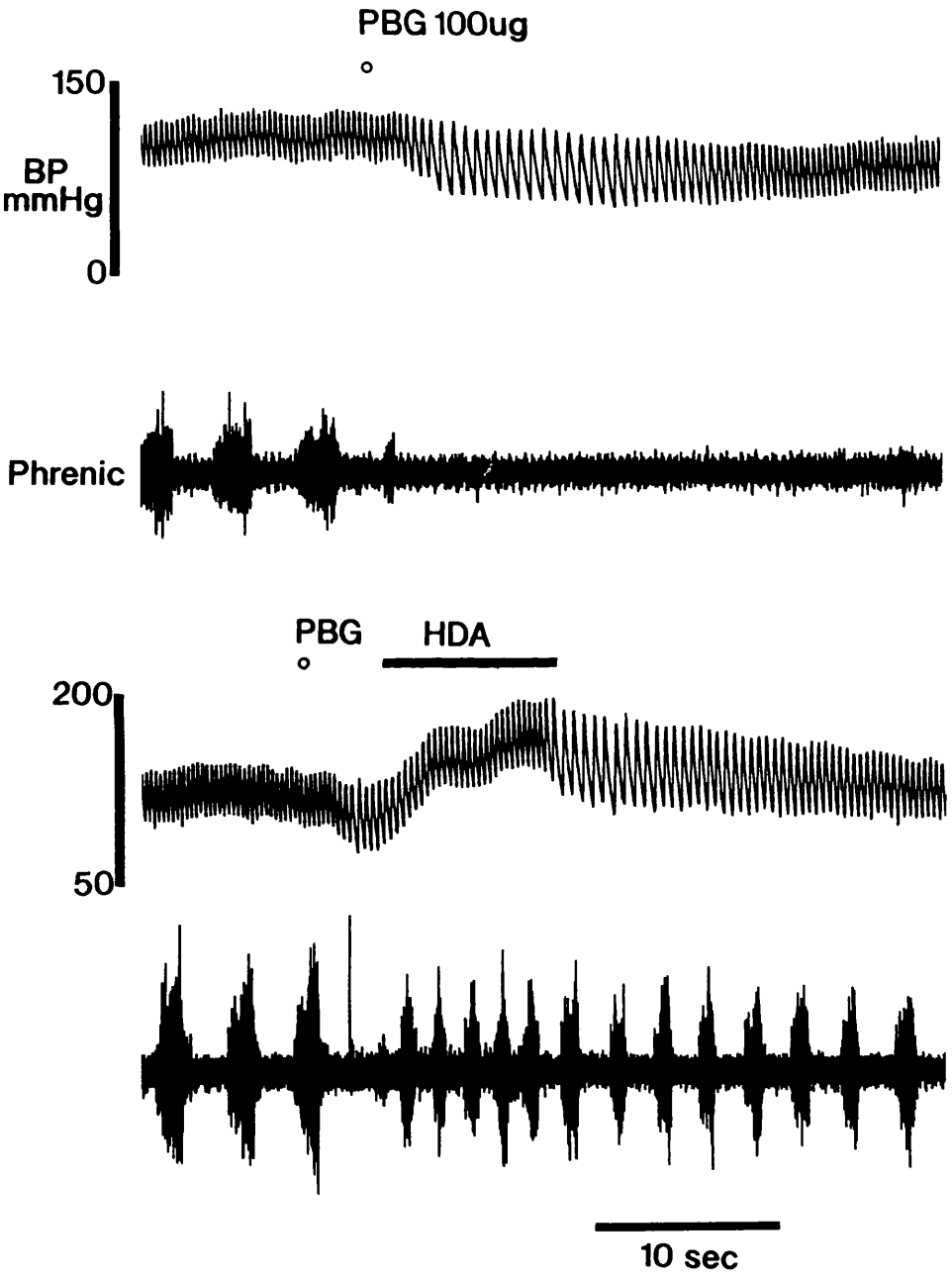

PBG 100ug
150
BP
mmHg
0
Phrenic
PBG
HDA
200
50
10 sec

FIGURE 5.8

(1) Recording of NTS cell receiving pulmonary C-fibre information. To the left are post-stimulus time histograms (5ms bins) (10s time slices). The dots signify the stimulus times (5 pulses delivered to the pulmonary branch, 10ms apart,each pulse was 15V and 1ms in duration)

