

Properties of presympathetic neurones in the rostral ventrolateral medulla in the rat: an intracellular study 'in vivo'

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1. Intracellular recordings were made in pentobarbitone-anaesthetized rats from sixty-eight neurones located in the rostral ventrolateral medulla (RVLM), which responded with inhibition (latency, 33.6 ± 9.3 ms) after stimulation of the aortic depressor nerve with short bursts of pulses. This inhibition was due to chloride- and voltage-dependent IPSPs.
2. Seventeen neurones could be excited antidromically after stimulation in the T2 spinal segment (conduction velocity, $1.9\text{--}8.5$ m s⁻¹) and were classified as RVLM presympathetic vasomotor neurones.
3. 'Spontaneously' active neurones ($n = 29$) displayed a largely irregular pattern of firing, with no clear relationship between the level of the membrane potential and cycles of phrenic nerve activity at end-tidal CO₂ < 5.0%. Cardiac cycle-related shifts of the membrane potential were not considered indicative of baroreceptor input as they could be due to movement artifacts.
4. All neurones displayed large synaptic activity (EPSPs and IPSPs, peak-to-peak amplitude > 5.0 mV). The depolarizing IPSPs observed during injection of chloride and/or negative current consisted of a phasic and a tonic component.
5. The on-going activity of these neurones resulted from synaptic inputs, with individual action potentials usually preceded by identifiable fast EPSPs.
6. No evidence was found for the presence of gradual depolarizations (autodepolarizations) between individual action potentials, and therefore under these experimental conditions the activity of RVLM presympathetic neurones did not depend on intrinsic pacemaker properties.
7. These results are consistent with the 'network' hypothesis for the generation of sympathetic vasomotor tone.

There is now considerable evidence that a distinct neuronal subpopulation located in the rostral and ventrolateral part of the medulla oblongata (the rostral ventrolateral medulla, RVLM) plays a critical role in maintaining the activity of vasomotor preganglionic sympathetic neurones and in evoking changes in arterial blood pressure by a variety of cardiovascular reflexes, including the arterial baroreceptor reflex (for review see Guyenet, 1990; Dampney, 1994; Reis, Golanov, Ruggiero & Sun, 1994). The major features of these neurones include sensitivity to activation or inactivation of arterial baroreceptors, and axonal projection to the spinal cord where they excite, probably monosynaptically, pre-

ganglionic sympathetic neurones. These barosensitive and bulbospinal RVLM 'presympathetic' neurones (also referred to as 'vasomotor', 'sympathoexcitatory' or 'subretrofacial') have been extensively studied with *extracellular* micro-electrodes in rats (e.g. Brown & Guyenet, 1984; Morrison, Milner & Reis, 1988; Kanjhan, Lipski, Kruszezwska & Rong, 1995), cats (e.g. McAllen, 1987) and rabbits (e.g. Terui, Saeki & Kumada, 1986), but so far only a few attempts have been made to study their intracellular properties *in vivo* (Dembowsky & McAllen, 1990; Granata & Kitai, 1992; Lipski, Kanjhan, Kruszezwska & Rong, 1995a).

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The aim of this study was to further examine the electrophysiological properties of RVLM presympathetic neurones using intracellular microelectrodes in anaesthetized rats. In particular, we wanted to find out whether synaptic inhibition of these neurones after stimulation of baroreceptor afferents is due to disfacilitation or active inhibition (IPSPs). The answer to this question should provide additional information regarding the organization of central baroreceptor pathways. The second goal was to examine the evidence for two possibilities: (a) that the activity of RVLM presympathetic neurones *in vivo* is due to synaptic inputs as proposed by Barman & Gebber (1987); and (b) that this activity is largely determined by intrinsic pacemaker properties. A pacemaker hypothesis of basal sympathetic tone generation was initially suggested by Sun, Guyenet and their colleagues (Sun, Hackett & Guyenet, 1988*a*; Sun, Young, Hackett & Guyenet, 1988*b*) mainly on the basis of the intracellular studies conducted in medullary slices, and extracellular recordings *in vivo* in the presence of a glutamate receptor antagonist.

METHODS

Animal preparation

Experiments were conducted on thirty-six male Wistar rats (380–500 g) anaesthetized with sodium pentobarbitone (Nembutal, 80 mg kg⁻¹, i.p.; supplementary doses, 4–6 mg h⁻¹, i.v.). The surgical techniques were similar to those described in recent studies conducted in our laboratory (Kruszezwska, Lipski & Kanjhan, 1994; Kanjhan *et al.* 1995). In brief, after cannulation of the trachea, femoral artery and external jugular vein, three nerves were dissected and prepared for standard bipolar stimulation or recording: the phrenic nerve, aortic depressor nerve and the mandibular branch of the facial nerve (the latter nerve was dissected on both sides). The animals were placed in a stereotaxic frame and the dorsal surfaces of the medulla oblongata and spinal cord (C1–T2) were exposed by interparietal and occipital craniotomy, laminectomy and suction of the cerebellum. All exposed tissues were covered with warm paraffin oil. The animals were paralysed with pancuronium bromide (Pavulon, 0.4 mg h⁻¹, i.v.). Analgesia was ensured by the absence of any significant changes in blood pressure, heart rate and of the rhythm of respiratory activity recorded from the phrenic nerve in response to paw pinch. In addition, the animals were allowed to recover from muscle paralysis at regular intervals, at which time the adequacy of anaesthesia was assessed by testing for the absence of withdrawal reflex. The protocol was approved by the University of Auckland Animal Ethics Committee and fully complied with.

The animals were ventilated with O₂-enriched air (frequency usually 70 min⁻¹) after a bilateral pneumothorax was made. An expiratory load of 2–3 cmH₂O was applied. The values of the end-tidal CO₂ were measured as described previously (Kanjhan *et al.* 1995) and maintained between 4 and 5% by adjusting tidal volume. As the vagus nerves were left intact, the cycles of phrenic nerve activity were synchronized (at 1:1 or 1:2 ratio) with the cycles of the ventilator.

At the end of each experiment the animals were killed with an overdose of anaesthetic, or deeply anaesthetized with an additional dose and perfused transcardially with a fixative.

Stimulation and recording

The activity of the phrenic nerve was amplified and filtered (60 Hz to 3 kHz). The aortic nerve, which in the rat contains only baroreceptor afferents (e.g. Saprú & Krieger, 1977), was stimulated at a frequency of 0.5–1 Hz with trains of three stimuli (pulse width, 0.2 or 0.5 ms), 5 ms apart (interelectrode distance, 2 mm). The stimulus intensity was adjusted to 5 times the threshold for evoking a depressor response during stimulation of the nerve at 100 Hz for approximately 5 s (threshold stimulus intensity was usually 0.1–0.5 V, corresponding to 2–10 μ A). The mandibular branch of the facial nerve was stimulated with 0.1 ms pulses, at a frequency of 5 Hz (2.0–5.0 V). A specially designed stimulating electrode was placed inside the oesophagus and was used for antidromic stimulation (1.0 ms pulses, up to 75 V, 2 Hz) of motoneurons of the compact formation of the nucleus ambiguus (Kruszezwska *et al.* 1994, and below). Two monopolar stimulating electrodes (Parylene-C insulated stainless steel; shaft diameter, 0.25 mm; tip exposure, 0.2 mm; Micro Probe Inc.) were placed in the lateral funiculus at the T2 segment ipsilaterally to the recording side in the RVLM, and were used for antidromic identification of neurones projecting to this spinal level (0.2 ms pulses, up to 1.0 mA).

Initial mapping of the positions of the facial nucleus and compact formation of the nucleus ambiguus was made in each experiment with low-impedance glass microelectrodes filled with 2 M NaCl (resistance, 4–8 M Ω). Tracking was done with electrodes orientated vertically to the dorsal medullary surface, at 75 or 100 μ m intervals in the mediolateral direction and 100 or 200 μ m in the rostrocaudal direction, using a high-speed microdrive (SCAT-1, Digitimer Ltd, Welwyn Garden City, UK). Intracellular recordings (NeuroLog NL102/NL104/NL125 amplifiers/filters, Digitimer Ltd; bandwidth: 0–10 kHz DC, 0.1 Hz to 10 kHz AC) were made with high-impedance bevelled microelectrodes containing either 2 M potassium methyl sulphate (resistance, 50–70 M Ω), 3 M KCl (resistance, 35–40 M Ω), 1 or 1.5% neurobiotin in 0.4 M KCl (resistance, 80–100 M Ω), or 3 or 5% Lucifer Yellow carbohydrazine (Molecular Probes) in 0.4 M lithium chloride (resistance, 75–85 M Ω). The peak-to-peak amplitude of the electrical ‘noise’ recorded extracellularly (with capacity compensation just below the level at which ‘ringing’ was observed) was less than 1.0 mV (less than 0.5 mV for microelectrodes filled with 3 M KCl). Tracking with intracellular microelectrodes was done at 50 or 75 μ m intervals in the mediolateral direction and 100 μ m in the rostrocaudal direction. After impalement, and a period of initial stabilization, two criteria had to be met before further analysis was made: (a) membrane potential greater than –40 mV; and (b) increased or decreased firing rate (with clear after-hyperpolarizations following each action potential) during the application of small depolarizing or hyperpolarizing currents. Axonal recordings were distinguished by the lack of synaptic ‘noise’, the inability to respond with a continuous train of action potentials during positive currents (or to reduce the firing frequency during negative currents) and lack of distinct after-hyperpolarizations. Input resistance was not measured systematically due to errors associated with the bridge balance technique when high-impedance microelectrodes are used to record from neurones located several millimetres (~3.0 mm) below the brain surface. Morphological properties of these neurones revealed after intracellular labelling with neurobiotin or Lucifer Yellow are the subject of a separate publication (Lipski, Kanjhan, Kruszezwska & Smith, 1995*b*).

