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Tachykinins and central respiratory activity: an in vitro study on the newborn rat

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Abstract

The newborn rat brainstem-spinal cord preparation was used to study the effects of tachykinins on the activity of the respiratory rhythm generator in vitro and to characterize the receptors involved. Substance P and tachykinin NK_1 and NK_3 receptor agonists induced a concentration-dependent increase in the respiratory frequency (10^{-9}–10^{-7} M), whereas the respiratory frequency was only slightly affected by the tachykinin NK_2 receptor agonist. Pre-treatments with tachykinin NK_1 receptor antagonists (SR140333, (*S*)1-{2-[3-(3.4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-*n*-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2]octane chloride; GR82334, pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-(S-S)Pro-Leu(spiro-γ-lactam)-Trp-NH_2) reduced the substance P-induced increases in the respiratory frequency but the tachykinin NK_2 receptor antagonists (SR48968, ((*S*)-*N*-methyl-*N*-[4-(4-acetylamino-4-phenylpiperidine)-2-(3,4-dichlorophenyl)butyl]benzamide); MEN 10376, Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2) had no effect; the increase in the respiratory frequency induced by the tachykinin NK_3 receptor agonist was not affected by a pre-treatment with tachykinin NK_1 and NK_2 receptor antagonists. These results indicate that tachykinin NK_1 and NK_3 receptors may be involved in the control of the respiratory frequency.

Keywords: Respiratory rhythm; Substance P; Tachykinin; (In vitro); (Newborn rat)

1. Introduction

Tachykinins are a family of peptides among which substance P was the first to be evidenced (Von Euler and Gaddum, 1931) and purified (Chang et al., 1971). Two other mammalian tachykinins were subsequently isolated in the 1980s and termed neurokinin A and neurokinin B. Three types of receptors, termed NK_1, NK_2 and NK_3, mediate the effects of tachykinins in the nervous system and other tissues (Otsuka and Yoshioka, 1993; Maggi et al., 1993); these receptors are members of the G-protein-linked family of metabotropic receptors (Nakanishi, 1991). Substance P displays a higher affinity for tachykinin NK_1 receptor, neurokinin A for the tachykinin NK_2 receptor and neurokinin B for the tachykinin NK_3 receptor, but all the tachykinins are able to act as agonists on each type of receptors (Regoli et al., 1987). There exists a large body of data suggesting that substance P plays a crucial role in the lower brainstem, particularly in the afferent nerve terminals and areas dealing with autonomic functions such as cardiovascular or respiratory regulation. Substance P is present in cranial nerves (9th and 10th) connected to arterial baroreceptors and chemoreceptors and at their projection sites within the nucleus of the tractus solitarius (Kalia et al., 1984; Gillis et al., 1980; Lindefors et al., 1986). Only a fraction of the substance P content of the nucleus of the tractus solitarius is provided however by afferents, as indicated by denervation experiments (Gillis et al., 1980). Several results have suggested that substance P might be involved in respiratory regulation: neurons and terminals containing substance P have been found to exist in the ventrolateral medulla (Liebstein et al., 1985) and the nucleus ambiguus (Holtman, 1988), both of which areas belong to the so-called 'respiratory centres'. The effects of substance P on the respiratory activity were first documented by Von Euler and Pernow (1956). Intraventricular injection of substance P (see also Hedner et al., 1984; Yamamoto and Lagercrantz, 1985) or microinjections of substance P into the nucleus of the tractus solitarius (Chen et al., 1990) induced an increase in the respiratory fre-

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quency. Furthermore, substance P applied by microiontophoresis exerted excitatory effects on neurons of the nucleus of the tractus solitarius (Morin-Surun et al., 1984) and on respiratory neurons (Rampin et al., 1993). Tachykinin receptor antagonists applied at the medullary level blocked the respiratory stimulating effect of hypoxia (Yamamoto and Lagercrantz, 1985) or induced respiratory depression or apnoea (Chen et al., 1988). Several results have suggested that substance P may play an important role during the perinatal period. Higher concentrations of substance P than those present in adults are to be found in the medulla of rabbit pups and human babies (Bergström et al., 1984; Yamamoto and Lagercrantz, 1985); the finding that the levels of preprotachykinin mRNA in the nucleus of the tractus solitarius of rabbit foetus drastically increase on the day of birth (Srinivasan et al., 1991) suggests that substance P may play a crucial role in the onset of breathing.

The in vitro brainstem-spinal cord preparation of the newborn rat makes it possible to study the central effects of various neurotransmitter systems (Hilaire et al., 1989; Morin et al., 1990a,b) after eliminating the afferent pathways. In this preparation, substance P has been found to enhance the respiratory rhythm, but some inhibitory effects were also reported to occur (Yamamoto et al., 1992). The present experiments were undertaken in order to investigate the effects of substance P on the medullary rhythm generator and to determine the type of tachykinin receptors involved. The results indicate that substance P may exert potent facilitatory effects on the respiratory rhythm generator which depend upon the activation of tachykinin NK_1 receptors, but the involvement of tachykinin NK_3 receptors cannot be ruled out.