(2) 12 conditioning pulses (20ms apart) to the HDA (28V, 1ms duration) inhibits the cell

(3) Note the transient rebound effect on the NTS cell.

This HDA reversed the hypotension and apnoea of the pulmonary chemoreflex but not the bradycardia. It completely blocked the baroreflex bradycardia, and completely blocked an NTS neurone receiving short latency (20ms) cardiac input.

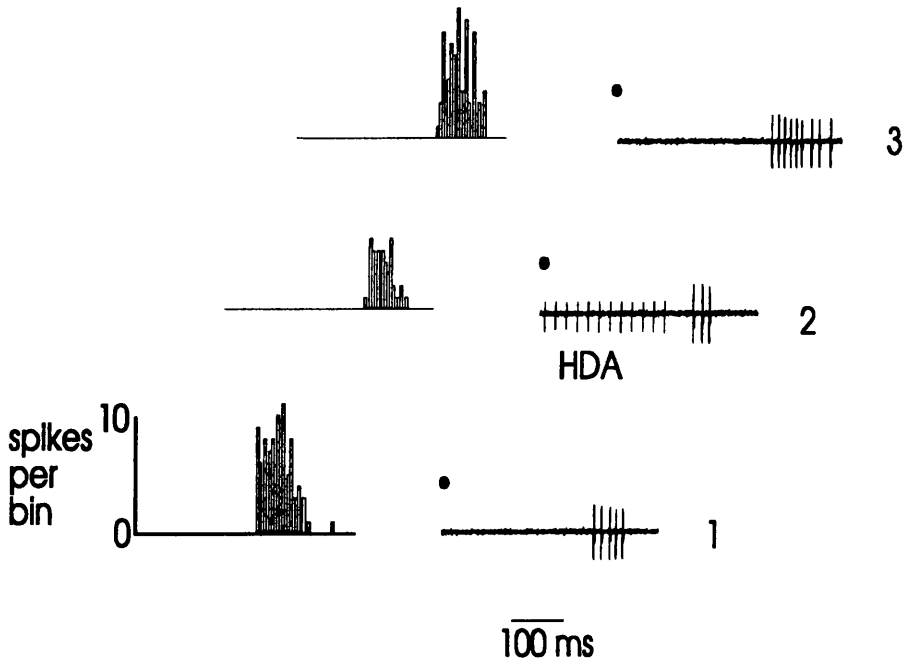
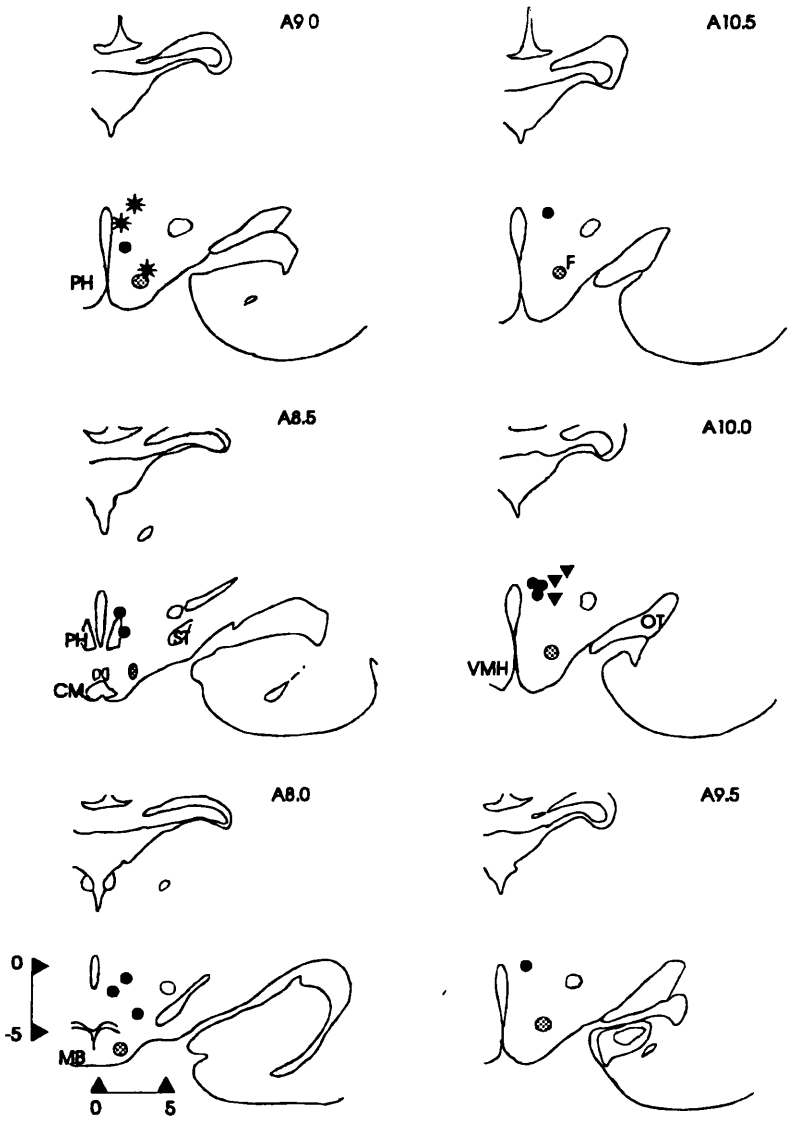

