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Heart rate responses to selective stimulation of cardiac vagal C fibres in anaesthetized cats, rats and rabbits

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1. The contribution of cardiac vagal C fibres to vagal chronotropic control in anaesthetized cats, rats and rabbits was analysed using electrical stimulation of the vagus nerve with a selective anodal block technique.
2. After bilateral vagotomy and pretreatment with atenolol, 10 Hz continuous selective stimulation of unmyelinated fibres in the cut peripheral end of the cervical vagus evoked a bradycardia in anaesthetized rats, cats and rabbits. With this stimulation protocol the three species exhibited a similar lengthening of the heart period (R–R interval) when expressed as a percentage of their basal cardiac interval.
3. The mechanism of action of the selective blocking technique was analysed by recording eighty-nine single A- ($n = 12$), B- ($n = 22$) and C-fibre ($n = 55$) vagal-projecting neurones in the medulla of the rat. This demonstrated that the technique can selectively block conduction in myelinated fibres and that 'break excitation' is seen mainly in unmyelinated fibres. Although thirty C fibres showed break excitation sixteen did not and this difference could not be correlated with their axonal conduction velocity, chronaxie or initial segment frequency following.
4. Using the anodal block technique the vagal effects on heart rate were reanalysed in the cat by incorporating a collision technique. B fibres were activated orthodromically to evoke cardioinhibition and simultaneously antidromically to collide with errant B-fibre spikes activated at the electrode producing anodal block. With this protocol it was noted that the B- and C-fibre bradycardias were not additive. Using a double anodal block and collision technique, it was demonstrated that this phenomenon was likely to be due to occlusion of the effects of B and C fibres.
5. In conclusion, in addition to the well-defined effects of vagal B fibres on heart rate, selective stimulation of vagal C fibres also had a cardioinhibitory effect in all three species studied. However, since the effects of cardiac C fibres on heart rate was small, these neurones alone cannot account for the cardioinhibition of the pulmonary chemoreflex. It is likely that activation of both B- and C-fibre cardiac vagal preganglionic neurones accounts for this reflex cardioinhibition.

Certain features of cardiac vagal control appear to be retained across a range of vertebrates (Withington-Wray, Taylor & Metcalf, 1987). Centrally, there are two populations of preganglionic neurones which project distally to ganglia located near the cardiac pacemaker. The functional role of the different groups of preganglionic neurones is uncertain. It has been proposed that the B-fibre group, located in the nucleus ambiguus, control chronotropy whilst the C-fibre group, located in the dorsal vagal motor nucleus, control inotropy (Geis & Wurster, 1980). In support of this argument, a number of studies have suggested that the chronotropic action of the vagus in the cat is mediated by the myelinated B-fibre population (Heinbecker & Bishop, 1935; Middleton, Middleton & Grundfest, 1950; Kidd & McWilliam, 1982). Electrical stimulation of myelinated fibres evoked a profound cardiac slowing and additional recruitment of unmyelinated fibres caused no further cardiac slowing. However, using a modified form of the anodal block technique (Accornero, Bini, Lenzi & Manfredi, 1977), it has been demonstrated in both rats and rabbits that stimulation of either B or C fibres alone can produce chronotropic effects (Nosaka, Yasunaga & Kawano, 1979;

Woolley, McWilliam, Ford & Clarke, 1987). This technique of selective anodal block has not, however, been applied in cats and so it is still generally accepted that in this species vagal C-fibre efferents do not have a chronotropic action. A recent proposal that the C-fibre efferents may mediate the bradycardia of the pulmonary chemoreflex (Daly, 1991) and the demonstration that both populations of cardiac vagal preganglionic neurones can be activated during the pulmonary chemoreflex (Jones, Wang & Jordan, 1994) has prompted a reinvestigation of the possible chronotropic role of cardiac vagal C fibres. In this study the effect of vagal efferent C fibres on heart period was studied in three species, rat, cat and rabbit, since there are similarities between the on-going and reflexly evoked activity of cardiac vagal C-fibre preganglionic neurones of rat and cat (Jones, Wang & Jordan, 1995) and the evidence for C-fibre efferent-mediated bradycardia is probably strongest in the rabbit (McWilliam & Wooley, 1990).

When attempting to resolve whether C-fibre stimulation can evoke bradycardia, it is essential to establish that the technique used is truly selective, since stimulation of only a few B fibres can evoke bradycardia in cats (McAllen & Spyer, 1978). Insufficient anodal block or anodal break excitation of B fibres may explain the small bradycardia ascribed to C fibres. Therefore central recordings were obtained from A-, B- and C-fibre vagal preganglionic neurones and motoneurones, in order to gain more information about the selective blocking technique.

A preliminary report of this work has already been published (Jones & Jordan, 1993).

METHODS

Preparation of the animals

A total of seventeen Sprague–Dawley rats (250–570 g), seventeen New Zealand White rabbits (1·8–2·4 kg) and nine cats (1·9–3·0 kg) were used. Rats were anaesthetized with pentobarbitone sodium (Sagatal, 60 mg kg^{-1}, I.P.), rabbits with urethane (Sigma, 1·4 g kg^{-1}, I.V.) and cats with a mixture of α-chloralose (Aldrich, 50 mg kg^{-1}) and urethane (0·5 g kg^{-1}) I.P. Following induction of anaesthesia, supplementary doses were given when necessary as assessed by the flexion withdrawal reflex, the blood pressure responses to paw pinch, and the state of the pupils. The temperature of the animals was monitored with a rectal temperature probe and maintained at 37 °C with a Harvard homeothermic blanket system. The left femoral artery and vein were cannulated for measurement of blood pressure with a Statham P23Db transducer (Statham Ltd, Puerto Rico) and injection of drugs and/or fluids, respectively. A precordial chest lead (V_3) was used to record ECG (Neurolog System; Digitimer Ltd, Welwyn Garden City, UK). The trachea was cannulated below the larynx with a polythene tracheostomy tube, fitted with two side-arms for continuous monitoring of end-tidal CO_2 (ADC fast response analyser) and tracheal pressure. Tracheal pressure was measured with a Validyne transducer and amplified by a Buxco Electronics Inc. Pulmonary Mechanics Analyser (Model 6; Sharon, CT, USA). Following placement in a stereotaxic frame the animals were artificially ventilated (Harvard Apparatus small animal ventilator) with oxygen-enriched air and 1 cm of water positive end-expiratory pressure. A tidal volume and frequency were chosen appropriate for the particular species and these were adjusted to maintain an arterial blood carbon dioxide pressure (P_{CO_2}) between 35 and 40 mmHg. All blood gas variables were measured by a Corning Blood Gas Analyser (Model 158 pH/blood gas analyser). The bladder had a catheter placed transurethrally in the cats to prevent accumulation of urine.