2. Materials and methods

2.1. Electrophysiological experiments

The methods used in the present study have been described previously in detail (Suzue, 1984; Hilaire et al., 1989). Newborn rats (0–3 days old) were anaesthetised with ether and decerebrated just rostrally to the Vth cranial nerves. Another section was performed at the level of the last cervical vertebra. The skin, muscles and bones were rapidly removed. The preparation was placed with the ventral surface upwards in a chamber permanently perfused with artificial cerebrospinal fluid (aCSF). The aCSF (in mM, NaCl 129, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.15, $NaHCO_3$ 21, NaH_2PO_4 0.58, glucose 30) was warmed to 27 ± 0.5°C and equilibrated with 95% O_2 and 5% CO_2.

The electrical activity of the cervical ventral roots (generally C4) was recorded using suction electrodes. The signals were filtered (5–3000 Hz), amplified, fed to leaky integrators and displayed on an oscilloscope and a paper recorder. The respiratory frequency was defined as the frequency of occurrence of the discharge from the cervical ventral roots.

Drugs (agonists and antagonists) were prepared just before the superfusion procedure by diluting stock solutions (1 ml, stored at −80°C) in 100 ml of aCSF. All the agonists were applied for 6 min. Antagonists were applied alone for periods ranging from 15–60 min, and then for 6 min in association with substance P or a tachykinin receptor agonist. One drug at one concentration (or one antagonist associated with one agonist) was tested in each experiment. In some experiments the recording chamber was divided by placing a barrier, improved with vaseline, at the level of the first cervical root in order to apply the aCSF containing a drug to either the brainstem or the spinal cord alone.

2.2. Drugs

Substance P, [Sar^9,Met(O$_2$)^{11}]substance P, [β-Ala^8]neurokinin A-(4–10), succinyl-[Asp^6,*N*-Me-Phe^8]substance P (senktide), and [pGlu^6,Pro^9]substance P-(6–11) (septide) were purchased from Sigma and GR 82334 (pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-(S-S)Pro-Leu(spiro-γ-lactam)-Trp-NH_2) and MEN 10376 (Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2) from Neosystem Laboratoire. SR140333 (*S*)1-{2-[3-(3.4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-*n*-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2,2,2]-octane chloride), SR48968 ((*S*)-*N*-methyl-*N*-[4-(4-acetylamino-4-phenylpiperidine)-2-(3,4-dichlorophenyl)butyl]-benzamide) and SR142801 ((*S*)-(*N*)-1-53-51-benzoyl-3-(3,4-dichlorophenyl)-piperidin-3-yl)propyl)-4-phenylpiperidin-4-yl)-*N*-methylacetamide were gifts from SANOFI Recherche.

2.3. Analysis of results

The number of respiratory discharges was measured during each minute to estimate the respiratory frequency (min^{-1}). From these measurements, the latency of drug effect, the time to peak effect, and the decay time were calculated. Furthermore, prior to the application of any drugs, the mean ($\pm$S.E.) duration of the respiratory cycle (and the mean respiratory frequency) or the mean amplitude of the integrated inspiratory activity were calculated by precisely measuring them during 10–20 cycles. Similar measurements were repeated during the period corresponding to the peack effect of the drug (i.e., for substance P and tachykinin receptor agonists between the 4th and the 6th min of drug application) and also when returning to baseline levels. The series of values were compared (using a non-parametric test for unpaired values). Any changes in the respiratory frequency or amplitude are expressed as a percentage of the control values. Each type of experiment was repeated on at least 5 different newborn rats from different litters. The series of control and test values for each type of experiment were compared (using a non-para-

metric test for paired values). The data were averaged to determine the mean effect of a drug at a given concentration. Any changes were taken to be significant at P values of less than 0.05.

3. Results

3.1. Effects of substance P

3.1.1. Effects of substance P on respiratory frequency

Fig. 1A illustrates the baseline respiratory activity of the in vitro brainstem-spinal cord preparation of the newborn rat. With the preparations ($n = 140$) used in this study, the mean control respiratory frequency was 3.8 ± 0.2 min^{-1}. Superfusing the preparation with aCSF containing substance P increased (latency: 40 ± 10 s) the respiratory frequency in a concentration-dependent manner. Substance P concentrations ranging between 10^{-10} and 10^{-7} M were used. At each of these concentrations, except for 10^{-10} M, substance P induced a significant increase in the respiratory frequency (Table 1). There was no significant difference between the increases in the respiratory frequency induced by concentrations of 10^{-7} and 10^{-8} M or by concentrations of 5×10^{-9} and 10^{-9} M; the increases induced by these two groups of concentrations were significantly different, however. Fig. 1B illustrates the mean effect of substance P (10^{-8} M) and the recovery which occurs when returning to the normal medium.

Fig. 2A shows the relationships between the baseline respiratory frequency and the increases in frequency (expressed as percentage of control frequency) induced by application of aCSF containing substance P. Although the excitatory effect of substance P is stronger when the control frequency is lower, substance P always exerts an excitatory effect. In no instance did the respiratory frequency ever decrease in response to substance P application. The basal respiratory frequency increases drastically as the result of a transection eliminating the pons (Hilaire et al., 1989). After this transection ($n = 8$), the mean basal respiratory frequency stabilized at 9.3 ± 0.9 min^{-1} and the application of aCSF containing substance P (10^{-7} M) still induced a limited (10.6 ± 1 min^{-1}) but significant (paired values) increase in the respiratory frequency (Fig. 2B). No inhibitory effects were ever observed in these preparations.

