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SYNAPTIC EVENTS IN VENTRAL RESPIRATORY NEURONES DURING APNOEA INDUCED BY LARYNGEAL NERVE STIMULATION IN NEONATAL PIG

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SUMMARY

1. Postsynaptic potentials evoked by electrical stimulation of superior laryngeal nerve (SLN) were recorded during SLN-induced apnoea from the respiratory neurones of the ventral respiratory group (VRG) in pentobarbitone-anaesthetized, vagotomized and artificially ventilated newborn piglets ($n = 14$, 4–7 days old). All recorded inspiratory ($n = 10$), post-inspiratory ($n = 10$) and expiratory ($n = 20$) neurones had a triphasic pattern of membrane potential and were identified for their projections to the spinal cord or cervical vagus nerve.

2. During long-lasting apnoea, induced by SLN stimulation, the membrane potential trajectory of each type of recorded neurone was held at the level corresponding approximately to the membrane potential reached during stage I of expiration. Compound postsynaptic potentials evoked in most respiratory-related neurones had an early short-lasting and a late long-lasting component.

3. Postsynaptic potentials in four out of seven inspiratory neurones, in which postsynaptic potentials were well demonstrated, were characterized by an early depolarization followed by long-lasting hyperpolarization. In three other inspiratory neurones only late hyperpolarization was present. The reversal of the late hyperpolarization by intracellular chloride injection was achieved to a different degree in the early and late portions of late hyperpolarization.

4. Postsynaptic potentials evoked in expiratory neurones were studied in sixteen neurones and displayed two patterns: early hyperpolarization followed by long-lasting hyperpolarization ($n = 7$, six were not antidromically activated after spinal cord stimulation) or early hyperpolarization followed by late depolarization ($n = 9$, eight projected to the spinal cord). The early hyperpolarization was readily reversed by chloride injection. The late hyperpolarization was more difficult to reverse and usually the reversal was not completed.

5. Postsynaptic potentials evoked in post-inspiratory neurones showed a pattern of two consecutive phases of depolarization.

6. The present study revealed that during long-lasting apnoea evoked by SLN stimulation each category of VRG respiratory neurones received a temporally synchronized combination of an initial fast input derived reflexly from laryngeal

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afferents, and of late inputs representing involvement of the whole respiratory network in the response.

INTRODUCTION

Mechanical and chemical activation of laryngeal receptors inhibits activity in the phrenic nerve and stops breathing, an effect which can be simulated by electrical stimulation of the superior laryngeal nerve (SLN; Biscoe & Sampson, 1970; Iscoe, Feldman & Cohen, 1979; Sica, Cohen, Donnelly & Zhang, 1984; Jodkowski & Berger, 1988). Several groups have attempted to determine cellular mechanisms leading to respiratory inhibition during activation of the laryngeal afferents. Using extracellular recording techniques, Pantaleo & Corda (1985) observed a silent period in discharges of rostral medullary expiratory non-bulbospinal neurones induced by SLN stimulation during phrenic nerve silence. Similar observations were reported by Jodkowski & Berger (1988) regarding caudal medullary expiratory bulbospinal neurones in the area of nucleus retroambiguus.

Intracellular recordings from different types of respiratory neurones in the ventral respiratory group (VRG) have shown complex effects of superior laryngeal nerve stimulation on different respiratory neurones, suggesting an involvement of the whole respiratory neuronal network in integrating the reflex response. Chemical and short-lasting electrical stimulation of laryngeal afferents prolonged the duration of the phase I of expiration (Remmers, Richter, Ballantyne, Bainton & Klein, 1986) and caused suppression of the after-discharge of phrenic motoneurones (Richter, Ballantyne & Remmers, 1987). Medullary post-inspiratory neurones were activated by SLN stimulation (Remmers *et al.* 1986). Also, under the same conditions respiratory-related hyperpolarization of membrane potential, which occurs during state I of expiration, was enhanced in both inspiratory (Richter *et al.* 1987) and expiratory neurones (Ballantyne & Richter, 1986). These results led to the hypothesis that stimulation of laryngeal afferents holds the whole respiratory network in stage I of expiration and the term of 'post-inspiratory apneusis' was proposed to describe apnoea reflexly evoked from laryngeal afferents (Remmers, Richter, Ballantyne, Bainton & Klein, 1985).

The present study was undertaken to investigate synaptic events in respiratory neurones of the ventral respiratory group during long-lasting apnoea induced by electrical stimulation of the superior laryngeal nerve in the neonatal pig. In newborns of several species (Harding, Johnson & McClelland, 1978; Boggs & Bartlett, 1982), including piglets (Downing & Lee, 1975; Lee, Stoll & Downing, 1977; Lawson, 1981), stimulation of laryngeal receptors causes protracted apnoea. The ventral respiratory group (VRG) of neonatal animal preparations (e.g. piglets) exhibits similar respiratory control mechanisms and contains similar populations of neurones (Lawson, Richter & Bischoff, 1989) as adult cats (Richter, 1982; Richter & Ballantyne, 1983). Therefore the model of the neonatal piglet may provide results which are likely to be comparable with those from adult cats.

Some of these results have been published in abstract form (Czyzyk-Krzeska, Lawson & Rudesill, 1990).

METHODS

Experiments were carried out on fourteen Yorkshire piglets (1·1–2·6 kg, age 4–7 days) obtained from a local commercial breeder on the day of each experiment. Animals were anaesthetized initially by intraperitoneal injection of 30 mg/kg of sodium pentobarbitone (Nembutal, Abbott Laboratories) and the adequacy of anaesthesia was accepted if animals did not show reaction to nociceptive stimuli. When necessary, additional doses (6 mg/kg) were supplemented intravenously during the experiment (when arterial pressure and/or respiratory output showed an increase or became irregular). The cervical trachea was cannulated and catheters were placed into the femoral vein and artery. Arterial pressure and end-tidal CO_2 were monitored continuously. Systolic arterial pressure was kept above 50 mmHg and, if necessary, 6% Dextran (Kodak) in 0·9% saline was administered intravenously. Animals were paralysed by continuous infusion of 1% gallamine triethiodide (Sigma) and artificially ventilated with room air enriched by oxygen. Positive end-expiratory pressure of 1–2 cmH_2O was applied to prevent lung atelectasis. Bilateral pneumothorax was created in order to decrease ventilator-related brain stem movements. End-tidal CO_2 was maintained in the range 40–45 mmHg. Rectal temperature was kept between 38 and 39 °C with a ventral heating pad controlled by a feedback circuit and additionally with a heat lamp. The animal was fixed in a stereotaxic apparatus with the head ventroflexed and was suspended by dorsal clamps at the thoracic and lumbar spines.

