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Chemosensory and cholinergic stimulation of fictive respiration in isolated CNS of neonatal opossum

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1. The aim of the present experiments was to characterize the central chemical drive of fictive respiration in the isolated CNS of the newborn opossum, *Monodelphis domestica.* This opossum preparation, in contrast to those of neonatal rats and mice, produces respiratory rhythm of high frequency *in vitro.*

2. Fictive respiration was recorded from C3–C5 ventral roots of the isolated CNS of 4- to 14-day-old opossums using suction electrodes. At room temperature (21–23 °C) the frequency of respiration was $43 \pm 5{\cdot}3\ \text{min}^{-1}$ (mean ± S.E.M., $n = 50$) in basal medium Eagle's medium (BMEM) equilibrated with 5% CO_2–95% O_2, pH 7·37–7·40. Respiratory discharges remained regular throughout 8 h experiments and continued for more than 20 h in culture.

3. Superfusion of the brainstem confirmed that solutions of pH 6·3–7·2 increased both the amplitude and frequency of respiration. High pH solutions (7·5–7·7) had the opposite effect and abolished the rhythm at pH 7·7. Addition of ACh (50–100 μM) or carbachol (0·01–10 μM) to the brainstem superfusion also increased the amplitude and frequency of respiratory activity, as did physostigmine (50–100 μM) or neostigmine (20–50 μM). Conversely, scopolamine (50–100 μM) reduced the amplitude and frequency of the basal respiratory rhythm by about 30%.

4. H^+- and cholinergic-sensitive areas on the surface of the isolated CNS were explored with a small micropipette (outer tip diameter, 100 μm) filled with BMEM (pH 6·5) or 1 μM carbachol. Carabachol applied to H^+- and cholinergic-sensitive areas in the ventral medulla mimicked the changes of respiratory pattern produced by low pH application. Responses to altered pH and carbachol were abolished by scopolamine (50 μM). Histochemistry demonstrated several medullary groups of neurons stained for acetylcholinesterase. The superficial location of one of these groups coincided with a functional and anatomically well-defined pH- and carbachol-sensitive area placed medial to the hypoglossal roots.

5. Exploration of chemosensitive areas revealed that application of drugs or solutions of different pH to a single well-defined spot could have selective and distinctive effects upon amplitude and frequency of respiratory activity.

6. These results show that fictive respiration in the isolated CNS of the newborn opossum is tonically driven by chemical- and cholinergic-sensitive areas located on the ventral medulla, the activity of which regulates frequency and amplitude of respiration. They suggest that a cholinergic relay, although not essential for rhythm generation, is involved in the central pH chemosensory mechanism, or that cholinergic and chemical inputs converge upon the same input pathway to the respiratory pattern generator.

Changes in carbon dioxide and hydrogen ions within the brain trigger ventilatory responses through activation of specific chemosensory areas of the mammalian CNS (Loeschcke, 1982; Bruce & Cherniack, 1987). These chemosensory areas provide tonic drive to the neural network that generates the respiratory rhythm. Central chemosensory areas have been localized on the surface of the ventral medulla (Mitchell, Loeschcke, Massion & Severinghaus, 1963*a*; Mitchell, Loeschcke, Severinghaus, Richardson & Massion, 1963*b*; Loeschcke, 1982; Bruce & Cherniack, 1987), and recently, in locus coeruleus and nucleus tractus solitarius of cats and rats (Coates, Li & Nattie, 1993).

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Central chemoreception at the surface of the ventral medulla may involve cholinergic mechanisms since topical application of ACh agonists or acid produce similar respiratory responses (Dev & Loeschcke, 1979*a*, *b*; Fukuda & Loeschcke, 1979). However, the identity of the receptors responsible for chemoreception in the CNS has not been resolved (Coates *et al.* 1993).

Experiments made *in vivo* have inherent problems that interfere with the precise localization of chemosensory areas: these include effects of anaesthesia, dilution of chemical stimuli by the circulation, secondary respiratory changes due to cardiovascular effects, and pulsations. Such problems can be mitigated by the use of preparations *in vitro* such as the isolated spinal cord lower brainstems of perinatal rats (Suzue, 1984; Harada, Kuno & Wang, 1985; Onimaru & Homma, 1987; Monteau, Morin & Hilaire, 1990; Smith, Greer, Liu & Feldman, 1990; Issa & Remmers, 1992). Isolated preparations made from perinatal rats do not, however, preserve the properties of respiration seen in the animal *in vivo*. The main differences are that the rhythm recorded *in vitro* has a different pattern and it is far slower than that recorded *in vivo*, achieving frequencies about one tenth of the normal rate or below (Smith *et al.* 1990; Wang, Fung, Darnall & St John, 1996). This slow rhythm could represent an alteration of underlying mechanisms of breathing. Brainstem–spinal cords isolated from neonatal spiny mice, which are born in a more mature state of CNS development than rats, do not generate a sustained respiratory activity (Greer, Carter & Allan, 1996).

In contrast, the isolated CNS of the newborn opossum (*Monodelphis domestica*) generates fictive respiration of high frequency (34–47 min^{-1}) which is close to the normal value of 60 min^{-1} in 4- to 8-day-old pups (Nicholls, Stewart, Erulkar & Saunders, 1990; Zou, 1994). Moreover, this marsupial preparation is pH sensitive, showing an increase in the rate of respiratory activity recorded from ventral roots after acidification of the medium (Zou, 1994). Since long-term studies lasting for a week or more are also practicable (Nicholls *et al.* 1990; Stewart, Zou, Treherne, Møllgard, Saunders & Nicholls, 1991; Zou, Treherne, Stewart, Saunders & Nicholls, 1991; Saunders *et al.* 1992; Treherne, Woodward, Varga, Ritchie & Nicholls, 1992) the opossum CNS seems favourable for exploring chemosensory ventilatory areas and the role of cholinergic mechanisms involved in central chemosensitivity.

The aims of this work were to characterize responses of the isolated CNS of the newborn opossum to chemical stimulation, to localize chemosensory areas on the brainstem surface, to establish whether cholinergic mechanisms are involved in these responses or in rhythm generation, and to define whether restricted activation of a well-defined chemosensory area generates specific patterns of respiratory responses.

Part of this work has been reported in abstracts (Eugenín, 1994, 1995, 1996).

METHODS

Newborn *Monodelphis domestica* (4–14 days old) were removed from their mothers in the breeding colony and anaesthetized with methoxyflurane (Metofane; Pitman-Moore). The pups were immersed in cooled (4 °C) BMEM (basal medium Eagle's medium; cat. no. 041-01010, Gibco) containing (mM): NaCl, 116·3; KCl, 5·4; $NaHCO_3$, 26·2; $NaH_2PO_4(H_2O)$, 1·0; $CaCl_2$, 1·8; $MgSO_4(7H_2O_2)$, 0·8; as well vitamins and amino acids. The medium was suplemented with D-glucose to a final concentration of 30 mM and equilibrated with 5% CO_2–95% O_2 (pH 7·37–7·40) at room temperature (22–25 °C). The CNS together with its cervical and thoracic dorsal root ganglia was dissected from the pup in order to obtain long ventral roots (Nicholls *et al.* 1990). The preparation was transferred to a recording chamber 0·5 ml in volume, fixed to Sylgard with fine insect pins and superfused continously with gassed BMEM (0·6–0·8 ml min^{-1}) at room temperature (21–23 °C).

Electrical recording

Spontaneous activity from C3–C5 ventral roots was recorded with glass suction micropipettes with tips of 30–50 μm inner diameter driven by a manipulator. In some experiments, simultaneous recordings from two ipsilateral or contralateral ventral roots or a ventral root and hypoglossal, vagal or glossopharyngeal nerve root were made. Single respiratory-related units from regions in the lower brainstem previously described by Zou (1994) were recorded from extracellularly by means of a 2–4 MΩ glass microelectrode filled with 1 M NaCl and driven by a micromanipulator. Electrical signals were amplified by low-noise differential amplifiers (Almost Perfect Electronics, Basel, Switzerland) and bandpass filtered at 3–3000 Hz. Multiunitary activity and integrated activity obtained by full-wave rectification (time constant, 50–100 ms) were displayed on an oscilloscope (Tektronix) and a chart recorder (Clevite Corporation, Cleveland, OH, USA). Signals were stored on videotape at a sampling frequency of 9 or 18 kHz through a VR-100 digital recorder system (Instrutech Corp., Mineola, NY, USA).

Stimulation

The brainstem and spinal cord were perfused separately. A thin partition sealed with Vaseline at the level of C1–C2 (Fig. 1*A*; see Zou, 1994) allowed BMEM, containing test substances or gassed with different CO_2–O_2 mixtures, to be superfused to one part of the chamber while the other was superfused with control BMEM (pH 7·4). With flow rates in both parts of the chamber of 1·2–1·5 ml min^{-1}, the test medium reached the chamber in about 25 s and complete exchange took an additional 10–15 s. Absence of leakage between compartments was checked by the addition of Methylene Blue or Fast Green.