3.1.2. Other effects of substance P

The amplitude of the inspiratory integrated activity was not significantly affected by the application of an aCSF containing substance P to the whole brainstem-spinal cord preparation. In the 12 experiments in which this parameter was determined (substance P: 10^{-7} M), the amplitude of the integrated inspiratory activity either remained unchanged, decreased significantly or increased significantly ($n = 7$, 3 and 2, respectively). Application of substance P ($n = 9$, 10^{-7} M) at the brainstem level alone induced: (i) a significant increase in the respiratory frequency which was similar to that induced when substance P was applied to the whole preparation, and (ii) a significant decrease in the amplitude of the integrated inspiratory activity ($19.9 \pm 5\%$; Fig. 3A). In response to substance P ($n = 13$) applied at the spinal level alone, (i) no change in the respiratory frequency (Fig. 3B), and (ii) a significant increase in the amplitude of the inspiratory integrated activity ($15.8 \pm 3.6\%$; Fig. 3B,C) were observed.

Besides its effects on the respiratory frequency or the amplitude, substance P (10^{-7}, 10^{-8} M) triggered non-respiratory motor activities in 36% of the experiments in the

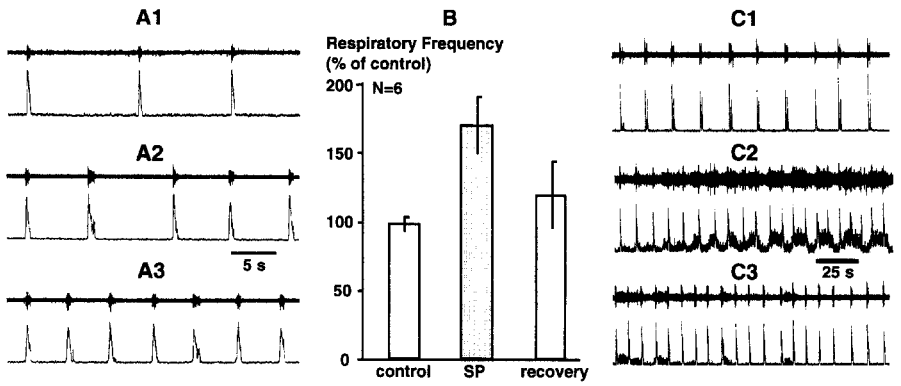

Fig. 1. Effects of substance P and tachykinin NK_1 receptor agonist on the respiratory frequency. Panels A and C give the raw (top) and integrated (bottom) inspiratory motor discharge from a cervical ventral root. (A) Effects of superfusion with aCSF containing substance P (10^{-7} M); A1, control activity; A2, A3, increase in the respiratory frequency (after 3 and 6 min, respectively). (B) The histograms represent the mean respiratory frequency (as a percentage of control values) measured under normal aCSF, during the 2 last min of substance P (10^{-8} M) application, and the recovery (4–6 min after returning to normal aCSF). Respiratory frequency during substance P application is different from the respiratory frequency during control and recovery ($P = 0.03$ in both cases). Respiratory frequency during control and recovery are not significantly different. (C) Effect of superfusion with aCSF containing the tachykinin NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]substance P (10^{-7} M). C1, control activity; C2 (4 min after changing the bathing medium); note the increase in the respiratory frequency and the emergence of another rhythmic motor pattern (sometimes a tonic activity without any discernible rhythm was evoked). After returning to normal medium (C3, 3 min) the tonic activity disappeared and the respiratory frequency slowly returned to control values.

Table 1
Effects of substance P (SP) and tachykinin receptor agonists on the respiratory frequency; mean increase in the respiratory frequency as compared to the control level

Drug	10^{-10} M	10^{-9} M	5×10^{-9} M	10^{-8} M	10^{-7} M
Substance P	NS	20 ± 7%	26 ± 7%	70 ± 20%	91 ± 11%
	$n = 5$	$n = 6$	$n = 10$	$n = 6$	$n = 5$
[Sar^9,Met(O$_2$)^{11}]SP	NS	29 ± 7%	NT	81 ± 16%	95 ± 18%
	$n = 4$	$n = 5$		$n = 5$	$n = 6$
[β-Ala^8]neurokinin A-(4–10)	NT	NS	NT	20 ± 7%	16 ± 5%
		$n = 4$		$n = 5$	$n = 5$
Succinyl-[Asp^6,*N*-Me-Phe^8]SP	NS	29 ± 7%	NT	95 ± 13%	106 ± 6%
	$n = 5$	$n = 8$		$n = 12$	$n = 5$

NS: non-significant effect; NT: drug not tested at this concentration.

form of either tonic discharges (as described by Murakoshi et al., 1985; Yamamoto et al., 1992) or rhythmic motor patterns (similar to those in Fig. 1C), which were clearly different from the respiratory activity.

