Modulation of the cough reflex by antitussive agents within the caudal aspect of the nucleus tractus solitarii in the rabbit

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Running Head: Modulation of the cough reflex within NTS

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ABSTRACT

We have previously shown that ionotropic glutamate receptors in the caudal portion of the nucleus tractus solitarii (NTS), especially in the commissural NTS, play a prominent role in the mediation of tracheobronchial cough and that substance P potentiates this reflex. This NTS region could be a site of action of some centrally acting antitussive agents, and a component of a drug-sensitive gating mechanism of cough. To address these issues, we investigated changes in baseline respiratory activity and cough responses to tracheobronchial mechanical stimulation following microinjections (30-50 nl) of centrally acting antitussive drugs into the caudal NTS of pentobarbitone anesthetized, spontaneously breathing rabbits. [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) and baclofen decreased baseline respiratory frequency because of increases in the inspiratory time only at the higher concentration employed (5 mM and 1 mM, respectively). DAMGO (0.5 mM) and baclofen (0.1 mM) significantly decreased cough number, peak abdominal activity, peak tracheal pressure and increased cough-related total cycle duration. At the higher concentrations, these agents suppressed the cough reflex. The effects of these two drugs were counteracted by specific antagonists (10 mM naloxone and 25 mM CGP-35348, respectively). The neurokinin-1 (NK₁) receptor antagonist CP-99,994 (10 mM) abolished cough responses, whereas the NK₂ receptor antagonist MEN 10376 (5 mM) had no effect. The results indicate that the caudal NTS is a site of action of some centrally acting drugs, and a likely component of a neural system involved in cough regulation. A crucial role of substance P release in the mediation of reflex cough is also suggested.

Keywords: µ-opioid receptors; GABA₉ receptors; neurokinin receptors; control of breathing; airway defensive reflexes.
INTRODUCTION

Previous studies led to the conclusion that some antitussive drugs act centrally since they inhibit cough when administered by intravertebral arterial or intracerebroventricular route in guinea pigs and cats (7, 8). These drugs comprise the opioid receptor agonist codeine, the µ-opioid receptor agonist morphine, the neurokinin-1 (NK₁) receptor antagonist (+)(2R,3R)-3-(2-methoxybenzylamino)-2-phenylpiperidine (CP-99,994), the NK₂ receptor antagonist {[(+) -N-methyl-[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-di-dichlorophenyl) butyl]benzamide} (SR48968), and the GABA₉ receptor agonist baclofen. A prominent central antitussive effect has been proved for the selective µ-opioid receptor agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) by using intracerebroventricular administration in the rat (30). However, the central responsive structures and the mechanism of action of these drugs remain to be investigated.

It is widely agreed that tracheobronchial rapidly adapting receptors (RARs) are involved in cough mediation, while the role of bronchopulmonary C-fibers and Aδ-nociceptive pulmonary afferent fibers in this reflex is controversial (see e.g. 36, 49, 62; see Ref. 43 also for further Refs.). We have recently provided evidence that ionotropic glutamate receptors located in the caudal aspect of the nucleus tractus solitarii (NTS) and, especially, within the commissural subnucleus of the NTS (comNTS) are involved in the mediation of the cough reflex evoked by the mechanical stimulation of the tracheobronchial tree in the rabbit (43). This medullary region has been identified as the predominant site of central projections of tracheobronchial RARs (32, 33) and, possibly, of the so-called “cough receptors” responsive to mechanical stimuli and acid, recently described in the larynx and rostral trachea of guinea pigs (16, 40). We have also shown that substance P microinjected into the same NTS regions markedly potentiates reflex cough responses (43), thus extending previous observations by Mazzone et al. (40).
The above mentioned evidence led us to hypothesize (43) that the caudal portion of the NTS: 1) plays an important role in the integration of peripheral inputs regulating the cough reflex; 2) could be a site of action of antitussive drugs; and 3) is one of the components of the central gating mechanism of cough proposed by Bolser et al. (8, 9). Our hypotheses are consistent with and extend previous suggestions (see e.g. 12, 20, 32, 38). In more detail, the caudal NTS has been considered a strategic site where excitatory and inhibitory neurotransmitters (12, 20, 32, 38) as well as plasticity phenomena (12, 38) may contribute to the modulation of the cough reflex.