3
2
HDA
spikes
per
bin
10
0
1
100 ms

FIGURE 5.9

The histology of the cats hypothalamus from 8-10.5mm anterior to hypothalamic zero. The stars indicate areas that completely block the pulmonary chemoreflex but do not effect the baroreflex. The triangles indicate areas that block the baroreflex but have little or no effect upon the pulmonary chemoreflex. The dot in section A9.0 blocks both the baroreflex and the pulmonary chemoreflex equally well.


A9.0
A10.5
PH
F
A8.5
A10.0
PH
ST
CM
VMH
OT
A8.0
A9.5
0
-5
MB
0
5

5.4 Discussion

It is clear that the hypothalamus can override the pulmonary chemoreflex. This appears a little odd if this reflex is considered a primitive defensive response to life-threatening stimuli. It is of interest that the other depressor reflexes the coronary chemoreflex (Wennergren et al. 1976) and the baroreflex (Humphreys et al. 1971) are known to be inhibited during HDA stimulation. Arterial baroreceptor afferents and cardiac C-fibre afferents (Wennergren et al. 1977) are both excited during defence stimulation (Thoren 1979) and if unchecked would produce powerful cardiac slowing. Perhaps like these other afferents, pulmonary C-fibres fire during HDA stimulation and must be gated in order to enable full expression of the visceral alerting response. This lends support to Paintal's hypothesis that pulmonary C-fibres are stimulated by large pulmonary bloodflow increases (Anand & Paintal 1980). According to Folkow et al. (1968) there can be a 200% increase in cardiac output during the defense response.

Part of the HDA inhibition of the pulmonary chemoreflex is explained by NTS block, the transmitter responsible for this is unknown. It is not known whether the HDA affects the cardiac vagal preganglionic neurones mediating the pulmonary chemoreflex. The significance of the HDA's ability to differentially affect cardioinhibitory reflexes is unknown.