After mid-dorsal incision and bilateral retraction of the scapulae, both vagi, the left phrenic nerve and the right superior laryngeal nerve were exposed. Each of the nerves was cut and the left-side phrenic nerve and the right-side vagus and right-side superior laryngeal nerves were placed on bipolar platinum electrodes under warm paraffin oil. The superior laryngeal nerve was identified at its entrance to the nodose ganglion. The activity of the phrenic nerve was amplified (bandpass filtered: 10 Hz–3 kHz) and integrated. For intraspinal stimulation, laminectomy was performed at the level of C2–C3 and two bipolar concentric electrodes (diameter 1 mm) were inserted bilaterally into the ventrolateral region of the spinal cord.

The dorsal aspect of the medulla was exposed by removing the occipital bone, the atlanto-occipital membrane, the dura and the arachnoid membrane. The cerebellum was displaced rostrally.

Stimulation

Stimulation of the vagus and superior laryngeal nerves and the spinal cord was accomplished via a constant-voltage isolation unit (Grass) with square-wave pulses of 0·05–0·5 ms duration. The threshold for stimulation of the superior laryngeal nerve was established as the minimal stimulus necessary to evoke silence in the phrenic nerve at the frequency 7–10 Hz, which was the frequency for experimental trials. In most cases threshold was in the range of 0·5–3 V. Trains (10–60 s) of individual pulses of a voltage 2–3 times greater than threshold were used (i.e. 1–6 V). In a few cases apnoea could be induced only by stimuli of 6–8 V, due probably to the deterioration of electrode–nerve coupling during the course of the experiment.

Vagal projections of respiratory units were determined by testing for antidromic invasion following stimulation of the ipsilateral cervical vagus nerve. The vagus was placed onto the stimulating electrode approximately 2–3 cm caudal to the nodose ganglion. The threshold for vagal stimulation was determined on the basis of inhibition of the phrenic nerve activity (Hering–Breuer reflex). The stimulus voltage used in tests for antidromic invasion of vagal motoneurones was in the range of 6–12 V. To test for an antidromic character of responses evoked by spinal cord stimulation, single pulses of amplitude 40–80 V and duration of 0·05 ms were delivered. The adequacy of this stimulation was accepted when evoked mass discharges were produced in the phrenic nerve at a latency to onset of 0·8–1·4 ms. The distance between the spinal cord stimulation site and the medullary recording site averaged $34 \pm 4{\cdot}3$ mm. Antidromic activation was accepted to occur when an action potential without a preceding synaptic potential could be evoked at a constant latency and, in case of spontaneously spiking neurones, when obliteration of the antidromically evoked spike occurred due to collision with a spontaneously generated spike.

Intracellular recordings

Search for respiratory-related units was performed in the ventral respiratory group (VRG) on the right side of the medulla (ipsilateral to the superior laryngeal and vagal nerves), 2–3·6 mm medio-

lateral and 0–3 mm caudal from the obex at a depth between 2·50–4·00 mm (Lawson *et al.* 1989). Recordings were made from within the circumference of a small pressure foot which was applied to the brain stem surface in order to improve stability. Glass microelectrodes were filled with solutions of 3 M-KCl or 2 M-potassium methylsulphate. Before use electrodes were bevelled (Sutter Instruments Co., model BV-10M) to a final DC resistance of 20–50 MΩ when measured in the brain tissue. The microelectrodes were mounted on a digital micropositioner with an electronic control unit (Nanostepper, type B, Adams & List Assoc. Ltd). Intracellular recordings were amplified (gain, 10 times; filter bandpass DC, 3–10 kHz, Axoclamp 2A, Axon Instruments) with capacitance neutralization (1–7 pF) in the bridge mode and continuously monitored on an oscilloscope (Tektronix). Only stable intracellular penetrations having membrane potential values more negative than −40 mV were accepted for analysis. Most neurones had membrane potentials between −50 and −60 mV. Vagal and laryngeal motoneurones were not included for analysis.

Membrane potential, phrenic nerve activity, blood pressure and end-tidal CO_2 were recorded continuously on magnetic FM tape (Vetter) or digital video cassettes (40 kHz sample rate, Vetter Digital). Data were displayed simultaneously on a chart recorder (Gould, TA 2000).

Off-line stimulus-triggered averaging of membrane potential was accomplished by a computer using an in-house developed program (Asyst Software Technologies, Inc., Rochester, NY, USA) with sampling at a rate of 2–4 kHz. Each average consisted of 30–200 responses of 50–100 ms duration. Sweeps containing action potentials were excluded from analysis. Average postsynaptic responses were displayed on an *X–Y* plotter. For statistical analysis, values were calculated from the averaged sweeps. The following properties of postsynaptic potentials were determined: latency to the onset and to the maximum of response, and the amplitude. Comparisons between mean values were determined using Student's *t* test. All values are reported as means ± S.D.

RESULTS

Respiratory-related activity and the membrane potential time course of recorded neurones were classified on the basis of the three-stage respiratory pattern (Richter, 1982). All recorded respiratory neurones showed a tri-phasic pattern of changes in the membrane potential trajectory during the respiratory cycle. A unit was defined as inspiratory, if it depolarized simultaneously with the burst in the phrenic nerve (Fig. 1*Aa* and *Ba*), expiratory if maximum depolarization occurred during the latter half (i.e. stage II) of the phrenic nerve silent period (Fig. 3*A*), and post-inspiratory if maximum depolarization occurred just after cessation of the phrenic burst (e.g. stage I of expiration; Fig. 7*A*).

