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Inhibitory synaptic inputs to the respiratory rhythm generator in the medulla isolated from newborn rats

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Abstract. Involvement of chloride-dependent, gamma-aminobutyric acid-(GABA-) like synaptic inhibition in the generation of respiratory rhythm was studied in brainstem-spinal cord preparations isolated from newborn rats. Primary respiratory rhythm is presumably generated within the rostral ventrolateral medulla, the site of Pre-I neurones, the firing of which precedes inspiration. Therefore, we examined the responses of Pre-I and inspiratory neurones to GABA antagonists (picrotoxin and bicuculline), a glycine antagonist (strychnine) and reduced chloride concentration in the perfusate. These antagonists (2–20 μM) and reduction of chloride concentration reversibly blocked the transient inhibition of Pre-I activity that occurred during the inspiratory phase. The rhythmic Pre-I and inspiratory neurone activity remained. Changes in the firing properties of Pre-I and inspiratory neurones in 10 μM bicuculline, 10 μM picrotoxin, 5 μM strychnine or reduction of chloride concentration to 40% of normal were analysed statistically. Burst rate of Pre-I neurones tended to increase during these treatments. Delay time from initiation of Pre-I firing to the peak of C4 motorneurone inspiratory activity tended to decrease except during reduced chloride concentration. Changes in mean intraburst firing frequency of Pre-I neurones were not consistent; increase (32%), no change (38%) or decrease (30%). Burst duration of inspiratory neurones decreased. Intraburst firing frequency of inspiratory neurones tended to increase except in 5 μM strychnine. GABA (0.1 mM) or glycine (0.2 mM) reduced the intraburst firing frequency and burst rate of Pre-I neurones, but did not affect the intraburst firing frequency of inspiratory neurones. The burst duration of inspiratory neurones increased during GABA and glycine treatment. The results suggest: 1. Inhibition of Pre-I activity during the inspiratory phase depends on chloride-dependent, GABA- (or glycine-) like inhibitory synaptic interaction (probably inhibitory synaptic inputs to Pre-I neurones), but Pre-I rhythm generation does not require this phasic inhibition. 2. Tonic $GABA_A$-like inhibition (where A denotes the receptor sub-type) might be involved in the modulation of rhythm generation and inspiratory pattern generation.

Key words: Chloride-dependent synaptic inhibition – Pre-inspiratory neurones – $GABA_A$ – Glycine – Rostral ventrolateral medulla – In vitro – Newborn rat

Introduction

The brainstem-spinal cord preparation isolated from newborn rat affords many advantages for physiological and pharmacological investigation of the respiratory centre and other parts of the central nervous system, due to the simple experimental conditions [11, 16, 25, 27]. We have postulated that respiratory rhythm in the newborn preparation in vitro is primarily produced by Pre-I neurones which are localized in the rostral ventrolateral medulla (RVL) [17–19]. Pre-I neurones, the firing of which precedes inspiration, are considered to trigger periodically an inspiratory pattern generator (IPG), which is proposed to be located more caudally in the medulla [19]. Pre-I firing is usually inhibited during inspiration. This may be due to phasic inhibitory inputs to Pre-I neurones from the IPG, since the inhibition disappears in association with depression of inspiratory discharge after lesion of the caudal ventrolateral medulla (CVL) [19].

Synaptic inhibition is present in the network of respiratory neurones [2–4, 6, 13, 14] and is suggested to contribute to the generation of respiratory rhythm [22]. Gamma aminobutyric acid- (GABA-)like and glycine-like inhibition are probably involved in the periodic inhibition of medullary respiratory neurones [5, 9]. It has been previously suggested, however, that generation of the basic respiratory rhythm in lamprey [23] and in newborn rat preparations [7, 24] does not require chloride-dependent synaptic inhibition and, therefore, that respiratory rhythm might be generated by a pacemaker-driven oscillator [26]. Recently, we presented indirect evidence that some Pre-I neurones might possess an intrinsic pacemaker property that can produce periodic burst activity [21].

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In the study reported here, we examine the effects of the antagonists of the $GABA_A$ sub-type receptor, picrotoxin and bicuculline, the glycine antagonist, strychnine, as well as reduced chloride concentration on the activity of Pre-I neurones and inspiratory neurones (Insp neurones) in the RVL. Characteristics of the phasic inhibition of Pre-I neurones during inspiration and involvement of $GABA_A$-like synaptic inhibition in respiratory rhythm generation are discussed. Some of results reported here have been previously reported in abstract form [20].

Materials and methods

The methods used have been described previously [17, 19, 27]. The brainstem and spinal cord of 0- to 4-day-old Wistar rats (46 preparations) were isolated under deep ether anaesthesia. The brainstem was rostrally decerebrated between the sixth cranial nerve roots and the lower border of the trapezoid body. The preparation was continuously perfused at a rate of 2.5–3.0 ml/min in a 2-ml chamber with the following standard solution (mM): NaCl, 124; KCl, 5.0; KH_2PO_4, 1.2; $CaCl_2$, 2.4; $MgSO_4$, 1.3; $NaHCO_3$, 26; glucose, 30; equilibrated with 95% O_2 and 5% CO_2; at 25–26 °C, pH 7.4. Chloride-free solution was made by substituting sodium isethionate, potassium, propionate and calcium propionate for the chloride salts of sodium potassium and calcium of the standard solution respectively. Low chloride solutions were prepared by mixing the standard solution and the chloride-free solution. Drugs were obtained from Sigma, (St. Louis, MO., USA) (bicuculline and GABA) or Wako Pure Chemical (Osaka, Japan) (picrotoxin, strychnine sulphate and glycine).