3.2. Effects of tachykinin receptor agonists

3.2.1. Tachykinin NK_1 receptor agonist

[Sar^9,Met(O$_2$)^{11}]substance P was used as tachykinin NK_1 receptor agonist and applied to the brainstem-spinal cord preparation at concentrations ranging between 10^{-10} and 10^{-7} M. The respiratory frequency increased significantly (mean latency: 40 s) at every concentration except for 10^{-10} M (Fig. 1C; Table 1). Another tachykinin NK_1 receptor agonist, [pGlu^6,Pro^9]substance P-(6–11) also induced a significant increase in the respiratory frequency (92 ± 31%; $n = 5$; 10^{-7} M). Non-respiratory motor activities were generally elicited by [Sar^9,Met(O$_2$)^{11}]substance P (Fig. 1C; mean latency: 3 min). The occurrence of these motor activities seems to be concentration-dependent (n = 0/5, 4/5 and 6/6 at 10^{-9}, 10^{-8} and 10^{-7} M, respectively). Application of [Sar^9,Met(O$_2$)^{11}]substance P to the spinal cord only ($n = 6$; 10^{-8} M) significantly increased the amplitude of the integrated inspiratory activity (5/6; 16 ± 2.7%; Fig. 3C) and triggered non-respiratory motor activities (3/6).

3.2.2. Tachykinin NK_2 receptor agonist

The tachykinin NK_2 receptor agonist [β-Ala^8]neurokinin A-(4–10) was used at concentrations ranging from

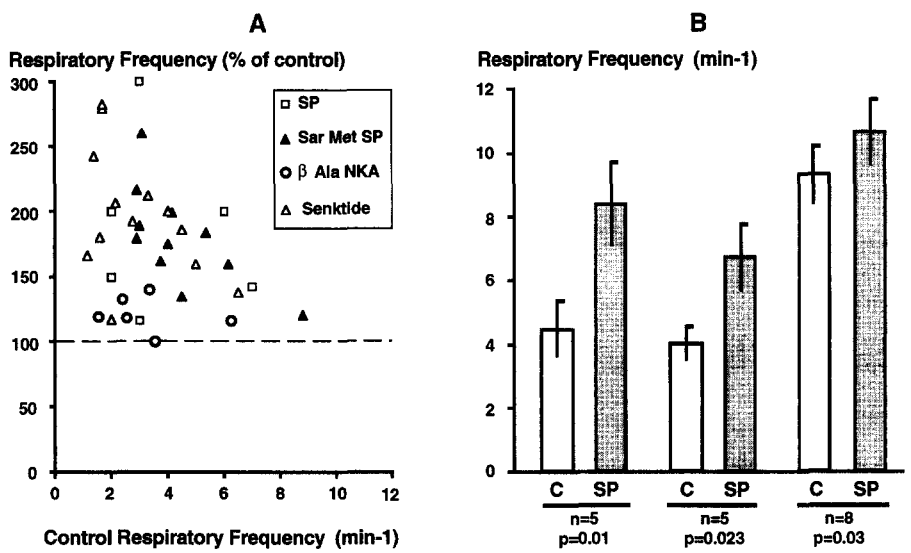

Fig. 2. (A) Relationships between control respiratory frequency and the increase in the frequency (as a % of the control level) induced by substance P and tachykinin receptor agonists ([Sar^9,Met(O$_2$)^{11}]substance P, [β-Ala^8]neurokinin A-(4–10) and senktide; 10^{-8} M). (B) Changes in the respiratory frequency (min^{-1}) induced by substance P (10^{-7} M) under various conditions as compared to the control respiratory frequency. The couples of vertical bars give the control respiratory frequency (C) and the respiratory frequency recorded during application of aCSF containing substance P under three different experimental conditions. From left to right. First couple: standard preparation (pons and medulla) and application of aCSF containing substance P to the whole preparation. Second couple: standard preparation where a barrier was placed at the level of the first spinal roots and the aCSF containing substance P was applied to the brainstem only. The increases in the respiratory frequency evoked in both cases were not significantly different. Third couple: medullary preparation (i.e. with a transection performed at the level of VIth cranial nerves to eliminate the pontine structures). This transection elicited a sustained increase in the respiratory frequency, which increased almost two-fold. Despite this fast control respiratory frequency, application of aCSF containing substance P still significantly increased the respiratory frequency (paired values).