Effect of SLN stimulation on phrenic nerve activity

The onset of superior laryngeal nerve stimulation was at random in relation to the respiratory phase. SLN stimulation resulted in abolition of phrenic nerve activity ('apnoea') lasting throughout the stimulus (10–60 s) and then persisted for another 2–10 s after its cessation. Occasionally very short irregular bursts of phrenic nerve activity could be observed during apnoea, especially at the beginning of stimulation. This phrenic 'breakthrough' activity was accompanied by transient shifts in the membrane potential of recorded units. In most piglets SLN stimulation did not affect the blood pressure; in a few cases we observed a transient decline of blood pressure at the onset of stimulation in the range of 5 mmHg.

Effect of SLN stimulation on inspiratory neuronal activity

Stable records were obtained from ten inspiratory units. Eight neurones were classified as projecting to the spinal cord (bulbospinal) on the basis of antidromic activation with a latency of 1·5–2 ms and a positive collision test. Single-point estimation (straight line) of the axonal conduction velocities averaged 21·2 ± 1·7 m/s.

Two inspiratory cells were not antidromically activated, either from the spinal cord or from the cervical vagus. Inspiratory units were characterized by an augmenting pattern of firing during inspiration and two distinctive phases of hyperpolarization during expiration: in most neurones the membrane potential was more negative during stage I than during stage II of expiration as is shown in Fig. 1*Aa* and *Ba*.

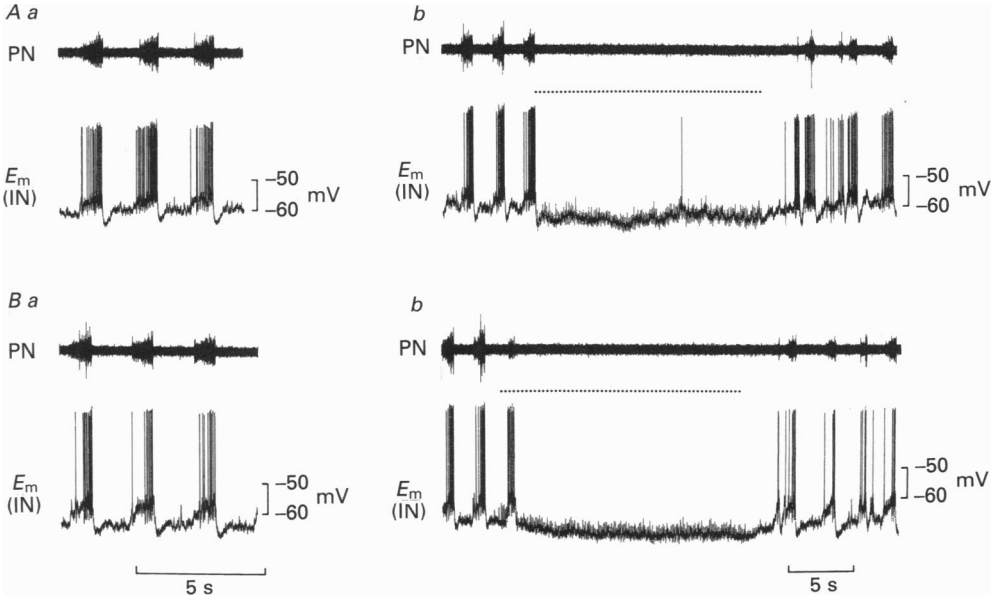

Fig. 1. Example of membrane potential (E_m) of an inspiratory bulbospinal neurone (IN) shortly after penetration (*Aa*) and 30 min later, after intracellular chloride injection (*Ba*). Effects of SLN stimulation (dotted lines, 10 Hz, 6 V) on the membrane potential trajectory at the beginning of recording (*Ab*) and after ionophoresis of chloride (−5 nA, 2 Hz, 10 min; *Bb*). PN, phrenic nerve activity. Time scales for *Aa* and *Ba*, and *Ab* and *Bb*, respectively.

Figure 1*Ab* and *Bb* shows examples of the membrane potential of a bulbospinal inspiratory neurone during two consecutive periods of apnoea produced by superior laryngeal nerve stimulation. The first stimulation was performed shortly after penetration of the neurone (Fig. 1*Ab*) and the second after intracellular injection of chloride (Fig. 1*Bb*). Stimulation of the SLN produced cessation of the three-stage rhythmic changes in the membrane potential. The membrane potential became hyperpolarized to or slightly below the post-inspiratory level of membrane potential before and after stimulation. In all inspiratory neurones during the course of a train of stimuli lasting for several seconds (10–60 s) membrane potential gradually became more negative, by approximately 2–7 mV, than at the very onset of stimulation (Fig. 1*Ab* and *Bb*). Upon cessation of the SLN stimulation, membrane potential of inspiratory neurones gradually depolarized to the level reached during the second part of expiration. Only after this depolarization, did the first recovery inspiratory burst occur.

Stimulus-triggered averages of membrane potential were analysed in seven out of ten inspiratory neurones. Figure 2 demonstrates examples of the pattern of changes

in membrane potential evoked by individual SLN stimuli in four different inspiratory neurones. Three of these cells (Fig. 2*Aa*, *Ab* and *C*) were recorded with potassium methylsulphate-filled electrodes. The cell shown in Fig. 2*B* and *D* was recorded with KCl-filled electrodes. In four of the seven neurones, all of which projected to the