6.General discussion

In the first volume of Pflugers Archives, Donders (1868) introduced a system to analyze the latency and time course of the sinoatrial pacemaker response to a single induction coil shock to the vagus. This response was depicted by plotting the lengthening of each cycle (expressed as a percent of basic cycle length, %BCL) against the interval that lies between the vagal stimulus and the end of that cycle. Brown and Eccles resurrected this system in 1934, and confirmed that a double inhibitory wave results when a single shock is delivered to the vagus of a cat in which both vagi and nervi accelerantes are cut. In order to study this phenomenon more closely Jalife and Moe (1979) recorded intracellularly from single sinoatrial pacemaker cells. After a latency of 100-200ms (the same value calculated by Brown and Eccles using the Donders method) the pacemaker potential is hyperpolarized but recovers quickly (first inhibitory wave), then the beating rate remains low for several cycles and this corresponds to a diminution of the slope of the pacemaker potential (second inhibitory wave). The major prolongation of the first cycle is due to the hyperpolarization itself not the reduction of the latter phase of depolarization. The long latency (200ms) to the first inhibitory wave puzzled Brown and Eccles and they concluded that only a small part of this latent period is occupied by the time of travel of impulses from the point of stimulation to the fibre terminals in the pacemaker. This conclusion was based on the fact that myelinated B-fibre cardiac vagal efferents conduct impulses rapidly to the ganglion where neurotransmission should also be rapid. It seemed probable at the time that most of the latent period is occupied in the liberation of the acetylcholine substance and its diffusion to the point of its action. The possibility that diffusion limitation is the cause of most of the latency of the vagal response was outruled by iontophoresis studies (Hill-Smith & Purves 1978; Osterrieder et al. 1981). In 1955 Hutter and Trautwein demonstrated that acetylcholine could increase a potassium conductance in the sinus venosus of a frog. It is now recognized that the coupling of heterotrimeric G-proteins (guanine nucleotide binding proteins) to the muscarinic receptor is the mechanism by which potassium conductance change leads to hyperpolarization of the pacemaker (Sakman 1983;Irisawa et al.,1993). The activation of

the acetylcholine gated potassium channel (I_kAch) in heart occurs with a lag of 100ms followed by an increase with a time constant of 100ms with 10 μmol acetylcholine (Hartzell et al. 1991). This adequately accounts for most of the latency to vagal stimulation. In fact this has to be the rate limiting step since there is no latency to slowing if a sinoatrial pacemaker is given a brief hyperpolarizing pulse in a sucrose gap preparation (Jalife & Moe 1979). Acetylcholine can differentially affect the pacemaker depending on the dose. Low dose acetylcholine causes a decrease in the slope of the pacemaker potential but elicits no hyperpolarization. However high dose acetylcholine triggers the hyperpolarization. This has been confirmed by many workers in different species (Campbell et al. 1989;DiFrancesco & Tromba 1988). There is no doubt that the I_kAch channel is responsible for the hyperpolarization (that is the M_2-muscarinic receptor G-protein linked response)(Irisawa 1993); but the explanation behind the low dose acetylcholine response (the change in the slope of the pacemaker) is currently still in dispute. This is due to the fact that no one theory concerning the genesis of pacemaking at the membrane level is universally accepted. Di Francesco has put forward a most attractive theory that advances the I_f (funny current) also called the I_h (hyperpolarization activated current) as the dominant current behind pacemaking and the current inhibited by low dose acetylcholine (DiFrancesco & Tromba 1988; Di Francesco 1993) . The acetylcholine action on I_f causes a shift in the current activation range to more negative voltages but causes no change in the fully activated amplitude (Di Francesco and Tromba 1988). The effect is mimicked by muscarine, blocked by atropine and opposite to that of isoprenaline. Many critics of the I_f theory of pacemaking have argued that the range of activation of the hyperpolarization activated I_f preclude its emergence in pacing. However it is clear from the work on multicellular preparations and single cell preparations that the activation range of I_f is extraordinarily variable. Since diastolic depolarization is 0.1mV/sec and SA node cells have 30pF capacitance it only requires a current of 3 pA to pace (DiFrancesco 1993). The theory would be more solid if a specific blocker of I_f could be found. However according to Di Francesco no such blocker has been discovered; even high dose caesium may leave a fraction of I_f unblocked. The conservative interpretation is that I_f along with a number of currents contributes to pacemaking (Noble 1984). Hirst et al. (1992) have attacked DiFrancesco's interpretation that I_f explains the responses to the vagal stimulation. The weakening of I_f with Cs^+ does