The pH of the superfusion medium was changed either by gassing with different CO_2–O_2 mixtures (2·5–97·5%, pH 7·7; 5–95%, pH 7·4; 7·5–92·5%, pH 7·1) or by adding bicarbonate to control BMEM equilibrated with 5% CO_2–95% O_2 (addition of 14 μM bicarbonate increases pH to 7·7). In topical application experiments (see below) pH was adjusted by supplementing BMEM with 5 mM Hepes (Sigma) and further titration of the medium with NaOH or HCl.

Drugs were added to gassed BMEM to the required final concentration: 1–100 μM ACh chloride, 0·001–10 μM carbamylcholine chloride (carbachol), 10–100 μM eserine (physostigmine), 1–100 μM neostigmine bromide, 10–100 μM atropine sulphate, 50–100 μM hexamethonium chloride and 10–100 μM scopolamine hydrochloride; all purchased from Sigma.

Ventral root activity was regular and stable for at least 3–5 min before application of drugs or pH solutions; washing between consecutive administrations lasted at least 10 min or 1 h in the case of scopolamine. In most of the experiments 3–5 min exposures to test solutions were used. Prolonged (more than 5 min) exposures to extreme alkaline or acidic media were avoided.

Local stimulation

Topical application of BMEM (pH 6·5) or carbachol to the surface of the CNS was performed through a glass pipette (100 μm outer diameter) (Fig. 1*B*); a triple barrelled electrode was placed inside the pipette about 70 μm from its tip; BMEM (pH 6·5) (supplemented with 5 mM Hepes and titrated with HCl) or 1 μM carbachol (in BMEM, pH 7·4) could be delivered to the interior of the tip of the glass pipette through two of the barrels. Internal suction was applied through the third barrel so as to renew medium inside the pipette and avoid leakage. Once the tip touched the surface of the CNS, slight and gentle suction was generated inside the pipette to produce a seal between the glass and the tissue; the test medium was released from one barrel to the pipette interior and the internal drain was stopped. Two other procedures were used for reducing leakage and diffusion of test medium into nearby tissue: the rate of superfusion was increased to 1·2–1·5 ml min^{-1} and an external drain was added, which sucked medium through a glass pipette tip (100–150 μm diameter) placed about 200 μm from the tip of the stimulating pipette. With these precautions, use of Methylene Blue in the pipette indicated that leakage to the surrounding tissue was minimal or undetectable. In some experiments pipette sites were marked with 2% Pontamine Sky Blue added to the stimulating solution.

Acetylcholinesterase histochemistry

To visualize cholinergic neuronal somata and processes at the medulla we used the acetylcholinesterase (AChE) staining procedure of Di Patre, Mathes & Butcher (1993), which is a modification of the sensitive method developed by Tago, Kimura & Maeda (1986). Under deep methoxyflurane anaesthesia, transcardiac perfusion of 5 ml of 0·1 M phosphate-buffered saline (PBS; pH 7·6, 4 °C) was followed by fixation with 10 ml 4% (w/v) paraformaldehyde containing 0·2% (v/v) saturated picric acid dissolved in 0·1 M PBS. The CNS was extracted and inmersed in cold fixative for 24–48 h, and then transferred to 30% (w/v) sucrose in 0·1 M PBS for 2–3 days and sectioned on a freezing microtome. Sections were collected in cold PBS and stored at 4 °C.

The staining procedure consisted of four successive incubations of samples. (1) The sections were first incubated for 30 min in 30 mM tetra-isopropylpyro-phosphoramide (iso-OMPA; Sigma) or in 10 mM 1·5-bis[4-allyldimethylammonium-phenyl]pentan-3-one dibromide (BW284c51; Sigma), which are inhibitors of butyrylcholinesterase and AChE, respectively. Both drugs were dissolved in 50 mM Tris–maleate buffer, pH 5·7. (2) The sections were then incubated for 15 min in Karnovsky–Roots (K–R) medium (Tago, Kimura & Maeda, 1986; Di Patre, Mathes & Butcher, 1993) diluted 1 : 1 or

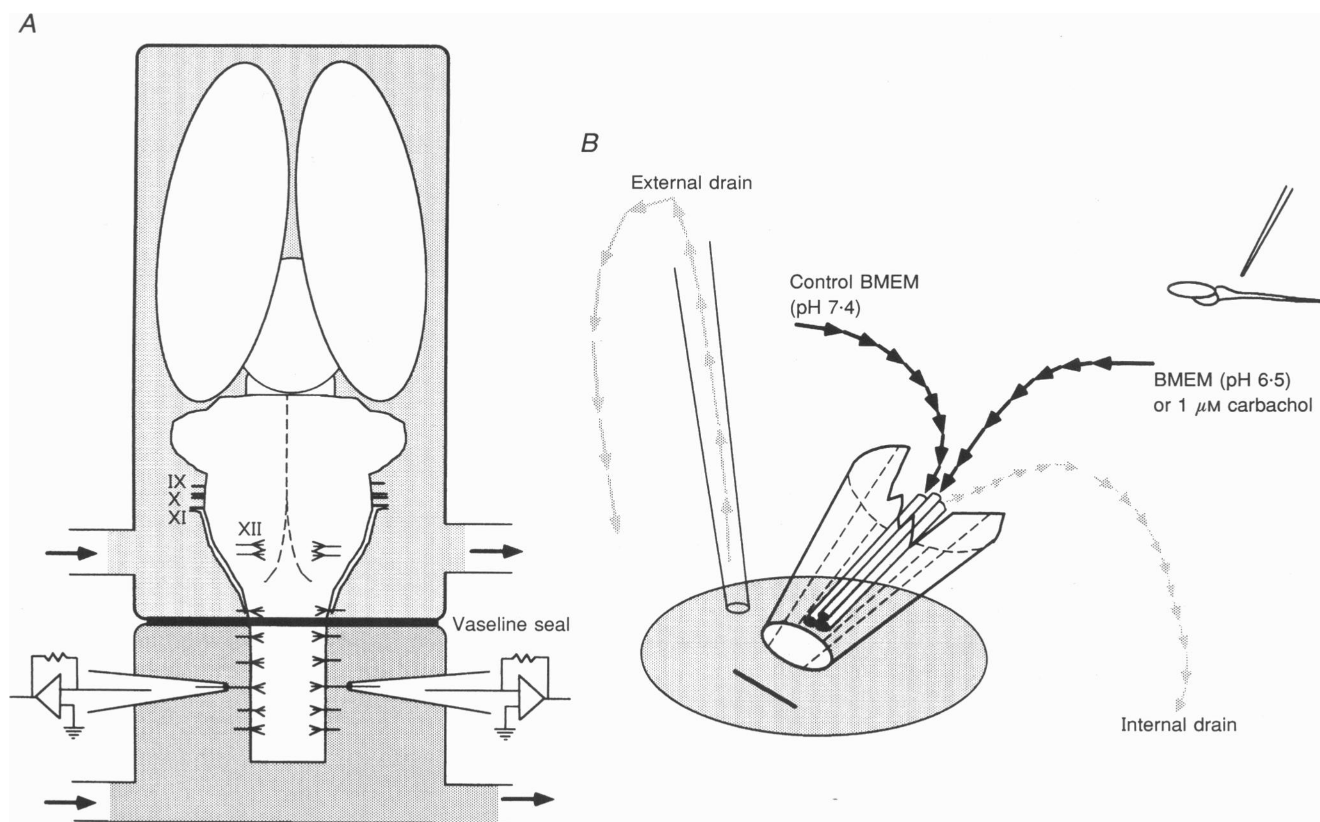

Figure 1. Diagram of the superfusion system

A, IX, X, XI and XII are cranial nerves (details in Methods). Direction of flow is indicated by arrows. Stimulation was done by switching superfusion from control to test medium or by local topical application of chemicals through a pipette of 100 μm diameter (*B*). Note that in *B* external and internal drains were aspirated (details in Methods). The bar represents 100 μm. The inset shows the placement of the pipette before it touches the surface of the CNS.

1 : 10 in 0·1 M PBS. (3) After washing with five changes of 50 mM Tris–HCl (pH 7·6), sections incubated in K–R (1 : 1) medium were incubated for 5 min in a solution of Tris–HCl buffer (pH 7·6) containing 20 mg diaminobenzidine (DAB; Sigma) and 10 ml 3% nickel ammonium sulphate (NAS) per 100 ml. Those sections which were incubated in K–R (1 : 10) were incubated for 5 min in a 0·8 mg ml^{-1} DAB–NAS solution. (4) Finally, sections were maintained for 10 min after adding 5 or 20 μl of 3% hydrogen peroxide per each 10 ml to the 0·2 or 0·8 mg ml^{-1} DAB–NAS solutions, respectively.

Sections were washed in 0·1 M PBS, mounted on glass slides coated with albumin and allowed to dry at room temperature. Sections were rinsed in distilled deionized water, air-dried again, immersed in xylene and mounted in Permount.

Data analysis

The period (time between the beginning of two consecutive cycles), instantaneous respiratory frequency (reciprocal value of the period), inspiratory duration and amplitude of each cycle were analysed from the full-wave rectified signal (time constant, 50–100 ms) with a PC IBM compatible computer (486 DX, 33 MHz) and a digital data adquisition system (Almost Perfect Electronics).