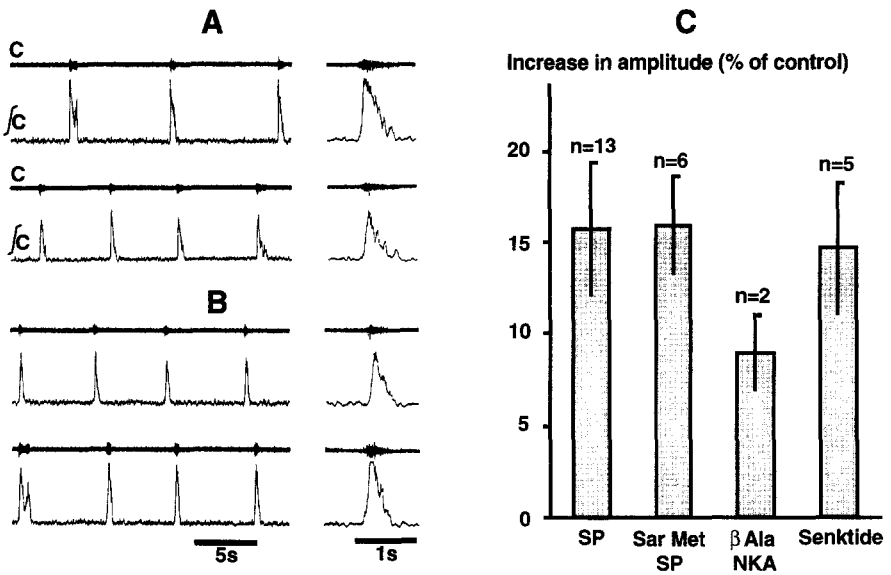

Fig. 3. Substance P and amplitude of the integrated respiratory motor output. (A,B) Raw and integrated cervical motor discharge (slow and fast time bases); top, control respiratory activity; bottom, respiratory activity during application of substance P (10^{-7} M). A barrier was placed at the level of the first cervical ventral roots (C1) in order to apply various aCSF to the spinal cord and the brainstem. (A) The aCSF containing substance P was applied to the brainstem (and normal aCSF to the spinal cord); note that the respiratory frequency increased, whereas the amplitude of the integrated inspiratory discharge decreased. (B) The aCSF containing substance P was applied to the spinal cord (and normal medium to the brainstem); note that the amplitude of the integrated inspiratory activity increased but not the respiratory frequency. (C) Diagram summarizing the changes in the amplitude of the integrated inspiratory activity induced by substance P and tachykinin receptor agonists ([Sar^9,$Met(O_2)^{11}$]substance P, [β-Ala^8]neurokinin A-(4–10) and senktide; 10^{-7} M) applied to the spinal cord.

10^{-9} to 10^{-7} M. Limited and delayed (mean latency: 2 min) increases in the respiratory frequency (Table 1) were elicited by concentrations of 10^{-8} and 10^{-7} M, respectively, whereas a concentration of 10^{-9} M had no effect. [β-Ala^8]neurokinin A-(4–10) triggered limited non-respiratory motor activities (0/5 and 3/5 at 10^{-8} and 10^{-7} M, respectively). Application of the tachykinin NK_2 receptor agonist to the spinal cord alone resulted in a limited

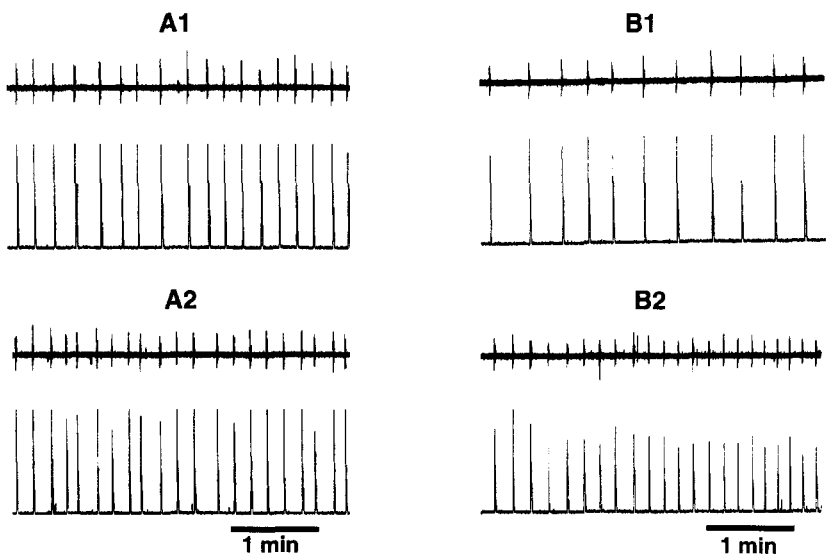

Fig. 4. Effects of tachykinin NK_1 and NK_2 receptor antagonists. (A,B) Raw (top) and integrated (bottom) respiratory activity. A1, respiratory activity recorded during application (60 min) of aCSF containing the tachykinin NK_1 receptor antagonist SR140333 (10^{-7} M); A2, the preparation was then bathed (6 min) with aCSF containing the tachykinin NK_1 receptor antagonist SR140333 (10^{-7} M) and substance P (10^{-8} M). Note that substance P induced a limited increase in the respiratory frequency (as compared with the 70 ± 20% increase recorded when no pre-treatment was applied). B1, respiratory activity recorded during application (15 min) of aCSF containing both tachykinin NK_1 (GR 82334, 10^{-7} M) and tachykinin NK_2 (MEN 10376; 10^{-7} M) receptor antagonists. B2, the preparation was then bathed (6 min) with aCSF containing both tachykinin receptor antagonists and the tachykinin NK_3 receptor agonist (senktide, 10^{-8} M). Note the increase in the respiratory frequency.

increase in the integrated inspiratory activity ($n = 2$, $9 \pm 2\%$; Fig. 3C), which was not accompanied by any non-respiratory motor output.

3.2.3. Tachykinin NK_3 receptor agonist

Succinyl-[Asp^6,*N*-Me-Phe^8]substance P was used at concentrations ranging from 10^{-10} to 10^{-7} M. Significant increases in the respiratory frequency occurred (mean latency: 70 s) in response to concentrations of 10^{-9}, 10^{-8} and 10^{-7} M (Table 1). Non-respiratory motor activities were frequently elicited (7/12 at 10^{-8} M; mean latency: 4 min) Application of succinyl-[Asp^6,*N*-Me-Phe^8]substance P to the cervical spinal cord ($n = 5$; 10^{-8} M) increased the amplitude of the integrated inspiratory activity ($14.5 \pm 3.6\%$; Fig. 3C) and induced some non-respiratory activity (2/5).

