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PULMONARY STRETCH RECEPTOR AFFERENTS ACTIVATE EXCITATORY AMINO ACID RECEPTORS IN THE NUCLEUS TRACTUS SOLITARII IN RATS

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SUMMARY

1. The goal of the present study was to identify potential neurotransmitter candidates in the Breuer–Hering (BH) reflex pathway, specifically at synapses between the primary afferents and probable second-order neurones (pump cells) within the nucleus tractus solitarii (NTS). We hypothesized that if activation of specific receptors in the NTS is required for production of the BH reflex, then (1) injection of the receptor agonist(s) would mimic the reflex response (apnoea), (2) injection of appropriate antagonists would impair the apnoea produced by either lung inflation or agonist injection, and (3) second-order neurones in the pathway would be excited by either lung inflation or agonists while antagonists would prevent the response to either.

2. Studies were carried out either in spontaneously breathing or in paralysed, thoracotomized and ventilated rats in which either diaphragm EMG or phrenic nerve activity, expired CO_2 concentration and arterial pressure were continuously monitored. The BH reflex was physiologically activated by inflating the lungs.

3. Pressure injections (0·03–15 pmol) of selective excitatory amino acid (EAA) receptor agonists, quisqualic acid (Quis) and *N*-methyl-D-aspartic acid (NMDA) into an area of the NTS shown previously to contain neurones required for production of the BH reflex produced dose-dependent apnoeas that mimicked the response to lung inflation. Injection of substance P (0·03–4 pmol) did not alter baseline respiratory pattern.

4. Injections of the EAA antagonists, kynurenic acid (Kyn; 0·6–240 pmol), 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) or 6,7-dinitroquinoxaline-2,3-dione (DNQX) into the BH region of the NTS reversibly impaired the apnoea produced by lung inflation. All three antagonists reduced or abolished the apnoeas resulting from injection of Quis or NMDA, and slowed baseline respiratory frequency. In contrast, injections of the highly selective NMDA receptor antagonist, D-2-amino-5-phosphonovaleric acids (AP5), in doses sufficient to block the apnoeic response to

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NMDA, neither altered the reflex apnoea evoked by lung inflation nor the baseline respiratory pattern.

5. Pump cells located within the BH region were excited by pressure injections of the broad spectrum EAA agonist, DL-homocysteic acid (DLH). Kyn reversibly blocked the excitation of pump cells in response to either lung inflation or DLH injection.

6. These findings suggest that EAAs mediate primary afferent excitation of second-order neurones in the Breuer–Hering reflex pathway, primarily through the activation of non-NMDA EAA receptor subtypes.

INTRODUCTION

Slowly adapting pulmonary stretch receptor afferents (SARs) are activated by lung inflation and transmit information about changes in lung volume to brainstem neurones controlling inspiratory and expiratory durations (Paintal, 1973; Bartoli, Cross, Guz, Huszuk & Jeffries, 1975). Lung inflation delivered during inspiration prematurely terminates inspiratory discharge (Cross, Jones & Guz, 1980; Cohen & Feldman, 1984); lung inflation during expiration prolongs the expiratory period and slows breathing (Clark & von Euler, 1972; Cross *et al.* 1980; Mitchell, Cross, Hiramoto & Scheid, 1980; Grippi, Pack, Davies & Fishman, 1985). The inspiratory-shortening and expiratory-lengthening responses are collectively referred to as the Breuer–Hering (BH) reflex.

While the BH reflex effects on respiratory timing are well characterized (Widdicombe, 1961), the central neural circuitry and the neurotransmitters mediating the reflex changes in respiratory motor output are only beginning to be understood. SARs give rise to myelinated vagal afferent fibres which course within the vagus nerve and monosynaptically activate pump cells as well as some inspiratory ($I\beta$) neurones within the NTS in cats (Backman *et al.* 1984; Berger & Dick, 1987). Pump cells do not receive a central respiratory drive, but synaptic inputs from SARs cause pump cells to discharge in phase with lung inflation (Berger & Dick, 1987). Davies, Kubin & Pack (1987) localized pump cells to regions immediately ventromedial and dorsolateral to the tractus solitarius in the cat. Pump cells in the dorsolateral group gave rise to axonal projections to the contralateral NTS but projections for neurones in the ventromedial group were not identified.

We recently found evidence that a group of pump cells within the NTS constitute part of the BH reflex circuitry in rats. These cells were concentrated in a region immediately medial to the NTS, and were co-extensive with the area postrema in the rostrocaudal direction. Within this region, (1) injection of picomole amounts of the excitatory amino acid (EAA), DL-homocysteic acid (DLH), produced a site-dependent inhibition of respiratory motor output that mimicked the BH reflex, and (2) transient interruption of synaptic transmission reversibly reduced the BH reflex and slowed respiratory frequency (Bonham & McCrimmon, 1990). This area will be referred to as the BH region.

EAAs are believed to mediate much of the excitatory neurotransmission within the central nervous system by activation of at least three ionotropic receptor subtypes. These receptors are identified by their selective agonists, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainic acid (KA), and *N*-methyl-D-

aspartic acid (NMDA); Monaghan, Bridges & Cotman, 1989). Glutamate, which activates all of these receptor subtypes, may be a transmitter for some vagal afferent fibres. Glutamate is released from vagal afferent fibres (Dietrich, Lowry & Loewy, 1982; Granata, Sved & Reis, 1984) and has been proposed as the neurotransmitter for baroreceptor afferent fibres (Talman, Perrone & Reis, 1980; Guyenet, Filtz & Donaldson, 1987). Injection of glutamate into the NTS mimics the reflex hypotension, bradycardia and apnoea elicited by baroreceptor activation (Talman *et al.* 1980), while injection of the broad spectrum EAA receptor antagonist, kynurenic acid (Kyn), or the selective NMDA-receptor antagonist, 2-amino-5-phosphonovaleric acid (AP5), blocks the arterial baroreflex (Guyenet *et al.* 1987; Kubo & Kihara, 1988). Potential roles for both NMDA and non-NMDA EAA receptors in mediating primary afferent input to NTS neurones is further supported by demonstrations that AP5 and the selective non-NMDA receptor antagonist, 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX), suppress EPSPs elicited in NTS neurones by stimulation of afferent fibres within the tractus solitarius of *in vitro* rat brain stem slices (Miller & Felder, 1988; Andresen & Yang, 1990). However, NMDA-receptor activation does not appear to be required for vagal afferent-mediated termination of inspiration. In vagotomized rats and cats, NMDA receptor blockade by the systemic administration of antagonists which cross the blood–brain barrier produces an apneustic breathing pattern but, at least in cats, does not prevent the vagal afferent-mediated termination of inspiration (Foutz, Champagnat & Denavit-Saubié, 1989; Monteau, Gauthier, Rega & Hilaire, 1990; Feldman, Windhorst, Anders & Richter, 1992). Thus, activation of vagal afferents appears to activate a heterogeneous group of EAA receptors in the NTS but only non-NMDA EAA receptors are likely candidates for mediation of the BH reflex termination of inspiration.

Substance P has also been localized within vagal afferent fibres and has been proposed as a candidate transmitter of chemo- and baroreceptor afferent fibres (Gillis, Helke, Hamilton, Norman & Jacobowitz, 1980; Helke, O'Donohue & Jacobowitz, 1980); Gallagher, Paxinos & White, 1985; Morilak, Morris & Chalmers, 1988). Consistent with this possibility, substance P excites single NTS neurones in cats (Morin-Surun, Jordan, Champagnat, Spyer & Denavit-Saubié, 1984). The finding that injection of substance P into the NTS causes a reduction in respiratory rate in rats is consistent with a role in the BH reflex (Carter & Lightman, 1985).

The aim of the present study was to identify neurotransmitters and associated receptors at the synapses between primary afferent fibres and second-order neurones in the BH reflex. We hypothesized that if activation of specific receptors in the NTS is required for production of the BH reflex, then: (1) injection of the receptor agonist(s) would mimic the reflex response (apnoea), (2) injection of the appropriate antagonist(s) would attenuate the apnoea produced by either lung inflation or local injection of agonist into the NTS, and (3) excitation of pump cells both by selective agonists and by physiological activation of SAR would be blocked by the same antagonists that prevented the reflex apnoea.

METHODS

General animal preparation

Experiments were performed on fifty-two male Sprague–Dawley rats (250–350 g) anaesthetized with urethane (1500 mg/kg I.P.) and given supplemental doses (50–100 mg I.V.) as needed.