All signals were monitored on a multichannel pen recorder or oscilloscopes, and selected signals were recorded on a thermal array recorder (Nikon Kohden, RTA-1100; frequency response, 0–10 kHz) or stored on digital tape (Vetter 3000, A. R. Vetter Co. Inc., Rebersburg, PA, USA; rise time of the channel for recording microelectrode signals, 25 μ s) for subsequent off-line analysis. Antidromic field potentials recorded from motoneurons of the compact formation of the nucleus ambiguus and synaptic potentials in RVLM neurones evoked by stimulation of the aortic nerve were routinely averaged (up to 100 averaged sweeps) using a computer-based data acquisition system (Axolab 1 and pCLAMP 5.5/Compaq 386; Axon Instruments Inc.). Peristimulus time histograms were computed using a separate acquisition system (Macintosh/MacLab 4), with 3.0 ms bin width and up to 500 accumulated sweeps. Values were expressed as means \pm s.d., and an independent Student's *t* test was used for statistical analysis.

RESULTS

Recording co-ordinates

The medullary area from which intracellular recordings were made was determined by initial mapping of the position of the facial nucleus and compact formation of the

nucleus ambiguus. The caudal and medial borders of the facial nucleus were identified by recording antidromic field potentials (latency, \sim 1.0 ms) evoked by stimulation of the ipsilateral mandibular branch of the facial nerve, as described by Brown & Guyenet (1984). The location of the compact formation of the nucleus ambiguus was mapped by recording antidromic field potentials (latency range of individual components, 5 to <100 ms) evoked by stimulation of the oesophageal wall, as described recently in our laboratory (Kruszewska *et al.* 1994). In rats, this part of the nucleus ambiguus extends rostrocaudally for approximately 800–1000 μ m, with the rostral end overlapping (by 100–200 μ m) with the caudal border of the facial nucleus. At this rostral level, antidromic potentials could be evoked from both the oesophagus and the facial nerve in single microelectrode tracks, with the oesophageal fields dorsal to the facial fields. At more caudal levels, only fields evoked by oesophageal stimulation could be recorded (Fig. 1).

The rostrocaudally orientated column of oesophageal motoneurons identified in this way served as a convenient

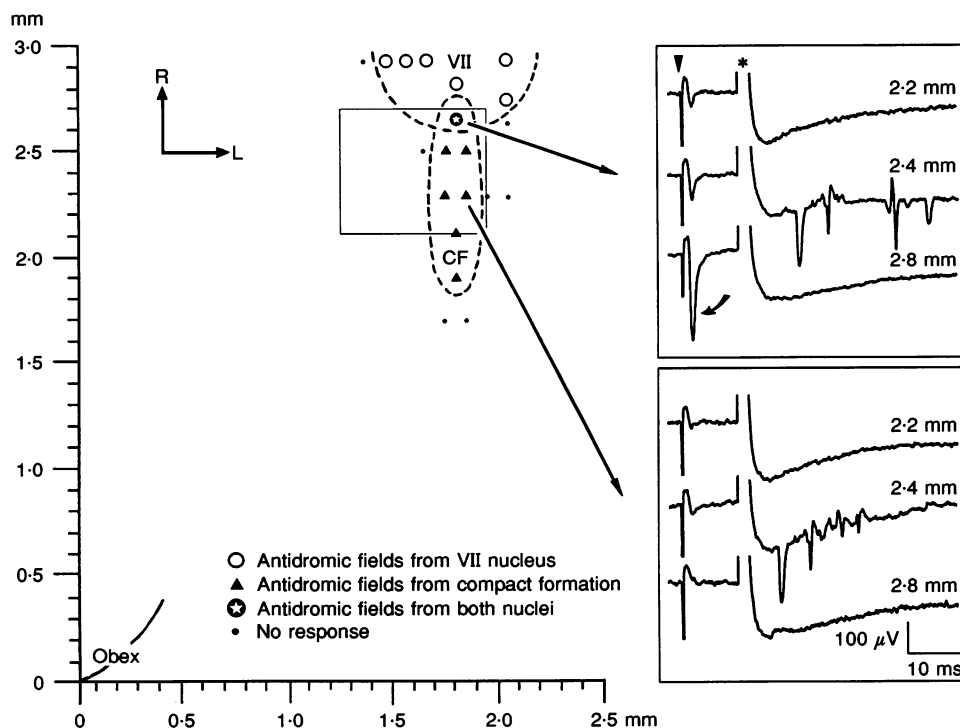


Figure 1. Example of a map showing microelectrode tracks in which antidromic field potentials could be recorded in the compact formation of nucleus ambiguus and the facial nucleus in one animal

Both nuclei are projected onto the dorsal medullary surface. Recordings made in two tracks, at three different depths (measured from the dorsal medullary surface), are shown on the right. In the rostral track, stimulation of the mandibular branch of the facial nerve (arrowhead) evoked antidromic responses within the facial nucleus at 2.8 mm (arrow). Stimulation of the oesophagus (*) evoked antidromic responses in both tracks at a depth of 2.4 mm. Each trace is an average of 40 sweeps. The boxed area behind the facial nucleus represents the region of subsequent intracellular recordings from RVLM neurones. Abbreviations: R, rostral; L, lateral; CF, compact formation of the nucleus ambiguus; VII, facial nucleus.

reference area for subsequent intracellular recordings from RVLm presympathetic neurones which are located ventral and ventromedial to this column (Kanjhan *et al.* 1995). In addition, this initial tracking allowed us to establish the depth of the column of units firing with a strong (on-off) expiratory activity forming, dorsally, parts of semi-compact formation of the nucleus ambiguus (motoneurons innervating muscles in the upper airways) and, more ventrally, interneurons of the Bötzing complex (Bryant, Yoshida, de Castro & Lipski, 1993). A map showing relative positions of the Bötzing and presympathetic neurones in the RVLm has been published elsewhere (Kanjhan *et al.* 1995).

Intracellular recordings were made in a region extending 100 μm rostral to 500 μm caudal from the caudal border of the facial nucleus, and mediolaterally from approximately the lateral edge of the compact formation to 700 μm medial (boxed area in Fig. 1). In the vertical plane, the recording area extended from 400 to 1000 μm below the compact formation.

Identification of RVLm presympathetic neurones

Neurones impaled in the region described above were tested for inhibition following stimulation of the ipsilateral or contralateral aortic depressor nerve. If such inhibition was recorded, some neurones were further examined for axonal projection to the T2 spinal segment.

Sixty-eight neurones (approximately 4% of all tested cells) responded with hyperpolarizing potentials following stimulation of the aortic depressor nerve (Figs 2A, B, 4, 6C, G, I, 7D and 9C). The mean level of membrane potential in these neurones, measured after a period of initial stabilization was 49 ± 8 mV (range, -40 to -71 mV). Some of these neurones ($n = 29$) displayed an on-going discharge throughout the period of recording (ranging from a few minutes to approximately 1 h), with the frequency of firing from 5 to 43 s^{-1} (Figs 5A, 6A, 7A, 8A and 9A). Stimulation of the aortic nerve evoked not only membrane hyperpolarization, but also a transient inhibition of the discharge in such neurones (Figs 2A and 9C). Action potentials were followed by after-hyperpolarizations (amplitude, 8.6 ± 4.5 mV; duration, 54 ± 21 ms), which were measured after the firing was slowed down by passing continuous negative current (≤ 0.2 nA; Fig. 5D). The remaining thirty-nine cells showed 'spontaneous' activity only immediately after impalement, with gradual deterioration of the membrane potential and increased firing frequency, followed by spike inactivation.