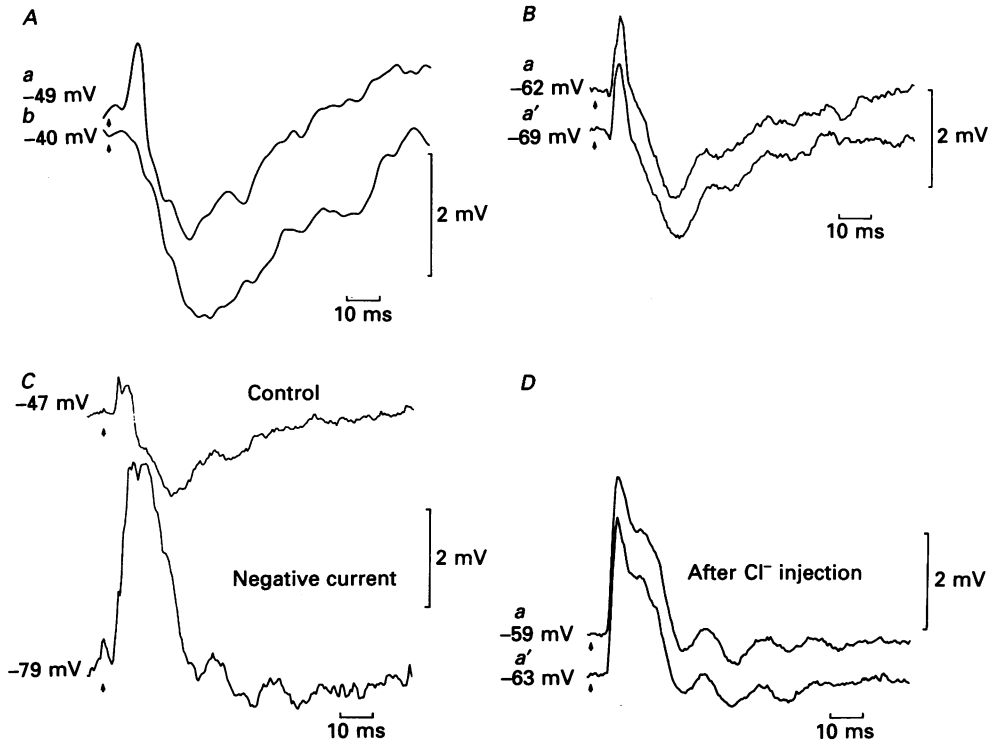

Fig. 2. Examples of postsynaptic potentials evoked by SLN stimulation. *A*, postsynaptic potentials recorded in two different inspiratory neurones by electrodes filled with potassium methylsulphate: *Aa*, inspiratory bulbospinal neurone (averaged 35 times); *Ab*, inspiratory non-antidromically activated neurone (averaged 40 times). Voltage and time scales are the same for both neurones. *B*, postsynaptic potentials recorded in an inspiratory bulbospinal neurone by KCl-filled electrode at the beginning (*Ba*) and at the end (*Ba'*) of the apnoea illustrated in Fig. 1*Ab* (both averaged 50 times). *C*, effects of passing negative current on the SLN-evoked changes in membrane potential; top, before any current was passed; bottom, during passage of negative current (5 nA; averaged 25 times). *D*, effects of chloride injection on SLN-evoked postsynaptic potentials to be compared with the control postsynaptic potentials shown in panel *B* (averaged 50 times); *Da* and *Da'* were collected at the beginning and end, respectively, of apnoea shown in Fig. 1*Bb*. Individual traces are separated by an arbitrary space for better visual demonstration.

spinal cord, biphasic postsynaptic potentials were recorded, the first phase being an early and short-lasting depolarization and the latter phase being a long-lasting hyperpolarization (Fig. 2*Aa*, *B* and *C*). In three other inspiratory neurones, two of which were not antidromically activated by spinal cord stimulation, only a late long-lasting hyperpolarizing potential was detectable (Fig. 2*Ab*). The latencies and amplitudes of these postsynaptic potential waves are given in Table 1. As the initial

TABLE 1. Latencies and amplitudes of postsynaptic potentials in each group of recorded respiratory neurones

Respiratory neurones	Response to SLN stimulation	n	Latency to onset (ms ± S.D.)	Latency to maximum of early response (ms ± S.D.)	Latency to maximum of late response (ms ± S.D.)	Amplitude of maximum of early response (mV ± S.D.)	Amplitude of maximum of late response (mV ± S.D.)
Inspiratory	Depolarization/		3·3 ± 0·9	7·2 ± 0·8	—	1·6 ± 0·2	—
	hyperpolarization	4	—	—	25·3 ± 2·1	—	2·3 ± 0·2
Inspiratory	Hyperpolarization	3	5·1 ± 1·8	—	23·2 ± 3·6	—	2·1 ± 0·9
Post-inspiratory	Depolarization/		3·6 ± 0·4	8·2 ± 0·9	—	2·1 ± 1·1	—
	depolarization	5	—	—	15·6 ± 1·0*	—	3·3 ± 2·9
Expiratory	Hyperpolarization/		2·8 ± 0·4	6·6 ± 1·0	—	1·0 ± 0·3	—
	depolarization	9	—	—	25·5 ± 8·5	—	1·2 ± 0·7
Expiratory	Hyperpolarization/		3·3 ± 0·8	8·0 ± 1·6	—	1·5 ± 1·3	—
	hyperpolarization	7	—	—	18·2 ± 4·9	—	2·2 ± 1·9

Latencies were measured from the end of the stimulus artifact to the onset and to the maximum response of the early and late phases (peak I, peak II, respectively).

* $P < 0·01$.

depolarization was recorded whether the electrodes were filled with potassium methylsulphate (Fig. 2*Aa* and *C*) or KCl (Fig. 2*B*), it most probably was not a fast-reversing hyperpolarization. The postsynaptic potentials labelled as *a* and *a'* in Fig. 2*B* had been collected, respectively, at the beginning and the end of SLN-induced apnoea demonstrated in Fig. 1*Ab* and they exemplify the gradual hyperpolarization of membrane potential during SLN stimulation (Fig. 2*B a–a'*).

In order to establish the character of the late hyperpolarizing phase the effects of passing hyperpolarizing DC current (5–10 nA) and of intracellular chloride injection were examined. The bottom panel of Fig. 2*C* shows the effect of application of negative current using a potassium methylsulphate-filled electrode. The differential reversal of the early and late portions of the hyperpolarizing response was observed. The polarity of the early portion of the late hyperpolarization was reversed to the depolarizing value. The latter portion of late hyperpolarization was reversed to a lesser degree. Similar differences in reversal of the two portions of late hyperpolarization were observed when chloride was injected intracellularly. This is demonstrated in Fig. 2*D* which shows postsynaptic potentials recorded from the same neurone as in Fig. 2*B*, but after chloride injection (−5 nA, 2 Hz, for 10 min). The two examples of postsynaptic potentials, labelled as *a* and *a'*, were collected at the beginning and end of the apnoea illustrated in Fig. 1*Bb*. Chloride injection resulted in reversal of the early portion of the hyperpolarizing phase and substantial reduction of the late portion of this phase compared to the control trials illustrated in Fig. 2*B*. Yet, at this time point the spontaneous post-inspiratory and expiratory inhibitory postsynaptic potentials were not reversed. Their reversal occurred after longer chloride injections, but then the pattern of reversal of both portions of late hyperpolarization was the same as shown in Fig. 2*D*. The gradual hyperpolarization of the membrane potential during apnoea induced by stimulation of superior laryngeal nerve did not disappear after chloride injection (Fig. 1*Bb*).