Values were normalized by expressing amplitude and instantaneous frequency of each respiratory cycle as a percentage of the respective average value obtained from ten to twenty cycles at basal conditions immediately before the onset of stimulation. Results are expressed throughout as means ± S.E.M. Statistical tests for significance were made with Student's t tests for paired samples.

RESULTS

Spontaneous activity recorded from C3, C4 and C5 ventral roots of newborn opossums *in vitro* displayed high or low frequency rhythms superimposed on a background of random activity. The high frequency rhythm ($43 \pm 5{\cdot}3$ min^{-1}, mean ± S.E.M.; $n = 50$) consisted of short duration (300 ms), high amplitude bursts of action potentials (Fig. 2*A* and *B*). The bursts recorded from ventral roots were regular in timing and amplitude and were preceded by similar bursts appearing at the hypoglossal, vagal or glossopharyngeal nerves a few milliseconds earlier. The onsets of the bursts recorded from both ventral roots of a single spinal cord segment were synchronous. Extracellular recordings from the ventral medulla showed single units that fired rhythmical bursts at the same frequency as this high frequency ventral root rhythm (Fig. 2*B*). As previously described (Zou, 1994) the pattern of discharge of these medullary units could be related to the phase of appearance of the ventral root bursts or the interval between them (Fig. 2*B*). The high frequency rhythm recorded from cervical and thoracic ventral roots disappeared after C1 spinal cord transection.

In contrast, the low frequency rhythm (less than 10 min^{-1}) consisted of high amplitude, long duration (1 or more seconds) bursts of action potentials; the shape of these bursts was highly variable from one animal to the other. This rhythm persisted after C1 spinal cord transection, indicating that it is an intrinsic spinal cord rhythm. Bursts recorded from contralateral ventral roots were either not synchronized or they showed an alternating pattern of discharge suggesting fictive locomotion.

The results described below deal only with the high frequency rhythm. Additional evidence that this high frequency rhythm in opossum CNS *in vitro* corresponds to fictive respiration has been given in a previous study (Zou, 1994) in which EMGs from respiratory muscles were recorded.

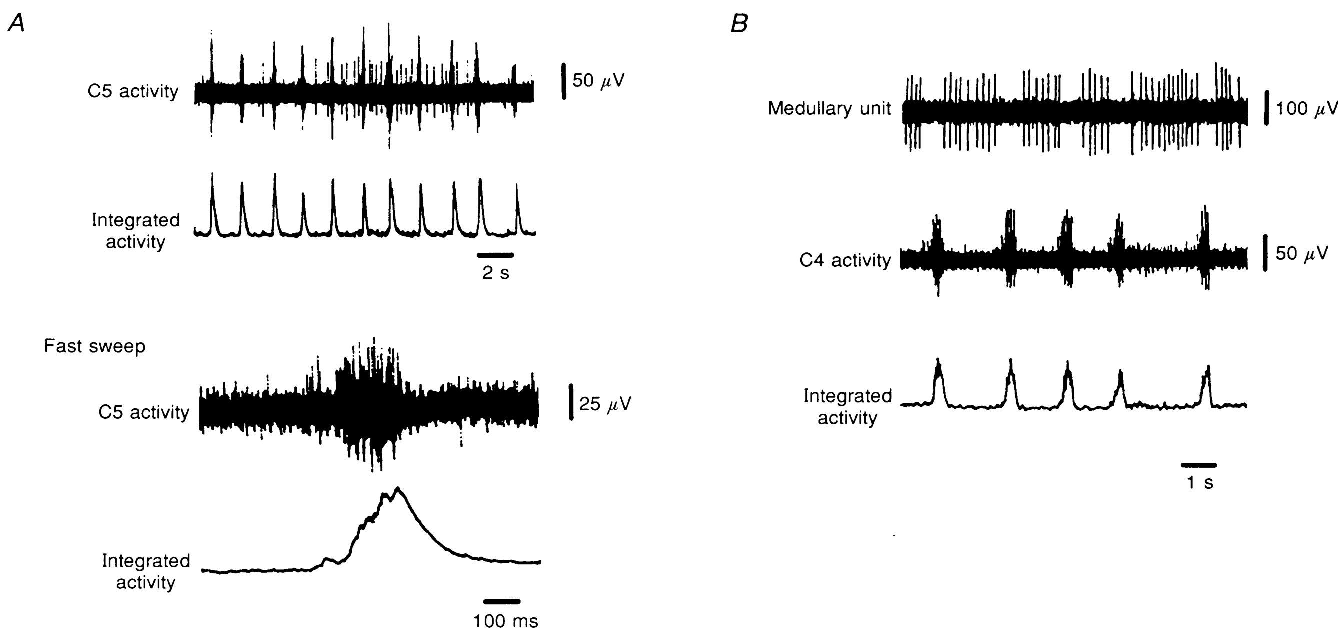

Figure 2. Fictive respiration recorded from C5 (*A*) and C4 (*B*) ventral roots in the isolated CNS preparation of 7- and 9-day-old opossums

C5 and C4 activities were integrated by a full-wave rectifier (time constants of 100 and 50 ms, respectively). The upper trace in *B* is an extracellular recording of an expiratory-related medullary unit.

Effects of pH

When the medium bathing the brainstem was acidified (pH 7·20 or lower), while the spinal cord was maintained in control conditions (pH 7·37–7·40), the amplitude and rate of respiration were increased (by about 120 and 50%, respectively; Fig. 3*A* and *C*). Alkalinization of the superfusion of the brainstem (pH 7·50 or higher) produced the opposite effect, reducing the amplitude and frequency (Fig. 3*B* and *C*). Extreme alkalosis (pH 7·70 or higher) abolished the respiratory rhythm. All pH effects were reversible after washing with control BMEM (Fig. 3*A* and *B*). Although there was some variability, in all twenty animals tested the change in frequency and amplitude started after 6–15 s and reached a steady-state 45–60 s after applying the test fluid. Effects of pH upon the frequency and amplitude of fictive respiration in a 10-day-old pup are illustrated in Fig. 3*C*. Modification of the pH of the spinal cord medium while superfusing the brainstem with control BMEM did not influence the pattern of fictive respiration.

Effects of ACh agonists and antagonists

Addition of ACh or carbachol to fluid superfusing the brainstem produced dose-dependent increases in amplitude and frequency of respiratory activity (Fig. 4*A*). The time courses of these responses were different from those obtained with pH stimulation. While the increases in amplitude and frequency started rapidly (6–16 s) after cholinergic agonist application, they often persisted for several minutes after washing. Repetitive stimulation with ACh or carbachol produced desensitization. High doses of ACh (100 μM) increased the frequency by 25·0 ± 7·9% ($P < 0·03$, $n = 5$) and the amplitude by 37·6 ± 13·2% ($P < 0·04$, $n = 5$). Carbachol (5 μM) increased the frequency by 50·3 ± 16·4% ($P < 0·03$, $n = 5$) and the amplitude by 88·6 ± 20·8% ($P < 0·01$, $n = 5$). The increases induced by these doses of ACh and carbachol were associated with a rise of background activity. This initial response was followed by the appearance of irregular large bursts of ventral root discharges that masked other kinds of activity in the multiunitary recording. After application of high doses in