Adequacy of anaesthesia was assessed at frequent intervals by the absence of a withdrawal reflex (in non-paralysed animals) and changes in blood pressure, heart rate or respiration rate in response to a noxious paw pinch. For experiments in which single unit recording was employed, rats were paralysed with gallamine triethiodide (15 mg/kg I.V., with supplemental doses of 10 mg/kg as required, $n = 5$), thoracotomized and ventilated with 100% O_2 or room air at an end-expiratory pressure of 2–2·5 cmH_2O. Phrenic nerve discharge or diaphragm electromyogram (EMG), the concentration of expired CO_2 and arterial pressure were monitored continuously in paralysed rats; expired CO_2 was maintained at 4–4·5% by adjusting the ventilator rate or volume. Because of the small tidal volume of the rat and low sampling rate of the CO_2 monitor the expired CO_2 underestimates alveolar CO_2 concentration by about 0·5% compared to arterial blood samples (F. Hayashi, S. K. Coles & D. R. McCrimmon, unpublished observations). In all experiments, baseline mean arterial pressure remained above 80 mmHg.

The diaphragm was exposed through an abdominal incision and two Teflon-coated silver wires (o.d. 0·11 mm), with ends bared for 2 mm, were inserted. For experiments in which phrenic nerve discharge was monitored, the fifth cervical (C5) branch of a phrenic nerve was isolated in the neck via a dorsolateral approach and cut distally. Its central end was placed on a bipolar silver hook electrode and covered with warmed mineral oil. The phrenic nerve discharge and diaphragm EMG signals were amplified and sent in parallel to an oscilloscope, Paynter filter for integration (time constant 15 ms), polygraph, and tape-recorder. Catheters were introduced into the femoral artery and vein for measurement of arterial pressure and administration of drugs, respectively. Body temperature was maintained at 38 ± 1 °C with a servo-controlled water blanket and heat lamp. The rats were placed in a stereotaxic frame, with the head ventroflexed such that the dorsal brainstem was horizontal, and an occipital craniotomy performed. The caudal portion of the fourth ventricle was exposed by removing the dura and arachnoid membranes.

Injections into the nucleus of the tractus solitarius

Using a pressure injection system previously described (McCrimmon, Feldman & Speck, 1986), we injected small volumes (0·5–18 nl) of chemicals into the NTS. Injections were made through a multibarrel glass pipette array; each barrel was connected by polyethylene tubing to a regulated, solenoid valve-controlled pressure source. The volume of each injectate was directly measured during every injection by monitoring the movement of the fluid meniscus in the pipette barrel using a compound microscope with an eyepiece reticule. Using a dissecting microscope we positioned the tip of the pipette on the midline dorsal surface of the brainstem at the level of calamus scriptorius; this point, approximately 0·7 mm caudal to the obex, defined a relative zero for subsequent rostrocaudal and lateral displacements of the pipette.

Unilateral injections were centred in the previously described BH region of the NTS (Bonham & McCrimmon, 1990). This region extended approximately from 0·1 to 0·6 mm rostral to calamus scriptorius, from 0·5 to 0·9 mm lateral to midline, and from 0·5 to 0·9 mm ventral to the dorsal surface. To define the optimal electrode position within this region, the effect on phrenic nerve activity was determined in response to injections of an excitatory amino acid. A multibarrel pipette containing agonists and antagonists was stereotaxically positioned on the surface of the brainstem 0·5 mm rostral to calamus and 0·7 mm lateral to the midline. This position was rostral to the geometric centre of the BH region but was a location from which maximal effects on phrenic nerve activity could be elicited with minimal changes in arterial pressure (Bonham & McCrimmon, 1990). The pipette was advanced through the brainstem in 50–100 μm steps and 0·5–3 nl DLH or Quis injected at each step. The pipette was considered to be appropriately located within the BH region when the EAA injection elicited an apnoea lasting at least 3 s. If an adequate response was not obtained on the first penetration, new penetrations were made after moving the electrode 0·1 mm in the horizontal plane. No more than four penetrations were made in a single animal.

Physiological activation of the Breuer–Hering reflex

To physiologically activate the BH reflex, a T-tube was inserted into the trachea and oxygen was delivered at a constant flow of 1–2 l/min. By submerging the expiratory outlet at specified depths (5–12·5 cmH_2O) in a column of water, lung inflation was maintained at a series of continuous positive airway pressures (CPAP). The BH reflex was defined as the shortening of inspiratory time (T_I) and lengthening of expiratory time (T_E). The duration of the BH reflex apnoea was expressed as the ratio of the duration of the maximum T_E during lung inflation ($T_{E,max}$) to the duration of

the T_E preceding lung inflation ($T_{E,\,initial}$; Widdicombe, 1961). T_E for each control period was averaged over twelve cycles.

Protocol for agonist injections

Agonists for endogenous neurotransmitters were screened for their ability to depress respiratory motor output in a concentration-dependent fashion. Since glutamate had been implicated as the neurotransmitter for other vagal afferent fibres (Talman *et al.* 1980; Guyenet *et al.* 1987), initial studies focused on agonists for the three major EAA receptor subtypes: the AMPA receptor agonist quisqualate (Quis), KA and NMDA. The selectivity of EAA responses was determined by comparison with response to the injection into the BH region of substance P.

Equal volumes of several different concentrations of each agent were pressure injected into the BH region of the NTS to determine (1) the threshold dose for lengthening T_E and (2) the dose just sufficient to produce apnoea, which we defined as an increase in T_E to at least 3 s. Typically three or four doses of each agonist were tested per animal by injecting 3 nl volumes of different concentrations from separate barrels of a four-barrel pipette. In some experiments, the duration of the apnoeas produced by equivolume injections of each of the different agonists was compared. The use of different concentrations served to establish that the response was concentration dependent rather than a result of pressure or volume artifacts. In addition, injection of up to 24 nl of 150 mM NaCl (pH 7·2–7·4, $n = 6$) had no effect on baseline phrenic nerve discharge.

Protocol for antagonist injections

Additional studies were carried out to determine whether pressure injection of EAA receptor antagonists would attenuate the physiologically evoked BH reflex and alter the spontaneous pattern of breathing in a manner consistent with interruption of SAR input. The antagonists studied were the broad spectrum EAA receptor blocker, kynurenic acid (Kyn), the selective NMDA receptor blocker, 2-amino-5-phosphonovaleric acid (AP5), and two selective AMPA/KA receptor blocking agents, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; Monaghan *et al.* 1989).

To optimize the ability of small localized injections of antagonist to impair the BH reflex, in initial studies the cervical vagus nerve contralateral to the NTS injection was cut. In these experiments, the BH reflex was activated by maintaining lung inflation at CPAP of 5, 7·5, 10, 12·5 and 15 cmH_2O. In later studies, both vagi were left intact; in these rats, SARs were activated at the lower CPAP of 2·5, 5 and 10 cmH_2O.

At least two injections of each agonist concentration were made to establish the reproducibility and duration of the control response. Lung inflations were performed to establish the baseline diaphragm EMG response to SAR activation. An EAA antagonist was then pressure injected from the pipette and the response to lung inflations and agonist injections was determined in the presence of the antagonist. The protocol was repeated at 10–30 min intervals until recovery from the antagonist was attained.

Extracellular recording and drug application to single neurones in the nucleus tractus solitarii

In the final series of experiments, we investigated the effect of pressure ejection of analogues of the candidate EAA transmitter on the firing of extracellularly recorded single units (pump cells) within the BH region of the NTS. To be designated as a pump cell a neurone had to discharge in phase with lung inflation. Since the central respiratory pattern was usually entrained to the ventilator, these pump cells usually began to discharge towards the end of a phrenic nerve burst and continued into the silent period between bursts (e.g. Fig. 9). Additionally, the neurone must either cease discharging when the ventilator was turned off at end-expiration for a period comparable to at least three consecutive respiratory cycles, or it must exhibit sustained activity when the ventilator was turned off at end-inspiration for a comparable period. The fact that a neurone increased its discharge rate in response to application of an excitatory amino acid was taken as evidence that the recording arose from the somato-dendritic membrane of a neurone rather than an axon.