Effects of SLN stimulation on expiratory neuronal activity

Twenty expiratory units were recorded. Ten could be antidromically activated from the spinal cord with a latency of 1·0–1·6 ms and mean conduction velocity 28·1 ± 5·7 m/s. The other ten expiratory neurones were not antidromically activated following either vagus nerve or spinal cord stimulation. Expiratory neurones had an incremental pattern of firing beginning shortly after cessation of the phrenic burst and the three phases of membrane potential during the respiratory cycle could be distinguished (Fig. 3*A*).

Figure 3*B* shows the effect of SLN stimulation on the membrane potential of a bulbospinal expiratory neurone when this stimulation was insufficient to induce apnoea. The control activity of this neurone is demonstrated in Fig. 3*A*. During this stimulation the expiratory time on the phrenic nerve recording was prolonged and the duration of the post-inspiratory phase was substantially increased. This finding is consistent with the results of similar experiments performed in cats (Ballantyne & Richter, 1986).

Figure 4 shows an example of the membrane potential of a bulbospinal expiratory neurone during apnoea induced by stimulation of the superior laryngeal nerve. Among all twenty recorded expiratory neurones the membrane potential during SLN-induced apnoea was similar to the level seen during post-inspiration in control

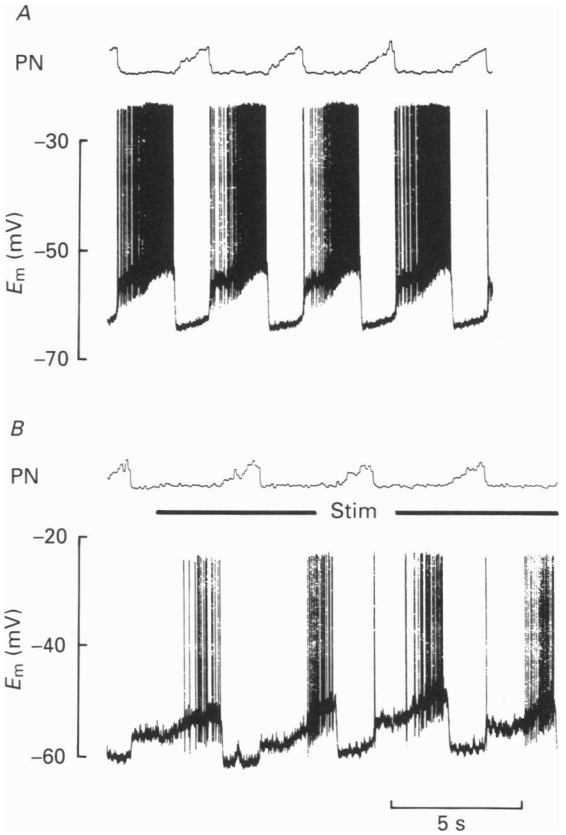

Fig. 3. Example of an expiratory bulbospinal neurone (*A*) and the same neurone (*B*) during subthreshold for apnoea SLN stimulation (Stim) (2 Hz, 6 V). Notice substantial prolongation of stage I of expiration. Time scale is the same for *A* and *B*. PN, phrenic nerve activity; E_m, membrane potential.

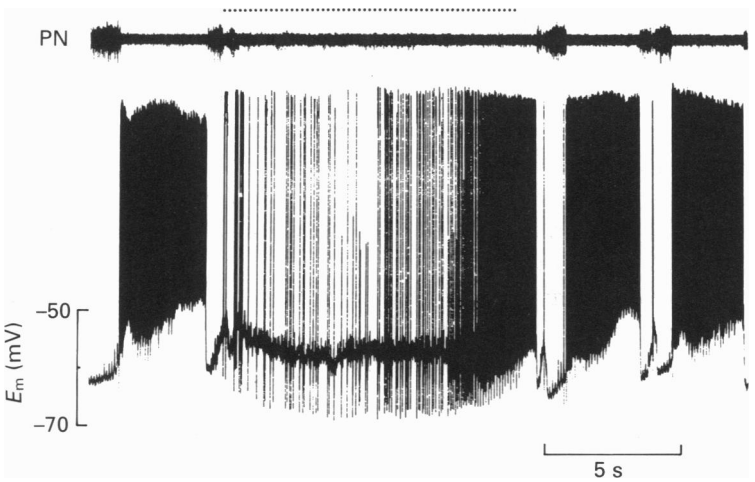

Fig. 4. Effects of SLN stimulation (dotted line, 7 Hz, 3 V) on the membrane potential (E_m) of an expiratory bulbospinal neurone and activity in the phrenic nerve (PN).

recordings. In some cases, however, membrane potential of expiratory neurones during apnoea was more negative than the polarity state observed in post-inspiration of control recordings. After the end of the stimulation the membrane potential immediately became depolarized to the level seen during stage II of expiration, followed by the first inspiration. The post-inspiratory portion of the expiratory neurone activity was prolonged over several following respiratory cycles.