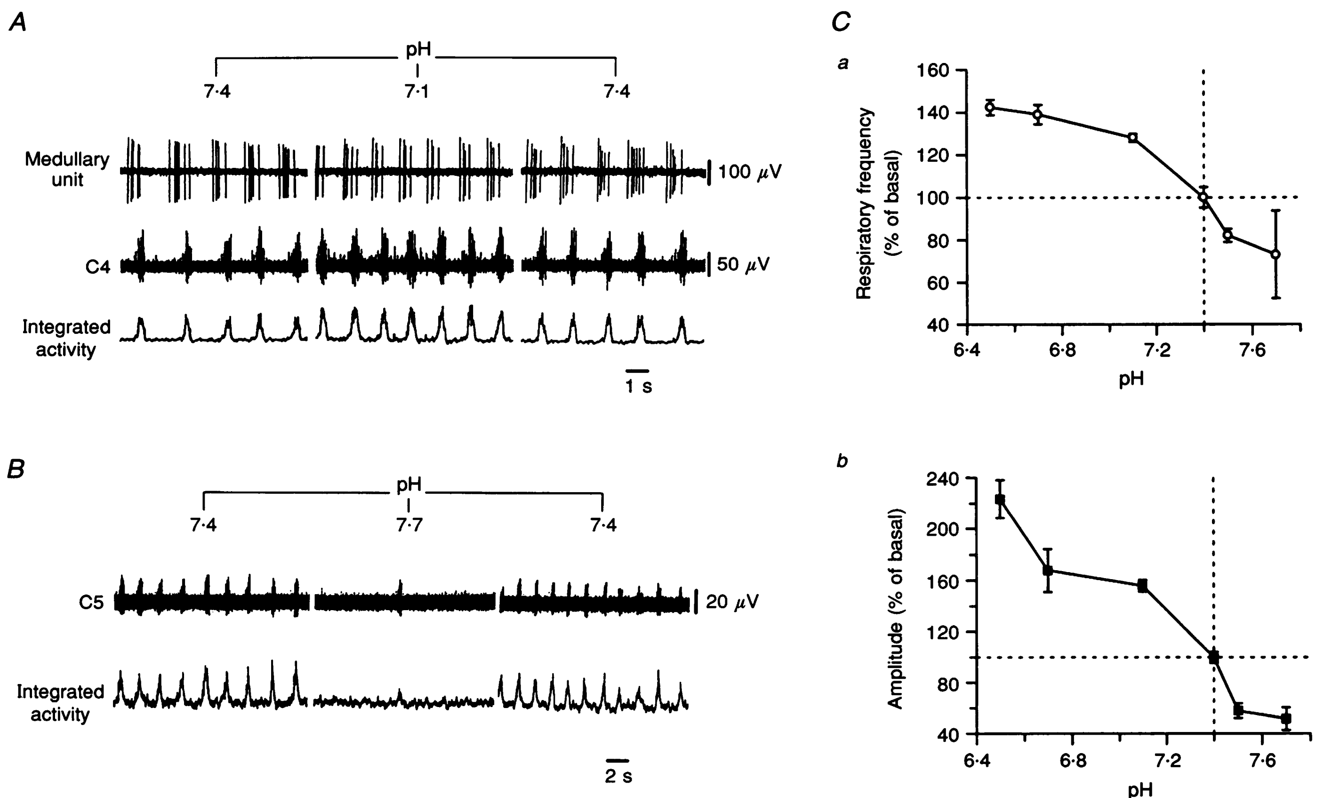

Figure 3. Changes of respiratory activity in response to brainstem superfusion of low (*A*) and high (*B*) pH

A, the amplitude and frequency of the respiration recorded from the isolated CNS of a 6-day-old opossum increased 1 min after switching from control BMEM (pH 7·4) to acid BMEM (pH 7·1) and recovered after returning to control BMEM (pH 7·4). *B*, respiratory cycles almost disappeared 1–2 min after alkalinization of the superfusion of the brainstem of a 13-day-old opossum (pH 7·7 achieved by addition of 14 mM $NaHCO_3$ to BMEM to a final concentration of 40 mM gassed with 5% CO_2–95% O_2. *C* shows respiratory frequency (*a*) and amplitude (*b*) dependence on pH of the brainstem bath in a 10-day-old opossum. Different pH values were obtained by adding 5 mM Hepes to BMEM followed by HCl or NaOH titration. Symbols are means ± S.E.M. from 4–10 cycles under steady-state conditions.

four out of five cases the respiratory rhythm declined, showing high variability in timing and amplitude.

ACh or carbachol administered to the spinal cord superfusion while the brainstem was bathed with control BMEM increased the background activity without changing the pattern of fictive respiration. At higher doses the background activity masked the respiratory rhythm.

To elucidate whether endogenous release of ACh affects the pattern of fictive respiration, acetylcholinesterase inhibitors were added to the brainstem bath (Fig. 4*B*). Physostigmine and neostigmine mimicked the effects of ACh administration. Physostigmine (50–100 μM) increased the respiratory frequency by $23{\cdot}4 \pm 2{\cdot}5\%$ ($P < 0{\cdot}001$, $n = 5$) and the amplitude by $28{\cdot}0 \pm 6{\cdot}8\%$ ($P < 0{\cdot}015$, $n = 5$). Neostigmine (20–50 μM) increased the respiratory frequency by $23{\cdot}7 \pm 2{\cdot}5\%$ ($P < 0{\cdot}019$, $n = 4$) and the amplitude by $11{\cdot}1 \pm 3{\cdot}18\%$ ($P < 0{\cdot}041$, $n = 4$). In other experiments antagonists were tested. Scopolamine (50–100 μM) reduced the amplitude of respiration by $22{\cdot}6 \pm 7{\cdot}2\%$ ($P < 0{\cdot}026$, $n = 6$) and the frequency by $20{\cdot}5 \pm 5{\cdot}5\%$ ($P < 0{\cdot}014$, $n = 6$) when administered to the brainstem superfusion (Fig. 4*C*). Nicotinic blockade with hexamethonium (50–100 μM) in four opossums did not produce any detectable change of the respiratory pattern, nor did atropine (100 μM) in seven out of nine pups. In the other two pups atropine slightly reduced respiratory activity.

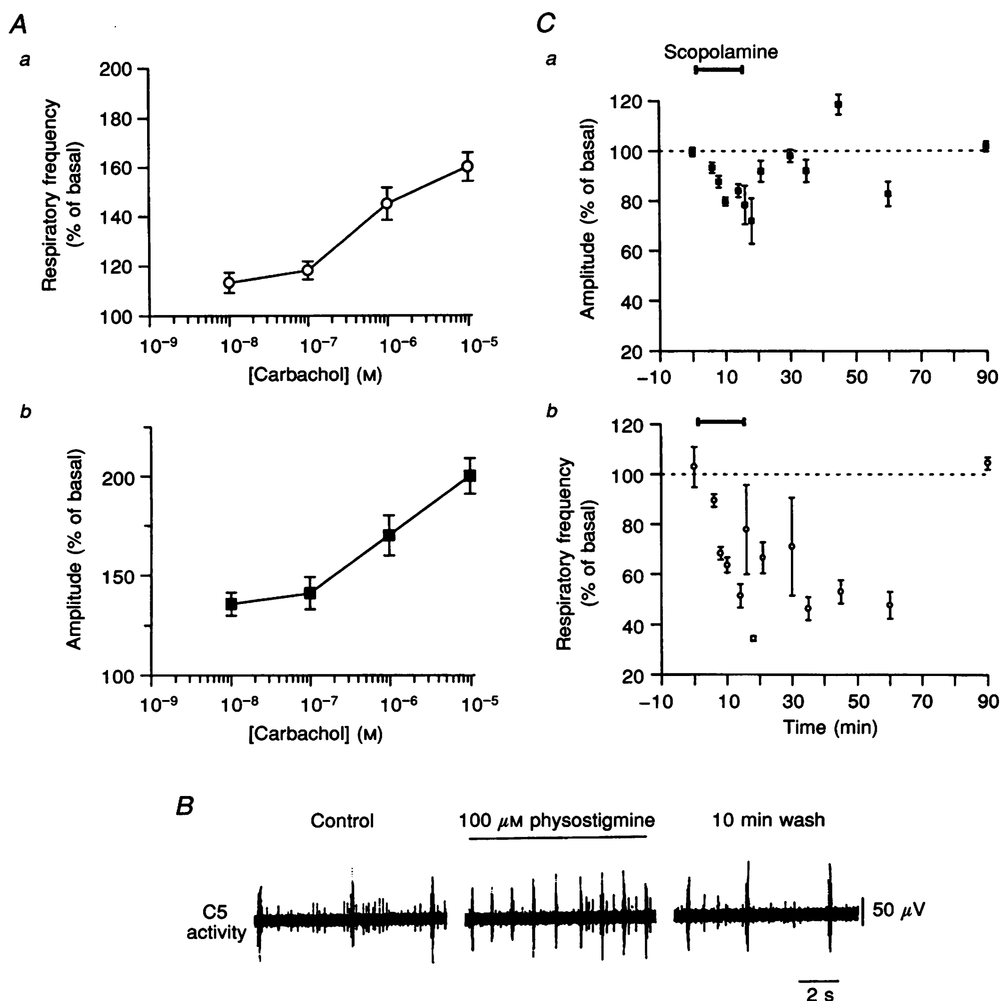

Figure 4. Effects of carbachol (*A*), physostigmine (*B*) and scopolamine (*C*) administered to the brainstem

A, dose–response curve for the increases in instantaneous frequency of respiratory activity (*a*) and amplitude (*b*) induced by increased doses of carbachol in an 8-day-old opossum. *B*, effect of the administration of 100 μM physostigmine on C5 recording in a 7-day-old opossum preparation. *C*, time course of the effects of 50 μM scopolamine upon fictive respiration recorded from C5 in a 9-day-old opossum; amplitude (*a*) and instantaneous respiratory frequency (*b*). Duration of superfusion with scopolamine is indicated by the horizontal bar. Symbols represent means ± S.E.M. expressed as a percentage of basal values during 1–2 min under steady-state conditions.