Because the use of standard multibarrel pipette arrays greatly increased the difficulty in isolating single pump cells within the NTS, compound electrodes were used. These were similar to electrodes described by Stone (1985) and consisted of a single-barrel recording pipette (tip o.d. $\leqslant 1\ \mu m$) glued to a double-barrel pipette (combined tip o.d. $\leqslant 5\ \mu m$). The tip of the recording barrel protruded 20–50 μm beyond that of the double-barrel assembly. The recording barrel was filled

with 3 M NaCl while one of the double barrels was filled with Kyn (10 mM in 150 mM NaCl, pH 7·4) and the other with DLH (1 mM in 150 mM NaCl, pH 7·4). Neural signals were fed through a high-impedance source follower to a second-stage amplifier, filtered (0·1–10 kHz), and fed in parallel to an oscilloscope, chart recorder, audio-monitor and tape-recorder. Despite the improvement in recording quality afforded by this electrode assembly, it was still very difficult to obtain stable recordings from well-isolated single pump cells. The difficulty presumably arose from the small size of these neurones since other NTS neurones could be routinely isolated. Typical pump cell recordings consisted of dual or multiunit activity from which single units could usually be isolated using a time–amplitude window discriminator (BAK instruments).

Pharmacological agents injected into the NTS included quisqualic acid (10–500 μM; Tocris Neuramin, Bristol, UK), D-2-amino-5-phosphonovaleric acid (Sigma, St Louis, MO, USA), kainic acid (5–500 μM; Sigma), *N*-methyl-D-aspartic acid (50–5000 μM, Sigma), kynurenic acid (3–10 mM; Sigma), DL-homocysteic acid (1 mM, Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20–100 μM; Tocris Neuramin), 6,7-dinitroquinoxaline-2,3-dione (DNQX 50–100 μM; Tocris Neuramin), glycine (20 μM; Sigma) and substance P (10–200 μM, Sigma). All pharmacological agents were dissolved in 150 mM NaCl and the pH adjusted to 7·2–7·4.

Data analysis

T_I, T_E, and the duration of apnoea produced by maintained lung inflation or by injection of agonists were compared before, during and after injection of antagonists by using analysis of variance (ANOVA) for repeated measures followed by Scheffe's F test, when appropriate. The slopes of the lines relating the apnoea duration to maintained lung inflation at different airway pressures were compared by testing for parallelism.

RESULTS

Respiratory effects of agonists

Injection of picomole or smaller quantities of the EAA agonists Quis, KA and NMDA into the BH region of the NTS produced dose-dependent periods of apnoea. Figure 1 shows examples of the lengthening of T_E in response to injections of increasing concentrations of Quis (Fig. 1*A*), KA (Fig. 1*B*) and NMDA (Fig. 1*C*). Equivolume (3 nl) injections of each agonist concentration were made from a four-barrel pipette assembly. The AMPA receptor agonist Quis was the most potent agonist; it slowed breathing at a dose of 30 fmol (3 nl of 10 μM) and produced an apnoea at doses $\geqslant$ 150 fmol (3 nl of 50 μM). KA produced a prolonged, modest slowing of breathing frequency at 10-fold greater amounts (1·5 pmol; 3 nl of 500 μM). The threshold dose for NMDA to slow breathing was 150 fmol (3 nl of 50 μM); doses $\geqslant$ 1·5 pmol produced apnoeas which lengthened as the concentration increased.

In twelve rats, Quis (3 nl of 500 μM; 1·5 pmol) produced apnoeas that averaged 6·6 $\pm$ 2·7 s (mean $\pm$ S.D.). Injections of the same concentration and volume of NMDA produced a similar period of apnoea (10·5 $\pm$ 4·6 s) in eight rats. In a ninth animal, NMDA (3 nl, 500 μM) elicited a considerably longer apnoea (40 s). The response to KA injections was variable ($n = 5$). Low concentrations lengthened T_E but a tachyphylaxis was frequently evident with repeated injections at concentrations exceeding about 200 μM, possibly due to a depolarization block. The concentrations of KA employed did not appear to be neurotoxic since the responses recovered over a period of several minutes.

In contrast to the effects elicited by EAA, baseline respiratory motor output was not affected by equal or larger injections of saline (150 mM, pH 7·2–7·4, 3–24 nl, $n =$ 6 rats) or substance P (10, 50 and 200 μM, 3–20 nl; $n = 4$ rats). Saline or substance P were injected from a multibarrel pipette in which at least one barrel was filled with

an EAA. Proper positioning of the electrode within the BH region was assured by eliciting an apnoea of at least 3 s in response to a 3 nl injection of DLH or Quis.

Although substance P did not modify baseline respiratory motor output, it potentiated the response to DLH ($n = 2$). As shown in the example in Fig. 2,

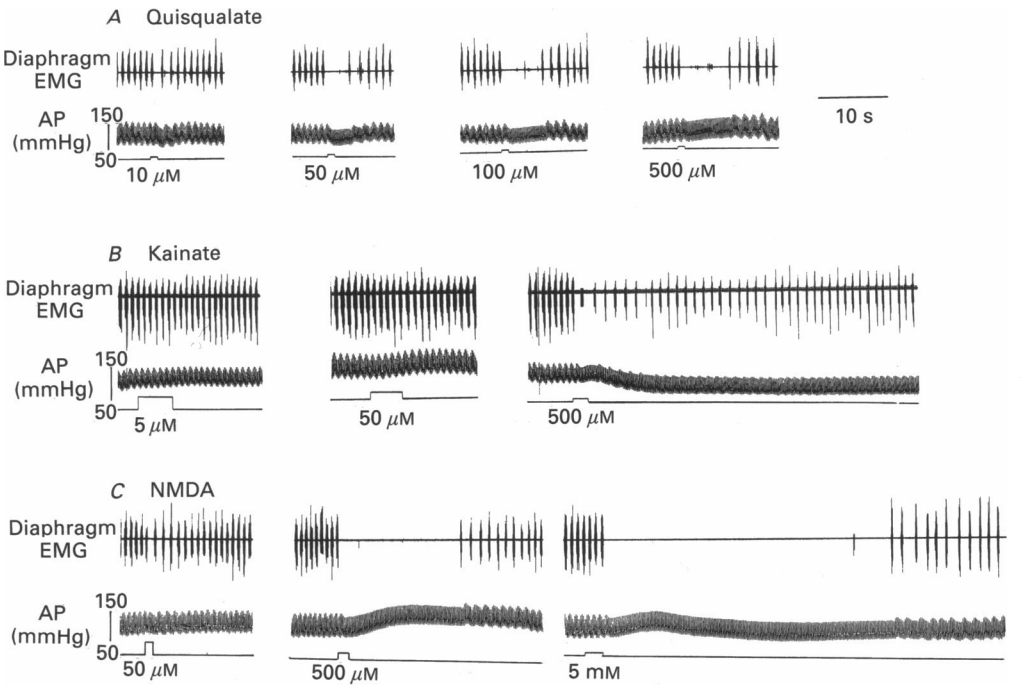

Fig. 1. Concentration-dependent changes in respiratory motor output elicited by equivolume (3 nl) injections of EAA agonists into the BH region of the NTS. Each agonist was injected at the indicated concentrations from separate barrels of a multibarrel pipette assembly in a single rat. One agonist was injected in each of three animals. Traces are, from the top, diaphragm EMG, arterial pressure (AP), and injection protocol. Total injection amounts were: quisqualate, 0·030, 0·150 and 1·5 pmol; kainate, 0·015, 0·150 and 1·5 pmol; NMDA, 0·15, 1·5 and 15 pmol.

injection of DLH (3 nl of 20 mM) produced a 12 s apnoea (Fig. 2*A*, left traces). A smaller volume (0·5 nl) elicited an apnoea of ~ 2·5 s (Fig. 2*B*, left traces). Substance P (2·4 pmol; 12 nl of 200 μM) injected through the second barrel of a double-barrel pipette had no effect on diaphragm EMG (Fig. 2*A*, right traces). However, when substance P (2 or 4 nl; 200 μM) was injected simultaneously with the smaller volume of DLH (Fig. 2*B*, middle pair of traces), the duration of the apnoea almost tripled from 2·5 to 8 s. About 2 min after the substance P injection was discontinued, the duration of the DLH-induced apnoea had returned to the control response.