The unit activity of neurones was recorded extracellularly using glass microelectrodes filled with 2% pontamine sky blue in 0.5 M sodium acetate (5–20 MΩ). The electrodes were inserted through the ventral surface into the left and/or right RVL, namely, the medullary reticular formation extending caudally and slightly medially to the facial nucleus, ventrally to the retrofacial nucleus and 50–250 μm deep from the ventral surface [1, 18]. Respiratory activity corresponding to inspiration was monitored at the C4 or C5 motorneurone ventral root, the activity of which is known to synchronize with phrenic nerve discharges and with the contraction of inspiratory intercostal muscles [16, 27]. Neuronal unit activity and C4 motorneurone activity were stored on magnetic tape for subsequent data analysis.

The firing pattern of Pre-I neurones typically consists of the following three parts: (1) firing preceding the inspiratory phase, (2) depression of firing during the inspiratory phase [referred to as inspiration-related inhibition of the Pre-I firing (IIPI)], and (3) firing after the inspiratory phase. Firing properties (Fig. 1) of Pre-I neurone activity that were analysed were: (1) intraburst firing frequency (f_p), which was mean firing frequency during Pre-I burst excluding the IIPI period; (2) Pre-I firing frequency in inspiratory phase (f_{pi}); (3) cycle period (CP) in seconds from which the burst rate (BR) in bursts per minute was calculated; and (4) delay time from initiation of Pre-I firing to the highest peak near the onset of C4 motorneurone inspiratory activity (DE).

Observed properties of inspiratory neuron activity were: (1) intraburst firing frequency (f_i), which was the mean firing frequency during the inspiratory phase; and (2) burst duration (DU), which closely corresponded to the duration of C4 inspiratory bursts.

Results form 10 μM bicuculline, 10 μM picrotoxin, 5 μM strychnine or reduction of chloride concentration to 40% of normal were statistically evaluated. Each firing property was measured from data indicated on a computer display, which was stored transiently in IC memory through an AD converter from a tape recorder. Mean and standard deviation (SD) were calculated from 10 cycles of each unit activity. For each neurone, statistical significance of values before and after drug application were calculated using the two-sample (Student's) t-test (when variance was equal) or Welch's t-test (when variance was unequal), after the F-test (significance level of 0.05) for analysis of variance. Group responses before and after drug perfusion were analysed statistically using paired sample t-test. $P < 0.05$ was considered significant.

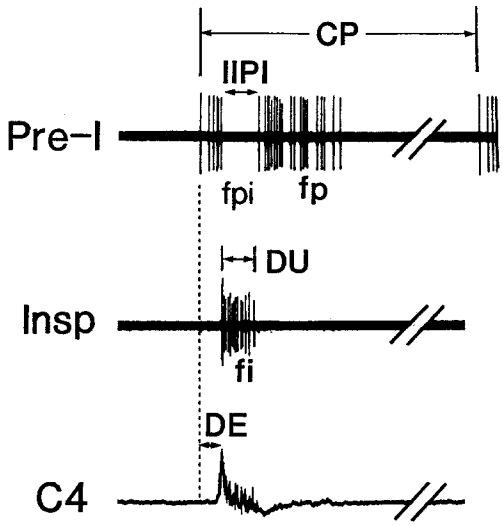

Fig. 1. Analysed firing properties of Pre-I (*Pre-I trace*) and inspiratory (*Insp trace*) neurone activity. IIPI, Inspiration-related inhibition of Pre-I firing; f_p, intraburst firing frequency of Pre-I neurones except in IIPI period; f_{pi}, Pre-I firing frequency during inspiratory phase; *CP*, cycle period of Pre-I activity; *DE*, delay time from initiation of Pre-I firing to peak at onset of C4 motorneurone inspiratory activity (*C4 trace*); f_i, intraburst firing frequency of Insp neurones; *DU*, burst duration of Insp neurones

Results

GABA and glycine antagonists

Effects of bicuculline perfusion (2–20 μM) for 4–12 min on Pre-I neurones (17 units), Insp neurones (11 units) and C4 motorneurone activity were examined in 12 preparations. The transient IIPI was negligible or absent after 5–6 min of 2–10 μM bicuculline perfusion. A typical result is shown in Fig. 2. In this neurone, IIPI almost completely disappeared after 8 min perfusion with 10 μM bicuculline (Fig. 2B). The Pre-I firing during the inspiratory phase was completely suppressed before bicuculline, and f_{pi} (spikes/s) was 9.2 ± 2.2 (mean ± SD in 10 cycles) after 4 min and 10.8 ± 1.3 after 8 min of bicuculline perfusion. IIPI reappeared with a slower time course after the perfusate was returned to normal (Fig. 2C, D). The value of f_{pi} was 9.0 ± 1.6 (spikes/s) 10 min after returning to the normal solution, 7.5 ± 1.0 (spikes/s) after 30 min and 3.1 ± 1.4 (spikes/s) after 50 min.

Seizure-like discharges were observed in the C4 motorneurone record (Fig. 2B). The discharges appeared with lower frequency (0.5–2 bursts/min) but longer duration (2–20 s) than the inspiratory C4 motorneurone activity. These seizure-like discharges occasionally affected respiratory rhythm by resetting Pre-I rhythm. In the result shown in Fig. 2, seizure-like discharges were followed by Pre-I firing with delay of about 100 ms from onset of the discharges, but no inspiratory neurone firing occurred and then the Pre-I burst rhythm was reset. When the seizure-like discharges occurred with a longer delay time from the preceding C4