not hyperpolarize the membranes of arrested cells whereas vagal stimulation does (Bywater et al. 1990). In the case of cardiac muscarinic stimulation, Hirst et al. (1992) discuss evidence based on different responses of pacemaker cells to externally applied and vagally released Ach, and in relation to studies on the action of perfused Ach in the heart. They show that vagal stimulation slows rhythm without affecting hyperpolarization, whereas perfusion of an intact preparation with 10μmol acetylcholine (Ach) slows the rhythm and also induces strong hyperpolarization. It is apparent that there is some confusion in the literature emanating from experiments conducted on the in vitro pacemaker preparation.

If it is assumed that the resynthesis and reuptake of acetylcholine and choline are not rate limiting and that the percent changes of cardiac period are proportional to the concentration of free acetylcholine; then the steady state period and the onset slope of the response are linear functions of vagal frequency (first order differential equation) (Celler 1989). In addition such a model predicts that the offset response should equal the onset response, in fact it does not, the offset response is always faster. This rapid rebound which is obvious in every vagal stimulation experiment is difficult to explain in terms of acetylcholine hydrolysis (Celler 1989). Boyett and Roberts (1987) have described a fade phenomenon of the response to acetylcholine at the rabbit isolated sino-atrial node. But this phenomenon takes many seconds to develop and cannot explain the trough in the double inhibitory wave. The mechanism that contributes to the trough in the Brown and Eccles inhibitory waveform is probably the same that shapes the phase response curve of the sino-atrial pacemaker cells to vagal stimuli i.e. a post-inhibitory rebound. What is the genesis of the second inhibitory wave? Jalife et al. (1983) suggested that the pacemaker cells may possess two populations of muscarinic receptors one with fast kinetics the other with slow kinetics. Administration of small doses of atropine (5μg) selectively diminishes the amplitude of the primary wave without affecting the second inhibitory wave (Brown and Eccles 1934). Levy and Martin (1984) have contended that since there is no hyperpolarization during the secondary inhibitory wave acetylcholine must have been completely hydrolysed, but since acetylcholine does not necessarily have to hyperpolarize the pacemaker in order to slow it down, this is incorrect. Furthermore, eserine affects both primary and secondary inhibitory waves (Brown and Eccles 1934). Spear (1979)

asserted that raised extracellular potassium is responsible for the secondary inhibitory wave due to massive permeability changes. However the evidence for this is not really convincing, since a direct correlation between secondary inhibition and external potassium activity has not been established (Jalife et al 1983). In summary, there appears to be uncertainty concerning the genesis of the second inhibitory wave of Brown & Eccles.