The hyperpolarizing potentials evoked by stimulation of the aortic nerve were recorded with microelectrodes containing a solution free of chloride ions or a low concentration of these ions (0.4 M). The onset latency and the latency to the peak of these potentials were 33 ± 8 and 56 ± 14 ms, respectively, when the ipsilateral aortic nerve

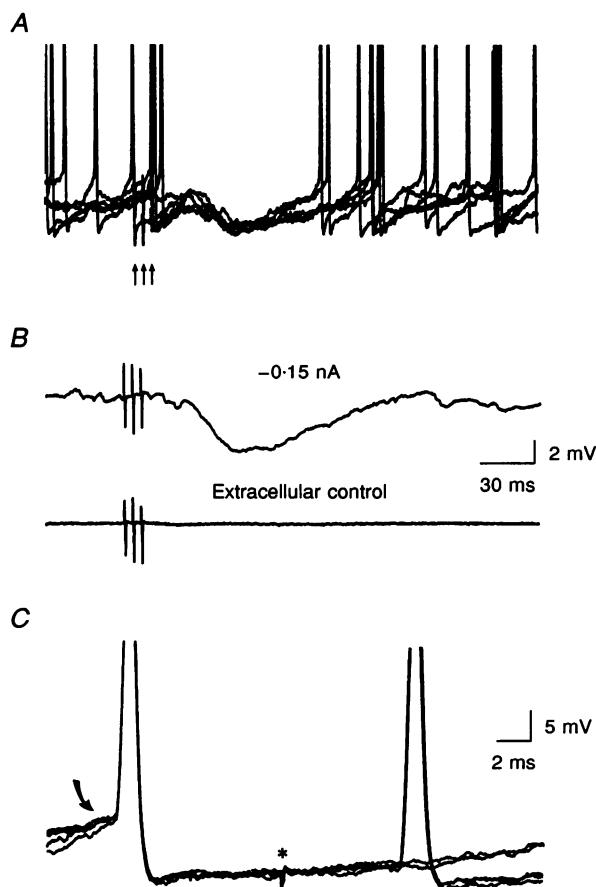
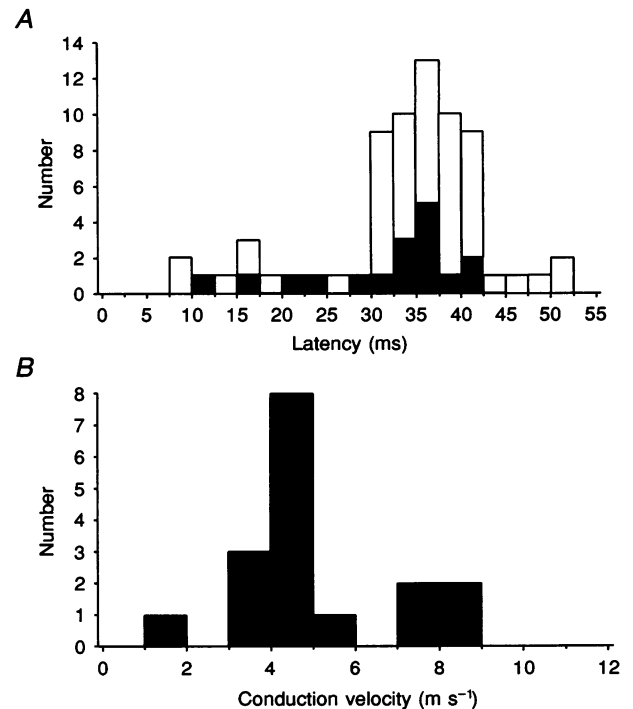


Figure 2. Electrophysiological identification of RVLm presympathetic neurones

A, hyperpolarizing potentials and inhibition of firing (6 superimposed sweeps; no polarizing current; membrane potential, -59 mV) evoked by stimulation of the ipsilateral aortic nerve with bursts of 3 pulses (arrows). B, the aortic nerve-evoked potentials (20 averaged sweeps) recorded intracellularly when the firing of the cell was stopped by a continuous negative current. The lower trace shows the extracellular control. C, antidromic action potentials evoked by near-threshold stimulation (*) in the T2 segment. Five superimposed sweeps pretriggerged by 'spontaneous' action potentials are shown. The arrow indicates EPSPs preceding 'spontaneous' spikes. Note that spinal stimulation was followed by antidromic responses only in 3 sweeps, and that the level of the membrane potential at which the action potentials occurred after spinal stimulation was about 7 mV more negative than the threshold at which 'spontaneous' action potentials were induced by on-going synaptic activity. Action potentials are truncated in A and C.

Figure 3. Histograms showing distribution of onset latencies of IPSPs evoked in RVLM neurones after stimulation of the aortic nerve (A), and conduction velocities of spinal axons of 17 of these neurones positively identified as bulbo-spinal (B)

The mean value of the onset latency of the IPSPs was 33.6 ± 9.3 ms for all neurones (□) and 31.8 ± 8.7 ms for bulbo-spinal neurones (■).



was stimulated ($n = 41$), and 35 ± 11 ms and 60 ± 22 ms, respectively, after stimulation of the contralateral nerve ($n = 27$). As the difference between these two groups was not significant ($P = 0.5$), the latency distribution is shown as a combined histogram (Fig. 3A, □). The amplitude of these potentials was 2.3 ± 1.7 mV when the ipsilateral nerve was stimulated and 2.0 ± 0.9 mV after stimulation of the contralateral nerve ($P = 0.5$). The duration was 72 ± 26 ms (ipsilateral) and 69 ± 37 ms (contralateral) ($P < 0.8$). In some cases (25%; e.g. Fig. 7D) the hyperpolarizing potentials were followed by a clear 'rebound' depolarization, which usually lasted until the end of the analysed sweeps (250 ms after the onset of stimulation).

The latency distribution and other properties of the hyperpolarizing responses, recorded in seventeen neurones which were positively identified as bulbo-spinal (see below) and fifty-one which were not, were similar (Fig. 3A, ■).

For stimulation of the aortic nerve, the choice of three-pulse trains with an amplitude of 5 times threshold was based both on the need to relate these results to our previous *extracellular* study in which a similar pattern of stimulation was used (Kanjhan *et al.* 1995), and on preliminary experiments demonstrating the effectiveness of such stimulation in evoking synaptic responses in RVLM neurones. Figure 4 illustrates the results of one of

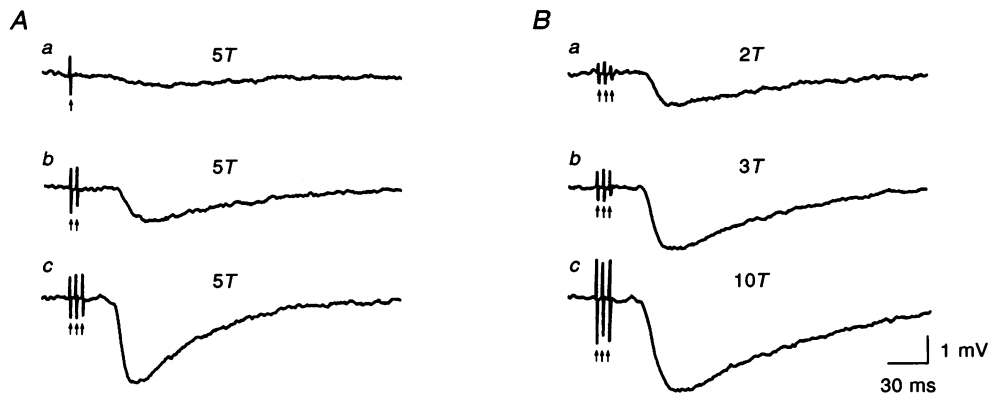


Figure 4. Example of temporal and spatial facilitation of IPSPs recorded in an RVLM presympathetic neurone after stimulation of the ipsilateral aortic nerve

Stimulus artifacts (arrows) occur near the beginning of each trace (average of 20–31 sweeps). Stimulus intensity in relation to threshold (T ; see Methods) is shown above each trace. A small continuous hyperpolarizing current (0.2 nA) was used to stop neuronal firing.

these early experiments in which temporal and spatial facilitation were tested. There was a clear increase in the amplitude of the hyperpolarizing synaptic responses when three pulses, 5 times threshold were used (Fig. 4A c) in comparison with the response evoked by three pulses, 3 times threshold (Fig. 4B b). Increasing the stimulus intensity to 10 times threshold (Fig. 4B c) resulted in only a small increase in the amplitude, and in an undesired larger drop of arterial blood pressure. The temporal summation was necessary as single pulses evoked little or no response (Fig. 4A a). Although bursts of four pulses resulted in a further increase of the response (not illustrated), using such bursts was not practical as the stimulus artifacts could interfere with measurements of responses occurring with short latencies.