We addressed these issues by using µ-opioid and GABA_B receptor agonists as well as NK_1 and NK_2 receptor antagonists. Bilateral microinjections of these centrally acting antitussive agents were performed into the caudal portion of the NTS, particularly into the comNTS, of pentobarbitone anesthetized, spontaneously breathing rabbits. Drug-induced changes in the cough motor pattern were evaluated by analysing selected cough-related variables.
MATERIALS AND METHODS

Animal preparation. This study concerns 33 successful experiments out of a total of 37. Experiments were performed on male New Zealand white rabbits (2.6-3.4 kg) anesthetized with sodium pentobarbitone (40 mg/kg iv, supplemented by 2-4 mg/kg every 30 min; Sigma-Aldrich, St. Louis, MO). Atropine (0.15 mg/kg im) was administered to reduce mucosal secretion in the airways. The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw; additional criteria were the presence of a stable and regular pattern of phrenic bursts and the absence of fluctuations in arterial blood pressure or phrenic nerve activity, whether spontaneous or in response to somatic nociceptive stimulation. All animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Community Council on the use of laboratory animals (Directive 86/609/EEC). The study was approved by the Animal Care and Use Committee of the University of Florence.

Experimental procedures and details about the methods employed have previously been described (10, 11, 42-45). After cannulation of the trachea, polyethylene catheters were inserted into a femoral artery and vein for monitoring arterial blood pressure and for drug delivery, respectively. The animal was allowed to breathe through a non-rebreathing valve (model No. 112095-2384A, Hans Rudolph Inc., Kansas City, MO) attached to the tracheal cannula. The C3 or C5 phrenic root either on the right or left side was dissected free, cut distally and prepared for recordings. The animal was placed in a prone position and fixed by a stereotaxic head holder and vertebral clamps; the head was ventroflexed for optimal exposure of the dorsal surface of the medulla by occipital craniotomy. Body temperature was maintained at 38.5-39 °C by a heating blanket controlled by a rectal thermistor probe.

Recording procedures. Efferent phrenic nerve activity was recorded using bipolar platinum electrodes from the central stump of the cut and desheathed C3 or C5 phrenic root. The
Electromyographic (EMG) activity of abdominal muscles was recorded by wire electrodes (Nichrome wires, insulated except for 1 mm at the tips, diameter 0.1 mm) inserted into the external or the internal oblique abdominal muscles. Phrenic and abdominal activities were amplified (x2000-10000), bandpass filtered (80-10000 Hz), full-wave rectified, and “integrated” (low-pass RC filter, time constant 100 ms). Arterial blood pressure and intratracheal pressure were recorded by strain-gauge manometers. End-tidal CO₂ partial pressure was measured by an infrared CO₂ analyzer (Datex, CD-102; Normocap, Helsinki, Finland). Integrated phrenic and abdominal activities as well as the signals of the other variables studied were recorded on an eight-channel rectilinearly writing chart recorder (model 8K20; NEC San-ei, Tokyo, Japan). Cardiorespiratory variables were also digitally acquired and analyzed using a personal computer, equipped with an analog-to-digital interface (Digidata 1200, Axon Instruments, Union City, CA) and appropriate software (Axoscope, Axon Instruments).

**Microinjection procedures.** Microinjection procedures have been described fully in previous reports (11, 42-45). Bilateral microinjections were performed into the caudal NTS and particularly into the lateral comNTS. The following antitussive drugs were used: DAMGO (0.5 and 5 mM), a µ-opioid receptor agonist; baclofen (0.1 and 1 mM), a GABA_B receptor agonist; CP-99,994 (10 mM), a NK₁ receptor antagonist; MEN 10376 (5 mM), a NK₂ receptor antagonist. Naloxone (10 mM), a largely prevailing µ-opioid receptor antagonist, and CGP-35348 (25 mM), a GABA_B receptor antagonist, were also employed in an attempt to prevent the effect of DAMGO and baclofen, respectively. All drugs were purchased from Sigma-Aldrich except CP-99,994 (gift from Pfizer Inc., Groton, CT) and MEN 10376 (Tocris Cookson, Bristol, UK). A single preparation was employed to test the effects of each antitussive drug. Drug concentrations were in the same range as those previously used in in vivo preparations (4, 13, 19, 21, 22, 35, 50, 56). All drugs were dissolved in 0.9 % NaCl solution. The pH of the solutions was adjusted to 7.4 using either 0.1 N NaOH or 0.1 N HCl. Control injections of equal volumes of the vehicle solutions were also made. Microinjections (30-50 nl) were performed via a glass micropipette.
(tip diameter 10-25 µm) by applying pressure using an air-filled syringe connected to the micropipette by polyethylene tubing. The volume of the injectate was measured directly by monitoring the movement of the fluid meniscus in the pipette barrel with a dissecting microscope equipped with a fine reticule. The time taken to inject the solution ranged from 5 to 10 s. In each experiment, bilateral microinjections were performed at two different sites along the rostrocaudal extent of the caudal NTS. The first was approximately at the level of the caudal-most end of the area postrema (obex level), 0.6-0.8 mm lateral to the midline and 0.7-0.8 mm below the dorsal medullary surface. The second was 0.5 mm more caudal, 0.4-0.5 mm lateral to the midline and 0.7-0.8 mm below the dorsal medullary surface. This procedure ensured to affect to the largest possible extent the population of second order neurons receiving cough-related afferent inputs (32, 33, 43). The stereotaxic coordinates were selected according to the atlas of Meessen and Olszewski (41). Microinjections at the two selected NTS sites were performed in succession using a single micropipette (see e.g. Refs. 11, 42, 43). The time taken to perform all the microinjections ranged from 4 to 5 min.