The exclusion of ganglionic mechanisms in the majority of theories, is hard to understand. Can the organization of the cardiac ganglion explain these phenomena and the double inhibitory wave of Brown and Eccles? The cardiac ganglion has never featured in the various theories behind the genesis of the double inhibitory wave although the concept of ganglionic "after discharge" is mentioned by Brown and Eccles (1934).
One of the most significant findings of these investigations is that a single vagal volley delivered through C-fibre cardiac efferents alone produced a response which resembles the second inhibitory wave described by Brown and Eccles (1934). Although not immediately obvious, this finding is of importance in understanding the pacemaker mechanism, the cardiac ganglion and the beat by beat control of the heart. A simple explanation of the events that follow a single vagal volley then are explained by postulating an initial brisk liberation of Ach in high dose which hyperpolarizes (Jalife and Moe 1979) the pacemaker (through I_kAch) and that this is followed by a slow sustained but smaller dose of Ach that slows the pacemaker through I_f. It is of interest that in this regard 0.013 μM Ach leads to half inhibition of I_f and 0.26μmol Ach causes half inhibition of I_kAch (DiFrancesco 1993).
It is now appreciated that, like any other autonomic ganglion, the cardiac ganglion has the machinery to provide long lasting principal cell discharge. The commonest mechanism that mediates this in autonomic ganglia is inhibition of the M-current (a simple time and voltage dependent K^+ channel that is switched off by muscarinic agonists). This causes phasic cells to become tonically firing. Such a mechanism has been described in mammalian cardiac ganglia of the dog (Xi et al. 1993) the rat (Selanyko & Skok 1992) and the guinea pig (Allen & Burnstock 1990). The inhibition of the M-current is linked to M_1 receptors and is responsible for a sEPSP. No physiological function has been ascribed to this mechanism; the results of the in vitro ganglion preparations have seldom been analyzed with regard to the function of whole cardiac vagal motor system.

The finding of this thesis that cardiac C-fibre efferents possibly acting through an M_1 sEPSP at the ganglion can cause the second inhibitory wave of Brown and Eccles raises several interesting possibilities. Is this a mechanism that contributes to the loss of respiratory modulation of the cardioinhibition of the pulmonary chemoreflex? It is possible to construct a hypothesis that integrates the brainstem ,the ganglion and the pacemaker and examine the possible evolutionary course of this coupling. To make this clear, it must first be shown that only myelinated B-fibres can control the heart beat by beat in mammals. The typical cardiac cycle of the cat is 300ms in duration. 80ms after the R-wave of the ECG, activity may be detected in the myelinated baroreceptive afferents of the carotid sinus (McAllen & Spyer 1978b). The delay to the ventrally placed cardiac vagal preganglionic neurones is bimodal: 40-100ms (natural delay is longer than electrically evoked latency), some processing occurs at hypothalamic level through long loops. The latency to ganglion through myelinated efferents is about 20ms. The cholinergic nicotinic receptor transmission is rapid (2 ms),the postganglionic fibre is unmyelinated but very short. The G-proteins take 50-100ms to effect a conductance change. The total closed loop baroreceptor feed forward system takes about 50 to 75 % of the resting cardiac cycle. Note for smaller species cardiac interval is progressively smaller but so is the vagal preganglionic length. A preganglionic C-fibre (conduction latency 140ms) coupled to a sluggish ganglionic G-protein (100ms) and a sluggish G-protein (100ms) at the pacemaker cannot control the heart beat by beat. Interestingly this theoretical expectation is fulfilled when the central recordings of C-fibre efferents are analysed for ECG related activity. There is none. This confirms the findings of Ford et al. (1990). By contrast B-fibre cardiac efferents fire with bursts of impulses at specified times of the cardiac cycle. The functional significance of this lies in the ability of the vagus to entrain the pacemaker node cells (Levy et al. 1969). Within a small bandwidth it is possible to accelerate the heart by increasing vagal stimulation. The bandwidth is calculated from a simple analysis of the phase response curve of the SA oscillator to vagal stimuli. This "paradoxical entrainment" was already a well recognized phenomenon in the sea slug (*Aplysia*) and the crayfish (*Procambarus*) (Perkel 1964) before Levy et al. (1969) described the phenomenon in the heart. The conditions for paradoxical entrainment are twofold: a functional dependence of the interval on the phase of the stimulus and the slope of this function must be positive. With more vagal impulses the heart rate is

entrained over greater ranges and harmonic and sub harmonic entrainment is demonstrable (Dong & Reitz 1970) The implication of these findings is that vagal regulation of cardiac frequency might be achieved by modulating both the frequency and the phase of incoming information. The phase shift depends on the timing the amplitude and the duration of the vagal hyperpolarization (Jalife et al. 1983). Levy's entrainment experiments were performed in open loop circumstances: involving a cut vagus or a cut carotid sinus nerve (Levy et al. 1972;Levy & Zieske 1972). Is in vivo entrainment really possible if there is closed loop feedback of baroreceptive information. In other words, how can the spontaneous lengthening of a cardiac cycle be readjusted by a shift in the relationship of the incoming vagal efferent traffic to the cycle when the vagal discharge itself is shifted?