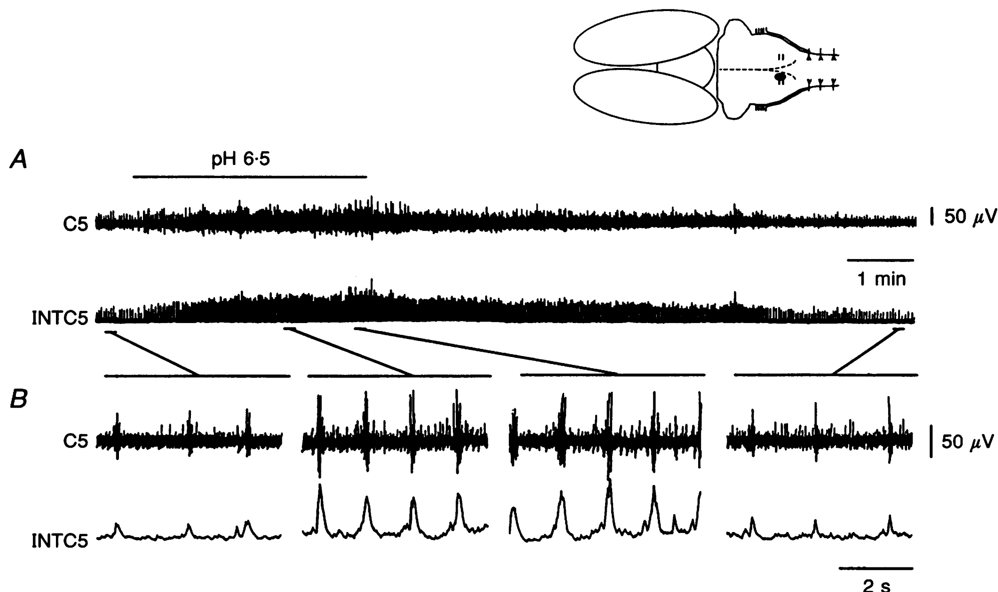

Figure 5. Changes of respiratory activity in response to local application of acidic medium to ventral medulla

A, effect of BMEM (pH 6·5) applied locally to the surface of the ventral medulla medial to the hypoglossal roots (black spot in CNS inset) upon C5 ventral root activity of a 5-day-old opossum *in vitro*. INTC5, integration of C5 activity. *B*, same recording at faster speed. Contact time of the pipette with the surface of the nervous tissue is indicated by the horizontal bar above the trace in *A*.

Local application of pH and carbachol

Ventral and dorsal surfaces of the isolated CNS of the newborn opossum were explored systematically by touching the pia mater with the tip of a glass micropipette (100 μm outer diameter) containing BMEM at pH 6·5 (detailed description in Methods). As illustrated in Figs 5 and 6, topical application of acid confined to discrete sites on the ventral medulla reproduced effects obtained with low pH superfusion. In this experiment the frequency increased by 15–120% ($51{\cdot}9 \pm 10{\cdot}6$%; $P < 0{\cdot}001$, $n = 11$) and the

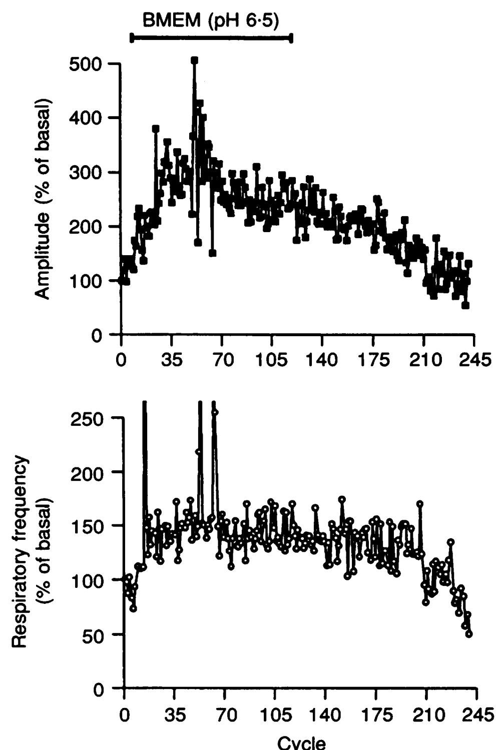

Figure 6. Local application of BMEM (pH 6·5) to ventral medulla at a site medial to the hypoglossal roots in a 5-day-old opossum

A, normalized amplitude of the integrated activity from C6 ventral root (time constant, 100 ms) expressed as a percentage of the mean values from 10 respiratory cycles before pH administration.
B, normalized instantaneous respiratory frequency. Each point represents a cycle; lines join consecutive cycles.

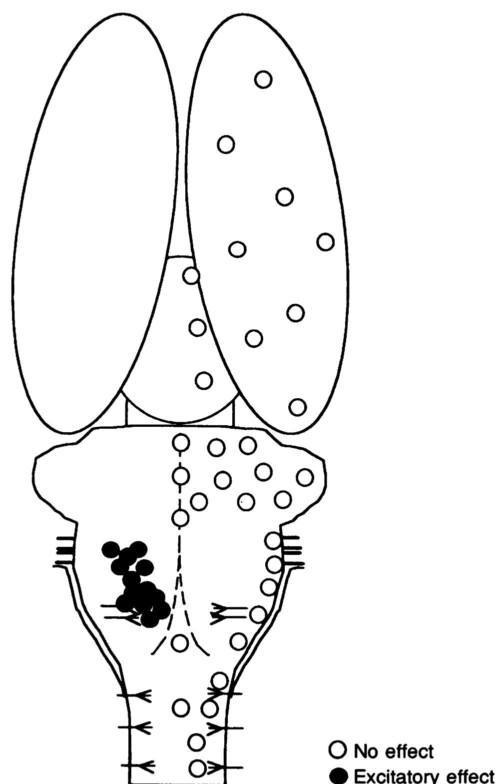

Figure 7. Diagram of pH 6·5-sensitive areas located superficially in 11 opossum neonates

Chemosensitive areas on the surface of the brainstem are confined to the ventral medulla and overlap carbachol-sensitive areas.

amplitude by 20–250% (69·0 ± 18·1%; $P < 0.003$, $n = 11$). The responses appeared 3–5 s after the electrode tip touched the ventral medulla surface, reached a maximum within a minute, and decayed to basal values 4–8 s after withdrawing the pipette. In five animals in which local stimulation failed to evoke any responses, superfusion of the brainstem with BMEM (pH 6·5) produced minimal responses (less than 15%, $n = 5$).

Chemosensitivity was confined to the ventral medulla. A diagram of the topography of the chemosensory areas found in eleven pups is shown in Fig. 7. The most sensitive area

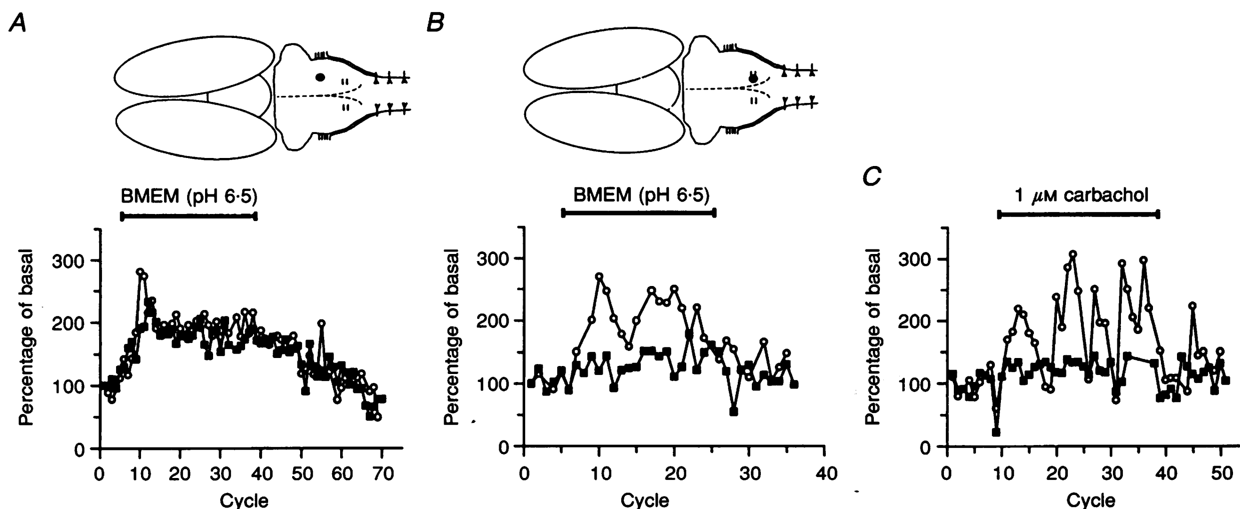

Figure 8. Different respiratory responses to stimuli applied topically to rostral and caudal medulla

Local stimulation with BMEM (pH 6·5) of rostral (*A*) and caudal (*B*) chemosensitive areas (indicated by black spots) of the ventral medulla of a 9-day-old opossum. *C*, 1 μM carbachol was applied topically to the caudal pH-sensitive area in the same opossum; compare pattern of response with *B*. Note selective effects on amplitude and frequency. Normalized amplitude (■) and instantaneous frequency (○) of the integrated activity from C6 (time constant, 100 ms) are expressed as a percentage of the mean value from 10 respiratory cycles before pH or carbachol administration. Each point represents a cycle.

corresponded to a region located medial to the hypoglossal roots.

Carbachol-sensitive areas were explored with a similar procedure. Local application of 1 μM carbachol to the surface of the ventral medulla increased respiratory amplitude by 15–110% (36·4 ± 14·7%; $P < 0{\cdot}048$, $n = 7$) and frequency by 20–150% in five out of seven preparations (49·2 ± 20·1%; $P < 0{\cdot}05$, $n = 7$). These carbachol-sensitive areas overlapped with pH-sensitive areas. The time courses of the responses were similar to those found during local pH stimulation except that effects persisted after removal of the pipette (minutes).