Preparation of the nerves

In the cats the right cardiac branch was accessed between the fourth and fifth ribs as described previously (McAllen & Spyer, 1976). In rats the second right rib was removed to gain access to the right cranial cardiac branch for recording purposes. In the neck the right vagosympathetic trunk was isolated and transected as near to the cranium as possible. The cervical sympathetic trunk was separated from the vagus, which was then desheathed and kept moist by cotton wool dipped in warm paraffin oil. The contralateral vagus was sectioned and the animal pretreated with atenolol (Sigma, 1 mg kg^{-1} I.V.) to ensure that there was no sympathetic component to evoked alterations in cardiac interval. In the rabbits the cervical vagus was similarly prepared but it was used for both recording and stimulating purposes.

Data capture

ECG activity was discriminated using a spike processor (Digitimer D130) and the resulting pulses used to generate instantaneous heart rate and R–R intervals (1401 interface and Spike 2 system, Cambridge Electronic Design). The vagal compound action potentials were amplified and filtered (500 Hz to 5 kHz) (NL104, NL125 Neurolog) and recorded on computer where they were averaged using a software package (SIGAVG, CED). Blood pressure, heart rate, end-tidal CO_2 and tracheal pressure were displayed on a second computer using a CHART CED system. The data was stored on videotape using a digital data recorder (Instrutech VR-100B).

The anodal block technique

In this study the circuit described by Accornero *et al.* (1977) was used to produce an exponentially decaying anodal block of nerve conduction. The technique relies on the fact that impulses travelling in myelinated fibres will quickly cross the interelectrode distance from the cathode to reach the anode at a time when the hyperpolarizing effect at the anode is still strong. However, impulses in slower conducting C fibres will reach the anode only after the current has decayed to a point that is ineffectual at blocking their conduction. The selectivity of the technique for fibres of different conduction velocities can be varied by switching between three capacitors in the circuit, which will alter the time constant of the exponential decay, and by adjusting the voltage applied. In these experiments the third capacitor was most commonly chosen and this gave a time constant of about 140 ms. It was claimed that exponential decay of the anodal current eliminated the problem of anodal break excitation, which had previously been a problem with this technique (Accornero *et al.* 1977). Both the stimulating and recording electrodes were made of silver wire (diameter 0·5 mm for cats and rabbits, 0·125 mm for rats). The distance between the anode and cathode of the

stimulating electrode was 2–3 mm. Stimuli were provided by stimulus isolation boxes (Digitimer DS2) controlled by a Master 8 digital programmer (AMPI).

Central recording in rats

The medulla was exposed by an occipital craniotomy. The central cut end of the mid-cervical vagus was stimulated using bipolar silver wire electrodes. When searching for vagal-activated neurones the central electrode was the cathode but this was reversed to the anode when testing the anodal block. Extracellular recordings from vagal-projecting neurones in the vicinity of the dorsal vagal motor nucleus and the nucleus ambiguus were made using single-barrelled borosilicate glass microelectrodes (Clarke Electromedical, GC150F-10) filled with Pontamine Sky Blue (20 mg ml^{-1} in 0·5 M sodium acetate) or 4 M saline. Neuronal activity was amplified (Dagan model 2400), filtered (Neurolog, NL 125; 0·1–3 kHz) and displayed on an oscilloscope (Tektronix 5103 N). The criteria for antidromic activation included the spike shape, constant latency and collision with an orthodromic spike. In some animals the vagus was left intact but in these cases a tripolar stimulating electrode was used to activate the motor axons since, with an intact nerve, it is not possible to produce anodal block with a bipolar electrode. The cathode was the central electrode of the three when applying anodal block.

Protocol

Three different stimulation and/or recording protocols were used to study the effects of vagal stimulation on the heart. Initially, the arrangement described by Accornero *et al.* (1977) was used (Fig. 1*A*). Bipolar electrodes were used to stimulate the cervical vagus and the compound action potentials recorded from the cardiac branch of the cat and rat and the cervical vagus in the rabbit. To overcome the possibility that activation of a few B fibres by the bipolar stimulating electrode was contaminating the C-fibre-evoked bradycardias the electrode arrangement illustrated in Fig. 1*B* was used in cats. The nerve was stimulated at low intensity with the symmetric tripolar stimulating electrode to activate action potentials in B fibres. These will conduct antidromically, where they will collide with any B-fibre action potentials which have either escaped anodal block or which have been evoked by break excitation at the bipolar electrode. In addition, however, they will also conduct orthodromically to evoke a B-fibre-mediated bradycardia so the arrangement was further modified (Fig. 1*C*). With this arrangement double anodal block is performed with the central electrode set to selectively activate C fibres. The peripheral electrode is stimulated at low intensity to activate only B fibres. The anodal block will prevent their conduction peripherally (this can be checked by assessing heart rate) but they will conduct antidromically to collide with any unwanted B-fibre activation at the central electrode. With this technique it is more likely that only C-fibre action potentials will reach the heart, compared with the technique of anodal block alone.

Data analysis

Changes in heart rate, cardiac interval and the percentage change of basal cardiac cycle length produced by stimulating the B and C fibres in the vagi of the three species were analysed for significance by using Student's unpaired t test, significance being taken as $P < 0·05$. Results are expressed as means ± S.E.M.

RESULTS

Cardiac chronotropic effects of selective stimulation of C-fibre vagal efferent fibres

In these experiments the changes in heart period and the compound action potentials evoked by stimulation of the cervical vagus were recorded simultaneously using the protocol illustrated in Fig. 1*A*. Conventional stimulation, with the cathode towards the heart, activates both the myelinated and unmyelinated fibres in the nerve, as can be seen in the compound action potential recorded peripherally (Fig. 2, top). In contrast, when the polarity of the electrode is reversed and anodal block applied, the myelinated component of the compound action potential is abolished, leaving the unmyelinated component (Fig. 2, bottom). Selective stimulation of C fibres with this technique was shown to evoke falls in heart rate in rats, rabbits and cats but these were substantially smaller than the bradycardias evoked by stimulation of both B and C fibres (Fig. 3). In order to make a quantitative comparison of the cardiac effects of selective C-fibre stimulation, a fixed stimulation protocol (1 ms, 5–15 V, 10 Hz for 20 s) was used in all three species. Selective stimulation of cardiac vagal C fibres slows the heart in all three species, the fall in heart rate being significantly greater in rabbits (21 ± 2·4 beats min^{-1}) than in cats (14 ± 1·7 beats min^{-1}). Although not statistically significant, there was also a tendency for the response to be greater in rats (24 ± 3·5 beats min^{-1}) than in cats (Fig. 4*A*). Since it has been shown previously that there is a straight line relationship between cardiac interval and frequency of vagal stimulation (Katona, Poitras, Barnett & Terry, 1970; Parker, Celler, Potter & McCloskey, 1984) the responses have also been expressed as the increase in cardiac interval. When the data are expressed in this way, the lengthening of the cardiac cycle evoked in cats (41 ± 6·2 ms) is now significantly greater than that seen in rats (20 ± 3·2 ms) with rabbits falling between the two values (29 ± 5·3 ms). Finally, the intrinsic cardiac pacemaker rate in each species (in the vagotomized and atenolol-pretreated state) is different (rat, 286 ± 10 beats min^{-1}; rabbit, 235 ± 19 beats min^{-1}; and cat, 162 ± 12 beats min^{-1}). Thus it might be expected that the apparent effectiveness of the stimulus might differ because of this. When the increase in cycle length evoked by selective C-fibre stimulation is expressed as a percentage of the basal cycle length (rat, 9·3 ± 1·5%; rabbit, 10·6 ± 1·3%; and cat, 10·4 ± 1·2%) there is no statistical difference between the responses evoked in the three species (Fig. 4*A*).