If phase information is important then one would have to postulate some kind of central phase shift detection system. This would presumably require a rhythmical internal reference clock beating very regularly at around cardiac frequency.

Why is there a beat by beat discharge in B-fibre cardiac preganglionic neurones? Does this reflect a beat by beat control? It is difficult to conceive of any reflex or behavioral adjustment that requires such rapid and precise control. Is the beat by beat discharge functionally related to dynamic baroreflex loops? Synchronization of the cardiac pacemaker is possible with repetitive stimulation of the carotid sinus nerve in the dog (Levy & Zieske 1972). The entrainment capability of cardiac vagal discharge could be of importance in synchronizing the activity of neighbouring automatic cells. The SA node has a low conduction velocity and there are no intercalated discs,and few gap junctions between P cells in the sinus node (James 1973). Although ultrastructural analysis reveals few gap junctions, Jalife (1984) has provided strong electrophysiological evidence for mutual entrainment and electrical coupling between rabbit sino-atrial pacemaker cells. The initiation of the heart beat should not be thought of as a result of a master pacemaker cell, but rather as a kind of democratic type of synchronous firing of SA pacemaker cells. If it is accepted that the fast cell speeds the slow, then the slow cell must also retard the fast. This is true for any group of coupled oscillators. If the SA cells are not in good direct connection to each other they are entrained indirectly through the cardiac vagus. However the autonomic susceptibility of pacemakers varies because innervation is not

spatially uniform (Negoescu 1992).

If the B-fibre preganglionic cardiac efferent system is beautifully designed to synchronize the SA pacemaker beat by beat what is the possible function of the slow C-fibre preganglionic system? The hypothesis that is first advanced is that the C-fibre efferent system is an active desynchronizing system that uncouples the respiratory and SA oscillators and that may have evolved before the synchronizing "B-fibre" system. In the medulla the C-fibre efferents do not migrate with branchiomotor neurones possibly because they have no part to play in respiratory synchrony. To analyze the possible evolutionary course of such a system it is necessary to study the early vertebrate life-forms. For this reason elasmobranchs serve as a useful model of early cardiac vagal control. It has recently been demonstrated that a gill chemoreflex may be elicited with capsaicin in dogfish (*Sycillorhinus canicula*) (Jones et al. 1993). The reflexogenic area is in the branchial circulation, the receptors appear to be at the blood-water interface. The response involves bradycardia, hypotension, inhibition of swimming movements and acceleration of respiration. The branchial bursts increase in amplitude and rate. This appears to be a species difference, mammals exhibit rapid shallow breathing, however it is not. Observe the response of an equivalent (i.e. branchiomeric) respiratory muscle in the mammal e.g. a post-inspiratory laryngeal motoneurone in the medulla of the cat. The respiratory bursts in fish are expiratory and occur at the mouth closing phase of the respiratory cycle. It may be of significance that the only class of respiratory cell to increase its activity during the pulmonary chemoreflex in mammals is the post-inspiratory cell. It is interesting to reflect that the dogfish evolved (Ordovician) before the capsicum fruit, this implies that sensory nociceptive afferents are highly conserved or that there is an endogenous capsaicin like molecule or that the capsaicin has a non-specific action. The important point about this gill chemoreflex is that the tonic units contained in the cardiac nerves so increase their activity that the phasic respiratory related units are swamped. The tonic activity could serve the useful task of uncoupling the increased respiratory bursts and the heartbeat. Synchronization has an increased likelihood of occurrence with increased respiratory bursting but this would have the effect of increased irritant flux across the exchanger. Did the desynchronizing function of the vagus evolve before the synchronizing function? Is this the primary function of the vagus? The thin-walled blood-