Respiratory effects of excitatory amino acid antagonists

In two groups of rats the BH reflex-lengthening of T_E was compared before, during, and after Kyn was injected in the NTS site where EAA injection produced apnoea. In one group both vagi were intact ($n = 6$); in the second group the vagus

nerve contralateral to the injection site was cut ($n = 4$). As expected from decreasing the vagal input, higher lung inflation pressures were required to produce equivalent changes in respiratory patter after section of one vagus nerve. Thus, 10 cmH_2O produced an apnoea lasting $4{\cdot}8 \pm 1{\cdot}4$ s in unilaterally vagotomized rats while

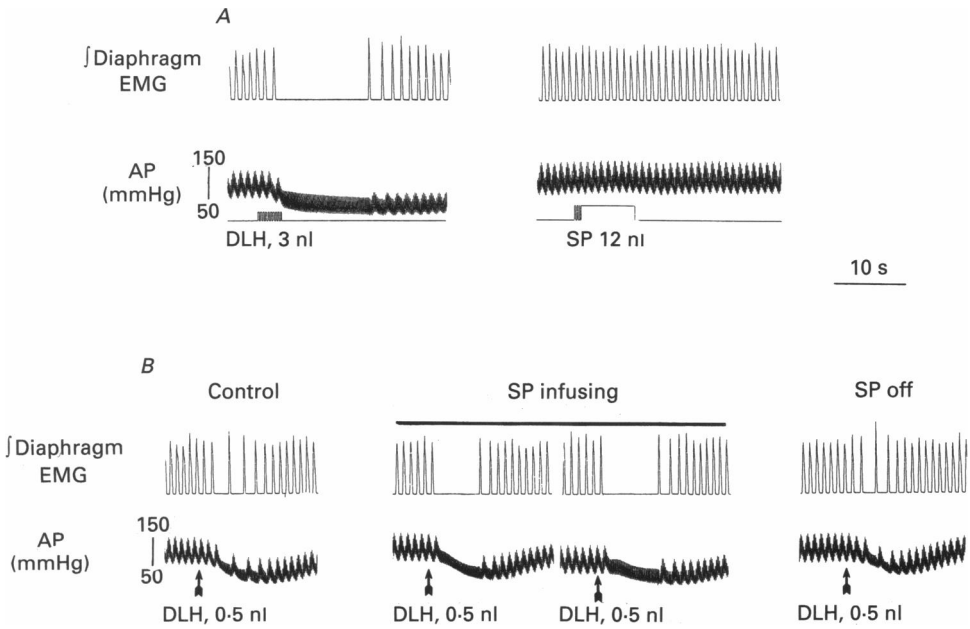

Fig. 2. The effects on diaphragm EMG of DL-homocysteic acid (DLH) and substance P (SP) injected separately (*A*) and simultaneously (*B*) into the BH region of the NTS. Traces are as indicated in Fig. 1. *A*, injection of the indicated volumes of DLH (20 mM, 120 pmol, left traces) and substance P (12 nl, 200 μM, 2·4 pmol, right traces) from a two-barrel pipette. *B*, left traces show injection of a smaller volume of DLH (0·5 nl, 20 mM); middle pairs of traces show simultaneous injections of substance P (2 nl in the left-hand traces and 4 nl in the right-hand traces) and DLH at the indicated volume (note the potentiation of the DLH response by simultaneous injection of substance P); right traces show return of the DLH response to pre-substance P levels about 2 min later.

7·5 cmH_2O elicited a longer apnoea ($16{\cdot}3 \pm 10{\cdot}4$ s; mean $\pm$ S.D.; $P < 0{\cdot}05$) in rats with both vagi intact.

Examples of the antagonism by Kyn of the reflex apnoea produced by lung inflation and that elicited by EAA injections into the NTS are shown in Fig. 3. In this vagally intact rat, the apnoea in response to lung inflation of 7·5 cmH_2O was impaired by Kyn (15 nl, 3 mM; total of 45 pmol) and recovered within about 40 min. The dose of Kyn required to diminish the BH reflex (Fig. 3*A*) also reversibly attenuated the apnoeas produced by injecting 3 nl (500 μM, 1·5 pmol) of either Quis (Fig. 3*B*) or NMDA (Fig. 3*C*).

The attenuation by Kyn of the BH-reflexive and EAA-mediated lengthening of T_E in rats with both vagi intact is shown in Fig. 4. As is evident from the figure, the duration of the apnoea produced by maintaining lung inflation at 7·5 cmH_2O was reversibly attenuated by Kyn (15–18 nl of 3 mM; total of 45–54 pmol; $P < 0{\cdot}05$ with

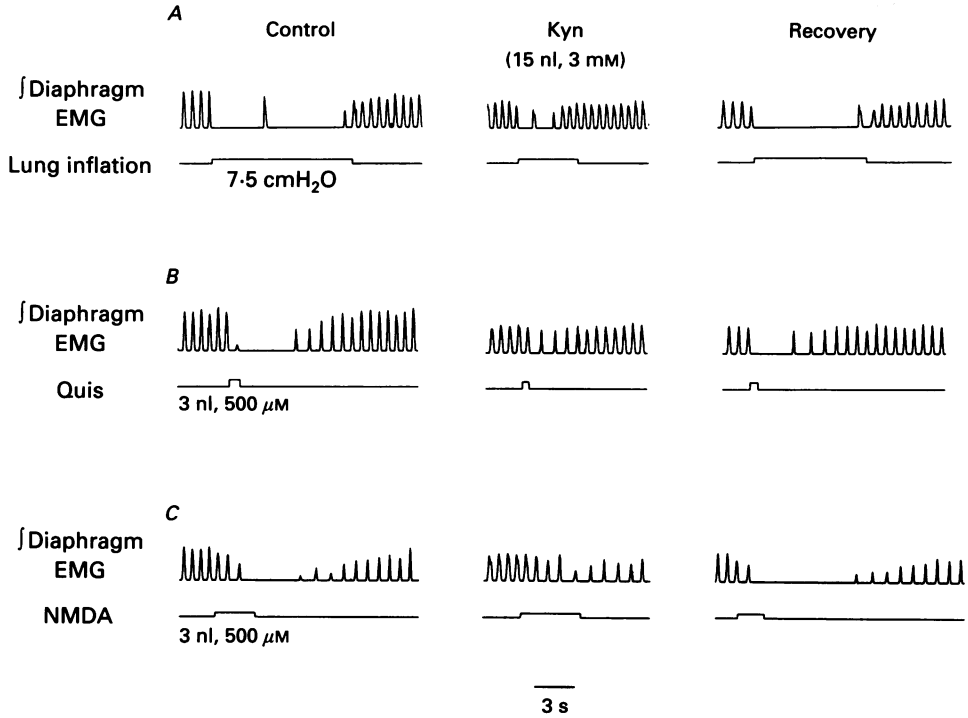

Fig. 3. Attenuation by kynurenic acid (Kyn) of the apnoeas produced by lung inflation (*A*), Quis (*B*), and NMDA (*C*) in a rat with both vagi intact. Upper trace, integrated diaphragm EMG; lower trace, lung inflation or drug injection protocol. Note the attenuation of the responses to lung inflation to 7·5 cmH_2O and injection of Quis or NMDA. Recovery was about 40 min post-Kyn injection.

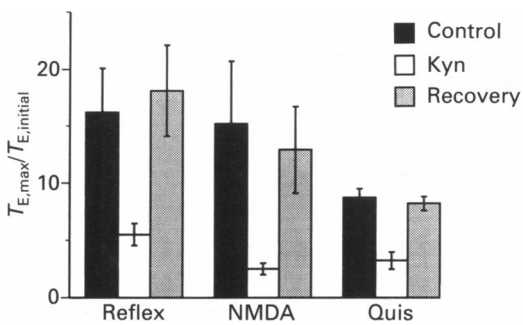

Fig. 4. Attenuation by Kyn (15–18 nl, 3 mM, 45–54 pmol) of the apnoeas produced by the BH reflex ($n = 6$) or by injections of Quis ($n = 6$) or NMDA ($n = 5$) in rats with intact vagi. The ordinate is the mean (±S.E.M.) of the normalized change in expiratory duration (see Methods). The BH reflex was activated by lung inflation to 7·5 cmH_2O.

respect to control and recovery). The durations of the apnoeas produced by Quis (3 nl, 500 μM or 6 nl, 250 μM; 1·5 pmol) and NMDA (3 nl, 500 μM; 1·5 pmol) were also reversibly attenuated ($P < 0.05$) by this dose of Kyn.