The synaptic potentials evoked by aortic nerve stimulation were chloride and voltage dependent. A reversal of the polarity of these potentials after passing a negative current through a microelectrode containing 0.4 M KCl is

illustrated in Fig. 6G–I. The trace in Fig. 6G is a hyperpolarizing potential recorded soon after impalement. A small continuous hyperpolarizing current (−0.1 nA) was used to stop the firing. When the membrane was hyperpolarized with a larger current (−1.0 nA), the potential reversed in polarity (Fig. 6H). When the large negative current was discontinued, stimulation of the aortic nerve again resulted in a hyperpolarizing potential, although of a smaller amplitude (Fig. 6I). As illustrated in Fig. 7D, reversals could also be observed when a smaller negative current was passed through microelectrodes containing 3 M KCl and time was allowed (~5 min) for loading the cells with chloride. In such cases, reversals were observed not only during current injection, but also after the polarizing current was stopped. The occurrence of depolarizing IPSPs evoked by aortic nerve stimulation was tested in fourteen cells. Five of these cells were impaled with microelectrodes containing 0.4 M KCl (maximum current, −2.0 nA), four with 3 M KCl (maximum current, −0.5 nA) and two with

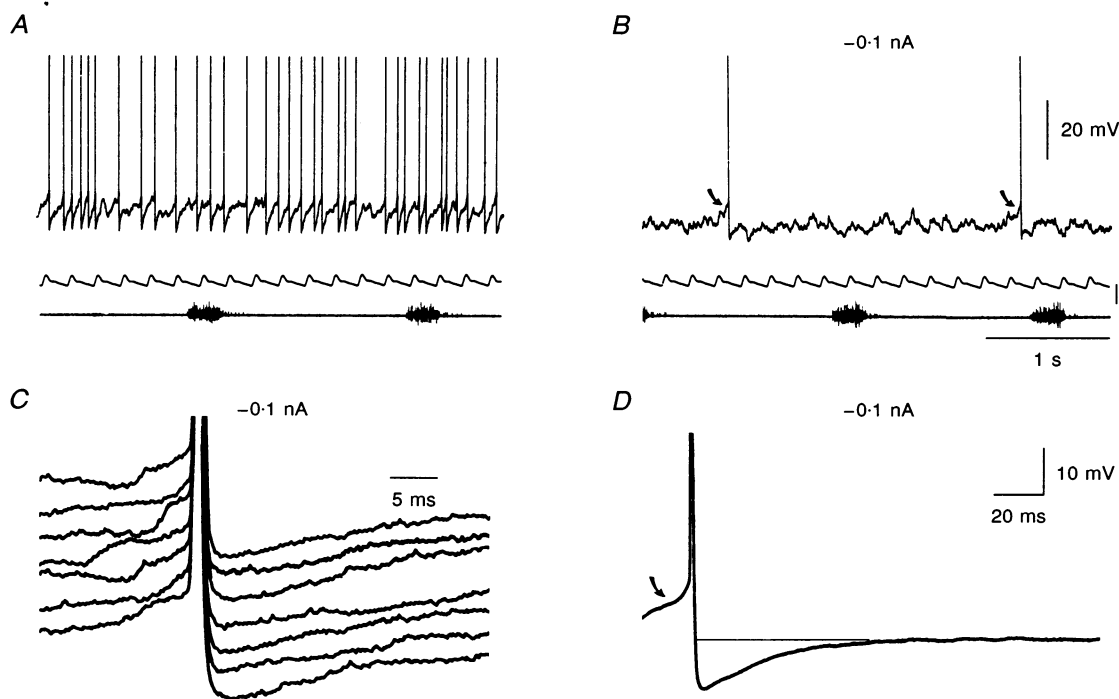


Figure 5. EPSPs initiating individual action potentials and after-hyperpolarizations following 'spontaneous' action potentials in an RVLm presympathetic neurone

Latency of IPSPs recorded in this neurone after stimulation of the ipsilateral aortic nerve, 35 ms; antidromic latency after stimulation in the T2 segment, 12.0 ms. *A*, synaptic activity and firing observed when no polarizing current was used (membrane potential, −65 mV). *B*, synaptic activity and firing during a continuous small polarizing current. Arrows indicate large components of the synaptic noise (EPSPs) initiating individual action potentials. Middle trace in *A* and *B*, arterial blood pressure (calibration bar, 0–100 mmHg); bottom trace in *A* and *B*, activity of the phrenic nerve. *C*, EPSPs preceding 'spontaneous' action potentials, and after-hyperpolarizations following each spike shown on a faster time base, with individual sweeps staggered vertically. *D*, time course of after-hyperpolarizations (the area below the thin horizontal line) following individual action potentials (50 averaged sweeps pretriggered with 'spontaneous' action potentials). The arrow indicates an averaged synaptic potential preceding 'spontaneous' action potentials. Note that action potentials are truncated in *C* and *D*.

2 M potassium methyl sulphate (maximum current, -2.0 nA). Complete reversals of polarity were observed in eleven of these cells, including five impaled with microelectrodes containing a low concentration (0.4 M) or no chloride ions. In the remaining cells, partial reversals were observed, in which only the initial part of the synaptic response reversed in polarity, while the remaining part of the hyperpolarizing response showed a smaller amplitude (not illustrated). These results indicate that the synaptic responses evoked by aortic nerve stimulation were IPSPs which reversed in polarity due to: (a) shifts of the membrane potential below the IPSP equilibrium potential, and (b) changes of the equilibrium potential towards less negative values after injection of chloride ions.

Seventeen barosensitive neurones (i.e. neurones showing IPSPs following stimulation of the aortic nerve) responded antidromically following stimulation in the T2 segment (Figs 2C, 7E and 9B). Antidromic responses were distinguished from synaptic excitation by using 'near-threshold' stimulation of descending axons. At this level of stimulation, short-latency EPSPs were usually absent. When such synaptic potentials were present, they were often below the threshold for triggering action potentials (particularly when the spinal stimuli were triggered with 'spontaneous' spikes after a delay which was longer than a 'critical delay' for collision; e.g. Figs 2C and 9B) and therefore the action potentials evoked in this group of neurones were clearly antidromic. Collisions between antidromic and 'spontaneous'

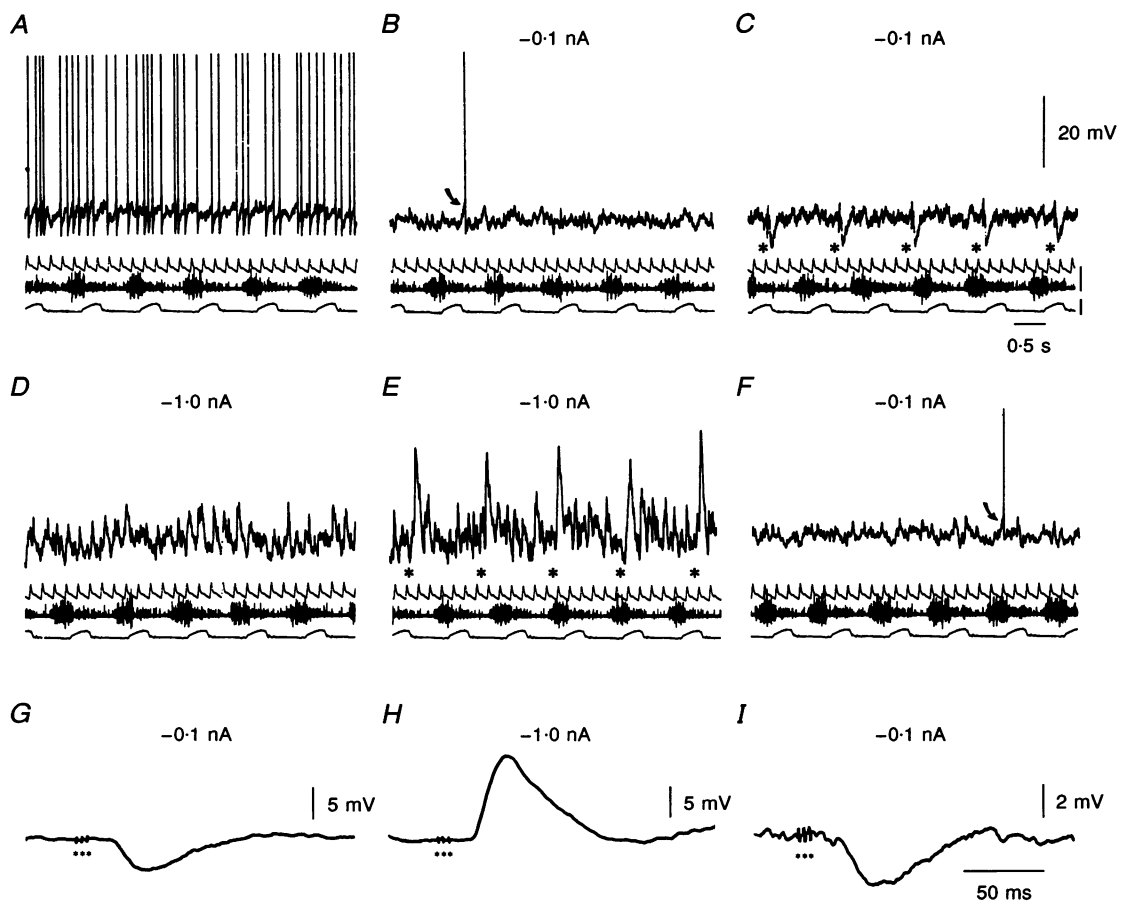


Figure 6. Reversal of 'spontaneous' and aortic nerve-evoked IPSPs during recording with a microelectrode containing a low concentration of chloride ions (0.4 M)

Traces from above in A–F: intracellular recording; arterial blood pressure (calibration bar, 0–100 mmHg); activity of the phrenic nerve; and tracheal pressure (calibration bar, 0–10 cmH₂O). A, no holding current (membrane potential, -68 mV). Values of the holding current in B–I are indicated above each panel. The arrows in B and F indicate EPSPs preceding action potentials. C, hyperpolarizing IPSPs following stimulation of the ipsilateral aortic nerve (*). D, depolarizing IPSPs synchronized with cardiac cycle (no aortic nerve stimulation). E, depolarizing IPSPs synchronized with aortic nerve stimulation (*) and cardiac cycle. F, recovery after reducing the value of the holding current. G–I, reversal of IPSPs evoked by stimulation of the aortic nerve and partial recovery, demonstrated by averaging (each trace is an average of 35 sweeps). The neurone responded antidromically after stimulation in T2 with a latency of 7.0 ms (not illustrated).

action potentials could be demonstrated, but the collision test was not considered necessary for identification of antidromic excitation during intracellular recording (see also Lipski *et al.* 1995a). The antidromic latencies from the T2 segment ranged from 4.0 to 18.0 ms (8.7 ± 3.5 ms) and the calculated axonal conduction velocity (single point stimulation and assumption of linear axonal trajectory) was $1.9\text{--}8.5$ m s⁻¹ (4.6 ± 1.9 m s⁻¹; Fig. 3B). The threshold for antidromic stimulation was 155 ± 132 μ A (range, 15–420 μ A).