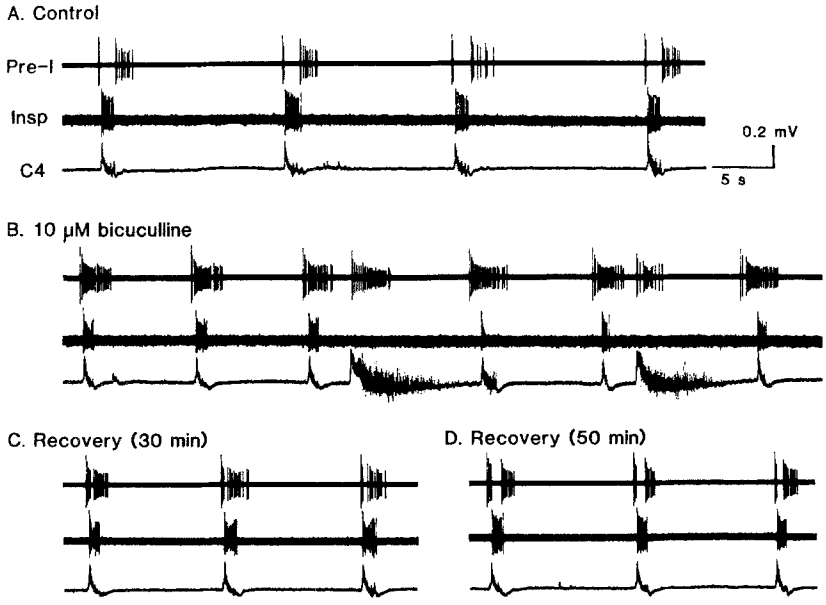

Fig. 2 A–D. Effects of 10 μM bicuculline on Pre-I and Insp neurones. **A** Pre-I activity from right rostral ventrolateral medulla (RVL) (*Pre-I trace*), Insp activity from left RVL (*Insp trace*) and C4 activity (*C4 trace*) recorded from a 4-day-old rat preparation in standard bath solution. **B** Pre-I, Insp and C4 activity 8 min after perfusion with 10 μM bicuculline. Note disappearance of IIPI. f_p and BR increased significantly to 137% and 149% of control respectively. DE decreased to 80% of control, but this was not significant. f_i increased significantly to 137% of control and DU decreased significantly to 60% of control. Seizure-like discharges appeared on C4 record. **C, D** Activity 30 min and 50 min respectively after perfusate was returned to standard solution after 9 min of bucuculline perfusion. Note recovery of IIPI

motorneurone inspiratory activity, Pre-I firing induced firing of the Insp neurone (not shown in this record). Resetting of Pre-I rhythm, in association with the seizure-like discharges, was observed in 10 of 13 neurones in 10 μM bicuculline.

Statistical analysis of changes in the firing properties were performed on 13 Pre-I units (9 preparations) and 6 Insp neurones (6 preparations), which were examined with 10 μM bicuculline. All statistically analysed results in this and other treatments are shown in Tables 1–3. A significant increase of f_{pi} is clearly shown in Table 1, which means significant removal of IIPI. The value of BR of Pre-I neurones significantly increased or did not change and the value of DE significantly decreased or did not change (Table 2). The value of f_p significantly increased in 6 units, but decreased in 4. Most Insp neurones increased f_i and decreased DU (Table 2). General tendencies as group responses are indicated by the total R values in Table 2 where R is the mean ratio in neurones that exhibited a change. However, increase of f_i during 10 μM bicuculline was not significant by the paired t-test (Table 3).

Effects of 5–13 min perfusion (2–20 μM) of picrotoxin on Pre-I neurones (20 units), Insp neurones (8 units) and C4 activity were examined in 14 preparations. After 5–6 min of picrotoxin, IIPI was negligible or absent as during bicuculline perfusion (Fig. 3, Table 1). This effect was reversible. Seizure-like discharges appeared along with the normal C4 motorneurone activity, although the discharges were not recorded in Fig. 3 because of their infrequent occurrence (0.2–2 bursts/min). Changes of firing properties of 8 Pre-I units (6 preparations) and 4 Insp neurones (4 preparations) which were treated with 10 μM picrotoxin were analysed. For Pre-I neurones, BR tended to increase and DE tended to decrease (Table 2, total R values), but not significantly according to the paired t-test (Table 3) and f_p increased (2 units), did not change (4 units), or decreased (2 units). For Insp neurons f_i tended to increase and DU tended to decrease, although these changes were not significant according to the paired t-test (Table 3). Since the sample number was small, we considered that statistical evaluation of group responses by the paired t-test might have been impractical, so we repeated the evaluation with the Wilcoxon signed-rank test and obtained the almost same results.

Table 1. Firing frequency in the inspiratory phase of Pre-I neurones (f_{pi}) during various interventions

Intervention	f_{pi} (spikes/s)
Control	3.1 ± 2.3
Bicuculline (10 μM)	9.2 ± 4.0*** (n=13)
Control	1.8 ± 1.2
Picrotoxin (10 μM)	7.9 ± 3.3** (n=8)
Control	4.0 ± 3.0
Strychnine (5 μM)	10.3 ± 3.3* (n=6)
Control	3.8 ± 2.8
40% Cl^-	9.9 ± 4.8* (n=7)

Values are means ± SD (spikes/s) before (control) and in the presence of gamma-aminobutyric acid (GABA) antagonists, glycine antagonists or reduced chloride concentration (Cl^-). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by paired t-test

Effects of strychnine perfusion (2–10 μM) for 4–8 min on Pre-I neurones (18 units), Insp neurones (5 units) and C4 motorneurone activity were examined in 12 preparations. IIPI was inconspicuous after perfusion with 2–5 μM strychnine (Fig. 4 and Table 1). This effect was reversible. Strong seizure-like discharges appeared along with the normal C4 activity. Firing properties of most Pre-I neurones did not change during 5 μM strychnine treatment, although BR tended to increase. For Insp neurones, f_i and DU did not change or decreased.