**Stimulation procedures.** Cough was induced by means of a 0.5-mm diameter nylon fibre inserted through a lateral port of the tracheal cannula until the tip was judged to be near the carina and main bronchi (10, 11, 43, 45). Back and forth movements of the fibre aimed at touching repeatedly (approximately 1 time every s) the carina or nearby airway walls were made over periods of 4-5 s. The manoeuvre was always conducted by the same experimenter in order to ensure consistency of stimulation intensity and frequency between trials. However, greater stimulation intensities and/or durations were occasionally employed following drug administration. Admittedly, our mechanical stimulation of the tracheobronchial tree may activate sensory endings innervated by Aδ- and C-fibers. However, RARs are far more mechanically sensitive to epithelial pressure than are C-fiber receptors, indicating that if relatively gentle stimuli are used they should cause cough via activation of RARs (e.g. 24, 49). In any case, stimulation of pulmonary and bronchial C-fiber receptors in anesthetized and
unanesthetized animals by selective stimuli has never been shown to cause cough, rather it has been reported to inhibit this defensive reflex (58, 59: for review see Ref. 49). Much care was taken to perform tracheobronchial stimulation always at least 8 min following each supplemental dose of pentobarbitone to avoid the possible immediate influences of the injected bolus on both breathing pattern and coughing. An interval of about 1 min was scheduled between cough stimulations. As a rule, 3 stimulation trials were performed before drug administration. These stimulation trials were also accomplished about 5 min after the completion of all the microinjections and repeated at appropriate intervals (at least 4-5 min) until complete recovery was observed or for a maximum of about 70 min.

Histology. At the end of each experiment, the animal was maintained on the stereotaxic head holder and the brain was perfused via a carotid artery with 0.9 % NaCl solution and subsequently with 10 % formalin solution. In order to obtain a brainstem block containing the pipette tracks, the brain was cut according to two transverse sections parallel to the frontal planes where the micropipettes had been placed. After at least a 48-h immersion in 10 % formalin solution, the brain was placed in a hypertonic sucrose solution. Frozen 20-µm coronal sections stained with cresyl violet were used for the histological control of pipette tracks and injection sites. In fact, as already illustrated in a previous study (43), sections made at the appropriate levels show, as a rule, the entire tracks along which microinjections were performed and, therefore, the localization of micropipette tips and injection sites. The atlas of Meessen and Olszewski (41) and the more recent atlas of Shek et al. (55) were used for comparison.

Data collection and analysis. Respiratory variables were measured during baseline respiration and cough efforts. The inspiratory (T₁) and expiratory (Tₑ) times, as well as the total duration of the respiratory cycle (Tₜ) were measured on recordings of raw phrenic nerve activity. The respiratory frequency was subsequently calculated (breaths/min). Peak amplitude (arbitrary units) of the phrenic nerve activity and abdominal EMG activity were measured on integrated traces. Normalization of the amplitudes of phrenic and abdominal activities was performed by
expressing them as a fraction (or percentage) of the highest achievable amplitude observed in each animal. The highest peak values were consistently obtained during coughing. Therefore, all amplitudes have been expressed in relative units (RU; see Refs. 8, 43). Breathing pattern variables were measured for an average of five consecutive breaths prior to and following drug bilateral microinjections into the NTS. In the same periods, systolic and diastolic blood pressures were measured at 2 s intervals; mean arterial pressure was calculated as the diastolic pressure plus one-third of the pulse pressure. Average values of cardiorespiratory variables observed in control conditions and at the time when the maximum response occurred were considered for statistical analyses (Sigma Stat, Jandel Scientific Software, San Rafael, CA). Owing to the small variations in respiratory and cardiovascular variables within each measurement period, average values were taken as single measurements for the purpose of analysis.