3.3. Effects of tachykinin receptor antagonists

Several drugs known to have potent antagonist effects on the various types of tachykinin receptors were used: SR140333 and GR 82334 as tachykinin NK_1 receptor antagonists, SR48968 and MEN 10376 as tachykinin NK_2 receptor antagonists, and SR142801 as tachykinin NK_3 receptor antagonist. The first result obtained here on these antagonists was that they had no effect on the baseline respiratory frequency. The mean respiratory frequency (3.5 ± 0.3 min^{-1}; $n = 42$) measured during the last minute of superfusion with the drug (whatever the antagonist: 10^{-7} M) was not significantly different from the mean control value (3.8 ± 0.2 min^{-1}).

3.3.1. Effects of tachykinin NK_1 receptor antagonists

The tachykinin NK_1 receptor antagonist SR140333 was used at two different concentrations (10^{-8} and 10^{-7} M). The pre-treatment was applied for periods ranging between 15 and 60 min, and substance P (10^{-8} M) was then applied for 6 min. The resulting changes in the respiratory frequency were compared to those induced by the same concentration of substance P applied without the pre-treatment. Pre-treatments of 15–30 min ($n = 5$, 10^{-8} M) failed to block the effects of substance P (mean increase in the respiratory frequency: 68% after the pre-treatment vs. 70% before the pre-treatment). When the pre-treatment was applied for 15 min at a concentration of 10^{-7} M, it resulted in a slightly significant decrease ($n = 5$, $P = 0.06$; $32 \pm 8\%$ vs. $70 \pm 20\%$). Further increasing the duration of the pre-treatment up to 60 min significantly reduced the substance P-induced increase in the respiratory frequency ($n = 5$, Fig. 4A; Fig. 5A; $21 \pm 4\%$ vs. $70 \pm 20\%$). Comparable results were obtained after a pre-treatment with another tachykinin NK_1 receptor antagonist (GR 82334; 10^{-7} M; 15 min, $n = 3$), which significantly decreased the substance P-induced increase in the respiratory frequency (Fig. 5A; $16 \pm 9\%$ vs. $70 \pm 20\%$).

When used as a pre-treatment (either alone or associated with the tachykinin NK_2 receptor antagonist MEN 10376), GR 82334 failed to reduce the increase in the

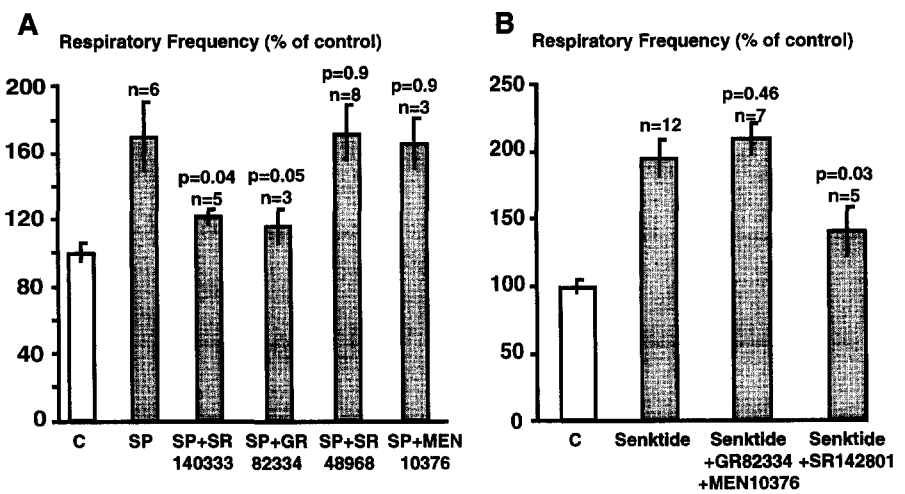

Fig. 5. Effects of tachykinin receptor antagonists. (A) Effects of tachykinin NK_1 and NK_2 receptor antagonists on the substance P- induced increase in the respiratory frequency; C: control respiratory frequency; SP: change in the respiratory frequency induced by the aCSF containing substance P. All the antagonists were applied at the same concentration (10^{-7} M) for 15 min (except for SR140333: 60 min) in the pre-treatment and then for 6 min concomitantly with substance P (10^{-8} M). Both tachykinin NK_1 receptor antagonists (SR140333; GR 82334) induced a significant decrease in the excitatory effects of substance P, whereas both tachykinin NK_2 receptor antagonists (SR48968; MEN 10376) failed to reduce the effects of substance P. (B) Effects of tachykinin receptor antagonists on the senktide-induced increase in the respiratory frequency. Simultaneous application of both tachykinin NK_1 and NK_2 receptor antagonists (GR 82334 and MEN 10376; 10^{-7} M; 15 min) failed to reduce the senktide-induced (10^{-8} M) increase in the respiratory frequency whereas a pre-treatment with the tachykinin NK_3 receptor antagonist (SR142801; 10^{-6} M) significantly ($P = 0.04$) reduced the excitatory effects of senktide.

respiratory frequency induced by a tachykinin NK_3 receptor agonist, senktide ($n = 7$, Fig. 4B; Fig. 5B; $110 \pm 11\%$ vs. $95.5 \pm 13\%$, with and without pre-treatment, respectively).

3.3.2. Effects of tachykinin NK_2 receptor antagonists

The two drugs SR48968 ($n = 8$) and MEN 10376 ($n = 3$), used as tachykinin NK_2 receptor antagonists (15 min, 10^{-7} M) failed to reduce the effects of substance P (10^{-8} M), which still significantly increased the respiratory frequency (Fig. 5A; $72 \pm 16\%$ and $66 \pm 15\%$ after application of SR48968 and MEN 10376, respectively). SR48968 also failed to block the increase of the respiratory frequency induced by the tachykinin NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]substance P ($n = 4$, $96 \pm 16\%$ after pre-treatment vs. $81 \pm 16\%$).