In four preparations, superfusion with 50 μM scopolamine abolished the responses induced by the topical local application of low pH and carbachol. These responses reappeared 90 min after ending scopolamine superfusion. Since atropine failed to influence the respiratory baseline, we did not test its effect upon the responses induced by local application of low pH solution or carbachol.

Patterns of response after local stimulation

To study whether different central chemosensory areas have similar or diverse functions, the rostral and caudal portions of ventral medulla were tested separately with BMEM (pH 6·5) and the pattern of the respiratory responses compared. In two out of five preparations, the topical application of acid to the rostral portion of the ventral medulla increased both frequency and amplitude with the same time courses and relative magnitudes (Fig. 8*A*). In contrast, when topical application of acid was restricted to the caudal portion of the ventral medulla, the increase in frequency was more pronounced than the increase in amplitude (Fig. 8*B*).

The differences in the pattern of response between rostral and caudal portions of ventral medulla were also found after topical application of carbachol 1 μM (Figs 8*C* and 9). However, not all the animals showed the same patterns of response in frequency and amplitude when specific regions of ventral medulla were stimulated (compare Figs 6 and 8). Nevertheless, it was clear that application to a single spot could influence the rhythm and the depth of inspiration separately and to different extents.

Acetylcholinesterase histochemistry

AChE staining was observed in various areas of neonatal opossum CNS. In the ventral medulla around hypoglossal roots, the area is well-defined anatomically and contained a clear boundary for a chemosensitive area. Figure 10 is a transverse section of caudal medulla showing AChE staining of a discrete group of neurons located medial to the hypoglossal roots. This location coincides with the area in which carbachol or low pH solution induced respiratory responses.

DISCUSSION

These experiments confirm that fictive respiration recorded from the isolated CNS of the newborn opossum is pH-dependent as in other preparations (Mitchell *et al.* 1963*a*,*b*; Cozine & Ngai, 1967; Schlaefke & Loeschcke, 1967; Trouth, Loeschcke & Berndt, 1973; Dev & Loeschcke, 1979*a*; Loeschcke, 1982; Harada, Wang & Kuno, 1985; Bruce & Cherniack, 1987). Since all sensory inputs from the periphery were cut, the responses can be attributed to central chemoreceptors. It is reasonable to think that pH sensors in an endothermic animal may operate according to the alphastat hypothesis (Nattie, 1990). This hypothesis

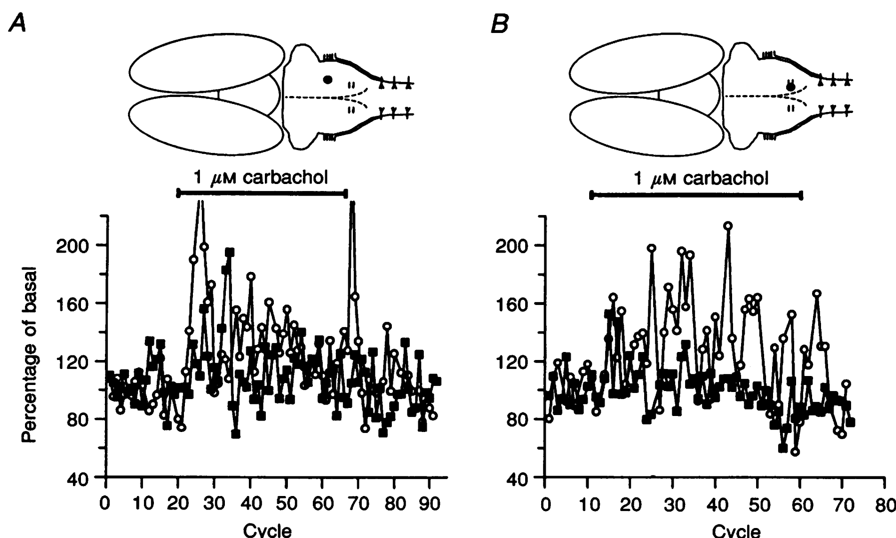

Figure 9. Stimulation of rostral (*A*) and caudal (*B*) chemosensitive areas with 1 μM carbachol also reveals differential effects upon the respiratory pattern of a 6-day-old opossum *in vitro*

Normalized amplitude (■) and instantaneous frequency (○) of the integrated activity from C4 (time constant, 100 ms) are expressed as a percentage of the mean value from 10 respiratory cycles before pH administration. Each point represents a cycle; lines join consecutive cycles.

implies that, at the temperature we did the present experiments, chemoreceptors would actually be sensing a more acidic pH. This should be considered when evaluating pH sensitivity of fictive respiration as well as the pH values over which the respiratory rhythm is abolished (see below).

The exploration by circumscribed, well-defined topical application of low pH solutions revealed a chemosensitive region on the surface of the ventral medulla which extended from zones analogous to the classical area of Mitchell (rostral) to the classical area of Loeschcke (caudal).

Two findings suggest that the chemical stimuli we applied to nervous tissue of opossum neonates were highly localized. First, only stimulation of very precise spots on the ventral medulla caused respiratory responses. Second, even prolonged application of acid (4 min or more) on the dorsal surface of the pons and medulla gave negative results. Our experiments do not rule out the existence of chemosensitive areas deep in the brainstem, which were not explored.

Role of endogenous ACh release

The present results suggest that endogenous release of ACh in opossum CNS exerts a tonic drive upon the respiratory activity by way of muscarinic receptors. As in cats and rats (Mitchell *et al.* 1963*a*; Dev & Loeschcke, 1979*a*, *b*; Monteau *et al.* 1990) ACh agonists produced excitation of respiration when applied to the ventral medulla as did AChE blockers. That this cholinergic pathway may provide a tonic input to

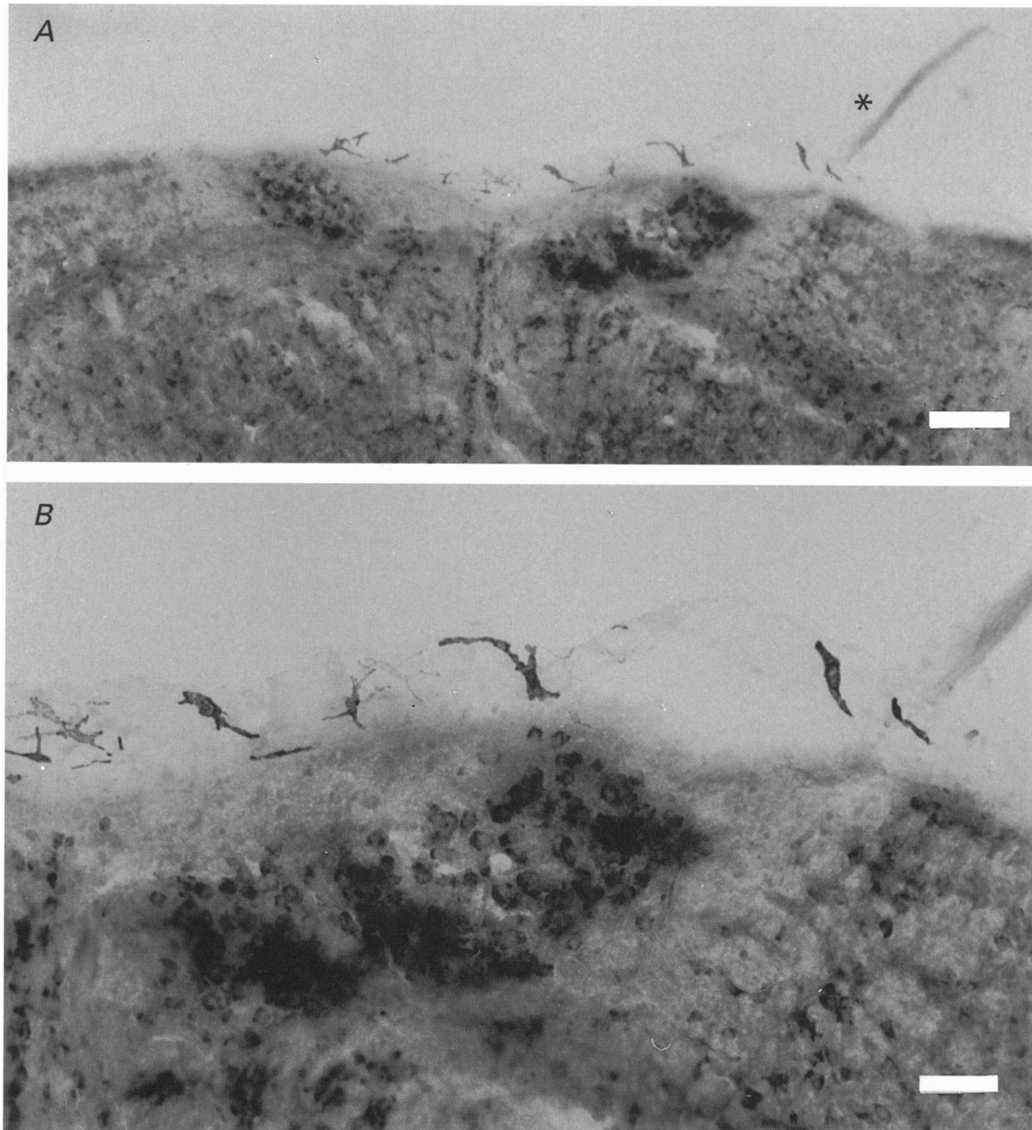

Figure 10. Transverse section of the ventral medulla at the level of left hypoglossal roots in 12-day-old opossum neonate

A, the asterisk indicates hypoglossal roots. Acetylcholinesterase staining revealed a group of intensely labelled neurons located medial to nerve roots about 30–200 μm in depth from the ventral surface. *B*, the area indicated by the asterisk in *A* is shown at a higher magnification. Bars correspond to 125 and 50 μm in *A* and *B*, respectively.

the respiratory pattern generator was suggested by the action of scopolamine, which produced a decrease in amplitude and frequency of fictive respiration.