Mechanism of action of the selective anodal block technique

Selective stimulation of C-fibre vagal efferents clearly slows the heart in all three species studied. However, since B-fibre efferents have a powerful effect on heart rate it

could be argued that the effects of activation of a few B fibres would not be seen within the compound action potential and these could contaminate the 'C-fibre bradycardias' described above. Alternatively, if some of the blocked B fibres were liable to 'break excitation', then these may have contributed to the apparent C-fibre response. To address these problems the response of single neurones during the selective anodal block was studied.

Brainstem recordings were made from vagal-projecting neurones (as shown by antidromic activation), some of which also received orthodromic vagal inputs (Jones *et al.* 1995). By varying the parameters of stimulation it was possible to block sequentially the conduction in fast, and then in slower inputs to the neurones. For example, in Fig. 5, early and late orthodromic (Ortho) responses, and a C-fibre antidromic (Anti) response are evoked by the

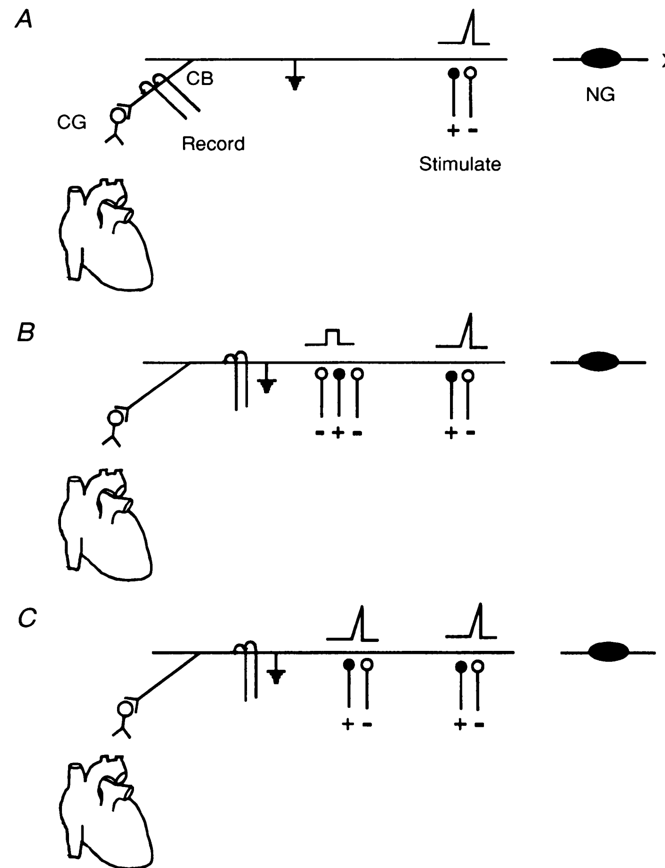

Figure 1. Positioning of recording and stimulating electrodes on the vagus nerve

A, a bipolar stimulating electrode was positioned on the cut cervical vagus nerve just below the nodose ganglion with the anode facing the heart. The recording electrode was placed on the right cranial cardiac nerve of rats and cats (peripherally on the cervical vagus of rabbits). Triangular-shaped pulses (1 ms, 5–15 V, 10 Hz) were used to activate C fibres selectively. *B*, the combined anodal block and collision technique performed on the cervical vagus of the cat. Only in the cats was the cervical vagus long enough to accommodate a recording and two stimulating electrodes. Square-shaped pulses were delivered to a symmetrical tripolar electrode (0·02–0·1 ms, 5 V, 5 Hz) to activate B fibres, orthodromically towards the heart and also antidromically towards the bipolar stimulating electrode where triangular-shaped pulses (1 ms, 5–6 V, 5 Hz) were applied to activate C fibres selectively as in *A*. When both stimulating electrodes were active (B stimulation at the tripolar electrode plus C stimulation at the bipolar electrode) any B fibres activated at the bipolar electrode would collide with those antidromically conducted from the tripolar electrode. *C*, the combined double anodal block and collision technique performed on the cervical vagus of the cat. The arrangement is similar to *B* but both stimulating electrodes produce triangular-shaped pulses. The peripheral stimulating electrode is set to activate action potentials in B fibres (0·02–1 ms, 5 V, 5 Hz) which will travel only antidromically, whilst the central electrode activated action potentials in C fibres (1 ms, 5–6 V, 5 Hz) which travel orthodromically. Coactivation of the two electrodes will produce a pure C-fibre effect at the heart. The interelectrode distance was set to at least ten times the distance between the tips of the electrodes activating the C fibres to allow sufficient time for collision to occur. CB, cardiac branch; CG, cardiac ganglion; NG, nodose ganglion; X, vagus nerve.