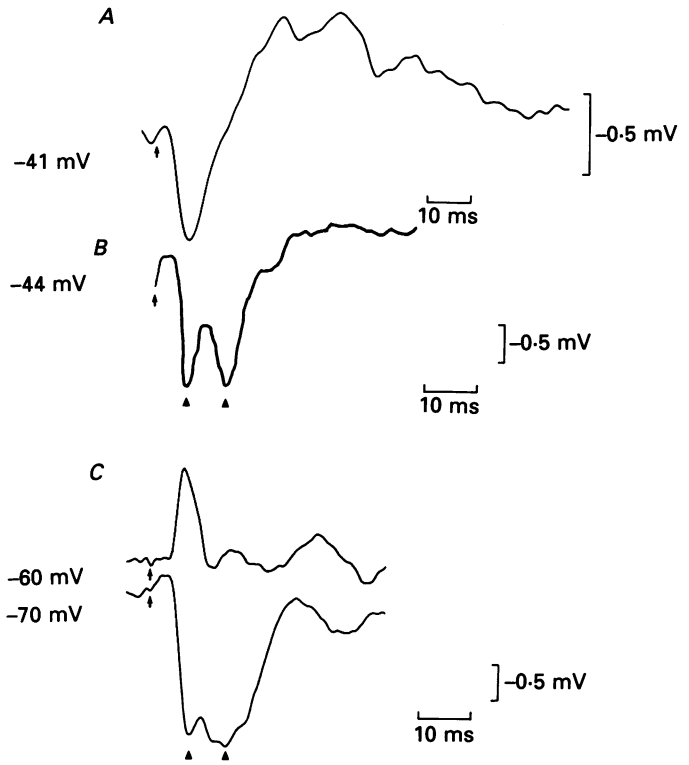

Fig. 5. Examples of stimulus-triggered (arrows) postsynaptic potentials in two expiratory neurones. *A*, early hyperpolarization followed by late depolarization (averaged 35 times), bulbospinal neurone; *B*, early and late hyperpolarization (averaged 25 times), non-bulbospinal neurone; *C*, pattern of reversal of early and late hyperpolarization in a different, non-bulbospinal neurone (averaged 50 times). ▲ indicate maxima of early and late responses. Traces are separated by an arbitrary space for better visualization.

Postsynaptic potentials were studied in sixteen out of twenty expiratory neurones. Figure 5 demonstrates examples of the stimulus-triggered postsynaptic potentials averaged during apnoea evoked by SLN stimulation. As for the inspiratory neurones, postsynaptic potentials evoked by SLN stimulation in the expiratory neurones were composed of two phases. The constant characteristic of these postsynaptic potentials was an early, short-lasting hyperpolarization (Table 1) observed in all sixteen analysed expiratory neurones. The second postsynaptic potential component was more variable. In nine neurones, eight projecting to the spinal cord, the second phase was a late, long-lasting depolarization (Fig. 5*A*). This late depolarization was present

when either potassium methylsulphate- and potassium chloride-filled electrodes were used. It could also be recorded immediately after neuronal penetration during the first test with SLN stimulation and its shape and size did not change during consequent trials. This late depolarization was more evident (in four neurones) when

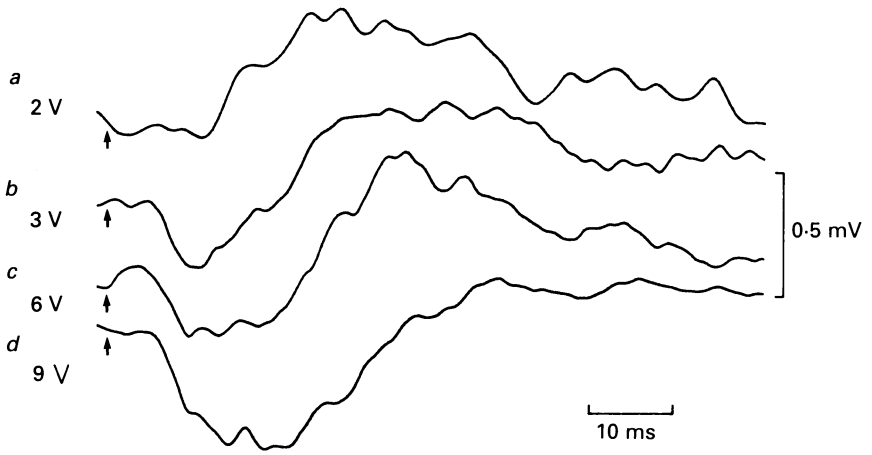

Fig. 6. Effects of increasing SLN stimulus strength (at 10 Hz) on the postsynaptic potentials evoked in an expiratory bulbospinal neurone. The voltage of stimuli is given at the beginning of each trace. Each trace shows an average of fifty to seventy-five individual postsynaptic potentials collected during consecutive SLN stimulus trains; arrows demonstrate stimulus artifacts. The initial membrane potentials were within the range −60 to −62 mV. Traces are separated by an arbitrary space for better visual demonstration.

SLN stimulation did not completely inhibit phrenic nerve activity but mainly prolonged the silent period in the phrenic nerve activity (Fig. 3*B*). In this regard, Fig. 6 demonstrates an example of the changes in the postsynaptic potentials evoked by increasing the strength of stimuli applied to the superior laryngeal nerve studied in four neurones. Stimulation of SLN (2 V, 10 Hz), which neither caused silence in the phrenic nerve nor stopped respiratory changes in the neuronal activity, evoked only a depolarization (Fig. 6*a*). However, this depolarization was gradually replaced and delayed by augmenting the voltage of the stimuli from 3 to 9 V (the threshold for the inhibition of the phrenic nerve activity and respiratory changes in the membrane potential of the neurone was 3 V at 10 Hz).

A different pattern of postsynaptic potentials, shown in Fig. 5*B* and *C*, was recorded in seven other expiratory neurones, six of these were non-antidromically activated from the spinal cord. The early hyperpolarization was followed by a late hyperpolarizing phase which was additionally distinguishable from the early hyperpolarizing component after injection of current or chloride (Fig. 5*C*). This pattern of synaptic potentials was observed mainly in the neurones in which membrane potential during SLN-evoked apnoea was more negative than the control post-inspiratory level. The latency to peak of this late hyperpolarization was not significantly different from that for the late depolarization (Table 1). The inhibitory

postsynaptic potentials were reversed by injection of chloride or by passing negative current (3–5 nA). Figure 5*C* demonstrates the typical pattern of reversal of both hyperpolarizing components in an expiratory, non-antidromically activated neurone. The complete reversal of early hyperpolarization was rapidly accomplished, often without an active injection of chloride, presumably due to leakage from the electrode or extracellular fluid. On the contrary, the late hyperpolarization could not be as successfully reversed as the early component.