The failure of atropine to produce an effect on the baseline of respiratory pattern raises the possibility that scopolamine might act through a non-cholinergic CNS-depressant mechanism. Other more specific muscarinic receptor blockers, such as pirenzepine, might be useful in establishing more decisively the mechanism of scopolamine action. In addition, a cholinergic relay may be involved in ventral medulla pH sensitivity since: (1) carbachol- and pH-sensitive areas overlapped; (2) the patterns of respiratory responses to cholinomimetics and pH were very similar; (3) scopolamine abolished respiratory responses to local and topical administration of 1 μM carbachol and BMEM (pH 6·5); and (4) AChE staining was limited to the chemosensitive area medial to the hypoglossal roots, which was defined by functional studies. Alternatively, cholinergic- and pH-sensitive pathways in the medulla could converge upon a common input to the respiratory pattern generator. Cholinergic synapses do not, however, appear to be essential for the genesis of the respiratory rhythm.

Although superfusion with BMEM at pH 7·7 or above abolished the respiratory activity, it is not clear whether chemical stimuli are essential for generating or maintaining the respiratory rhythm in the newborn opossum. Possibly cholinergic and chemosensory pathways partially share the same mechanisms. The opossum preparation is more sensitive than that of neonatal rats in which the rhythm is still maintained at pH 7·9 (Monteau *et al.* 1990).

Regional differences of chemosensory areas

Different chemosensitive areas of the CNS may produce different patterns of respiratory responses. Changes in pH at constant P_{CO_2} produce changes in the frequency of the rhythm recorded from C3–C5 ventral roots of isolated brainstem–spinal cord preparations of neonatal rats, while changes in P_{CO_2} at constant pH mainly affect the amplitude of the integrated activity (Harada *et al.* 1985). Similar results were obtained using the isolated brainstem of adult guinea-pigs *in vitro*. Superfusion of the preparation with hypercapnic Krebs solution increased the inspiratory burst amplitude whether the solution was acidic or non-acidic. Effects on respiratory burst frequency were, however, seen only with acidic solutions. Thus, normocapnic acidic Krebs solution increased the respiratory frequency with no change in burst amplitude (Morin-Surun, Boudinot, Schäfer & Denavit-Saubié, 1995). These data suggest the existence of two mechanisms of central chemosensitivity acting selectively upon the respiratory pattern generator structures responsible for the depth and timing of the rhythm.

In anaesthetized, paralysed and mechanically ventilated cats, local cooling of chemosensory areas of the ventral medulla produces diverse and selective effects upon the respiratory pattern. Bilateral cooling of the intermediate area abolishes the respiratory phrenic activity. Cooling of rostral areas decreases the respiratory frequency and reduces the amplitude of respiratory phrenic activity, while cooling of caudal areas decreases the amplitude and increases the respiratory frequency (Cherniack, von Euler, Homma & Kao, 1979).

In the present experiments, low pH topically applied to the caudal ventral medulla (medial to the hypoglossal roots) predominantly increased frequency, whereas rostral application (at the level of vagus and glossopharingeal roots) increased both frequency and amplitude. However, the respiratory pattern responses evoked from a defined anatomical location showed variability. We have not been able to define a precise correlation of specific ventral medullary areas to specific functional patterns. Nonetheless, two facts indicate that these selective topographic responses were not artefacts: (1) the pattern of response was highly reproducible – it was attained each time low pH solution was applied to the same position on the ventral medulla in the same animal; and (2) the pattern of respiratory responses elicited from a given spot on the surface of the ventral medulla was similar whether the stimulus was low pH solution or carbachol.

Pattern of respiratory response

The pattern of respiratory response of neonatal opossum *in vitro* to modification of H^+ concentration of the medium involved changes in both amplitude and frequency. This pattern in neonatal opossum is similar to the increase in tidal volume and respiratory frequency reported in adult animals *in vivo* (Mitchell *et al.* 1963*a*,*b*; Clark & von Euler, 1972). In contrast, different patterns of responses to chemical stimulation have been obtained in the neonatal rat brainstem–spinal cord preparation, which can show changes in frequency (Suzue, 1984; Issa & Remmers, 1992; Okada, Mückenhoff & Scheid, 1993), amplitude (Monteau *et al.* 1990) or both (Harada *et al.* 1985).

In neonatal rats, ACh agonists administered to the brainstem *in vitro* modified only the frequency of respiration, and ACh blockers had no clear-cut effect (Monteau *et al.* 1990). It is worth noting that the frequency of the bursts recorded by Monteau *et al.* (1990) from ventral roots (3–5 min^{-1}) is far lower than observed in intact neonatal rats. In addition, there is evidence that multiple pattern generators (e.g. for vomiting and gasping) coexist in the mammalian medulla with the generator of eupnoeic breathing (St John, 1990; Zhou, Wasicko, Hu & St John, 1991). In anoxia, the pattern of phrenic activity can change from eupnoea to gasping (Lumsden, 1924; Wang *et al.* 1996). This has led to the suggestion that the low frequency rhythm recorded from the rat preparation *in vitro* really corresponds to a gasp pattern generator (St John, 1990; Fung, Wang & St John, 1994; Wang *et al.* 1996).

Even though recordings were made under similar experimental conditions (room temperature, peripheral denervation etc.) the neonatal opossum preparation maintains a far higher rate of fictive respiration than that of neonatal rat

CNS *in vitro*. The rate at 40 min^{-1} is close to the respiratory rate of about 60 min^{-1} observed in intact pups at a higher body temperature of 29 °C. It should be mentioned that differences in breathing rate between neonatal opossum and neonatal rat *in vitro* preparations may be due in part to different dependence on vagal influences. Vagotomy prolongs inspiratory duration by a factor of two and causes a sixfold increase in expiratory duration in newborn rats (Fedorko, Kelly & England, 1988). By contrast, vagotomy in young North American opossums produces little or no change in respiratory pattern. With respect to survival, growth and development the isolated CNS of the opossum is remarkable when compared with neonatal rat preparations for which only hours of survival have been reported (Suzue, 1984). Moreover, the isolated CNS of neonate opossums can be cultured successfully for more than a week, maintaining electrical excitability, nerve conduction properties, reflexes and responses to amino acid transmitters such as GABA, glutamate and glycine (Nicholls *et al.* 1990; Stewart *et al.* 1991; Zou *et al.* 1991; Treherne *et al.* 1992). This preparation even shows some development of the spinal cord *in vitro*. Blast cells continue to divide and the histological fine structure is normal after 5 days in culture (Stewart *et al.* 1991; Møllgard, Balslev, Stagaard-Janas, Treherne, Saunders & Nicholls, 1994). Furthermore, the viability of the nervous tissue is so well conserved that spinal cord fibres are able to grow through a lesion and rapidly repair the traumatic area (Saunders *et al.* 1992; Treherne *et al.* 1992; Woodward, Treherne, Knott, Fernandez, Varga & Nicholls, 1993; Varga *et al.* 1996). Hence, the general state of the preparation *in vitro* is close in many respects to that of the CNS in the animal.

Conclusions

These results demonstrate that fictive respiration of the isolated CNS of the newborn opossum is tonically driven by inputs from pH- and ACh-sensitive areas located superficially on the ventral medulla. A cholinergic relay acts synergically with the central pH chemosensory mechanisms controlling the respiratory pattern generator. These chemosensitive areas control amplitude and frequency of the respiratory rhythm. One can hope that detailed anatomical and electrophysiological analysis, including intracellular recording in the neonatal opossum, will prove useful for understanding how respiratory rhythmicity is generated and regulated.

BRUCE, E. N. & CHERNIACK, N. S. (1987). Central chemoreceptors. *Journal of Applied Physiology* **62**, 389–402.

CHERNIACK, N. S., VON EULER, C., HOMMA, I. & KAO, F. F. (1979). Graded changes in central chemoreceptor input by local temperature changes on the ventral surface of the medulla. *Journal of Physiology* **287**, 191–211.

CLARK, F. J. & VON EULER, C. (1972). On the regulation of depth and rate of breathing. *Journal of Physiology* **222**, 267–295.

COATES, E. L., LI, A. & NATTIE, E. E. (1993). Widespread sites of brain stem ventilatory chemoreceptors. *Journal of Applied Physiology* **75**, 5–14.