Table 2. Changes in firing properties of Pre-I and Inspiratory (Insp) neurones during treatment with GABA, glycine, and their antagonists as well as reduced chloride concentration

Intervention	Parameter	Pre-I neurones (n)						Insp neurones (n)			
		f_p	R	BR	R	DE	R	f_i	R	DU	R
Bicuculline	+	6	1.42	7	1.42	0	–	5	1.94	0	–
10 μM	N	3	1.01	6	1.05	9	0.97	1	0.93	1	1.09
	–	4	0.69	0	–	4	0.51	0	–	5	0.47
	Total	13	1.10		1.25		0.83	6	1.78		0.57
Picrotoxin	+	2	1.34	3	1.28	0	–	2	1.50	0	–
10 μM	N	4	0.96	5	1.03	5	0.95	2	1.10	1	1.06
	–	2	0.64	0	–	3	0.53	0	–	3	0.63
	Total	8	0.97		1.12		0.79	4	1.30		0.74
Strychnine	+	1	1.19	3	1.50	0	–	0	–	0	–
5 μM	N	4	1.04	2	1.03	5	0.99	2	1.04	2	1.10
	–	1	0.81	1	0.88	1	0.60	1	0.74	1	0.42
	Total	6	1.03		1.24		0.93	3	0.91		0.81
40% Chloride	+	2	1.23	4	1.47	1	5.01	2	1.55	0	–
	N	2	1.03	2	0.88	5	1.11	2	1.11	1	1.14
	–	3	0.81	1	0.82	1	0.80	1	0.80	4	0.65
	Total	7	0.99		1.21		1.22	5	1.22		0.75
GABA	+	0	–	1	1.38	1	1.72	1	1.20	2	1.22
0.1 mM	N	1	0.86	0	–	2	0.91	3	0.95	2	1.13
	–	3	0.57	3	0.55	1	0.45	0	–	0	–
	Total	4	0.64		0.76		1.00	4	1.01		1.18
Glycine	+	0	–	1	1.35	0	–	0	–	2	1.31
0.2 mM	N	1	1.01	2	0.78	3	1.03	6	1.00	4	1.14
	–	3	0.58	1	0.36	1	0.21	0	–	0	–
	Total	4	0.69		0.82		0.82	6	1.00		1.17

+, Number of neurones exhibiting a significant increase ($P < 0.05$); N, number of neurones exhibiting no change; –, number of neurones exhibiting a significant decrease ($P < 0.05$); R, mean ratio of neurones that exhibited a change; R (total), arithmetic mean of R calculatd in each neurone; f_p, intraburst firing frequency of Pre-I-neurones; BR, burst rate; DE, delay time from initiation of Pre-I firing to the highest peak near the onset of C4 motorneurone inspiratory activity; f_i, intraburst firing frequency of inspiratory (Insp) neurones; DU, burst duration
Values shown are the ratio of the value measured during indicated treatment to that measured in control

Table 3. Average values of firing properties analysed in neurones in control and during experimental interventions

Intervention	Pre-I neurones			Insp neurones	
	f_p (spikes/s)	BR (bursts/min)	DE (ms)	f_i (spikes/s)	DU (ms)
Control	8.1 ± 3.1	6.0 ± 1.8	636 ± 505	16.1 ± 4.7	1023 ± 345
Bicuculline	8.7 ± 3.9	7.2 ± 1.4**	418 ± 235*	26.0 ± 9.4	514 ± 267*
(10 μM)	(n=13)			(n=6)	
Control	8.6 ± 4.1	7.3 ± 1.9	882 ± 131	12.5 ± 2.6	802 ± 445
Picrotoxin	8.4 ± 4.3	8.1 ± 2.3	679 ± 271	16.5 ± 5.9	518 ± 146
(10 μM)	(n=8)			(n=4)	
Control	12.4 ± 2.7	6.2 ± 2.5	871 ± 691	15.6 ± 8.2	942 ± 497
Strychnine	12.7 ± 2.8	7.1 ± 1.5	691 ± 495	13.4 ± 4.5	660 ± 197
(5 μM)	(n=6)			(n=4)	
Control	10.5 ± 4.8	5.6 ± 1.9	532 ± 573	13.8 ± 2.9	820 ± 212
40% Chloride	9.8 ± 4.5	6.3 ± 1.0	666 ± 403	16.2 ± 3.6	620 ± 304
	(n=7)			(n=5)	
Control	8.1 ± 1.2	6.2 ± 1.7	1076 ± 793	15.2 ± 8.5	1112 ± 301
GABA	5.2 ± 1.4*	4.4 ± 1.8	863 ± 372	15.1 ± 8.0	1302 ± 335*
(0.1 mM)	(n=4)			(n=4)	
Control	9.6 ± 2.6	6.6 ± 1.9	1215 ± 494	14.7 ± 4.1	512 ± 140
Glycine	6.2 ± 2.6	5.2 ± 1.8	1072 ± 791	14.8 ± 4.6	615 ± 176*
(0.2 mM)	(n=4)			(n=6)	

* $P < 0.05$; ** $P < 0.01$ by paired t-test
Values are means ± SD

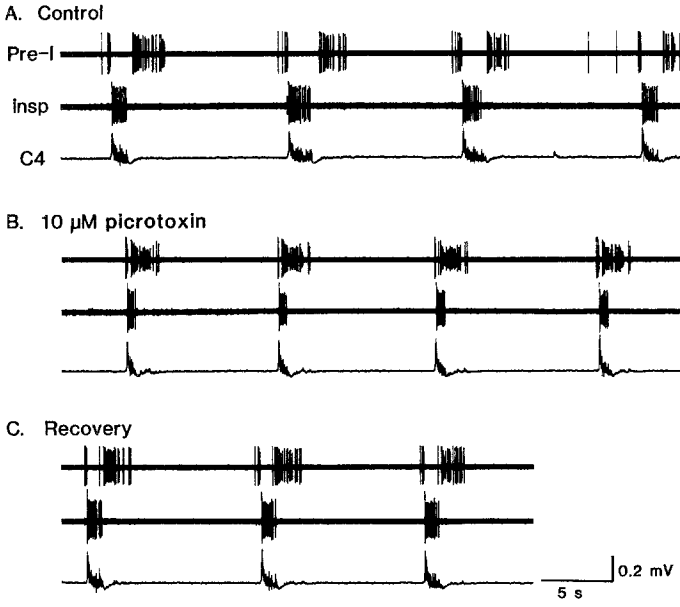

Fig. 3 A–C. Effects of 10 μM picrotoxin on Pre-I and Insp neurones. **A** Pre-I activity from left RVL (*Pre-I trace*), Insp activity from right RVL (*Insp trace*) and C4 activity (*C4 trace*) recorded from a 1-day-old rat preparation in standard bath solution. **B** Pre-I, Insp and C4 activity 10 min after perfusion with 10 μM picrotoxin. IIPI greatly diminished and f_p and BR increased to 137% and 117% of control respectively. DE decreased to 42% of control, f_i increased to 163% of control and DU decreased to 40% of control. All changes were significant. **C** Activity 20 min after perfusate was returned to standard solution