In two unilaterally vagotomized rats, Kyn was injected into adjacent regions of the NTS to determine whether the antagonist attenuated the reflex when injected outside the agonist-responsive area. In both rats, Kyn injections made outside the region in which DLH produced apnoea did not impair the Breuer–Hering lengthening

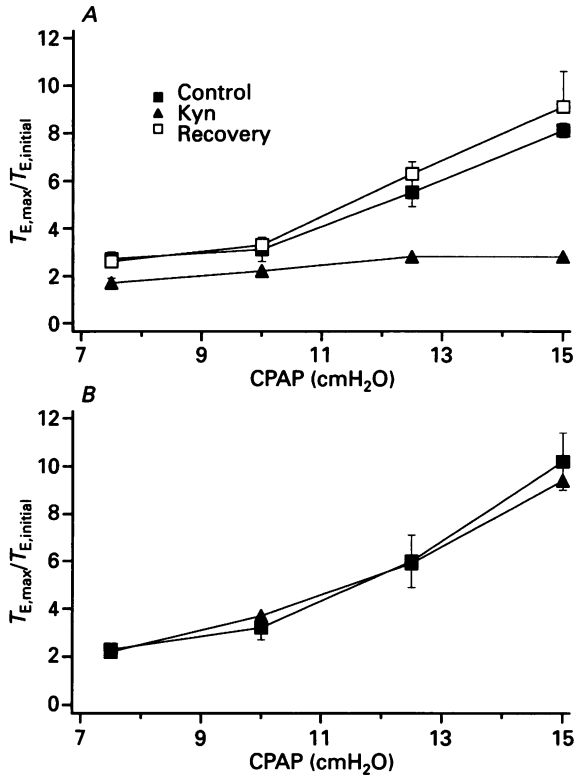

Fig. 5. Effect of Kyn (15 nl, 3 mM, 45 pmol) on the Breuer–Hering reflex when injected within and outside the DLH-responsive site in the same rat. *A*, the slope of the line relating the duration of apnoea to airway pressure decreased ($P < 0{\cdot}05$ with respect to control and recovery) when Kyn (45 pmol) was injected in the site where DLH produced an apnoea. *B*, an equal amount of Kyn injected 300 μm lateral to the DLH-responsive site did not alter the reflex lengthening of T_E.

of T_E. An example of the effect of a Kyn injection made within the BH region compared to an injection made 300 μm lateral to the DLH-responsive site in one rat is shown in Fig. 5. The reflex lengthening of T_E in response to maintained lung inflation at airway pressures of 5–15 cmH_2O was compared before, during and after recovery from Kyn. As shown in the Fig. 5*A*, the slope of the line relating the duration of apnoea to airway pressure was decreased significantly by Kyn (15 nl of 3 mM; total of 45 pmol; $P < 0{\cdot}05$ with respect to both Control and Recovery). The baseline reflex response recovered in about 40 min. DLH (3 nl of 5 mM) injected in this site produced a 3 s apnoea (not shown). As shown in the Fig. 5*B*, when the pipette was moved 300 μm outside the DLH-responsive region, an equivolume injection of Kyn did not impair the BH reflex.

TABLE 1. Effect of Kyn on T_I and T_E in rats with only one or both vagi intact

	Predrug control		Kynurenate		Postdrug recovery	
	T_I (s)	T_E (s)	T_I (s)	T_E (s)	T_I (s)	T_E (s)
One vagus cut ($n = 4$)	0·39±0·01	0·46±0·03	0·47±0·02*	0·62±0·03*	0·38±0·01	0·49±0·03
Both vagi intact ($n = 5$)	0·23±0·01	0·40±0·01	0·28±0·01*	0·39±0·02	0·23±0·01	0·41±0·02

* Different from predrug control and postdrug recovery ($P < 0·05$).

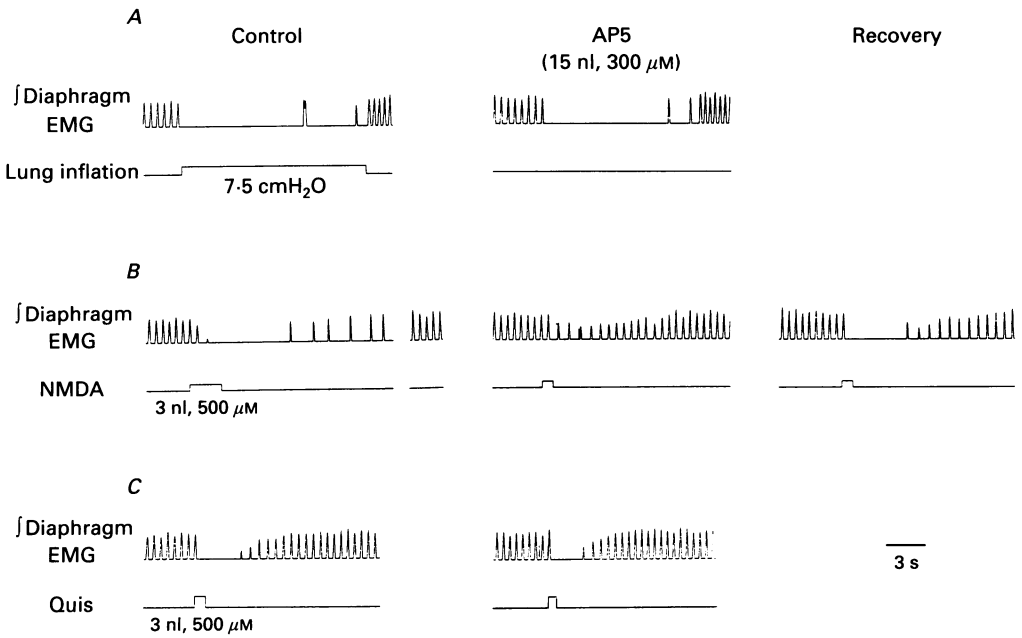

Fig. 6. Example of the effect of the NMDA receptor-preferring antagonist AP5 (4·5 pmol) on the apnoea produced reflexly by maintaining lung inflation at 7·5 cmH_2O, or by injecting NMDA or Quis. Traces are integrated diaphragm EMG and a pulse indicating the duration of lung inflation or pressure injection. The dose of AP5 which selectively abolished the apnoea produced by NMDA (*B*) and spared that produced by Quis (*C*), did not alter the BH reflex-induced apnoea (*A*).

Kyn also changed baseline respiratory rhythm, increasing both the control T_I and T_E in four unilaterally vagotomized rats ($P < 0·05$ compared to predrug control and recovery; Table 1). In five rats with both vagi intact, Kyn significantly prolonged T_I ($P < 0·05$), but not T_E (Table 1).

In contrast to the effects of Kyn, AP5 did not alter the BH reflex or baseline respiratory rhythm (Fig. 6). Injection of AP5 (15 nl, 300 μM, 4·5 pmol) blocked the NMDA-induced apnoea (Fig. 6*B*) but spared that produced by either lung inflation (Fig. 6*A*) or Quis (Fig. 6*C*). A summary of the effects of AP5 on the apnoea produced by maintained lung inflation or injection of NMDA or Quis in six rats is shown in Fig. 7. Doses of AP5 (1·5–4·5 pmol) which significantly attenuated the apnoea produced

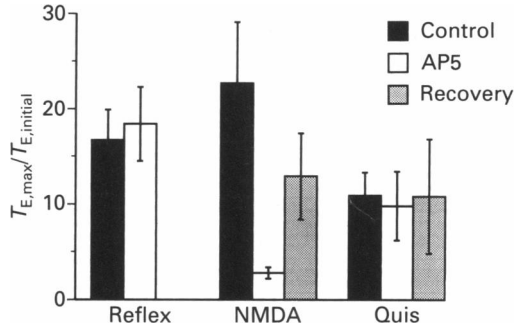

Fig. 7. Summary of the effect of AP5 on the Breuer–Hering reflex produced by maintaining lung inflation at 7·5 cmH_2O ($n = 5$) and the apnoeas produced by NMDA (750–1·5 pmol, $n = 6$) or Quis (1·5 pmol, $n = 6$).