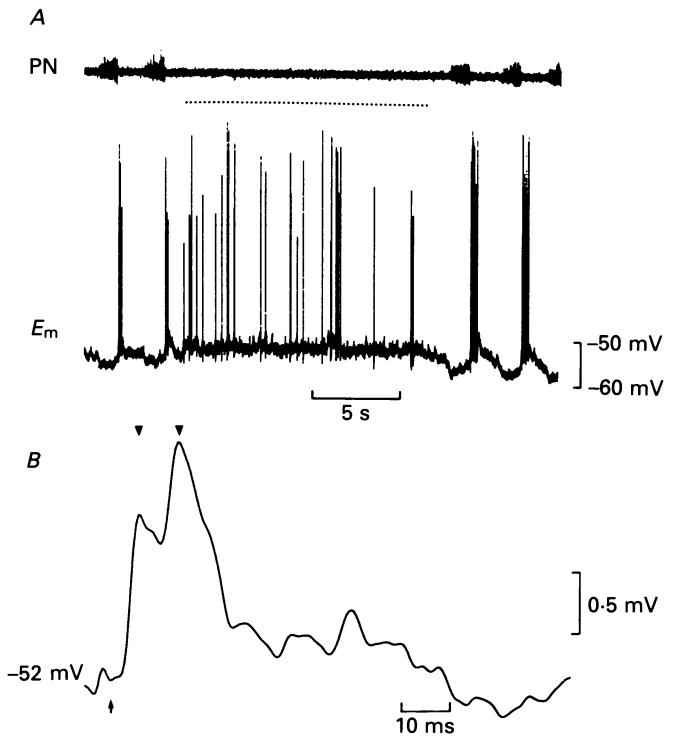

Fig. 7. Effects of SLN stimulation (3 V, 7 Hz) on post-inspiratory neurone activity (*A*) and averages (25 responses) of postsynaptic potentials collected during this stimulation (*B*). ▼ indicate maxima of early and late response. PN, phrenic nerve activity.

Effect of SLN stimulation on post-inspiratory neuronal activity

Successful recordings were performed in ten post-inspiratory neurones which were not antidromically activated by stimulation of the spinal cord or the vagus nerve. These neurones were found in the most rostral part of the recording area, i.e. at the level of the obex. Post-inspiratory neurones were characterized by a peak of depolarization which occurred shortly after cessation of phrenic nerve activity, resulting occasionally in spikes. Two levels of relative hyperpolarization were apparent during the other phases of the respiratory cycle. These two phases were a less negative phase during expiration and a more negative one during inspiration (Fig. 7). Stimulation of the superior laryngeal nerve caused depolarization of the membrane potential to the post-inspiratory level and spiking accompanying apnoea

(Fig. 7*A*). The evoked postsynaptic potentials were studied in five out of ten neurones and they consisted of two excitatory components (Fig. 7*B*). The latency of the early component was similar to the latency of early postsynaptic potentials recorded in inspiratory and expiratory units (Table 1). The depolarization following SLN stimulation resulted in action potentials with increasing stimulus strength. The late depolarizing phase in the post-inspiratory neurones reached its peak significantly earlier ($P < 0{\cdot}01$) than the peak of late hyperpolarization in the inspiratory neurones (Table 1). However, the time to the peak of the late depolarization of post-inspiratory neurones and the peak of the late postsynaptic phase in expiratory neurones (either depolarizing or hyperpolarizing) were not different.

DISCUSSION

This report provides an analysis of evoked postsynaptic potentials and membrane potential trajectories in ventral group respiratory neurones during long-lasting apnoea induced by electrical stimulation of the superior laryngeal nerve.

Many of the recorded inspiratory and expiratory neurones were identified as projecting to the spinal cord. However, some respiratory neurones of all three categories could not be antidromically activated from either the spinal cord or the cervical vagus nerve. In this regard the post-inspiratory neurones may represent propiobulbar neurones, as described earlier (Richter, 1982; Richter & Ballantyne, 1983; for review see Duffin & Aweida, 1990). Alternatively, these post-inspiratory neurones could have sent their axons to the pharyngeal branch of the vagus which separates from the main vagal trunk rostral to the nodose ganglion and therefore could not be antidromically activated by stimulation of the more distal cervical part of the vagal nerve. Support for this latter possibility was presented by Grelot, Barillot & Bianchi (1989) who showed that some vagal pharyngeal motoneurones had post-inspiratory patterns of activity. The non-antidromically activated expiratory and inspiratory neurones in the region of the nucleus retroambiguus have been identified earlier on the basis of their morphology (Kreuter, Richter, Camerer & Senekowitsch, 1977). Again the possibility exists that these neurones may be vagal or glossopharyngeal motoneurones innervating pharyngeal muscles (Grelot *et al.* 1989) rather than propiobulbar.

In each of the three major categories of neurones, SLN stimulation, sufficient to induce apnoea, evoked compound postsynaptic potentials in which separate components were distinguished on the basis of latencies, durations and patterns of reversal by current or chloride injection. The early and late components began with similar latencies in each type of recorded neurone indicating synchrony of the responses within the respiratory network to SLN stimulation.

The early response may represent a relatively direct oligosynaptic input relayed to the VRG neurones from interneurones in the region of the nucleus of the solitary trace (NTS) which receive input from SLN afferents. The SLN afferents terminate in the NTS (Kalia & Mesulam, 1980) and the projections from the NTS to the ipsilateral nucleus ambiguus are identified (Loewy & Burton, 1978; Bystrzycka, 1980). Also, second-order sensory neurones excited by laryngeal afferents were demonstrated in the NTS and adjacent regions (Sessle, 1973; Berger, 1977; Bellingham & Lipski,

1988). However, the actual identity of the neurones relaying early postsynaptic potentials to the respiratory neurones in the nucleus ambiguus remains to be determined. In the neonatal piglet afferent fibres from the SLN have been reported to end also in the proximity of the nucleus ambiguus (Goding, Richardson & Trachy, 1987) and therefore a shorter pathway from SLN afferents to neurones of the VRG may exist in neonates.