3.3.3. Effects of tachykinin NK_3 receptor antagonist

Due to the relatively low affinity of the tachykinin NK_3 receptor antagonist SR142801 for the rat tachykinin NK_3 receptors (Emonds-Alt et al., 1995), a high concentration (10^{-6} M) of this drug was used in the pre-treatment prior to application of the tachykinin NK_3 receptor agonist senktide. When applied for 15 min, the pre-treatment failed to reduce the increase in the respiratory frequency (93% vs. 95%). After a 30 min pre-treatment (i) a limited but significant increase in the respiratory frequency was induced by the tachykinin NK_3 receptor antagonist SR142801 (5.4 ± 0.6 vs. 3.8 ± 0.2 min^{-1}), (ii) the tachykinin NK_3 receptor agonist (10^{-8} M) still significantly increased the respiratory frequency, but (iii) this increase was significantly smaller than that recorded in the control experiments ($n = 5$, $41 \pm 18\%$ vs. $95 \pm 13\%$; Fig. 5B).

4. Discussion

4.1. Substance P and respiratory frequency

The results presented here confirm and extend the knowledge available about the effects of substance P on respiratory activity (Murakoshi et al., 1985; Yamamoto et al., 1992). Substance P increases the respiratory frequency of the in vitro brainstem-spinal cord preparation in a concentration-dependent manner; significant changes can be induced by substance P at a concentration of 10^{-9} M, and the maximum effect seems to occur at a concentration of 10^{-8} M since the response observed at 10^{-7} M was not significantly different. The changes induced by substance P are due to its action on medullary structures since they were still present after elimination of the pons and did not occur in response to a superfusion restricted to the spinal cord. This conclusion is in agreement with the finding that substance P had a direct excitatory effect on the so called pre-inspiratory neurons located in the rostral ventro-lateral medulla after the synaptic transmission had been blocked by applying low Ca^{2+}, high Mg^{2+} aCSF (Yamamoto et al., 1992). These neurons, which are considered as the 'primary rhythm generator' (Onimaru, 1995) may be one of the targets of substance P in the medulla, but the possibility that substance P may act on other neurons, including those which receive and relay the afferent inputs relating to autonomic functions cannot be ruled out. Even in reduced preparations, the effects induced by a neuroactive substance applied via the bathing medium are far from being simple and the changes observed can be the sum of multiple effects on several targets.

The effects of substance P have been reported to depend on the basal respiratory rate, and inhibitory effects of substance P have been described in preparations exhibiting a high respiratory frequency (Yamamoto et al., 1992). Our results do not confirm this point. Although the changes in the respiratory frequency (as a percentage of the control) were greatest in preparations with a slow basal rate, the respiratory frequency never decreased in response to substance P. Furthermore, after eliminating of the pons, which increases the respiratory frequency two-fold (Hilaire et al., 1989), substance P was still found to be able to significantly increase the respiratory frequency.

None of the tachykinin receptor antagonists used in this study triggered a significant decrease in the basal respiratory frequency. This finding is in agreement with the negative result obtained with another tachykinin receptor antagonist (spantide, Yamamoto et al., 1992). This lack of effect indicates that no facilitatory tonic influence depending on endogenous substance P is likely to participate in maintaining the basal respiratory activity under these experimental conditions. Endogenous effects have previously been described in the case of catecholamines (Hilaire et al., 1989) and serotonin (Morin et al., 1990b) with this preparation. The possible existence of an endogenous excitatory effect of substance P has been suggested on the basis of in vivo experiments performed on adult rats, since the application of substance P receptor antagonists in the ventrolateral medulla resulted in a respiratory depression (Chen et al., 1988).

4.2. Effects of substance P on inspiratory motoneurons

Superfusing the whole brainstem-spinal cord preparation with aCSF containing substance P brought about no significant changes in the amplitude of the integrated inspiratory activity. This negative result might mean that substance P has no effect on the spinal respiratory motoneurons. This view is challenged, however, by the fact that opposite effects were induced by superfusing substance P on either the brainstem or the spinal cord. Superfusion at the brainstem level increased the respiratory frequency and reduced the amplitude of the inspiratory motor output, whereas superfusion at the spinal level increased the amplitude of the inspiratory motor output

without changing the respiratory frequency. The latter result indicates that substance P exerts facilitatory effects at the spinal level either directly on the motoneurons or via a network of interneurons. Terminals containing substance P (Holtman et al., 1984) as well as substance P receptors (Helke et al., 1986) are present within the phrenic nucleus. The effects of substance P on respiratory motoneurons have never been studied, but at the lumbar level, substance P (Otsuka and Yanagisawa, 1980), like tachykinin NK_1 and NK_3 receptor agonists (Ireland et al., 1992), induces a depolarization in the ventral roots. It is worth noting that substance P applied either to the whole preparation or restricted to the cervical spinal cord induced either a tonic motor output or a non-respiratory rhythmic motor activity at the cervical level. Intracellular recording of the activity of motoneurons and interneurons at the cervical level (C4) will be necessary to determine the part played by respiratory and non-respiratory neurons in these tonic or rhythmic non-respiratory activity elicited by SP or tachykinin receptor agonists. Similar non-respiratory activities were also evoked by serotonin at the spinal level and involve (i) inspiratory and non-respiratory motoneurons (Morin et al., 1991), (ii) $5\text{-}HT_{2A}$ receptors (Monteau et al., 1994) located on the motoneurons (Lindsay and Feldman, 1993). Intracellular data have confirmed that tachykinin NK_1, NK_2 and NK_3 receptor agonists are able to depolarize the lumbar motoneurons and to increase their membrane input resistances (Fisher et al., 1994). These agonists act either directly on the motoneurons or indirectly by activating interneurons. As far as we know, comparable experiments have not been performed at the cervical level.