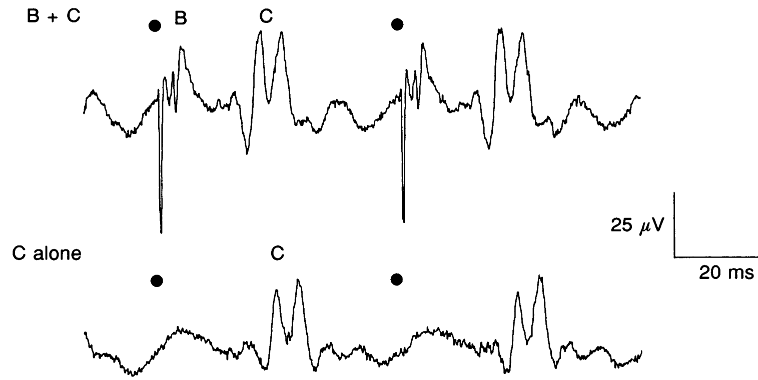

Figure 2. Compound action potentials recorded from the cardiac branch of a rat

The upper trace shows single sweeps of the cardiac compound action potential (electrode arrangement as in Fig. 1*A*) when all the fibres of the cut peripheral end of the cervical vagus are stimulated with conventional polarity (cathode towards the heart) (0·2 ms, 2 V, 20 Hz). The lower trace shows the effect of selective anodal block, which spares the action potentials corresponding to the C-fibre component of the compound action potential. ●, stimulation times.

stimulus. With anodal block the fast orthodromic input can first be blocked (not shown) followed by blockage of the antidromic response (Fig. 5, bottom) leaving the longer latency orthodromic input. These remaining spikes were due to the original orthodromic input and not to 'break excitation' spikes of the antidromic input since they did not exhibit cancellation when spontaneous action potentials occurred. Clearly, the technique can distinguish myelinated from unmyelinated input, and can even discriminate between C-fibre inputs of different conduction velocities. However, as more neurones were studied over greater voltage ranges, it became apparent that the technique does have problems related to break excitation. To study the phenomenon of anodal break, neurones antidromically activated from the vagus nerve were tested for the presence of anodal break excitation by slowly increasing the stimulating voltage up to 24 V (1 ms, 1 Hz). As shown in Fig. 5, when anodal block was applied, the neurones did not exhibit a break excitation at short latency. However, when the antidromic spike was blocked by using higher

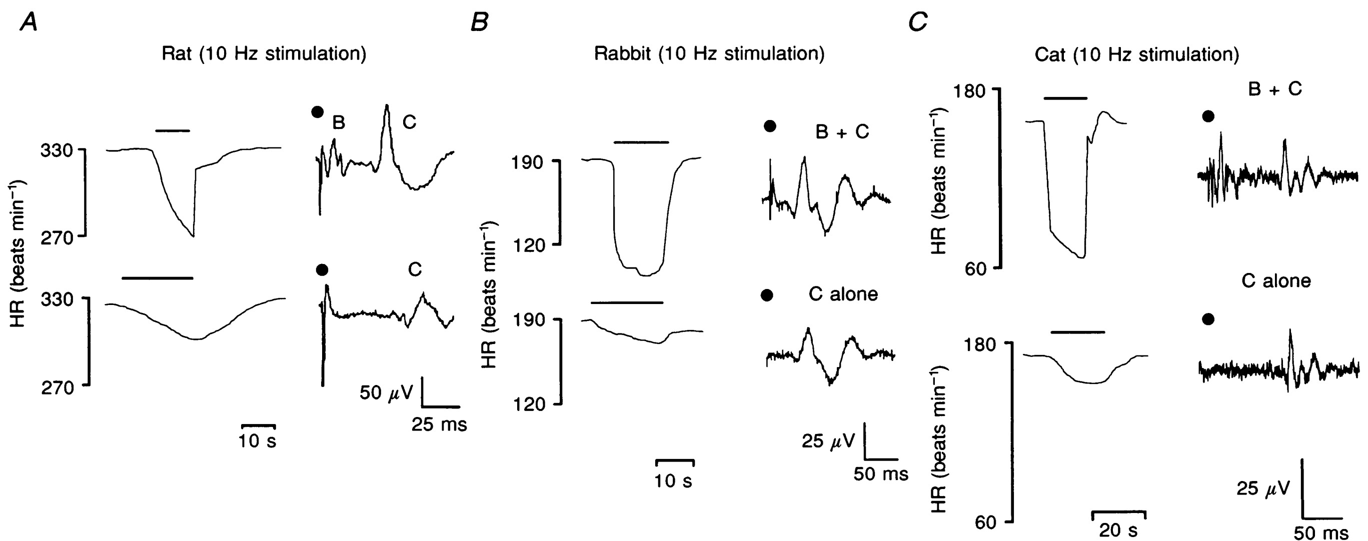

Figure 3. Heart rate responses to selective vagal stimulation in rat, rabbit and cat

Traces show the heart rate responses produced by electrical stimulation of the cervical vagus (1 ms, 10 V, 10 Hz). Responses to stimulating B + C fibres were evoked with the cathode facing the heart and no anodal block. Responses to stimulating C fibres alone were evoked with the anode facing the heart and the anodal block technique applied. Electrode arrangement as in Fig. 1*A*.

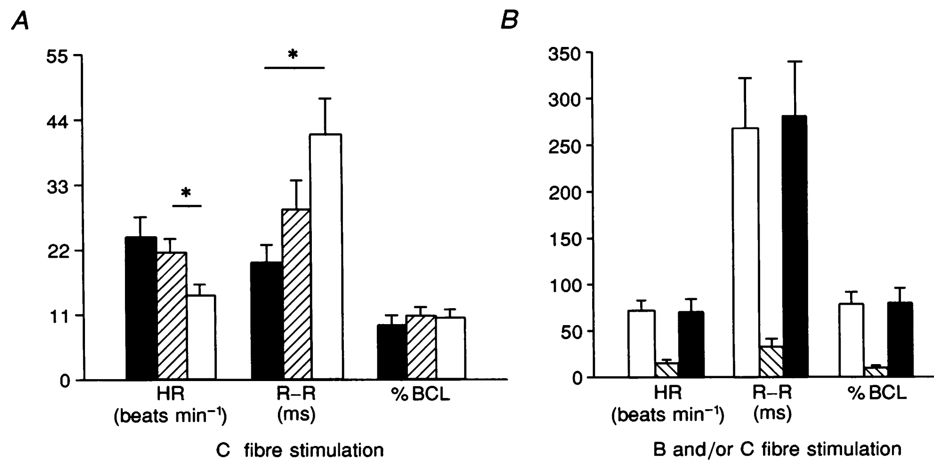

Figure 4. Cardioinhibition obtained by selective vagal stimulation

A, a comparison of the cardioinhibition evoked by selective C-fibre stimulation (1 ms, 5–15 V, 10 Hz for 20 s) in rats (■) $n = 5$, rabbits (▨) $n = 8$ and cats (□) $n = 8$ (electrode arrangement as in Fig. 1*A*). The same data are expressed as the decrease in heart rate (HR) in beats per minute (beats min^{-1}), the absolute increase in cardiac interval (R–R) in milliseconds and as the increase in cycle length as a percentage of the baseline cardiac cycle (%BCL). Values on the abscissa refer to the relevant units of each data set. $*P < 0{\cdot}05$, unpaired t test ($n =$ number of animals). *B*, a comparison of the cardioinhibitory effects evoked in 5 cats by stimulating B or C fibres individually, or simultaneously; B (□), C (▧) and B + C (■); (0·02–1 ms, 5–10 V, 5 Hz for 10 s). These results were obtained using the experimental approach outlined in Fig. 1*B*.

voltages (e.g. 23 V), a long latency input was sometimes observed (Fig. 6*Ab*). Since this response was shown to collide with spontaneous activity (Fig. 6*Ad*) it was clearly a break excitation of the antidromic neurone.