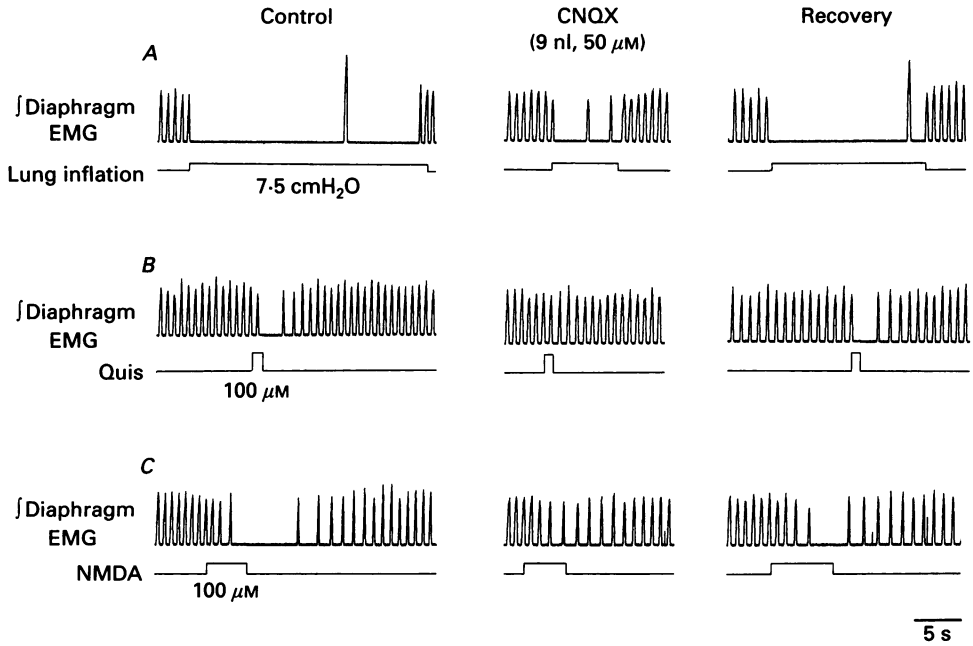

Fig. 8. The effect of the AMPA receptor-preferring antagonist CNQX (0·45 pmol) on the apnoea produced reflexly by maintaining lung inflation at 7·5 cmH_2O (*A*) or by injecting Quis (0·6 pmol; *B*) or NMDA (0·9 pmol; *C*). Glycine (20 μM) was included in the CNQX pipette. Note that CNQX attenuates the apnoea in response to all three treatments. Traces are integrated diaphragm EMG and a pulse indicating the duration of lung inflation or pressure injection.

by NMDA (750 fmol–1·5 pmol; $P < 0·05$ with respect to control and recovery) did not alter the duration of the apnoea produced by lung inflation to 7·5 cmH_2O, or by injection of Quis (1·5 pmol).

Both CNQX (20–100 μM, $n = 11$) and DNQX (50 or 100 μM, $n = 2$ per dose) behaved similarly to Kyn as broad spectrum EAA antagonists. Doses of CNQX sufficient to produce a reversible, dose-dependent antagonism of the apnoea induced by lung inflation, attenuated the apnoeas elicited by either Quis or NMDA. In the

example shown in Fig. 8, injection of CNQX (9 nl, 50 μM, 0·45 pmol) blocked the apnoeas induced by either Quis (Fig. 8*B*) or lung inflation (Fig. 8*A*) but also substantially attenuated the response to NMDA (Fig. 8*C*). At a concentration of 100 μM, CNQX attenuated the apnoea in response to lung inflation, Quis and NMDA

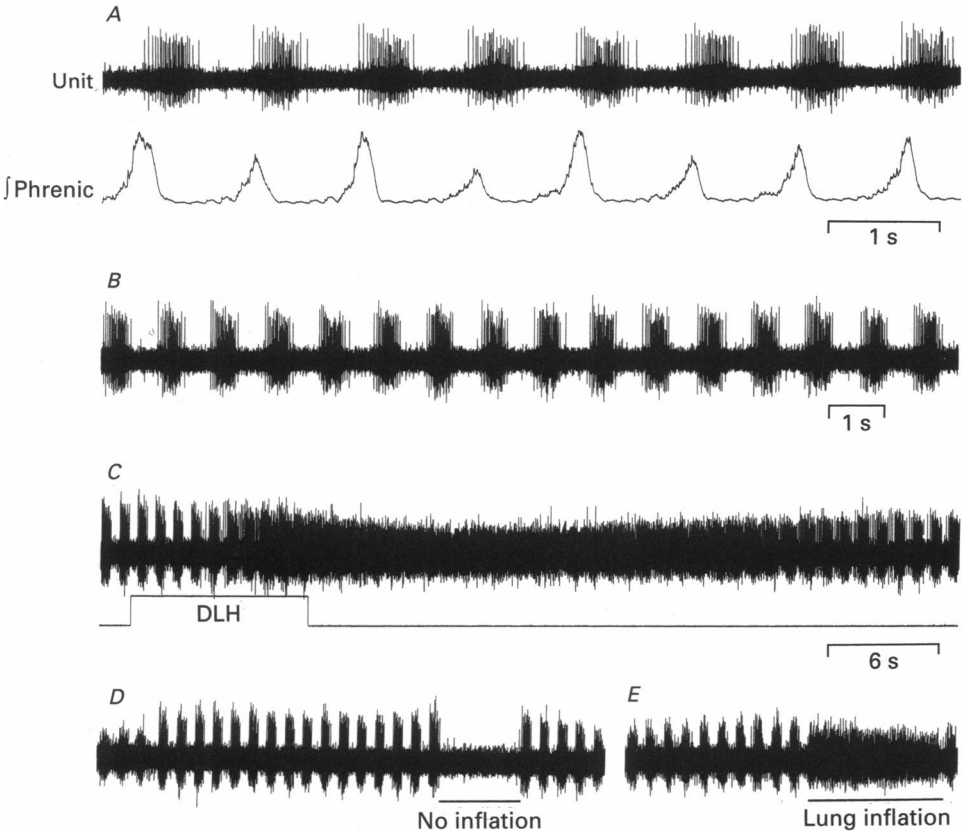

Fig. 9. Excitation of a pump cell by DLH (1 mM). *A*, control discharge, the unit discharges during each inflation cycle of the ventilator; *B*, pump cell is excited by DLH injected from a three-barrel compound electrode; *C*, neurone ceases discharging when the ventilator is turned off at end-expiration; *D*, maintains its discharge when the ventilator is turned off at end-inspiration. Time elapsed between panels *B* and *C* was 1·3 min, and between *C* and *D* it was 1 min.

by $\geqslant 80\%$. Inclusion of 20 μM glycine with CNQX in the delivery pipettes failed to improve the selectivity of CNQX (20, 40 and 50 μM) for Quis receptors ($n = 2$). Injection of DNQX (50 μM) had little effect on reflexive or EAA-induced apnoeas. At the higher concentration (100 μM), DNQX reduced the apnoeas in response to lung inflation, Quis and NMDA by $\geqslant 75\%$.

Single unit pharmacology

Neurones in the BH region were tested for their responsiveness to DLH. Seven of these were identified as pump cells since, as shown in Fig. 9, they exhibited a sustained discharge during maintained lung inflation (Fig. 9*E*) and were silent, or

had a low tonic rate of discharge, when the ventilator was turned off at end-expiration (Fig. 9*D*). Pressure ejection of small amounts of DLH ($\leqslant$ 1 nl, 1 mM) excited these neurones (Fig. 9*C*) without eliciting an observable change in phrenic nerve discharge.

The effect of Kyn on the pump cell responses evoked by lung inflation and DLH was determined in two rats. An example of one of these is shown in Fig. 10. The unit