4.3. Receptors involved

As far as we know, this is the first attempt to characterize the receptors involved in the central respiratory effects of substance P using tachykinin receptor agonists and antagonists. The drugs used in this study show a high selectivity for only one receptor subtype and have been generally recognized as standard tools in reviews dealing with the pharmacology of tachykinin receptors (Maggi et al., 1993; Regoli et al., 1994). Tachykinin NK_1 receptor agonist ([Sar^9,$Met(O_2)^{11}$]substance P; Drapeau et al., 1987) and substance P itself are almost equipotent in terms of the changes they induce in the respiratory frequency, and the corresponding dose-effects curves are very similar. The tachykinin NK_3 receptor agonist senktide (Wormser et al., 1986) induces changes in the respiratory frequency which are comparable to those induced by substance P or by [Sar^9,$Met(O_2)^{11}$]substance P (i.e., ranging between 20% at 10^{-9} M and 90% at 10^{-7} M). The tachykinin NK_2 receptor agonist [β-Ala^8]neurokinin A-(4–10) (Rovero et al., 1989) is a distinctly less potent means of inducing respiratory changes (no effect at 10^{-9} M, maximum changes of about 20%). The results obtained here using tachykinin receptor agonists suggest that tachykinin NK_1 and NK_3 receptors may have been involved in the responses recorded.

Two tachykinin NK_1 receptor antagonists, SR140333 (Emonds-Alt et al., 1993) and GR 82334 (Birch et al., 1992) have been used, both of which drastically reduced the substance P-induced increase in the respiratory frequency. The results obtained with SR140333 confirm that this drug needs a long pre-treatment time to fully develop its antagonist properties, as already found to be the case in other preparations (Croci et al., 1995). These authors reported that the antagonist inhibited [Sar^9,$Met(O_2)^{11}$]substance P-induced contractions of guinea-pig ileum equally effectively at 2 and 4 h of incubation time, but was less efficient at 30 min. In the brainstem-spinal cord preparation, pre-treatments applied for a period ranging from 15 to 30 min proved to induce only slightly significant effects or no effects at all, whereas pre-treatments applied for 60 min significantly reduced the effects of substance P.

Both of the tachykinin NK_2 receptor antagonists used in this study, SR48968 (Emonds-Alt et al., 1992) and MEN 10376 (Maggi et al., 1991), failed to reduce the substance P- or the [Sar^9,$Met(O_2)^{11}$]substance P-induced increase in the respiratory frequency. The results are consistent with those obtained with tachykinin NK_2 receptor agonists and confirm the involvement of tachykinin NK_1 receptors. The tachykinin NK_3 receptor agonist proved to be as potent as substance P or [Sar^9,$Met(O_2)^{11}$]substance P as a means of increasing the respiratory frequency. As this drug is thought to have a high affinity for tachykinin NK_3 receptors, these receptors may be involved in modulating the respiratory rhythm generator. The results obtained here using tachykinin receptor antagonists confirm this idea. The senktide-induced changes in the respiratory frequency were neither blocked nor reduced by a pre-treatment with tachykinin NK_1, NK_2 or NK_1 and NK_2 receptor antagonists, thus ruling out the possibility that senktide might act on these two types of tachykinin receptors. Furthermore, the use of the novel non-peptide tachykinin NK_3 receptor antagonist (SR142801; Emonds-Alt et al., 1995) confirms the involvement of the tachykinin NK_3 receptors. This antagonist has been reported to have a high affinity for tachykinin NK_3 receptors in several preparations of different species, but unfortunately a much lower affinity for rat brain tachykinin NK_3 receptors (Emonds-Alt et al., 1995). Given this lower affinity for rat brain tachykinin NK_3 receptors, an increased concentration of SR142801 was used for the pre-treatment (10^{-6} M). The basal respiratory activity and the changes induced by senktide were not affected by a pre-treatment applied for 15 min. When a longer incubation time of 30 min was used, some agonistic effects were observed and the respiratory frequency increased. The senktide-induced increase in the respiratory frequency was still present but significantly lower than in the control experiments. Despite the low affinity of SR142801 for rat brain NK_3 receptors, this result argues in

favour of the idea that tachykinin NK_3 receptors may be involved in modulating the respiratory frequency.

In conclusion, tachykinins may exert a facilitatory modulation on the respiratory rhythm generator by acting on tachykinin NK_1 and NK_3 receptors, the location of which remains to be determined. No such modulation via endogenous tachykinins operates in the in vitro preparation, however. Tachykinins may also facilitate the activity of the cervical respiratory network and increase the amplitude of the respiratory motor output.

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