The mechanism by which a late anodal break excitation is produced could be examined. There are two possibilites. First, the break excitation may actually occur at a short latency but because of a slowed rate of conduction around the anode it actually appears at a much later time. This is unlikely to be the explanation of the late break since either decreasing the time constant of the anodal decay, or increasing the voltage applied at the anode shortened the latency of the break spikes. Second, when a prolonged anodal current is applied to the axon (Fig. 6*B*, continuous line) the axonal excitability might accommodate (Fig. 6*B*, dashed line). The late break spike would then occur when the anodal decay intersects the altered time course of

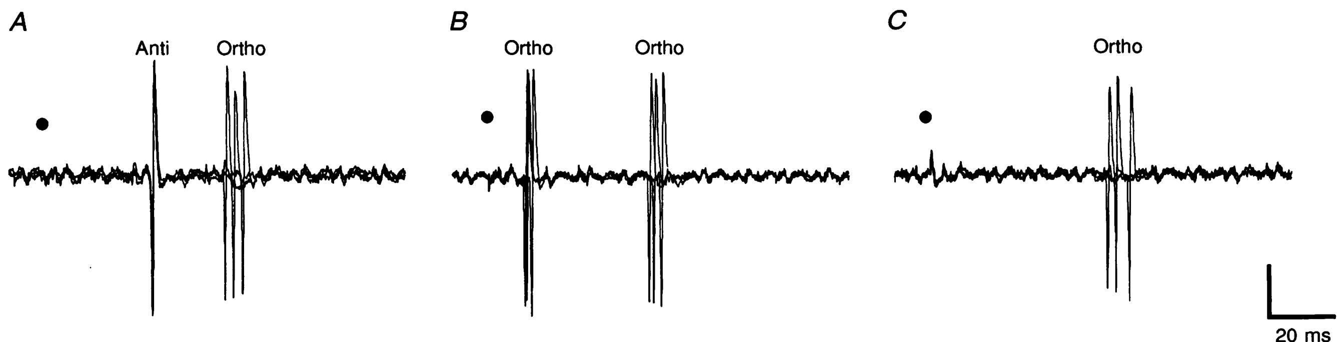

Figure 5. Brainstem recording from a single C-fibre vagal preganglionic neurone in rat

Antidromic (Anti) and orthodromic (Ortho) activity evoked in a vagal preganglionic neurone with C-fibre axons by a single pulse stimulation to the vagus (3–10 V, 1 ms, 1 Hz) at ●. *A* and *B*, conventional stimulation; the cathode was central and all vagal fibres were stimulated. Orthodromic input with a long latency is observed and in some sweeps a shorter latency orthodromic input is also evoked. When this occurs, the antidromically evoked activity is cancelled. *C*, anodal block; the cathode was peripheral and anodal block applied so that the faster conducting inputs were blocked and only the long latency orthodromic input is seen. Each panel shows 3 superimposed sweeps.

axonal excitability. In the example shown in Fig. 6 there was a period from 21 ms before the stimulus to 75 ms following it, during which the presence of an orthodromic spike prevented the appearance of the break spike. Since the antidromic conduction time was 25 ms (Fig. 6*Aa*), the break spike must have been evoked at 50 ms (dashed line). The usual 'critical period' for collision of this spike would be expected to be from ~25 ms to 75 ms (twice the conduction time). This leaves a period of 46 ms unaccounted for, which may represent the resetting of the axonal excitability.

Recordings were obtained from eighty-nine vagal antidromically activated neurones. Twelve had estimated conduction velocities indicating that they had A-fibre axons ($27{\cdot}9 \pm 1{\cdot}4$ m s^{-1}), twenty-two B-fibre axons ($5{\cdot}6 \pm 0{\cdot}4$ m s^{-1}) and fifty-five C-fibre axons ($1{\cdot}2 \pm 0{\cdot}0$ m s^{-1}). A late excitatory input was seen in only two of the A-fibre neurones (mean spike latency, 40 ms) and in only five of the B-fibre neurones (mean latency, $64{\cdot}0 \pm 14{\cdot}8$ ms). In contrast, of the forty-six C-fibre neurones analysed, thirty demonstrated a break excitation at long latency (mean latency, $63{\cdot}0 \pm 3{\cdot}3$ ms). The mean voltage which elicited break spikes in the unmyelinated fibres was $11{\cdot}2 \pm 0{\cdot}9$ V ($n = 30$) and $5{\cdot}0 \pm 1{\cdot}2$ V in the myelinated fibres. Whilst break excitation was more common in the C-fibre population, sixteen of the C-fibre axons could not be induced to evoke break spikes. It is not clear why this should be so. There was no significant difference between the conduction velocity of these fibres and those that did show break excitation ($1{\cdot}1 \pm 0{\cdot}1$ and $1{\cdot}2 \pm 0{\cdot}1$ m s^{-1}, respectively), nor is there any difference in the strength–duration curves for activation of these fibres