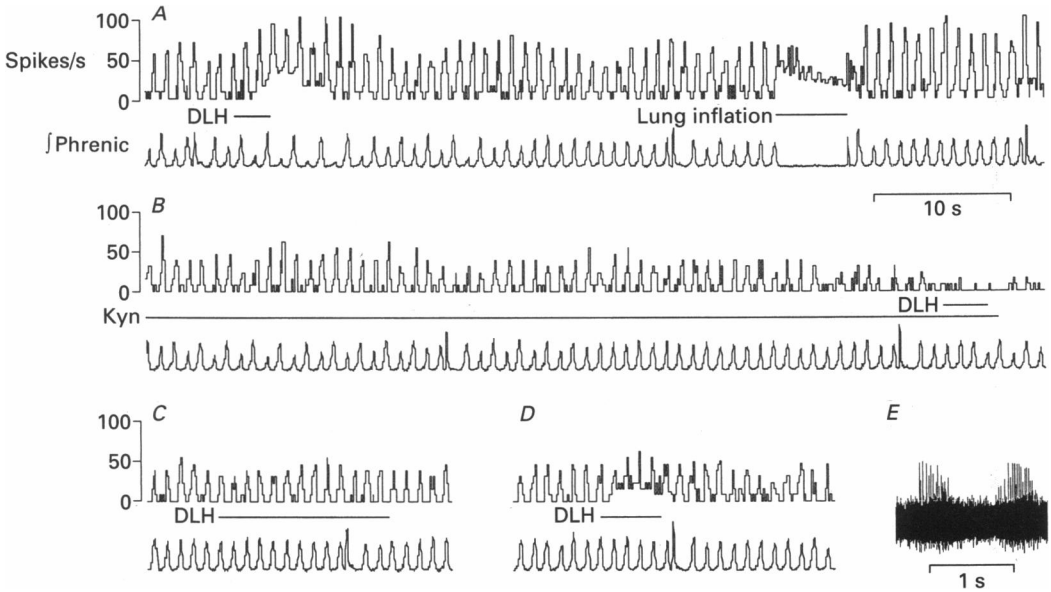

Fig. 10. Kyn block of spontaneous discharge and the response to DLH in a multiunit recording of pump cell activity. Top trace in each panel is a rate-meter record of the discharge of pump cell activity using a time amplitude window discriminator. *A*, excitation of neurone activity by DLH (1 mM) application and by maintained lung inflation while the ventilator was turned off at end-inspiration; *B*, inhibition of unit activity and the DLH response by continuous injection of Kyn (3 nl, 10 mM, total injection = 30 pmol) beginning about 30 s prior to the start of the record; *C*, recovery of unit activity but not of the response to DLH 0·7 min after Kyn injection; *D*, recovery of the DLH response 2 min after Kyn injection; *E*, extracellular recording of the multiunit pump cell activity in *A*.

was excited by DLH injection and maintained its discharge for several seconds when the ventilator was turned off at end-inspiration (Fig. 10*A*). Slow injection of Kyn (3 nl, 10 mM, Fig. 10*B*) over 70 s progressively reduced the neuronal discharge rate and blocked the response to DLH. After the Kyn injection, spontaneous activity recovered more rapidly than the unit's responsiveness to DLH (Fig. 10*C* and *D*).

Two neurones exhibited discharge patterns characterized by a small and variable number of action potentials per burst, suggesting an input from pulmonary rapidly adapting receptors (Knowlton & Larrabee, 1946; Fig. 11*A*). Kyn also inhibited the spontaneous discharge of these neurones. In the example shown in Fig. 11, the administration of Kyn (10 mM, Fig. 11*B*) gradually reduced and then abolished the spontaneous discharge. In Fig. 11*C* the unit is initially silent as a result of the Kyn application but still responds to the application of DLH.

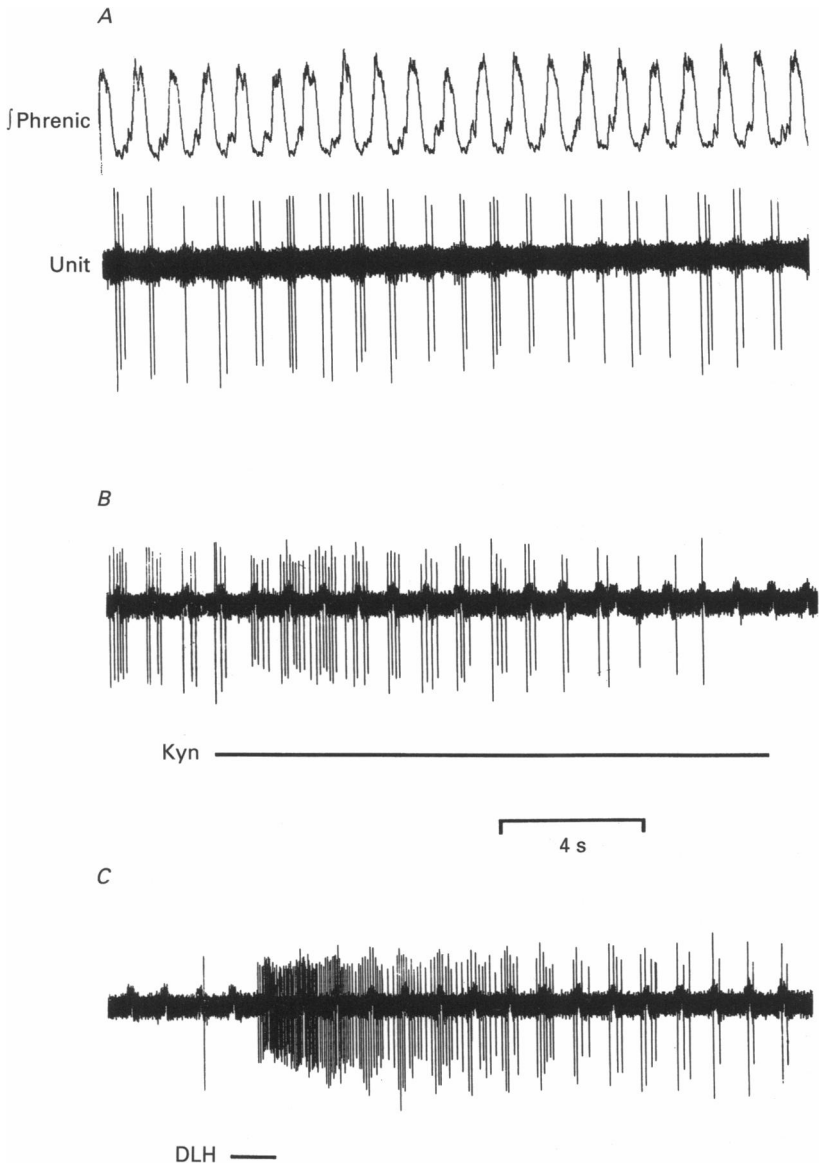

Fig. 11. Kyn block of spontaneous discharge in a non-pump cell unit in the BH region of the NTS of mechanically ventilated rat. *A*, control activity, unit discharges a few spikes during each lung inflation. The pattern of activity is consistent with that of a unit receiving input from pulmonary rapidly adapting receptors; *B*, inhibition of spontaneous activity by injection of Kyn (2 nl, 10 mM, total injection = 30 pmol); *C*, during the Kyn-induced inhibition of spontaneous activity application of DLH (1 mM) produces a marked excitation. Time elapsed between *A* and *B* = 2 min. Traces in *B* and *C* form a continuous record.

DISCUSSION

The major findings of this study were: (1) injection of picomole amounts of the EAA agonists Quis and NMDA into the BH region of the NTS depressed respiratory motor output, an effect that mimicked the BH reflex apnoea elicited by lung inflation, (2) substance P had no effect on baseline respiratory pattern, although it potentiated the apnoea elicited by EAA, (3) picomole amounts of the EAA antagonists Kyn, CNQX and DNQX reduced the BH reflex reversibly, and slowed breathing frequency, (4) the selective NMDA antagonist AP5 did not alter baseline respiratory pattern or the BH reflex, and (5) Kyn reversibly blocked excitation of pump cells in response to lung inflation and to picomole amounts of an EAA agonist.

Critique of pressure injection and chemical stimulation

In order to reproducibly deliver nanolitre volumes of drug solutions, we used a delivery system with a resolution of 0·5 nl (McCrimmon *et al.* 1986). Marked differences in drug responses observed following relocation of the delivery pipette by as little as 50 μm support our contention that drug actions were confined to a small region close to the pipette tip. Furthermore, the theoretical calculations of Nicholson (1985) indicate that the concentration of drug present at a radius of 300 μm from the site of a 10 nl injection is probably no more than 20% of that at the pipette tip. Although we cannot entirely discount the possibility that the responses are due to pressure-induced artifacts, this seems unlikely since the EAA drug-induced alterations in respiratory parameters were dependent upon drug concentration, and not volume. Moreover, the failure of equal or larger volumes of substance P to mimic the responses induced by pressure injections of EAA agonists strongly suggests a degree of pharmacological specificity in our results.

When studying behavioural responses to CNS injections of neuroactive agents, the physiological relevance of the responses should be considered (Lipski, Bellingham, West & Pilowsky, 1988). Since EAA receptors are located on most CNS neurones, agonist injection may depolarize groups of cells that do not generally discharge in synchrony, or may increase their neuronal firing rates beyond the usual range. However, in the present study, predictable reflex and pump cell responses to physiological activation of SARs were mimicked by injections of selective agonists and prevented by antagonists.