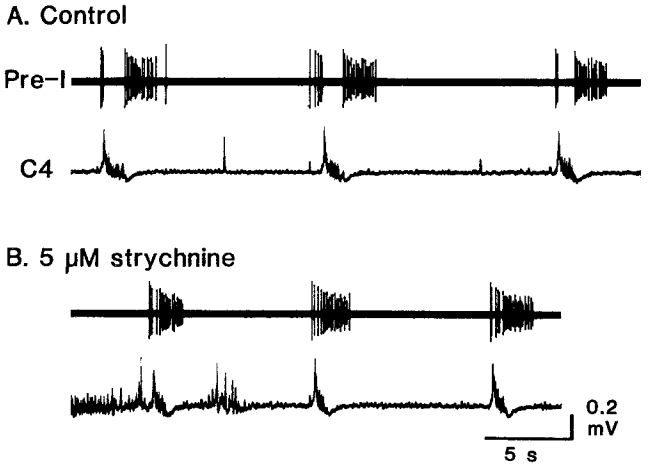

Fig. 4 A, B. Effects of 5 μM strychnine on Pre-I neurones. **A** Pre-I activity in RVL (*Pre-I trace*) and C4 activity (*C4 trace*) recorded from a 2-day-old rat preparation in standard bath solution. **B** Pre-I and C4 activity 4 min after perfusion with 5 μM strychnine. Note inconspicuous IIPI and seizure-like discharges during 5 μM strychnine perfusion. BR increased significantly to 130% of control

Reduced chloride concentration

Effects of reduced chloride concentration (to 0–80%) for 4–11 min perfusion on Pre-I neurones (23 units), Insp neurones (12 units) and C4 motorneurone activity were examined in 15 preparations. When chloride concentration was reduced below 50% of that in normal perfusate, IIPI was negligible or absent (Fig. 5, Table 1). Changes in the firing properties of Pre-I neurones (7 units, 6 preparations) and Insp neurones (5 units, 5 preparations) during perfusion of 40% chloride solution were analysed. The Pre-I BR significantly increased in 4 units, whereas f_p increased (2 units), did not chnage (2 units), or decreased (3 units). In Insp neurones, f_i tended to increase and DU tended to decrease. In chloride-free solution, strong tonic activity was induced in C4 motorneurones (4 preparations) and Pre-I neurones (4 units) tended to exhibit more or less tonic firing while their burst rhythmicity remained. All effects of reduced chloride concentration were reversible.

GABA and glycine

It was previously reported that GABA and glycine decreased the C4 (or C5) motorneurone burst rate [16]. Perfusion with 0.1 mM GABA for 2–5 min decreased both f_p and BR in 3 of 4 Pre-I units tested (4 preparations), and C4 motorneurone activity was then inhibited. In 1 unit, f_p decreased but BR increased and not every Pre-I burst was followed by a C4 motorneurone burst, therefore the C4 motorneurone BR decreased. In Insp neurones (4 units in 4 preparations), f_i was nearly constant but the DU increased. A specimen record is shown in Fig. 6. The seizure-like discharge in C4 observed in this figure (Fig. 6B) did not appear in three other preparations.

Perfusion with 0.2 mM glycine for 3–7 min significantly decreased f_p in 3 of 4 Pre-I units tested (4 preparations), and BR tended to decrease. Not every Pre-I burst was followed by a C4 burst. In 6 Insp neurones, there was no significant change of f_i, and DU tended to increase (Tables 2, 3). A specimen record is shown in Fig. 7.

Discussion

Firing properties of Pre-I neurones

IIPI consistently disappeared after application of the GABA (or glycine) antagonists or the reduction of chloride concentration. This result was verified from statistical analyses of f_{pi}, which increased significantly and then approximated to the mean values of f_p with these antagonists or reduced chloride concentration (Tables 1, 3). The results indicate IIPI dependence on some $GABA_A$- (or glycine-) like inhibitory mechanism. It has been previously reported that the

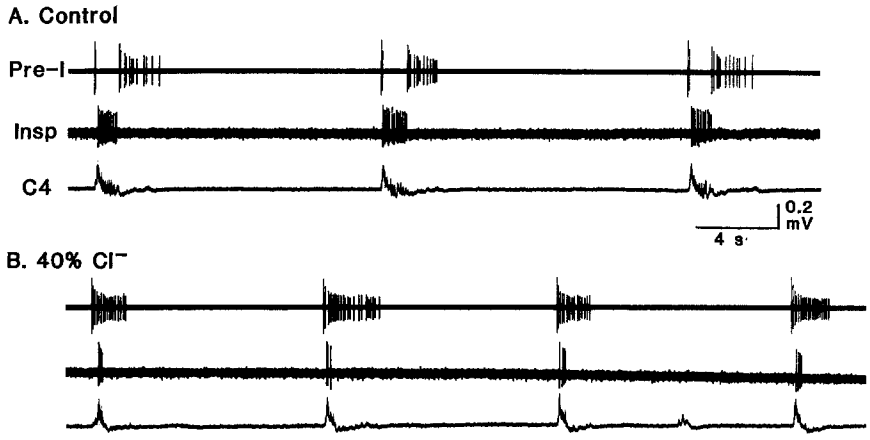

Fig. 5 A, B. Effects of reduced chloride concentration on Pre-I and Insp neurones. **A** Pre-I activity from right RVL (*Pre-I trace*), Insp activity from left RVL (*Insp trace*) and C4 activity (*C4 trace*) recorded from a 4-day-old rat preparation in standard bath solution. **B** Pre-I, Insp and C4 activity 4 min after perfusion with chloride solution at 40% of control. Note disappearance of IIPI. BR increased significantly to 145% of control, DU decreased significantly to 45% of control