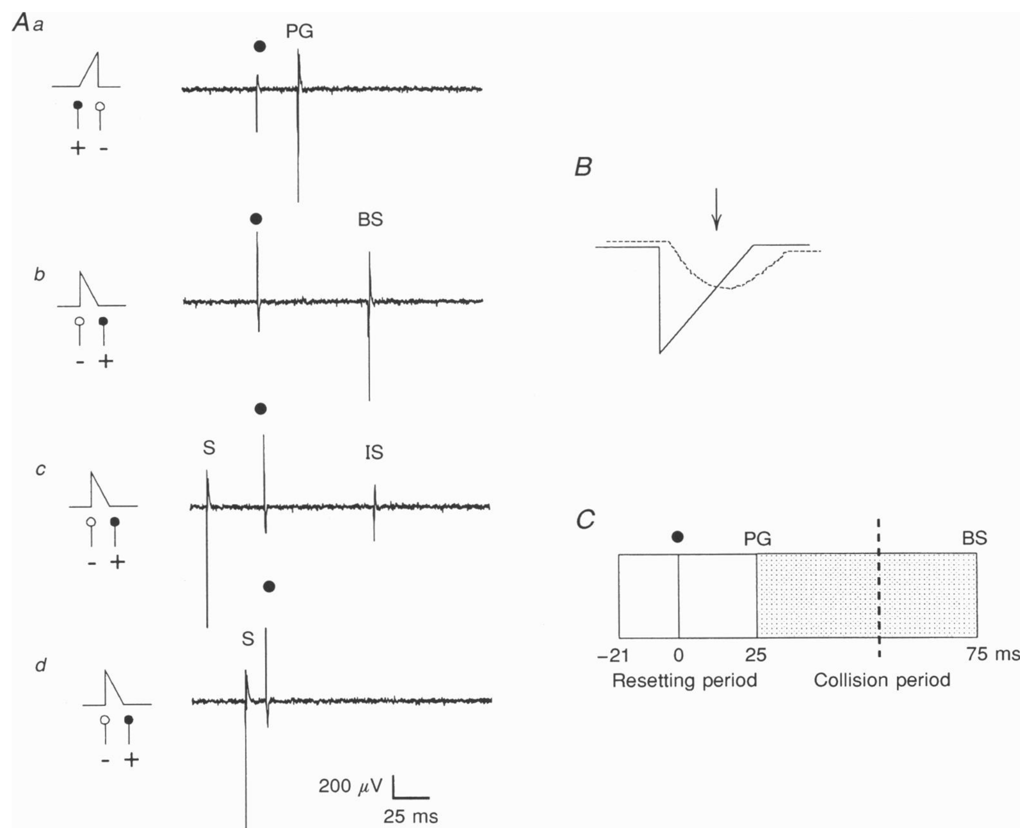

Figure 6. Mechanism of anodal break excitation

A, rat C-fibre vagal preganglionic neurone (PG) activated in the first sweep (*a*) by conventional stimulation at ●, with the cathode central and then in subsequent sweeps at a higher voltage (1 ms, 23 V) with anodal block (anode central). In the second sweep (*b*) the antidromic response has been blocked but a break spike (BS) has been evoked. In sweep *c*, a spontaneous spike (S) fails to collide with the break spike (seen here as the initial segment potential, IS, but cancellation does occur in sweep four (*d*) when the spontaneous spike arrives within the critical period. *B*, a possible interpretation of the anodal break phenomenon. The continuous line is the time course of the anodal current and the dashed line represents axonal excitability. The arrow indicates where the curves cross, when a late break excitation would occur. *C*, the time course of cancellation of the break spike by spontaneous spikes of the neurone illustrated in *A*. The dashed line represents the moment that corresponds to the arrow in *B*. Spontaneous spikes which cause cancellation of the break spike before 25 ms arrive before even the anodal break has occurred and must be resetting the excitability of the axon.

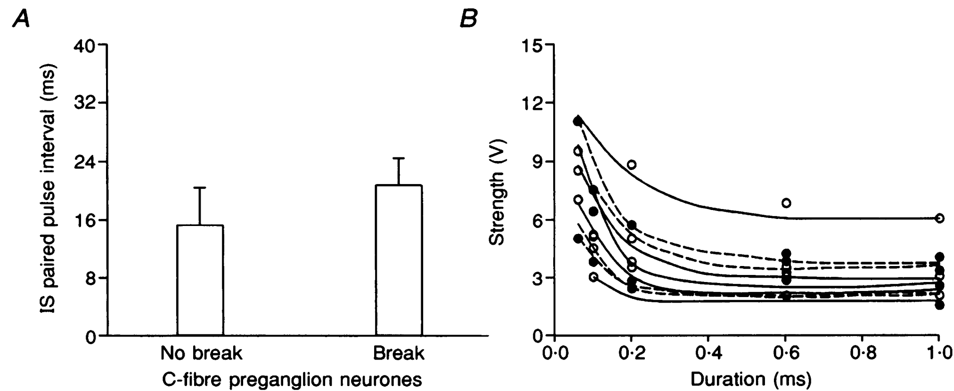

Figure 7. A comparison of the properties of C-fibre preganglionic neurones with and without break excitation

A, initial segment frequency following of rat C-fibre preganglionic neurones to conventional vagal stimulation (cathode central); break, $n = 7$; no break, $n = 6$. There is no significant difference between these neurones (unpaired t test). *B*, strength–duration curves for activation of 9 C-fibre preganglionic neurones, of which 4 showed break excitation. ○, no break, $n = 5$; ●, break, $n = 4$.

(Fig. 7). It is possible that one population of cells receives a strong inhibitory input, which would shunt break-evoked spikes, but if this were the case then their frequency following might be expected to differ. As Fig. 7 shows there was no significant difference in the frequency following of C fibres that showed break excitation and those that did not.

Combined collision and anodal block techniques

Although the majority of axons showing break excitation were C fibres, the present findings demonstrate that some myelinated axons also show a late break excitation. Since these would occur during the time of the C-fibre component of the compound action potential, they would be masked by it and would not be detected. To assess the possibility

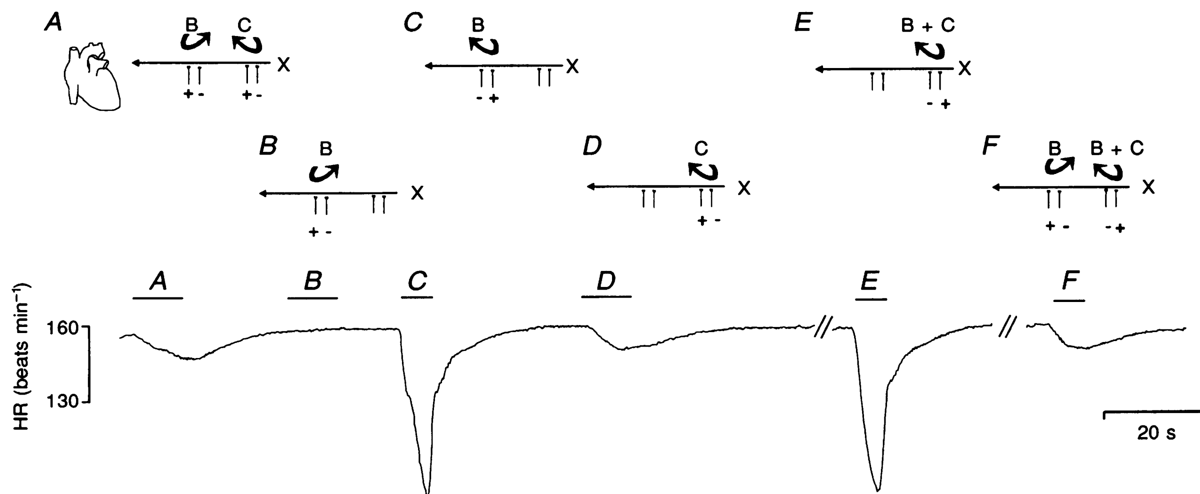

Figure 8. Heart rate responses to stimulation of B or C fibres of the vagus nerve in cat