COZINE, R. A. & NGAI, S. H. (1967). Medullary surface chemoreceptors and regulation of respiration in the cat. *Journal of Applied Physiology* **22**, 117–121.

DEV, N. B. & LOESCHCKE, H. H. (1979*b*). Topography of the respiratory and circulatory responses to ACh and nicotine on the ventral surface of the medulla oblongata. *Pflügers Archiv* **379**, 19–27.

DEV, N. B. & LOESCHCKE, H. H. (1979*a*). A cholinergic mechanism involved in the respiratory chemosensitivity. *Pflügers Archiv* **379**, 29–36.

DI PATRE, P. L., MATHES, C. W. & BUTCHER, L. L. (1993). Differential visualization of cholinesterasic neuronal somata and fibers by use of modifications of acetylcholinesterase pharmacohistochemistry. *Journal of Histochemistry and Cytochemistry* **41**, 129–135.

EUGENÍN, J. (1994). pH sensitivity and cholinergic modulation of fictive respiration of the newborn opossum (*Monodelphis domestica*) *in vitro*. *Society for Neuroscience Abstracts* **20**, 544.

EUGENÍN, J. (1995). Localization of areas of respiratory chemosensory drive in the isolated CNS of neonate opossum (*Monodelphis domestica*). *XXXVIII Annual Meeting of Society of Biology of Chile* **3**, 139.

EUGENÍN, J. (1996). Central pH chemosensitivity in the newborn opossum *Monodelphis domestica*. *XIII International Symposium on Arterial Chemoreceptors*, p. 23.

FEDORKO, L., KELLY, E. N. & ENGLAND, S. J. (1979). Importance of vagal afferents in determining ventilation in newborn rats. *Journal of Applied Physiology* **65**, 1033–1039.

FUKUDA, Y. & LOESCHCKE, H. H. (1979). A cholinergic mechanism involved in the neuronal excitation by H^+ in the respiratory chemosensitive structures on the ventral medulla oblongata of rats *in vitro*. *Pflügers Archiv* **379**, 125–135.

FUNG, M. L., WANG, W. & ST JOHN, W. M. (1994). Medullary loci critical for expression of gasping in adult rats. *Journal of Physiology* **480**, 597–611.

GREER, J. J., CARTER, J. E. & ALLAN, D. W. (1996). Respiratory rhythm generation in a precocial rodent *in vitro* preparation. *Respiration Physiology* **103**, 105–112.

HARADA, Y., KUNO, M. & WANG, Y. Z. (1985). Differential effects of carbon dioxide and pH on central chemoreceptors in the rat *in vitro*. *Journal of Physiology* **368**, 679–693.

HARADA, Y., WANG, Y. Z. & KUNO, M. (1985). Central chemosensitivity to H^+ and CO_2 in the rat respiratory center *in vitro*. *Brain Research* **333**, 336–339.

ISSA, F. G. & REMMERS, J. E. (1992). Identification of a subsurface area in the ventral medulla sensitive to local changes in P_{CO_2}. *Journal of Applied Physiology* **72**, 439–446.

LOESCHCKE, H. H. (1982). Central chemosensitivity and the reaction theory. *Journal of Physiology* **332**, 1–24.

LUMSDEN, T. (1924). Effects of bulbar anaemia on respiratory movements. *Journal of Physiology* **59**, lvii–lx.

MITCHELL, R. A., LOESCHCKE, H. H., MASSION, W. H. & SEVERINGHAUS, J. W. (1963*a*). Respiratory responses mediated through superficial chemosensitive areas on the medulla. *Journal of Applied Physiology* **18**, 523–533.

MITCHELL, R. A., LOESCHCKE, H. H., SEVERINGHAUS, J. W., RICHARDSON, B. W. & MASSION, W. H. (1963*b*). Regions of respiratory chemosensitivity on the surface of the medulla. *Annals of the New York Academy of Sciences* **109**, 661–681.

Møllgard, K., Balslev, Y., Stagaard-Janas, M., Treherne, J. M., Saunders, N. R. & Nicholls, J. G. (1994). Development of spinal cord in the isolated CNS of a neonatal mammal (the opossum *Monodelphis domestica*) maintained in longterm culture. *Journal of Neurocytology* **23**, 151–165.

Monteau, R., Morin, D. & Hilaire, G. (1990). Acetylcholine and central chemosensitivity: *in vitro* study in the newborn rat. *Respiration Physiology* **81**, 241–254.

Morin-Surun, M. P., Boudinot, E., Schäfer, T. & Denavit-Saubié, M. (1995). Localization of chemosensitive structures in the isolated brainstem of adult guinea-pig. *Journal of Physiology* **485**, 203–212.

Nattie, E. E. (1990). The alphastat hypothesis in respiratory control and acid–base balance. *Journal of Applied Physiology* **69**, 1201–1207.

Nicholls, J. G., Stewart, R. R., Erulkar, S. D. & Saunders, N. R. (1990). Reflexes, fictive respiration and cell division in the brain and spinal cord of the newborn opossum, *Monodelphis domestica*, isolated and maintained *in vitro*. *Journal of Experimental Biology* **152**, 1–15.

Okada, Y., Mückenhoff, K. & Scheid, P. (1993). Hypercapnia and medullary neurons in the isolated brain stem spinal cord of the rat. *Respiration Physiology* **93**, 327–336.

Onimaru, H. & Homma, I. (1987). Respiratory rhythm generator neurons in medulla of brainstem spinal cord preparation from newborn rat. *Brain Research* **403**, 380–384.

St John, W. M. (1990). Neurogenesis, control, and functional significance of gasping. *Journal of Applied Physiology* **68**, 1305–1315.

Saunders, N. R., Balkwill, P., Knott, G., Habgood, M. D., Møllgard, K., Treherne, J. M. & Nicholls, J. G. (1992). Growth of axons through a lesion in the intact CNS of fetal rat maintained in long term culture. *Proceedings of the Royal Society* B **250**, 171–180.

Schlaefke, M. E. & Loeschcke, H. H. (1967). Lokalisation eines and der Regulation von Atmung und Kreislauf beteiligten Gebietes and der ventralen Oberfläche der Medulla oblongata durch Kalteblockade. *Pflügers Archiv* **297**, 201–220.

Smith, J. C., Greer, J. J., Liu, G. & Feldman, J. L. (1990). Neural mechanisms generating respiratory pattern in mammalian brain stem spinal cord *in vitro* I. Spatiotemporal patterns of motor and medullary neuron activity. *Journal of Neurophysiology* **64**, 1149–1169.

Stewart, R. R., Zou, D. J., Treherne, J. M., Møllgard, K., Saunders, N. R. & Nicholls, J. G. (1991). The intact central nervous system of the newborn opossum in long term culture: fine structure and GABA mediated inhibition of electrical activity. *Journal of Experimental Biology* **161**, 25–41.

Suzue, T. (1984). Respiratory rhythm generation in the *in vitro* brain stem spinal cord preparation of the neonatal rat. *Journal of Physiology* **354**, 173–183.

Tago, H., Kimura, H. & Maeda, T. (1986). Visualization of detailed acetylcholinesterase fiber and neuron staining in rat brain by a sensitive histochemical procedure. *Journal of Histochemistry and Cytochemistry* **34**, 1431–1438.

Treherne, J. M., Woodward, S. K., Varga, Z. M., Ritchie, J. M. & Nicholls, J. G. (1992). Restoration of conduction and growth of axons through injured spinal cord of neonatal opossum in culture. *Proceedings of the National Academy of Sciences of the USA* **89**, 431–434.

Trouth, C. O., Loeschcke, H. H. & Berndt, J. (1973). A superficial substrate on the ventral surface of the medulla oblongata influencing respiration. *Pflügers Archiv* **339**, 135–152.

Varga, Z. M., Fernandez, J., Blackshaw, S., Martin, A., Muller, K. J., Adams, W. B. & Nicholls, J. G. (1996). Neurite outgrowth through lesions of neonatal opossum spinal cord in culture. *Journal of Comparative Neurology* **366**, 600–612.

Wang, W., Fung, M. L., Darnall, R. A. & St John, W. M. (1996). Characterizations and comparisons of eupnoea and gasping in neonatal rats. *Journal of Physiology* **490**, 277–292.

Woodward, S. K., Treherne, J. M., Knott, G. W., Fernandez, J., Varga, Z. M. & Nicholls, J. G. (1993). Development of connections by axons growing through injured spinal cord of neonatal opossum in culture. *Journal of Experimental Biology* **176**, 77–88.

Zhou, D., Wasicko, M. J., Hu, J. M. & St John, W. M. (1991). Differing activities of medullary respiratory neurons in eupnea and gasping. *Journal of Applied Physiology* **70**, 1265–1270.

Zou, D. J. (1994). Respiratory rhythm in the isolated central nervous system of newborn opossum. *Journal of Experimental Biology* **197**, 201–213.

Zou, D. J., Treherne, J. M., Stewart, R. R., Saunders, N. R. & Nicholls, J. G. (1991). Regulation of $GABA_B$ receptors by histamine and neuronal activity in the isolated spinal cord of neonatal opossum in culture. *Proceedings of the Royal Society* B **246**, 77–82.

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