In thirty-nine barosensitive neurones, spinal projection was not tested due to deterioration of the membrane potential and spike inactivation. In the remaining twelve cells, the spinal stimulation test was not conclusive due to

the occurrence of short-latency (< 10 ms) suprathreshold EPSPs following spinal stimuli applied at even relatively low intensity (< 100 μ A).

Other properties of RVLM presympathetic neurones

A prominent feature of all barosensitive neurones, including the seventeen identified as bulbospinal, was a high level of synaptic 'noise'. The peak-to-peak amplitude of this noise, measured when a small continuous negative current (≤ 0.2 nA) was injected to slow down the firing, or after spike inactivation occurred, was 8.2 ± 3.0 mV (Figs 5B, 6B, C and F, and 7B). The noise was composed of irregularly occurring depolarizing and hyperpolarizing components of variable amplitude and duration, resulting from summation of tonically occurring synaptic potentials.

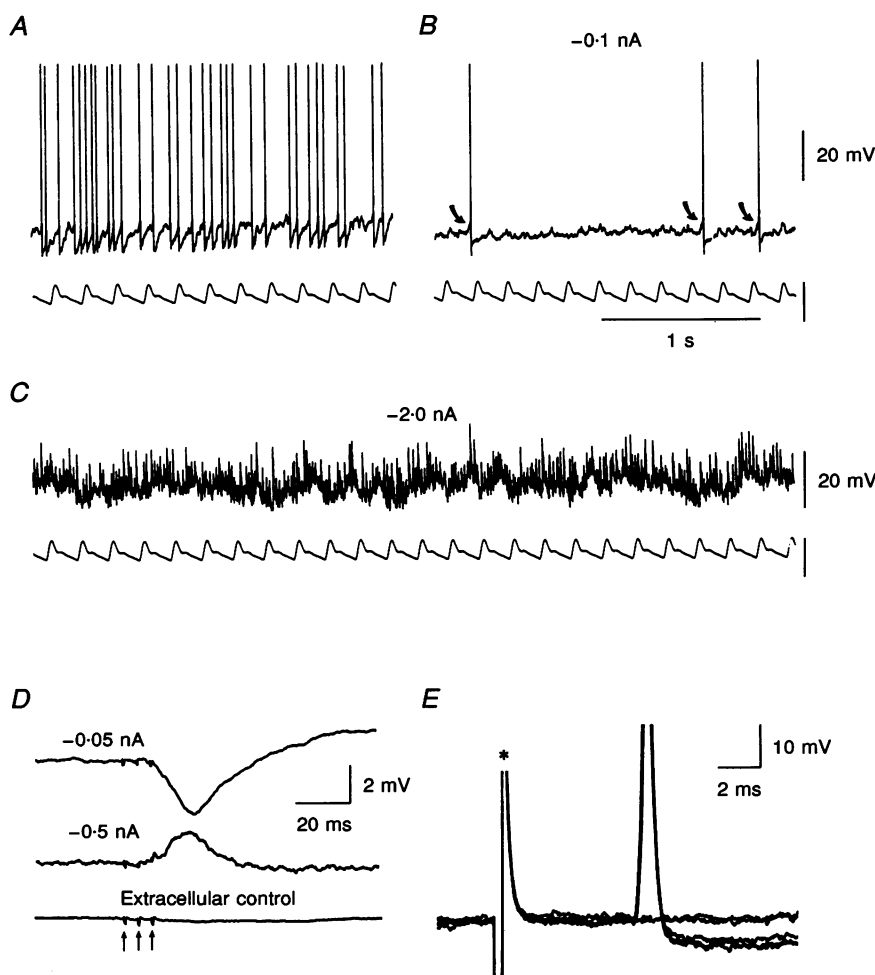


Figure 7. Reversal of IPSPs during recording with a microelectrode containing 3 M KCl

A, no holding current (membrane potential, -62 mV). *B*, synaptic activity and firing during a continuous small polarizing current (-0.1 nA). Arrows indicate EPSPs initiating the action potentials. *C*, record taken approximately 5 min after the beginning of a large continuous hyperpolarizing current (-2.0 nA). Bottom trace in *A*–*C*, arterial blood pressure (calibration bar, 0–100 mmHg). Time calibration bar in *B* applies to *A* and *C* also. *D*, hyperpolarizing IPSP evoked by stimulation of the contralateral aortic nerve (top trace), and its reversal during the passage of a larger polarizing current (-0.5 nA, middle trace). The bottom trace shows the extracellular control. Stimulus artifacts are indicated by arrows. *E*, antidromic action potentials evoked by near-threshold stimulation in T2 (*). Five superimposed sweeps are shown.

When the neurones were firing, such postsynaptic potentials were less conspicuous during the initial periods of after-hyperpolarizations, but developed larger amplitudes during the period with a relatively stable mean level of the membrane potential (before one of the depolarizing components reached the firing threshold; e.g. Fig. 5C). In each 'spontaneously' active neurone, action potentials were usually preceded by depolarizing potentials of variable amplitude and duration, with the features of fast EPSPs (Figs 2A, 5A–D, 6A and B, 7A and B, 8A and 9A). Such EPSPs were seen more clearly when faster sweeps (pre-triggered with 'spontaneous' action potentials) were used (Figs 2C, 5C and 9B).

During periods of recording with no holding current, the largely irregular pattern of 'spontaneous' action potentials could be demonstrated by triggering oscilloscope sweeps with random spikes (Fig. 8A*b*). No regularly occurring ramp-like 'pacemaker' potentials (autodepolarizations) leading to action potentials were observed. However, when the membrane potential was deteriorating (before spike inactivation), the action potentials occurred with a high and regular frequency and were not preceded by

identifiable EPSPs. The trajectories of the depolarizations observed between these action potentials appeared to be entirely determined by after-hyperpolarizations (Fig. 8B*b*). A similar conversion from a largely irregular to regular firing was observed when depolarizing current pulses were used (Fig. 8C).

When the firing was slowed down by a small continuous hyperpolarizing current (≤ 0.2 nA), the mean level of the membrane potential observed between individual action potentials was stable (with a superimposed synaptic noise), with no evidence of pacemaker potentials (Figs 5B, 6B, C and F, and 7B). Also, no pacemaker-like depolarizations were observed during larger hyperpolarizations evoked with current pulses (duration, 80–500 ms; up to 2 nA). During such hyperpolarizations, a clear pattern of irregular postsynaptic potentials with mainly depolarizing components could be seen (Fig. 9D).

Passing a continuous hyperpolarizing current of a sufficient amplitude resulted in reversal of not only IPSPs evoked by stimulation of the aortic nerve (see above), but also of some other tonically occurring IPSPs. When the mean level of arterial blood pressure was higher than 100 mmHg, such

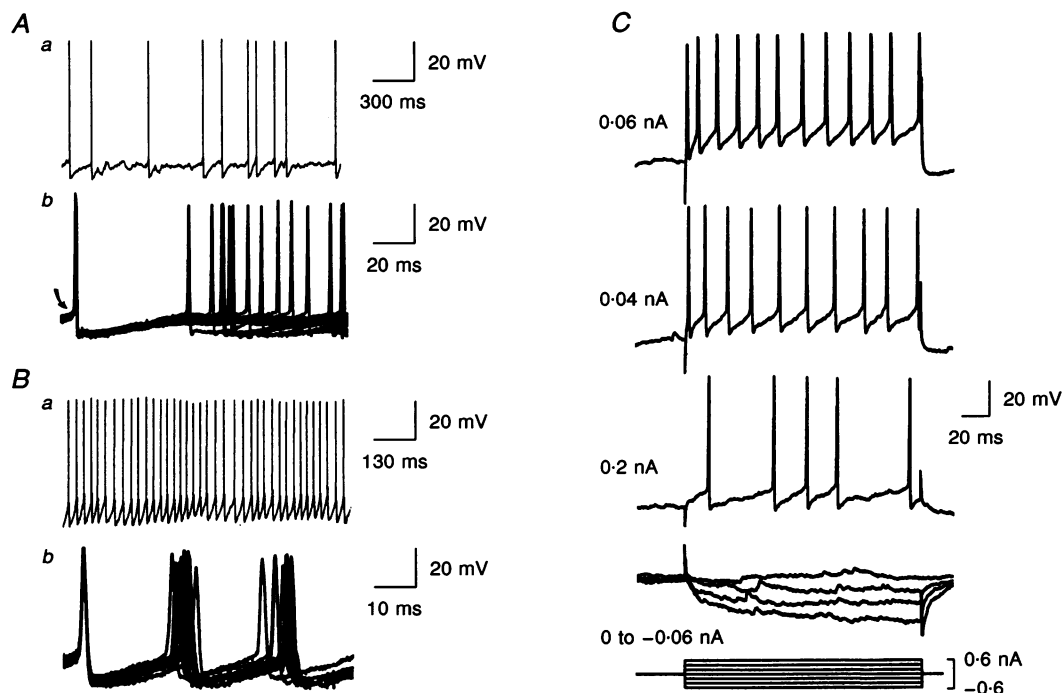


Figure 8. Firing induced in one RVLM neurone due to damage and depolarization, prior to spike inactivation (A and B), and in another neurone during depolarizing current pulses (C)