filled secondary lamellae represent an accessible part of the body surface. The fish respiratory system includes its extensive area of respiratory epithelium within or adjacent to the chamber through which the food must pass on its way to the oesophagus. The possibility exists that toxic dissolved substances or refluxed digestive contents may pass to the respiratory epithelium and gain access to the bloodstream. In addition there are many genera of gill parasites in the Copeopod orders,cyclopedea,Caligidea and Lerneopodidea that attack the gill lamellae (van Duijin 1967).

Vertebrates with aneural hearts have mechanical contrivances (eg rigid pericardium) to synchronize movement of blood with movement of water. Respiratory sinus arrhythmia (RSA) due to purely mechanical events must be more marked in exercise. In this regard vagal control for cardiorespiratory coupling may be dispensable. The primary function of the cardiac vagus may have been protective, to guard against absorption or ingestion of noxious agents. Elasmobranchs have impressive coughing and vomiting reflexes and gill chemoreflexes but no evidence of baroreflexes (Taylor 1993). The apparent regulatory roles ascribed to the cardiac vagus in mammals operate beat by beat or breath by breath. The behaviour of cardiac vagal preganglionic neurones appears to be different in the baroreflex and the pulmonary chemoreflex. Whereas C-fibre preganglionic neurones are reported to be mostly unaffected by carotid sinus inflation (Ford et al. 1990), they are excited during the pulmonary chemoreflex. Whether this explains the findings of Daly (1990) cannot be deduced because the function of the recorded neurones is unknown. However selective electrical stimulation of cardiac C-fibres points to a role of these efferents in chronotropy. Nevertheless, the amount of R-R interval lengthening produced is small and cannot per se account for all the powerful cardioinhibition of the pulmonary chemoreflex. In this regard the finding that B-fibre preganglionic neurones are also excited during this reflex suggests that the loss of respiratory modulation occurs at the level of the cardiac ganglion. Perhaps the B-fibres converge on the same neurones as the C-fibres. In the rabbit, the C-fibre post synaptic specialisations must be purely muscarinic, resistant to hexamethonium. It is known that in rat and dog every ganglionic cell expresses both nicotinic and muscarinic receptors. The functional significance of the sEPSP may then be to produce sustained discharge, increased probability that fEPSPs trigger action potentials and thereby desynchronize the heart from breathing. Such a

hypothesis neatly explains the findings of Daly (1991) but does not require the C-fibre preganglionic group to be involved. The single vagal volley experiment to B-fibres alone also shows a secondary inhibitory wave. If there is a special group of ventrally placed cardiac vagal preganglionic neurones that respond to pulmonary C-fibre stimulation with sustained discharge that overwhelms the inhibitory inspiratory IPSPs then this will yield the same end result. Unfortunately it is difficult to simultaneously record and hold a B-fibre preganglionic in the particular cats (10%) which shows no apnoeic response to PBG. The argument that implicates the dorsal medullary group is essentially a phylogenetic one. If this theory is correct, there may be a unifying theory for the vagal control of the heartbeat across vertebrates. In this regard certain zoological groups are of more interest than others. The salamanders of the Plethodontidae family lack gills and lungs; their gas exchanges are almost entirely cutaneous (Gatz et al. 1974). If there is no respiratory motor output, is there no respiratory rhythmogenesis? Is there only one population of cardiac vagal preganglionic neurones? What are the autonomic outflows like? How are the waveforms of the electroencephalogram influenced by the potential lack of a major biological rhythm? There must be many facets of natural philosophy which cannot be understood, without considering their emergence through time.

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