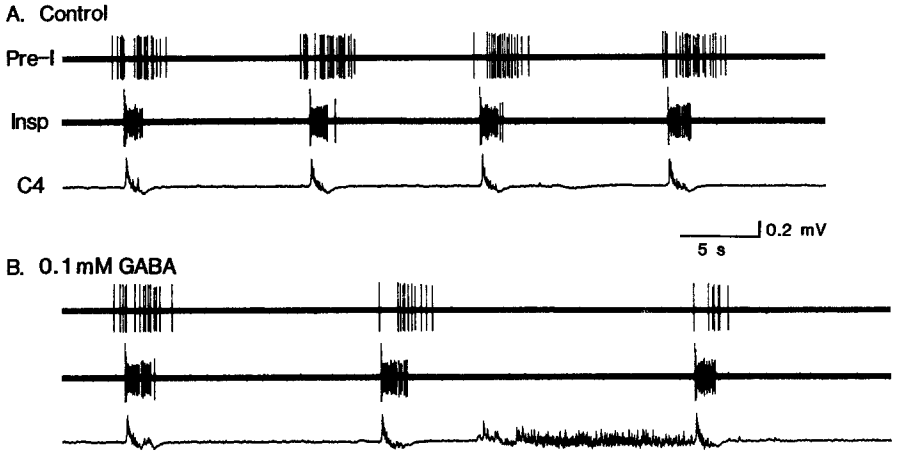

Fig. 6 A, B. Effects of 0.1 mM gamma aminobutyric acid (GABA) on Pre-I and Insp neurones. **A** Pre-I activity from left RVL (*Pre-I trace*), Insp activity from right RVL (*Insp trace*) and C4 activity (*C4 trace*) recorded from a 4-day-old rat preparation in standard bath solution. **B** Pre-I, Insp and C4 activity 1 min after perfusion with 0.1 mM GABA. f_p and BR decreased significantly to 49% and 65% of control respectively. The seizure-like discharge seen in C4 in this example is not commonly observed

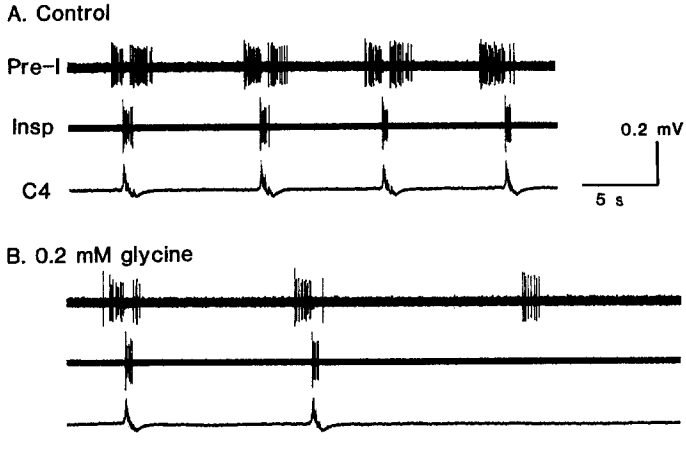

Fig. 7 A, B. Effects of 0.2 mM glycine on Pre-I and Insp neurones. **A** Pre-I activity from right RVL (*Pre-I trace*), Insp activity from left RVL (*Insp trace*) and C4 activity (*C4 trace*) recorded from a 2-day-old rat preparation in standard bath solution. **B** Pre-I, Insp and C4 activity 3 min after perfusion with 0.2 mM glycine. f_p decreased significantly to 66% of control. BR also decreased to 57% of control, but this was not significant

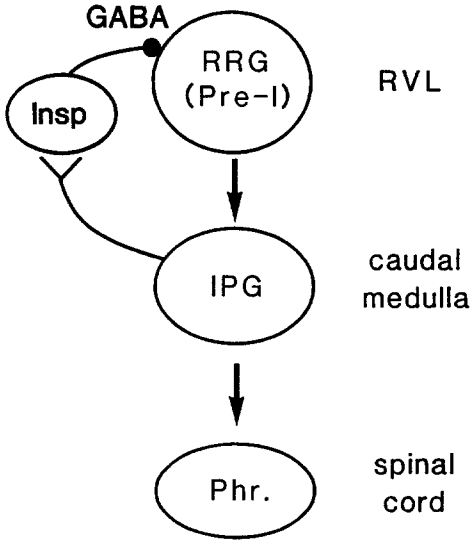

Fig. 8. Hypothetical scheme of the generation of rhythmic respiratory activity in the brainstem-spinal cord preparation from newborn rat. *RRG*, Respiratory rhythm generator; *IPG*, inspiratory pattern generator; *Phr*, phrenic motoneurones. RRG, composed of Pre-I neurones in the RVL, produces primary rhythm of respiration and triggers IPG. IPG, possibly located in the caudal ventral medulla, generates patterned inspiratory activity. Insp receive excitatory synaptic inputs from IPG and inhibit Pre-I activity during inspiration through GABA receptors of Pre-I neurones

specificity of GABA and glycine antagonists was unclear in brainstem-spinal cord preparations [15].