A, combined stimulation of B and C fibres using the protocol outlined in Fig. 1*C* to block B-fibre conduction to the heart. *B*, no cardioinhibition is evoked when there is anodal block of B fibres when stimulating B fibres alone. *C*, conventional stimulation of B fibres alone slows the heart. *D*, selective stimulation of C fibres alone evokes a bradycardia. *E*, conventional stimulation of B+C fibres slows the heart. *F*, conventional stimulation of B + C fibres at the distal electrode and collision of B fibres by antidromic activity elicited at proximal electrode produces a bradycardia which resembles the stimulation of C fibres alone, as *D*. B = 0·02 ms, 5 V, 5 Hz; C = 1 ms, 6 V, 5 Hz.

that these break spikes could account for the C-fibre bradycardias evoked by vagal stimulation the experimental arrangement of Fig. 1*B* was devised. The bipolar electrode was used to selectively stimulate C fibres whilst the tripolar electrode was used with conventional polarity to activate B fibres. These action potentials will conduct both orthodromically, to slow the heart and antidromically, where they will collide with any action potentials in B fibres that have either escaped anodal block or showed break excitation. In five cats, such low frequency stimulation of B fibres alone (5 Hz for 10 s) evoked a marked fall in heart rate (72 ± 11·2 beats min^{-1}) and additional recruitment of C fibres by low frequency stimulation at the bipolar electrode produced no significant additional fall in heart rate (73 ± 12·8 beats min^{-1}) (Fig. 4*B*). This is still true when the data are calculated as increases in cardiac interval (Fig. 4*B*). This result would be obtained if the effects of B- and C-fibre stimulation were subject to occlusion. A method of unmasking such occlusion was designed (Fig. 1*C*), in which two bipolar electrodes were placed on the vagus nerve. The peripheral electrode is stimulated at an intensity which will stimulate action potentials only in B fibres, which will then travel antidromically towards the second electrode, peripheral conduction being prevented by the anodal block. The central electrode is stimulated at a higher intensity to selectively stimulate C fibres. However, if any B-fibre activity passes the block, or is evoked at the anodal break, then it will collide with the antidromically evoked B-fibre activity, preventing any contaminant B-fibre activity reaching the heart. This protocol was carried out repeatedly in two cats and the same results were obtained in each animal. As illustrated previously (Fig. 4*A*), selective stimulation of C fibres evoked a fall in heart rate of 9 beats min^{-1} (Fig. 8*D*) whereas stimulation of B fibres produced a larger bradycardia of 69 beats min^{-1} (Fig. 8*C*), which is similar to the fall produced by stimulation of B and C fibres together (67 beats min^{-1}; Fig. 8*E*). Antidromic stimulation of B fibres evoked no heart rate response when applied alone (Fig. 8*B*) and had no effect on the size of the bradycardia evoked by stimulating C fibres (compare *A* and *D* of Fig. 8). In contrast, such antidromic stimulation of B fibres reduces the size of the bradycardia evoked by simultaneous stimulation of B and C fibres to the magnitude of that seen when C fibres are stimulated alone (compare *D*, *E* and *F* of Fig. 8). The degree of cardioinhibition obtained by selectively activating C fibres (using anodal block; 10·7 ± 1·7 beats min^{-1}, $n = 9$) was not reduced by incorporating B-fibre collision with the anodal block (11 ± 1·8 beats min^{-1}, $n = 6$ observations, respectively). This cardioinhibition was also not significantly different from conventional stimulation of both B and C fibres combined with B-fibre collision (10 ± 2 beats min^{-1}, $n = 3$ observations). These experiments were also performed using different delays (0, 5 and 10 ms) between stimuli applied at the two electrode pairs with the stimulation rate kept at 5 Hz throughout, for each electrode. The same results were obtained in each case. Taken together, these results provide strong evidence that C fibres in the vagus of the cat can indeed slow the heart.

DISCUSSION

The present experiments have demonstrated that in cats stimulation of non-myelinated vagal fibres can evoke falls in heart rate and confirms the work of others for rat and rabbit (Nosaka *et al.* 1979; Wooley *et al.* 1987). The relative efficacy of the species' cardiac C fibres depends on the method of analysis. Selective stimulation of non-myelinated fibres evokes a greater fall in heart rate in rabbits than in cats, but in contrast the increase in cardiac interval evoked by such stimuli is greatest in cats. These differences are due to the different baseline heart rates in the different species. If the results are normalized, by expressing them as changes in cardiac interval as a percentage of their baseline cardiac intervals, then all three species show a similar response. Although the present study has not addressed the question of whether these vagal C fibres are afferent or efferent, McWilliam & Wooley (1990) provided evidence that at least in the rabbit they are probably efferent, since chronic supranodose vagotomy abolishes the C-fibre bradycardia.

Previous studies on cats have suggested that vagal control of heart rate is mediated exclusively (Middleton *et al.* 1950; Kidd & McWilliam, 1982) or mainly (Heinbecker & Bishop, 1935) by the small myelinated efferent fibres, which also appear to mediate the reductions in atrial contraction and slowing of atrio-ventricular conduction evoked by vagal stimulation (Kidd & McWilliam, 1982). There are several problems with these earlier studies. In order to assess the contribution of non-myelinated fibres to cardiac responses the vagi were stimulated at intensities and frequencies sufficient to activate only myelinated axons and the effects recorded. The stimuli were then increased to recruit non-myelinated fibres and any additional effects noted. Thus, this protocol compared the effects of stimulating myelinated fibres with those of stimulating both myelinated and non-myelinated fibres, the contribution of the non-myelinated component being derived by subtraction. This protocol has two major inherent problems. First, if stimulation of myelinated fibres produces a near-maximal response, then effects evoked by recruitment of non-myelinated fibres may well be missed. This could be due to occlusion of convergent myelinated and non-myelinated preganglionic input to postganglionic neurones, or the production of a near-maximal postganglionic response at the effector organ. Second, those studies have usually measured changes in heart rate evoked by vagal stimuli. However, the relationship between heart rate and cardiac interval is alinear, and at low heart rates, when cardiac interval is

long, it is possible to evoke large changes in cardiac interval without changing heart rate significantly. These conditions actually prevail at rest in cats and also following myelinated fibre stimulation. Therefore, if only heart rate is monitored, small effects of C-fibre stimulation will be less easy to detect. However, in the present study it was confirmed in cats that recruitment of C fibres produced no further cardioinhibitory effect over and above that evoked by B-fibre stimulation alone (Fig. 4*B*), whether the responses were expressed either as changes in heart rate or as cardiac interval.