In inspiratory and post-inspiratory neurones the early depolarizing response is an evoked excitatory postsynaptic potential. The pattern of early excitatory postsynaptic potentials evoked during SLN-induced apnoea in the VRG inspiratory neurones in this study was similar to the short-lasting excitation preceding inhibition shown in some of the phrenic motoneurones (Bellingham, Lipski & Voss, 1989), although these authors used lower stimulation levels. Also, extracellular recordings of inspiratory neurones in the nucleus of the solitary tract (Donnelly *et al.* 1989) and recordings of the whole phrenic nerve activity (Berger & Mitchell, 1976; Iscoe *et al.* 1979; Sica *et al.* 1984; Donnelly *et al.* 1989) demonstrated an early excitation followed by late inhibition in response to SLN stimulation.

In contrast to the inspiratory and post-inspiratory neurones, the early response in expiratory neurones was a hyperpolarization. Its fast reversal by chloride or negative current shows that it is a chloride-mediated postsynaptic inhibition.

The late, longer lasting wave of postsynaptic potentials may be a result of polysynaptic effects exerted on the neuronal circuits of the respiratory network by SLN stimulation and is probably responsible for the long-lasting effects of SLN stimulation on neuronal membrane potential.

The late hyperpolarization in inspiratory neurones was composed of two different inputs. The earlier represented a chloride-mediated postsynaptic inhibition, as hyperpolarization was clearly reversible by chloride. The latter part of the late hyperpolarization was affected by intracellular chloride injection to a lesser degree and therefore the possibility exists that this latter part represents a remote dendritic synaptic site or non-chloride-mediated inhibitory responses. Such a non-chloride-mediated inhibitory mechanism may be an increased potassium conductance or presynaptic disfacilitation. The present experiments do not allow differentiation of these possibilities. However, the negative shift in the membrane potential observed during prolonged post-inspiration accompanying apnoea did not disappear after both portions of late hyperpolarization were reversed to the point achieved in this study. This fact supports the possibility that a slow, long-lasting, and non-chloride-mediated inhibition was activated in inspiratory neurones at the post- and/or presynaptic level during SLN-induced apnoea.

The late postsynaptic potentials induced by SLN stimulation in expiratory neurones were much less uniform in character than inspiratory and post-inspiratory neurones. In some expiratory neurones the late phase was an excitatory postsynaptic potential while in others it was hyperpolarization. Similar to inspiratory neurones, the mechanism of this late hyperpolarization included other non-chloride-mediated inhibitory responses, as it could not be reversed by chloride ionophoresis.

Activation of some expiratory neurones during apnoea evoked from laryngeal receptors has been previously reported. Bongianni, Corda, Fontana & Pantaleo (1988) demonstrated tonic activation of expiratory muscles and VRG expiratory-

related neurones in response to SLN stimulation. Jodkowski & Berger (1988) found that some expiratory bulbospinal neurones were inactivated and some had only diminished activity in response to SLN stimulation. The responsiveness of expiratory neurones to SLN stimulation was proposed to depend on the initial functional state of the neurone (Jodkowski & Berger, 1988). The non-uniformity of the late postsynaptic potentials and post-inspiratory membrane potential profile induced by SLN stimulation in expiratory neurones in our experiments may therefore be due to the functional state of individual neurones. The more so, that the different responses seemed to be related to the anatomic connections among recorded expiratory neurones, where most non-antidromically activated neurones displayed late hyperpolarization while bulbospinal expiratory neurones displayed late depolarization. In addition, the diminished activity of bulbospinal expiratory neurones in response to SLN stimulation described by Jodkowski & Berger (1988) may correspond to the reduced firing rate of expiratory neurones during the post-inspiratory phase, similar to that shown in Fig. 4. Ballantyne & Richter (1986) demonstrated that the spontaneous post-inspiratory stage in caudal expiratory neurones is not uniform. The post-inspiratory membrane potential in expiratory neurones represents a net effect of simultaneous incrementing excitatory post-synaptic potentials and decrementing inhibitory postsynaptic potentials and the final result is due to the predominance of one of these inputs (Ballantyne & Richter, 1986). Our results showed that during apnoea induced by SLN stimulation a similar situation may occur. SLN stimulation induces both excitatory and inhibitory postsynaptic potentials in expiratory neurones and by increasing the stimulus intensity the late excitatory response can be overcome by a late inhibition. Hence, the membrane potential of expiratory neurones during SLN-induced apnoea represents a net effect of excitatory and inhibitory inputs activated by SLN afferents.

Recently a hypothesis was proposed that the apnoeic state in phrenic nerve induced by stimulation of laryngeal receptors may originate from activation of post-inspiratory neurones by the same stimulation (Remmers *et al.* 1985, 1986). This hypothesis is based on the observation that stimulation of SLN with brief pulses or of laryngeal receptors with adequate stimuli in turn activated post-inspiratory neurones and enhanced the post-inspiratory phase in other medullary respiratory neurones (Richter & Ballantyne, 1983; Remmers *et al.* 1985, 1986; Richter *et al.* 1987; E. E. Lawson, D. W. Richter, M. F. Czyzyk-Krzeska, A. Bishoff & R. C. Rudesill, unpublished). The results of the present paper are in agreement with these observation as both activation of post-inspiratory neurones and prolongation of the post-inspiratory phase of other respiratory neurones was seen. In addition, the early and late synaptic inputs to the post-inspiratory neurones, evoked by laryngeal nerve stimulation, were excitatory. It may be therefore hypothesized that the late phase of hyperpolarization seen in inspiratory, and some expiratory neurones, in response to SLN stimulation may be due to synaptic inhibition originating from the excitation of post-inspiratory neurones by the same stimulus. This possibility, in the case of inspiratory neurones, is supported by the observation that the late postsynaptic component evoked by SLN stimulation in post-inspiratory neurones occurred significantly earlier than in inspiratory neurones.

The mechanism of late hyperpolarization in inspiratory and some expiratory neurones seemed to consist of several different synaptic components including chloride-mediated inhibition and potentially potassium-mediated inhibition or presynaptic disfacilitation. The present results do not allow discrimination among these mechanisms but suggest complex multisynaptic connections from post-inspiratory to other VRG respiratory neurones.

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