In normal solution, the initiation of IIPI always corresponds to the onset of inspiration despite fairly wide variation in DE (Table 3). Furthermore, depression of inspiratory activity, which is caused by lesion of the CVL [19] or stimulation of the vagus nerve [17], prevents IIPI. Therefore, we believe that IIPI depends on inhibitory synaptic input to Pre-I neurones, although this possibility should be verified by providing more direct evidence to show the presence of synaptic inhibition of the Pre-I neurones.

The BR value of Pre-I neurones tended to increase during treatment with GABA antagonists or during reduction of chloride concentration. On the other hand, GABA caused reduction of BR and f_p. These results suggest that Pre-I neurone activity might be influenced directly and/or indirectly by tonic (nonphasic) $GABA_A$-like synaptic inhibition. However, it has been reported that bicuculline produced direct membrane depolarization by reducing membrane potassium conductance in mouse spinal cord neurons [12]. This nonsynaptic effect appeared at a higher bicuculline concentration (5–200 μM) than for antagonism of postsynaptic GABA responses (0.2–10 μM). Therefore, effects of 10 μM bicuculline on Pre-I and Insp neurones in the present study seemed to be due mainly to GABA antagonism, although the effects might partly include nonsynaptic action. An increase of f_p is consistent with GABA antagonism, but a decrease in some other neurones cannot be interpreted so simply. These results may indicate pharmacological heterogeneity, such as different resistance to antagonists of Pre-I neurones, and/or involvement of other inhibitory systems besides $GABA_A$ (or glycine) in the modulation of Pre-I activity.

Firing properties of Insp neurones

The f_i value of Insp neurones tended to increase, and DU tended to decrease during treatment with GABA antagonists and the same tendencies appeared during reduction of chloride concentration. These changes may be a result of membrane depolarization of Insp neurones due to the treatment, since the depolarization and increase in firing frequency presumably cause faster inactivation of ion channels in Insp neurones [10]. Further, decrease of DE implies that the IPG was more easily triggered by Pre-I firing during treatment with GABA antagonists. Despite marked reduction of the C4 BR during perfusion with GABA or glycine, Insp neurones fired with nearly a normal f_i, but had rather long DU value when the IPG was triggered. Our preliminary observations show that the amplitude and duration of C4 bursts did not decrease, whereas the C4 BR diminished to 5–30% of control during 10–30 min perfusion with 0.2 mM GABA or 0.5 mM glycine. This suggests that the Insp neurones in the RVL and probably the Insp neurones composing the IPG possess few GABA or glycine receptors. Therefore, the effects of GABA antagonists on Insp neurones might be produced by disinhibition of excitatory inputs to, rather than by direct action on, the Insp neurones.

These results imply that $GABA_A$-like inhibitory synaptic interaction is less involved in termination of the inspiratory phase in newborn rat preparations (see also [26]). On the other hand, in adult animals (in vivo), phasic inhibitory synaptic inputs to Insp neurones are probably important to this termination [2, 5, 6, 10, 22]. This difference might be attributable to developmental changes in synaptic connections of neuronal networks in the respiratory centre.

The present results seem to differ in some respects from those by Smith and Feldman [24, 26], in which blockage of chloride-dependent synaptic inhibition increased the duration and amplitude of inspiratory motor bursts recorded from cranial and spinal roots. This may be due to different experimental conditions such as concentration of antagonists used (e.g. 0.1–1 mM bicuculline in their experiments), time of treatment and/or perfused area. That is to say, Smith and Feldman [24, 26] only altered the medium bathing the brainstem, whereas we changed the medium around the brainstem and the spinal cord.

Seizure-like discharges

Treatment with GABA or glycine antagonists induced seizure-like discharges in C4 motorneurones, which affected the respiratory rhythm to a greater lesser degree. Since these discharges are considered to occur synchronously in the brainstem and spinal cord [23], this acitivity could affect Pre-I firing and consequently the respiratory rhythm. Tonic C4 discharges induced by low chloride concentration suggest tonic synaptic inhibition of C4 motorneurones. However, the origin of these discharges and their relation to activity of the phrenic motorneurones is still unclear.

Synaptic inhibition in respiratory rhythm generation

Generation of respiratory rhythm under blocking chloride-dependent synaptic inhibition was first reported in the isolated brain preparation of the lamprey [23]. In brainstem-spinal cord preparations isolated from newborn rats, it was suggested that chloride-dependent ($GABA_A$ or glycine receptor-mediated) synaptic inhibition [24, 26] and $GABA_B$-mediated potassium-dependent inhibition [8] are not essential for respiratory rhythm generation, but the synaptic inhibition may contribute to the modulation. They proposed the involvement of an intrinsic pacemaker-driven oscillator in rhythm generation [26].

On the other hand, we hypothesized previously that respiratory rhythm in the newborn rat preparation is basically generated by a neuronal network composed of excitatory synapses among Pre-I intrinsic pacemaker cells [19, 21]. Present results, showing preserved Pre-I rhythm after the disappearance of IIPI, suggest that this phasic postsynaptic inhibition is not required for the generation of Pre-I rhythm. The results also suggest that a pathway to the phrenic motorneurone group through an IPG from a rhythm generator in the medulla could still function, even though $GABA_A$- (or glycine-) like inhibitory synaptic interaction is depressed.

A hypothetical model of the generation of rhythmic respiratory activity in a brainstem/spinal cord preparation from newborn rat is shown in Fig. 6. The IPG is periodically

triggered by a rhythm generator which is composed of Pre-I neurones in the RVL. The role of the Insp neurones in the RVL (RVL-I neurones) examined in the present study is unknown. However, firing of RVL-I neurones should reflect IPG activity and at least some RVL-I neurones are presumed to inhibit Pre-I neurones during the inspiratory phase. The possibility still remains that some RVL-I neurones are involved in inspiratory pattern generation.