Respiratory effects of agonists

We previously reported that neurones in a discrete region of the NTS, presumably pump cells, are necessary for the BH reflex (Bonham & McCrimmon, 1990). In that study, we injected the broad spectrum EAA receptor agonist DLH (1–6 nl; total of 20–120 pmol) to increase neuronal activity. In the present study, we injected much smaller amounts of specific EAA receptor agonists to identify specific receptors activated by pulmonary stretch receptor afferent input. Both Quis and NMDA elicited reproducible, dose-dependent apnoeas that resembled the BH reflex prolongation of T_E. These findings suggest that both AMPA and NMDA receptors are present on neurones mediating the BH reflex. The presence of both receptor types on the same neurones is common in other areas of the CNS, including phrenic

motoneurones (McCrimmon *et al.* 1989; Greer *et al.* 1991), and may reflect a mechanism modulating the strength of the reflex response to SAR activation. At normal resting membrane potentials, activation of NMDA receptors is prevented by physiological concentrations of magnesium (Monaghan *et al.* 1989). However, the block is voltage dependent and decreases as a neurone is depolarized. As a result, the activation of NMDA receptors on pump cells may have a greater physiological impact under conditions where the activity of SARs is tonically increased leading to a greater depolarization of pump cells (e.g. if functional residual capacity is increased).

KA injected at the same concentrations and volume as Quis or NMDA often slowed respiratory frequency without eliciting an apnoea; repeated injection of KA frequently elicited successively smaller changes in respiration. While the mechanism of these effects is not clear from the current data, the findings are consistent with a KA-induced depolarization block. Similar problems were never encountered with the other agonists.

Substance P had no observable effect on baseline respiratory pattern, suggesting that it is not a primary transmitter in the BH reflex pathway. However, this peptide excites many NTS neurones (Morin-Surun *et al.* 1984) and augmented the DLH-induced apnoea. It is possible that substance P receptors are located on interneurones within the BH reflex pathway and their activation potentiates responses to glutamate. This possibility is consistent with a recent demonstration that substance P potentiates a glutamate-induced inward current in rat spinal neurones (Randic, Hecimovic & Ryu, 1990). Alternatively, substance P could activate other neurones within the BH region that either directly activate or disinhibit neurones within the BH pathway. The physiological role for substance P modulation of the BH reflex is not yet clear, but could be related to changes in reflex gain, for example, during chemoreceptor activation (Mitchell & Vidruk, 1987).

Respiratory effects of antagonists

Mimicry of the BH reflex apnoea by Quis or NMDA is not by itself a compelling argument for EAA neurotransmission from SAR primary fibres. Activation of interneurones in other afferent pathways, such as those in the laryngeal chemoreflex (Lawson, Richter, Czyzyk-Krzeska, Bischoff & Rudesill, 1991), baroreflex (Talman *et al.* 1980), and the pulmonary C fibre afferent pathways (Green, Schmidt, Schultz, Roberts, Coleridge & Coleridge, 1984; Bonham & Joad, 1991) can also produce apnoea. In addition, eliciting an apnoea only indicates that involved interneurones have EAA receptors, a trait shared with most other neurones (Monaghan *et al.* 1989). The argument supporting a role for a neurotransmitter or receptor in a pathway is considerably strengthened by the ability of the appropriate antagonist(s) to block the physiologically activated reflex.

Interruption of synaptic transmission in the BH region by cobalt injection reversibly attenuates the BH reflex (Bonham & McCrimmon, 1990). In the current study, the broad spectrum EAA antagonist, Kyn, reversibly blocked both the physiologically activated BH reflex and the apnoea produced by either Quis or NMDA. In addition, Kyn slowed breathing frequency by lengthening T_I in rats with intact vagi, and by lengthening both T_I and T_E in rats with one vagus cut. These

respiratory pattern changes are similar to those occurring in paralysed cats if SAR afferent inputs are reduced by withholding lung inflation (Cohen & Feldman, 1984). Thus, the present findings are consistent with the interpretation that SAR primary afferent fibres release an EAA transmitter which activates second-order neurones in the BH reflex.

AP5 selectively blocked the apnoea induced by NMDA, but not the responses to lung inflation or Quis. This finding is consistent with previous reports that the systemic administration of NMDA receptor antagonists which cross the blood–brain barrier do not block the BH reflex termination of inspiration in cats (Foutz *et al.* 1989; Feldman *et al.* 1992). Collectively, the effectiveness of Kyn in blocking the BH reflex, and the lack of effect of AP5 suggest that non-NMDA EAA receptors mediate the BH reflex. This conclusion is consistent with reports that CNQX blocks excitatory postsynaptic potentials in NTS neurones elicited by stimulation of afferent fibres in the tractus solitarius (Andresen & Yang, 1990).

In this study, both CNQX and DNQX blocked the BH reflex responses to lung inflation. However, this antagonism cannot be ascribed to a selective blockade of non-NMDA receptors since both compounds attenuated the apnoeic responses to both NMDA and Quis. Since it is known that CNQX antagonizes NMDA responses by a competitive block of the glycine site on the NMDA receptor (Harris & Miller, 1989), in some experiments glycine was included in the pipette barrel with CNQX, but this did not improve the selectivity of the CNQX-induced block. A likely explanation for the lack of selectivity arises from the relatively high concentration which must be put in the pipette to ensure adequate blockade at receptors several tens to hundreds of micrometres from the pipette tip. Nevertheless, blockade of the BH reflex by Kyn and CNQX, but not by AP5, suggests that SAR afferent fibres release glutamate or a related substance which primarily activates non-NMDA receptors on NTS neurones in the BH reflex pathway.

Single unit pharmacology

The effects of EAA agonists and antagonists on the BH reflex-induced changes in phrenic nerve discharge were consistent with the effects of DLH and Kyn on single NTS neurones. Within the BH region, pump cells form the largest group of cells with spontaneous activity and are the most likely candidates for eliciting the BH reflex (Bonham & McCrimmon, 1990). Kyn blocked the response of pump cells to both lung inflation and DLH application, consistent with the effects of Kyn on the BH reflex. Although these findings support the hypothesis that an EAA released by SAR primary afferent fibres activates pump cells in the BH region, other explanations are possible. For example, if Kyn blocked an excitatory input to pump cells (other than from SARs) it could reduce the excitability of pump cells below the threshold for excitation by SARs. This seems unlikely, however, since pump cells in the cat are strongly depolarized by SAR afferent inputs (Berger & Dick, 1987). Alternatively, it is possible that activation of neurones other than pump cells was responsible for the reflex apnoea. Other candidates include Iβ neurones which have been identified in cats. These neurones receive a monosynaptic input from SARs as well as a centrally generated respiratory drive (Backman *et al.* 1984; Berger & Dick, 1987). However, an equivalent population has not been identified in rats and the general consensus in the literature is that there are few if any respiratory neurones in the NTS of the rat

(Saether, Hilaire & Monteau, 1987; Ezure, Manabe & Yamada, 1988; Zheng, Barillot & Bianchi, 1991). Consistent with these studies in the rat, we found only scattered neurones with a centrally generated respiratory pattern in the NTS, primarily lateral to the tractus. Unlike pump cells, however, preliminary studies suggest that these respiratory neurones are inhibited during maintained lung inflation (A. C. Bonham, S. K. Coles & D. R. McCrimmon, unpublished observations). Hence, although the participation of other NTS neurones in the BH reflex cannot be ruled out, pump cells are the most likely candidates.

Other afferent pathways to this region of the NTS are also likely to employ an EAA transmitter. A small number of neurones with discharge patterns suggesting that they received input from pulmonary rapidly adapting receptors (see Results) were amongst the neurones recorded within the BH region. Like pump cells, they were excited by DLH and both their spontaneous activity and their response to DLH were antagonized by Kyn. This finding is consistent with that of Andresen & Yang (1990) who reported that the fast excitatory input from stimulation of the tractus solitarius could be antagonized by EAA antagonists. Thus, it seems likely that many large myelinated afferent fibres terminating in this region of NTS utilize EAA transmitters. However, the efficacy of this transmission may be modulated by other agents such as substance P that may be co-released with an EAA from vagal afferent fibres or could be released from other NTS neurones.

In summary, the ability of broad spectrum EAA antagonists to (a) reduce the reflex apnoea in response to maintained lung inflation, (b) slow baseline respiratory rate, and (c) inhibit the response of pump cells to lung inflation provides evidence that glutamate or another EAA is necessary for production of the BH reflex. Further, the lack of effect of selective blockade of NMDA receptors on the BH reflex is consistent with an action primarily on non-NMDA receptors in the NTS. Thus, as a working hypothesis, we propose that SAR primary afferent fibres release EAA-activating non-NMDA receptors on pump cells, thereby initiating activity in the central Breuer–Hering reflex pathway.

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