A*a*, firing soon after impalement (no holding current; membrane potential, -54 mV). A*b*, interspike intervals observed when individual sweeps were pretriggered with random spikes (7 superimposed sweeps, arrow indicates EPSPs preceding action potentials which were used as trigger source). B*a*, firing during a spontaneous decay of the membrane potential resulting from cell damage. B*b*, interspike intervals observed when individual sweeps were pretriggered with every fourth spike. C, response to hyperpolarizing and depolarizing current pulses, with a holding current of -0.1 nA (the same neurone shown in Fig. 6). The input resistance of the cell in C was approximately 42 M Ω . The neurone in A and B was inhibited by stimulation of the aortic nerve but not tested for spinal projection.

reversed potentials occurred with a clear cardiac rhythm (Fig. 6*D* and *E*). In animals in which the mean blood pressure was below 80 mmHg, the reversed IPSPs were still pronounced but occurred without any clear relationship to cardiac cycle (Fig. 7*C*). Analysis of synaptic potentials evoked by 'naturally' occurring excitation of baroreceptors was complicated by the fact that in some cases it was not clear whether these potentials were genuine, or were due to movement artifacts. On some occasions, cardiac cycle-related shifts of the membrane potentials (and corresponding changes in firing frequency) were also observed in neurones which did not respond to stimulation of the aortic nerve, particularly when continuous polarizing current was used. For that reason such shifts were not used for assessment of baroreceptor input.

No clear relationship between the level of the membrane potentials and cycles of phrenic nerve activity were observed at end-tidal CO₂ below 5% (but above the threshold for rhythmical activity of the phrenic nerve). Occasionally observed shifts of the baseline occurring in synchrony with cycles of the ventilator (and cycles of the phrenic nerve which were linked to the ventilator; see

Methods) were not considered indicative of respiratory modulation of synaptic input for two reasons: (a) similar baseline shifts were occasionally observed with microelectrodes in the extracellular position, and (b) in contrast to respiratory neurones of the Bötzing complex (Bryant *et al.* 1993), such shifts were not clearly associated with changes in the amplitude of synaptic potentials. Such shifts could have resulted, therefore, from ventilator-induced movement artifact. However, a genuine relationship was observed in two presympathetic neurones (microelectrodes containing 2 M potassium methyl sulphate or 0.4 M KCl) during hypoxia evoked by substituting nitrogen for inspired air for 10 s. In one of the two cells, there was a strong hyperpolarization during each phrenic burst and depolarization during postinspiration (associated with clear changes of the synaptic noise) resulting in pronounced respiratory modulation of the firing (Fig. 9*A*). In the second neurone, a similar hypoxic test resulted in membrane depolarization and increased firing frequency without clear respiratory modulation (not illustrated). Attempts to repeat this test during recordings from other presympathetic neurones were unsuccessful, due to cell damage associated with parallel changes in arterial blood pressure.

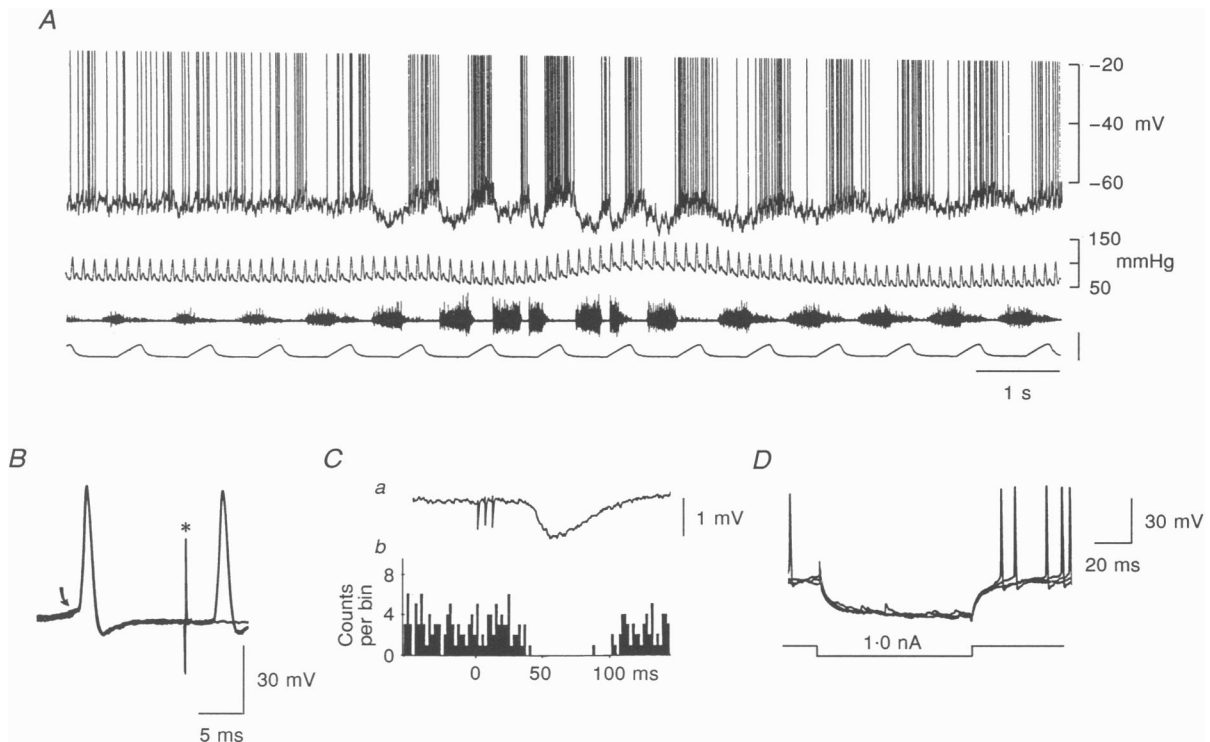


Figure 9. Response of an RVLm presympathetic neurone to short lasting hypoxia

A, traces from above: intracellular recording (no holding current); arterial blood pressure; activity of the phrenic nerve (period of hypoxic excitation associated with an increased amplitude of phrenic bursts); and tracheal pressure (calibration bar, 0–10 cmH₂O). *B*, antidromic action potentials evoked by near-threshold stimulation (*) in T2 (4 superimposed sweeps pretriggered by 'spontaneous' action potentials). The arrow indicates EPSPs preceding 'spontaneous' action potentials. *C*, inhibitory response after stimulation of the contralateral aortic nerve. *a*, averaged IPSPs (average of 30 sweeps); *b*, peristimulus time histogram. *D*, response to a large hyperpolarizing current pulse (3 superimposed sweeps). The input resistance of this cell was approximately 25 M Ω .

Other impalements in the RVLM

In addition to presympathetic neurones (and barosensitive neurones not confirmed as bulbospinal), numerous impalements were made from other 'spontaneously' active or silent axons and neuronal cell bodies. At the dorsal part of the examined region, neurones of the Bötzing complex (e.g. Bryant *et al.* 1993) were frequently impaled. They did not respond to stimulation of the aortic nerve with short bursts of stimuli (see also Kanjhan *et al.* 1995) and were not analysed further. Many impalements were made into the cell bodies which showed a relatively low level of synaptic noise (peak-to-peak amplitude < 3 mV) and were either silent or displayed a largely irregular firing pattern. No evidence of synaptic responses of these neurones after stimulation of the aortic nerve was found. Finally, on some occasions, neurones which displayed a regular firing pattern and a low level of synaptic activity were impaled (Fig. 10). Such neurones showed slow, ramp-like depolarization between action potentials, with no clear EPSPs initiating individual action potentials. Although no evidence of synaptic input from the aortic nerve was found, they were of interest due to the similarity of some of their features with the RVLM 'pacemaker' neurones described by others (see Discussion). The discharge of such regularly firing neurones could be reset by brief depolarizing current

pulses (Fig. 10A). When a small continuous hyperpolarizing current was used to slow down the firing, the mean level of the membrane potential was stable, with no evidence of pacemaker potentials (Fig. 10C).