Conclusion

The results reported here suggest that inspiration-related inhibition of Pre-I activity was induced by chloride-dependent GABA- (or glycine-) like inhibitory interaction (probably inhibitory synaptic inputs to Pre-I neurones). Pre-I rhythm generation does not require this phasic inhibition. The present results were consistent with previous studies [23, 26] suggesting that rhythmic respiratory motor output could be generated without chloride-dependent synaptic inhibition. However, the rhythm generator and IPG are variably influenced by blocking chloride-dependent inhibitory interaction. Therefore, tonic $GABA_A$-like inhibition, which might be synaptic, is probably involved in the modulation of these functions.

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References

1. Arata A, Onimaru H, Homma I (1990) Respiration-related neurons in the ventral medulla of newborn rats *in vitro*. Brain Res Bull 24: 599–604
2. Ballantyne D, Richter DW (1984) Post-synaptic inhibition of bulbar inspiratory neurones in the cat. J Physiol (Lond) 348: 67–87
3. Ballantyne D, Richter DW (1986) The non-uniform character of expiratory synaptic activity in expiratory bulbospinal neurones of the cat. J Physiol (Lond) 370: 433–456
4. Bianchi AL, Grélot L, Iscoe S, Remmers JE (1988) Electrophysiological properties of rostral medullary respiratory neurones in the cat: an intracellular study. J Physiol (Lond) 407: 293–310
5. Champagnat J, Denavit-Saubie M, Moyanova S, Rondouin G (1982) Involvement of amino acids in periodic inhibitions of bulbar respiratory neurons. Brain Res 237: 351–365
6. Ezure K, Manabe M (1988) Decrementing expiratory neurons of the Bötzinger complex. II. Direct inhibitory synaptic linkage with ventral respiratory group neurons. Exp Brain Res 72: 159–166
7. Feldman JL, Smith JC (1986) Separate generation of oscillation and motor burst pattern for mammalian respiration: *in vitro* studies. Soc Neurosci Abstr 12: 791
8. Feldman JL, Smith JC (1989) Cellular mechanisms underlying modulation of breathing pattern in mammals. In: Davis M, Jacobs BL,Schoenfeld RI (eds) Modulation of defined vertebrate neural circuits. Ann NY Acad Sci 563: 114–130
9. Grélot L, Iscoe S, Bianchi AL (1988) Effects of amino acids on the excitability of respiratory bulbospinal neurons in solitary and para-ambigual regions of medulla in cat. Brain Res 443: 27–36
10. Haji A, Connelly C, Schultz SA, Wallace J, Remmers JE (1987) Postsynaptic actions of inhibitory neutrotransmitters on bulbar respiratory neurons. In: Euler C von, Langercrantz H (eds) Neurobiology of the control of breathing. Raven Press, New York, pp 187–194
11. Harada Y, Kuno M, Wang YZ (1985) Differential effects of carbon dioxide and pH on central chemoreceptors in the rat *in vitro*. J Physiol (Lond) 368: 679–693
12. Heyer EJ, Nowak LM, MacDonald RL (1981) Bicuculline: a convulsant with synaptic and nonsynaptic actions. Neurology 31: 1381–1390
13. Lindsey BG, Segers LS, Shannon R (1987) Functional associations among simultaneously monitored lateral medullary respiratory neurons in the cat. II. Evidence for inhibitory actions of expiratory neurons. J Neurophysiol 57: 1101–1117
14. Merrill EG, Lipski J, Kubin L, Fedorko L (1983) Origin of the expiratory inhibition of nucleus tractus solitarius inspiratory neurones. Brain Res 263: 43–50
15. Murakoshi T, Otsuka M (1985) Respiratory reflexes in an isolated brainstem-lung preparation of the newborn rat: possible involvement of γ-aminobutyric acid and glycine. Neurosci Lett 62: 63–68
16. Murakoshi T, Suzue T, Tamai S (1985) A pharmacological study on respiratory rhythm in the isolated brainstem-spinal cord preparation of the newborn rat. Br J Pharmacol 86: 95–104
17. Onimaru H, Homma I (1987) Respiratory rhythm generator neurons in medulla of brainstem-spinal cord preparation from newborn rat. Brain Res 403: 380–384
18. Onimaru H, Arata A, Homma I (1987) Localization of respiratory rhythm-generating neurons in the medulla of brainstem-spinal cord preparations from newborn rats. Neurosci Lett 78: 151–155
19. Onimaru H, Arata A, Homma I (1988) Primary respiratory rhythm generator in the medulla of brainstem-spinal cord preparation from newborn rat. Brain Res 445: 314–324
20. Onimaru H, Arata A, Homma I (1988) Inhibitory synaptic inputs to respiratory rhythm generator in medulla isolated from newborn rats. J Physiol Soc Jpn 50: 584
21. Onimaru H, Arata A, Homma I (1989) Firing properties of respiratory rhythm generating neurons in the absence of synaptic transmission in rat medulla in vitro. Exp Brain Res 76: 530–536
22. Richter DW (1982) Generation and maintenance of the respiratory rhythm. J Exp Biol 100: 93–107
23. Rovainen CM (1983) Generation of respiratory activity by the lamprey brain exposed to picrotoxin and strychnine, and weak synaptic inhibition in motoneurons. Neuroscience 10: 875–882
24. Smith JC, Feldman JL (1986) Role of chloride-dependent synaptic inhibition in respiratory pattern generation. Studies in an *in vitro* mammalian brainstem-spinal cord preparation. Fed Proc 45: 518
25. Smith JC, Feldman JL (1987) In vitro brainstem-spinal cord preparations for study of motor systems for mammalian respiration and locomotion. J Neurosci Methods 21: 321–333
26. Smith JC, Feldman JL (1987) Central respiratory pattern generation studied in an in vitro mammalian brainstem-spinal cord preparation. In: Sieck GC, Gandevia SG, Cameron WE (eds) Respiratory muscles and their neuromotor control. Liss, New York, pp 27–36
27. Suzue T (1984) Respiratory rhythm generation in the *in vitro* brain stem-spinal cord preparation of the neonatal rat. J Physiol (Lond) 354: 173–183