Evidence for a possible role for non-myelinated fibres in heart rate control has been provided previously in other species, such as rat (Nosaka *et al.* 1979), rabbit (Heinbecker & Bishop, 1935; Wooley *et al.* 1987) and guinea-pig (McWilliam & Wooley, 1987). The data presented here confirm this role in rats and rabbits and extend it to include cats. The validity of the data presented in the present report is dependent upon the ability of the anodal block technique to reliably and completely distinguish between stimulation of myelinated and non-myelinated fibres. Other techniques have been used to activate selectively or block particular groups of fibres but they are less suitable than the anodal block technique used in this study. Local anaesthetic block (Heavener & De Jong, 1974) does not discriminate adequately between non-myelinated and small myelinated fibres (Nathan & Sears, 1961). Gradual cooling (Paintal, 1965) will block large myelinated fibres before it affects smaller fibres and would be a possible method to use. However, the size of the probe required makes this technique less suitable for smaller animals where the length of the nerve is limited and, in this respect, anodal block has an advantage over cooling. In addition, with anodal block there is less chance that C-fibre discharge will also be reduced at the stage when myelinated fibres are blocked. This difference could be important if the effects of C fibres are weak. One problem with the traditional anodal block technique, which uses a square-wave blocking current, was the liability for an anodal break excitation to occur when the block was removed. In addition, if the block was applied continuously the nerve was likely to deteriorate quite rapidly (Whitwam & Kidd, 1975). The modification of the technique used in this study, using a triangle-shaped blocking current that decays exponentially, is said to reduce the problem of anodal break excitation and is less likely to damage the nerve, since the block is applied phasically at the time of stimulation (Accornero *et al.* 1977).

The problem of break excitation is particularly relevant in the present experiments, since a break excitation of B fibres might occur during the C wave of the compound action potential and thus go unobserved. Since stimulation of only a few B fibres can have a significant effect on heart rate (McAllen & Spyer, 1978), such a break excitation could evoke a change in heart rate which was wrongly attributed to stimulation of C fibres. This has been tested in the present experiments by recording the activity of single vagal neurones with myelinated and non-myelinated axons. With the stimulation parameters used to selectively activate C fibres, B-fibre neurones were rarely activated. When higher stimulating voltages were used (> 12 V), a long latency-break excitation was observed in some C-fibre neurones. In cats, a final experiment was performed (Fig. 8) whereby B fibres were activated from one electrode and conducted antidromically to the stimulating electrode evoking the cardiac response (Fig. 1*C*) where they would collide with any errant activity evoked in B fibres, however they were produced. There were no differences between the cardiac effects evoked by selectively stimulating C fibres with, and without the collision of B fibres, suggesting that even if a few B fibres had been activated by break excitation this is unlikely to account for the bradycardia evoked. It is still possible that break excitation did take place at the right-hand pair of electrodes (Fig. 8) but that this was in B fibres that were not activated by the stimulus applied to the left-hand pair of electrodes. The action potentials in these B fibres would not have collided out and they may therefore be the cause of the observed bradycardia. This possibility is considered unlikely because if all the B fibres had not been recruited at the left-hand pair of electrodes it is to be expected that the C-fibre bradycardia would be at least significantly reduced. However, there was no significant difference between the bradycardias obtained using anodal block alone and those seen when combining this with a collision technique. Evidence is also presented which suggests that all the B fibres had indeed been recruited for collision purposes. When B fibres were antidromically activated at the same time as the B and C fibres from the right-hand electrode (Fig. 8*F*) the bradycardia obtained by stimulating both B and C fibres conventionally (which would be expected to recruit all B fibres) was reduced to the same size as that evoked by C-fibre stimulation alone. Due to the remaining doubt, the experimental arrangement of Fig. 1*C* does not yield definitive proof that C fibres slow the heart of the cat, but rather it represents a failure to disprove that these fibres have a negative chronotropic action.

The cardiac changes evoked by selective stimulation of B or C fibres are quantitatively and qualitatively different. The bradycardias evoked by activating C fibres are smaller in magnitude (Figs 4 and 8) and have a less rapid onset (Figs 3 and 8) than those evoked by B-fibre stimulation. Even stimulation with a single pulse evokes a double inhibitory effect on the heart with C fibres contributing to the second component (J. F. X. Jones, Y. Wang & D. Jordan, unpublished observations). This might suggest that the responses are mediated by different functional pathways and/or different neurotransmitters. In all the species studied the chronotropic effects of both B- and C-fibre responses are blocked by the muscarinic receptor

antagonist, atropine; but there are reports in rabbits that the bradycardia evoked by non-myelinated vagal axons is resistant to the nicotinic ganglion blocker, hexamethonium (Ford & McWilliam, 1986; Wooley *et al.* 1987; McWilliam & Wooley, 1990). More recently, Seabrook, Fieber & Adams (1990) reported a component to transmission in cardiac ganglia which is not sensitive to nicotinic antagonists. It has now been demonstrated that excitatory synaptic transmission in cardiac ganglion cells from guinea-pigs (Allen & Burnstock, 1990), rats (Selyanko & Skok, 1992) and dogs (Xi-Moy, Randall & Wurster, 1993) is mediated by both fast and slow EPSPs which act via nicotinic and muscarinic M_1 receptors, respectively. Using the techniques described in the present paper, preliminary studies have confirmed that in rabbits the bradycardia evoked by selectively stimulating non-myelinated vagal fibres is resistant to the nicotinic ganglion blockers hexamethonium and chlorisondamine but can be attenuated by low doses of pirenzipine, an antagonist at muscarinic M_1 receptors (Jones & Jordan, 1993). A full neuropharmacological investigation of ganglionic neurotransmission of the B- and C-fibre responses has not yet been performed.

Stimulation of pulmonary C fibres evokes a large bradycardia that is not modulated in phase with respiration, and Daly (1991) suggested that this might be mediated by activation of the C-fibre efferents. Indeed, Dawes, Mott & Widdicombe (1951) demonstrated that the bradycardia induced by phenyldiguanide is not abolished until the vagi are cooled to 3 °C, suggesting that both afferent and efferent limbs of the reflex involve non-myelinated fibres. Although, in the present study it has been demonstrated that selective activation of C-fibre efferents can indeed slow the heart, the magnitude of the response is not sufficient to account for the pulmonary C-fibre bradycardia. Indeed, there is evidence that both B- and C-fibre cardiac vagal preganglionic neurones are activated when pulmonary C fibres are stimulated (Jones *et al.* 1994). Preganglionic neurones with B-fibre axons fire phasically with both beat-by-beat and breath-by-breath rhythms (McAllen & Spyer, 1978) whereas those with C-fibre axons fire tonically (Ford, Bennett, Kidd & McWilliam, 1990; Jones *et al.* 1995). A convergence of these inputs at the level of the ganglion may govern the final vagal control of the heart.

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