DISCUSSION

Identification of RVLM presympathetic neurones and their baroreceptor inhibition

All recordings in this study were made from neurones located at the level of the caudal end of the facial nucleus or immediately behind it, and ventral or ventromedial to the compact formation of the nucleus ambiguus. This location corresponds well with maps of barosensitive, bulbospinal neurones published in previous extracellular studies in the rat (Brown & Guyenet, 1984; Morrison *et al.* 1988; Kanjhan *et al.* 1995). One of the main difficulties with recording from these small medullary neurones relates to proper identification of baroreceptor input. The changes in the membrane potential and firing frequency occurring in phase with the cardiac cycle, or during pressor or depressor responses, can result from movement artifacts and therefore are not reliable as a means of demonstrating this input. We have recently proposed that two features are sufficient to classify RVLM neurones as presympathetic: (a)

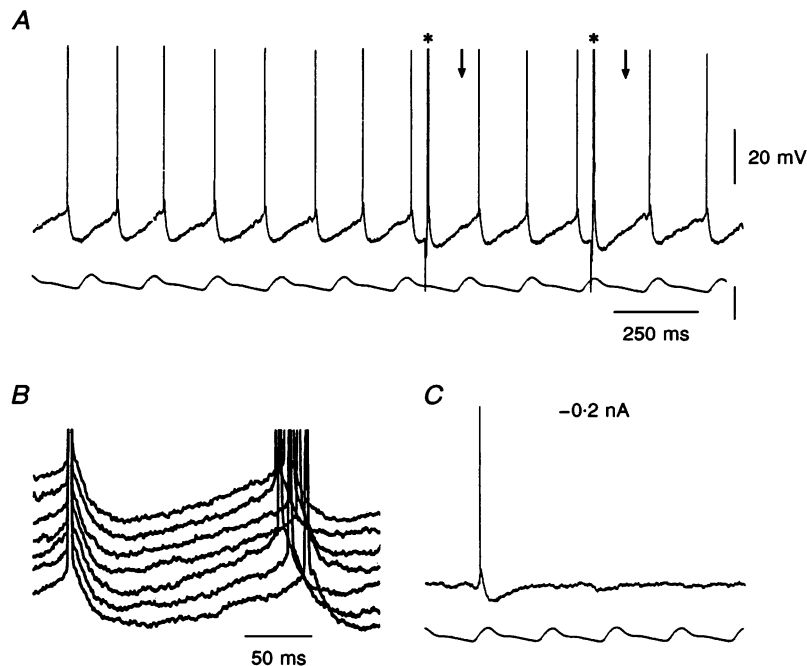


Figure 10. Regular firing in a non-barosensitive neurone which showed a low level of synaptic activity and large after-hyperpolarizations

A, 'spontaneous' activity (no holding current; the level of potential during peak after-hyperpolarization, -58 mV) with resetting of the rhythm following action potentials evoked by short depolarizing current pulses (*). Arrows indicate approximate times at which action potentials would be expected without resetting. *B*, interspike intervals observed when individual sweeps were pretriggered with every second spike. Note the absence of discrete EPSPs initiating individual spikes. *C*, recording during a small holding current which slowed down the firing. Bottom trace in *A* and *C*, arterial blood pressure (calibration bar, 0–100 mmHg).

inhibitory response to stimulation of the aortic nerve with short bursts of pulses applied at low frequency to avoid large changes in blood pressure; and (b) antidromic responses evoked by *near-threshold* stimulation of the descending axons to minimize both the changes of blood pressure associated with such stimulation, and the amplitude of short-latency synaptic responses which can interfere with antidromic identification (Lipski *et al.* 1995a). The same criteria were used in the present study.

The RVLM presympathetic (barosensitive and bulbospinal) neurones presumably had myelinated spinal axons (conduction velocity, 1.9–8.5 m s⁻¹). It is not clear why a subpopulation with slower conducting axons, reported in some previous extracellular studies (e.g. Terui *et al.* 1986; Morrison *et al.* 1988), was not found. One possible explanation is that our intracellular recordings were biased towards larger cells, with faster conducting axons. The possibility that neurones with unmyelinated spinal axons may receive an inhibitory input from the carotid/cardiac but not aortic baroreceptors is unlikely, as the results of a previous extracellular study (Jeske, Morrison, Cravo & Reis, 1993) showed that stimulation of the aortic nerve also inhibited the activity of RVLM neurones with spinal axons conducting at < 1.5 m s⁻¹.

Only a relatively small proportion (25%) of all RVLM neurones inhibited by stimulation of the aortic nerve were positively identified as bulbospinal. This contrasts with the results of the extracellular study by Jeske *et al.* (1993) in which 63% of neurones inhibited by similar stimulation were spinally projecting. The difference can be explained by the facts that in our study: (a) spinal projection was not tested when spike inactivation occurred; and (b) the test was not conclusive in some other neurones in which the lack of antidromic responses could have been due to collision with synaptically induced action potentials evoked with short latencies. Therefore, in the present study, the proportion of spinally projecting barosensitive neurones was underestimated to a degree higher than in previous extracellular studies in which only the second of the two factors was likely to play a role (e.g. Haselton & Guyenet, 1989).

Until now, the most convincing evidence for direct involvement of inhibitory interneurons in the reflex pathway from baroreceptors to RVLM presympathetic neurones was provided by Sun & Guyenet (1985), who reported the total inhibition of the blood pressure sensitivity of these neurones by ionophoretic application of bicuculline. Our study demonstrating chloride- and voltage-dependent IPSPs in these neurones following stimulation of the aortic nerve is entirely consistent with that result, and provides additional evidence that the baroreceptor-induced inhibition of RVLM neurones is postsynaptic (i.e. results from IPSPs rather than disfacilitation). The onset latency and duration of these IPSPs were similar to the latency and duration of

inhibition of firing observed in our previous extracellular study in which an identical pattern of aortic nerve stimulation was used (Kanjhan *et al.* 1995). The findings that the onset latency was relatively long (mean, 33.6 ms) and that both spatial and temporal facilitation were required are compatible with the general concept of an oligosynaptic reflex baroreceptor pathway involving second-order excitatory interneurons in the nucleus of the solitary tract, and third-order inhibitory interneurons in the caudal ventrolateral medulla (CVLM) (e.g. Guyenet, Filtz & Donaldson, 1987).

No clear respiratory modulation of the on-going firing or synaptic activity was observed in these neurones under our experimental conditions. This observation differs from the results of some previous extracellular studies which reported a large proportion of RVLM presympathetic neurones with activity synchronized with respiratory cycles (e.g. McAllen, 1987; Haselton & Guyenet, 1989). This difference could be due to the lower baseline level of arterial P_{CO_2} maintained during our experiments.

Origin of the on-going activity of RVLM presympathetic neurones 'in vivo'

All impaired barosensitive and bulbospinal neurones (as well as the neurones inhibited by stimulation of the aortic nerve but not positively identified as bulbospinal) displayed a high level of synaptic activity. Although the time course and voltage dependence of the individual components of this activity were not examined in detail, the observations that both the depolarizing and hyperpolarizing potentials occurred when no polarizing current was used, and that mainly depolarizing components were seen during large membrane hyperpolarizations, strongly indicate that these components were the fast IPSPs and EPSPs.

A number of studies have provided evidence of a tonic *inhibitory* control of these neurones. For example, it has been demonstrated that local application of GABA_A or GABA_B receptor antagonists results in pressor responses (e.g. Dampney, Blessing & Tan, 1988; Avanzino, Ruggeri, Bianchi, Cogo, Ermirio & Weaver, 1994) and excitation of barosensitive bulbospinal neurones (Sun & Guyenet, 1985). The pressor effects were also observed after elimination of baroreceptor input, which indicates that the disinhibition has a baroreceptor-independent component (e.g. Dampney *et al.* 1988). The results of our study support the concept of an additional inhibitory input which is independent of baroreceptors. Such an input was demonstrated by membrane hyperpolarization and chloride ion injection, which disclosed large, irregularly occurring reversed IPSPs even in animals with blood pressure below the threshold for activating most of the baroreceptors. As GABA- and glutamic acid decarboxylase-immunoreactive cell bodies have been identified in the general area of RVLM (e.g. Ruggiero, Meeley, Anwar & Reis, 1985; Millhorn, Hökfelt, Serogy & Verhofstad, 1988), these IPSPs may originate

from local GABAergic interneurons. The inhibition may also originate from distant neurones, e.g. those located in the CVLM (Willette, Krieger, Barcas & Sapru, 1983; Guyenet *et al.* 1987; Cravo & Morrison, 1993).

Electrophysiological and pharmacological studies have identified a number of central sites and peripheral nerves from which synaptic excitation of RVLM presympathetic neurones can be evoked following electrical or chemical stimulation (e.g. Sun & Guyenet, 1986; Cechetto & Chen, 1992). However, so far only two brainstem regions have been identified, which may provide a 'tonic' excitatory drive to these neurones: lateral tegmental field in the dorsal reticular formation of the medulla oblongata (Barman & Gebber, 1987), and the pontine reticular formation (Hayes, Calaresu & Weaver, 1994). Further investigations are required to determine the major source(s) of this excitatory input.

The EPSPs responsible for eliciting tonic but largely irregular outflow from presympathetic neurones are likely to be mediated by excitatory amino acids. This conclusion is not in conflict with previous studies which reported either an increase, no change, or decrease in arterial blood pressure or sympathetic nerve activity following a bilateral microinjection into RVLM (or intracisternal administration) of a non-selective excitatory amino acid antagonist kynurenic acid, glutamate diethyl ester, blockers of the non-NMDA receptor, or cobalt (e.g. Guyenet *et al.* 1987; Sun *et al.* 1988a; Trzebski & Baradziej, 1992; Abrahams, Hornby, Chen, Dasilva & Gillis, 1994). The variability of the response may be due to the fact that these drugs (with the exception of cobalt which can block transmission in all synapses) not only block excitatory amino acid receptors on presympathetic neurones, but also on other local (and distant, in the case of intracisternal administration of kynurenate) interneurons, including *inhibitory* neurones. If these interneurons are also tonically excited by synapses utilizing excitatory amino acids, then such chemical *deafferentation* would not only lead to disfacilitation of presympathetic neurones, but also to their disinhibition (cf. Kiely & Gordon, 1993). The overall effect will depend on the balance between direct excitatory and inhibitory inputs to presympathetic neurones, and to antecedent interneurons. It is likely that this balance will be affected by experimental conditions, e.g. the kind of anaesthesia.

Two lines of evidence indicate that, under our experimental conditions, the on-going activity of RVLM presympathetic neurones results from synaptic inputs and not from intrinsic pacemaker properties as suggested by others (see below). First, the action potentials were normally preceded by depolarizing potentials showing features of fast EPSPs. Second, no evidence was obtained for the existence of regular, ramp-like depolarizations between action potentials in neurones which showed no obvious signs of injury or were not excited by large depolarizing current pulses. Also,

no pacemaker potentials (autodepolarizations) were seen when the action potentials were slowed down (or stopped) by injection of small negative currents. In agreement with the general principle that many neurones are capable of generating a rhythmic discharge when appropriately depolarized (Connor, 1985), we observed that depolarization due to injury, or induced with large depolarizing current pulses, resulted in conversion from a largely irregular firing pattern induced by synaptic potentials, to firing with a higher frequency and with nearly constant interspike intervals, with no clear EPSPs preceding the action potentials. As expected, in such situations synaptic inputs play a smaller role in controlling the occurrence of individual action potentials due to a decreased amplitude of EPSPs (resulting from both the decrease in their electrochemical driving force, and an increase in membrane conductance following individual action potentials). In such cases, the action potentials occurred at the end of after-hyperpolarizations triggered by preceding spikes, and the trajectory of the membrane potential between individual spikes appeared to depend largely on the ratio of the outward current associated with the after-hyperpolarizations and the inward current associated with cell damage or induced by current pulses.

Our results, therefore, are consistent with the 'network' hypothesis for the generation of sympathetic vasomotor tone, which implies that the activity of presympathetic neurones *in vivo* depends on their antecedent excitatory inputs (Barman & Gebber, 1987). As an extension of this hypothesis, we propose that the on-going activity of presympathetic neurones is also controlled by tonic (presumably GABAergic) inhibitory input which is primarily responsible for setting the level of excitability of these neurones. We believe that future studies examining the origin of synaptic excitation of presympathetic neurones should focus not only on identification of the sources of EPSPs, but also on mechanisms leading to disinhibition.

Comparison with previous studies describing 'pacemaker' properties of RVLM neurones

In brief, the 'pacemaker' hypothesis of basal sympathetic tone states that the activity of RVLM presympathetic neurones (at least those with the non-adrenergic phenotype; but see Kangrga & Loewy, 1995) is largely determined by their ability to generate action potentials by auto-depolarizations, and that the synaptic and other extrinsic factors only modulate the basic rhythm generated by these neurones (for review see Guyenet, Haselton & Sun, 1989; Guyenet, 1990). The main arguments are the following. (a) No fast postsynaptic potentials were observed during intracellular recording in medullary slices (roughly in the cardiovascular portion of the RVLM) when the membrane was hyperpolarized and the firing stopped. (b) Intracellular recording in slices demonstrated the presence of ramp-like depolarizations which followed each action potential and led to subsequent action potentials, with no evidence of EPSPs

preceding the spikes. (c) The regular pattern of firing, which was also observed *in vivo* after kynurenic acid administration, could be reset by action potentials evoked by short depolarizing (or hyperpolarizing) current pulses, or by antidromic stimulation. (d) *Indirect* evidence has been provided that the neurones with such properties in slices (or *in vivo* after kynurenate administration) correspond to barosensitive and bulbospinal neurones in 'intact' animals. In addition, other *in vitro* studies (Lewis & Coote, 1993; Kangrga & Loewy, 1995) revealed that the on-going activity of some RVLM neurones was not blocked in Ca^{2+} -free (or low- Ca^{2+} -high- Mg^{2+}) solution. However, the observation that the depolarizing 'ramps' were abolished when action potentials were eliminated by a continuous hyperpolarizing current or tetrodotoxin (Guyenet *et al.* 1989; Kangrga & Loewy, 1995) indicates that the ramps were related to preceding action potentials and that the pacemaker properties were not expressed at subthreshold voltages. In contrast, in a well documented model of an invertebrate pacemaker neurone *in vitro* (R15 neurone in the abdominal ganglion of *Aplysia*), oscillations of the membrane potential occur in the absence of action potentials (e.g. Mathieu & Roberge, 1971).

We propose that the regular pattern of activity observed in tissue slices (and *in vivo* after kynurenate administration) may be the *result* of deafferentation, and that the pacemaker-like properties observed in such preparations play little, if any, role in controlling the activity of presympathetic neurones under standard conditions *in vivo* (cf. also Dembowski, 1995, who observed pacemaker-like activity in preganglionic sympathetic neurones *in vitro*, but not *in vivo*). The key element of our hypothesis is an assumption that the *in vitro* isolation or intracisternal administration of kynurenate are associated with reduction or elimination of not only EPSPs but also of inhibitory inputs from both distant and local inhibitory interneurones (see above). If the inhibitory input normally prevails, then *in vitro* surgical isolation or *in vivo* administration of kynurenic acid could lead to disinhibition, threshold depolarization and firing. This is supported by the observation of Sun *et al.* (1988a) that, following intracisternal kynurenate administration *in vivo*, there was some increase in the mean firing rate of presympathetic neurones. Following deafferentation and resulting threshold depolarization, action potentials would be expected to occur regularly, with the trajectory of the membrane potential and the interspike intervals mainly governed by after-hyperpolarizations (Ca^{2+} -activated potassium current; $I_{\text{K}(\text{Ca})}$). Such a regular firing pattern would be expected to reset after an extra spike induced by intracellular current pulses, or antidromically. Interestingly, the duration of after-hyperpolarizations observed in our experiments (mean, 54 ms) closely corresponds to the mean interspike interval reported in tissue slices at 37 °C, and *in vivo* after kynurenate administration (mean, 21–22 spikes s^{-1} ; Sun *et*

al. 1988a, b; see also Lewis & Coote (1993), who reported a mean duration of after-hyperpolarizations at 34 ± 1 °C of 47 ms). The involvement of other membrane channels (in addition to those responsible for $I_{\text{K}(\text{Ca})}$) remains to be established.

A regular pattern of discharge was observed in our *in vivo* experiments in some RVLM neurones which were non-barosensitive. Such unidentified neurones displayed only a low level of synaptic noise and their firing could be reset by intracellular current pulses. As discussed before, this firing pattern may not be due to the 'intrinsic' pacemaker properties, but to the fact that their membrane potential was close to the firing threshold, with interspike intervals mainly determined by after-hyperpolarizations. However, it is not clear if this depolarized level of the membrane potential reflected the general features of such neurones, was due to disinhibition caused by experimental conditions, or was simply due to the impalement. A similar explanation can be offered for the interpretation of previous *in vivo* results obtained by Granata & Kitai (1992). These authors recorded, in the general area of RVLM, some regularly firing neurones with no obvious EPSPs during current-induced hyperpolarizations. However, no convincing evidence was presented that these neurones were either barosensitive or bulbospinal (see Lipski *et al.* 1995a for discussion).

In conclusion, our study demonstrates that presympathetic neurones in anaesthetized rats are synaptically driven, and therefore these results are consistent with the 'network' hypothesis of generation of sympathetic vasomotor tone. No evidence for intrinsic pacemaker properties was obtained. We suggest that the pacemaker-like, regular discharge observed by others in slices and *in vivo* after kynurenate administration may result from a partial or complete deafferentation (surgical or chemical) and threshold depolarization due to elimination of prominent tonic inhibitory inputs.

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Acknowledgements

This study was supported by the Health Research Council of New Zealand and the Lottery Health Board. We thank Lisa Christmas for her secretarial assistance and Drs L. Kubin and D. de Castro for their helpful comments on the manuscript.

Received 6 January 1995; accepted 21 July 1995.