Cough responses to mechanical stimulation of the tracheobronchial tree were characterized by repeated coughs consisting of coordinated bursts of inspiratory activity and expiratory activity (10, 11, 43, 45). In agreement with our previous results in rabbits and cats (10, 11, 43, 45), repeated coughs usually started during stimulation and continued shortly after stimulus cessation. Each cough consisted of an augmented phrenic burst (preparatory inspiration) immediately followed by a burst of expiratory abdominal activity (11, 43, 45). A comparison between respiratory variables during control breathing and cough responses in the rabbit has been recently reported (43). Cough-related variables were measured and averaged (3 stimulation trials) before and 10 min after bilateral microinjections of antitussive drugs at the two selected sites, i.e. in the period when maximum effects on the cough reflex were present. These variables included cough-related T\textsubscript{T}, peak phrenic amplitude, peak abdominal activity, peak tracheal pressure and the cough number, i.e. the number of coughs that followed each stimulation. Average values of cough-related variables were taken as single measurements for subsequent statistical analysis. In some cases, the first obvious response following mechanical stimulation of the tracheobronchial tree was a small-amplitude expiratory effort without a preceding
preparatory inspiration (see also Refs. 10, 11, 43). Accordingly, recordings of tracheal pressure show that this expiratory effort clearly occurs after the beginning of the expiratory phase of a control breath (see Fig. 1). This pattern could fit more appropriately the definition of expiration reflex that is typically evoked by mechanical stimulation of the vocal folds (31), but that can be also produced by mechanical stimulation of the tracheobronchial tree (61, see also Ref. 57 for further Refs.). The distinction between cough and the expiration reflex is important. Different neural mechanisms underlie the two reflexes (3) which have different physiological and pharmacological properties as well as different functions (23, 31; see Ref. 57 also for further Refs.). However, in our study an expiration reflex only occurred as the first motor event in a cough epoch, and its appearance was limited to a few occasions. Therefore, these expiratory responses were not considered for data analysis. In each preparation, microinjections of DAMGO and baclofen were performed using two concentrations. In three experiments for each drug the microinjections at the higher concentration were executed when a complete recovery was observed after the injections at the lower concentration. In the other three preparations, only 20-min intervals were scheduled between microinjections at the two different concentrations. Since with the higher concentrations cough responses were always suppressed, data obtained with these different procedures were pooled (see Results). Comparisons between cardiorespiratory variables recorded during baseline breathing before and after administrations of DAMGO or baclofen were performed by means of the one way repeated measures analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) tests. Changes in cardiorespiratory variables during baseline breathing as well as changes in cough-related variables observed in all the other experimental conditions were evaluated by Student’s paired t tests. All reported values are means ± SE; P < 0.05 was taken as significant.
RESULTS

Drug-induced responses in cardiorespiratory variables during eupneic breathing. Changes in respiratory variables during eupneic control breathing were observed only following bilateral microinjections of DAMGO or baclofen. They consisted of decreases in respiratory frequency mainly due to increases in $T_I$; these changes attained the level of statistical significance only with the higher drug concentrations (Table 1). Changes in respiratory activity reached their maximum within 6-10 min, while recovery to control levels occurred within 60 min. None of the antitussive drugs significantly affected mean arterial blood pressure (Table 1).

Drug-induced effects on the cough reflex. Bilateral microinjections of 0.5 mM DAMGO (30-50 nl; 15-25 pmol) at the two selected NTS sites were performed in 6 animals. They induced within about 10 min consistent and marked reductions in the cough number, peak abdominal activity and peak tracheal pressure accompanied by increases in the cough-related $T_T$ due to a rise in both $T_I$ and $T_E$. As shown in Fig. 1, DAMGO at 5 mM (30-50 nl; 150-250 pmol) always abolished the cough reflex. The suppression of the cough reflex occurred within 5 min and persisted for 20-25 min (Table 2). In 4 additional experiments, microinjections of 10 mM naloxone (30-50 nl; 300-500 pmol) performed into the responsive sites caused no change in either the respiratory activity or the cough reflex. However, microinjections of 5 mM DAMGO performed at the same sites 10 min after the completion of naloxone microinjections failed to alter both baseline respiratory activity and cough responses (data not shown).

Bilateral microinjections ($n = 6$) of 0.1 mM baclofen (30-50 nl; 3-5 pmol) provoked within about 10 min significant decreases in the cough number, peak abdominal activity and peak tracheal pressure; cough-related $T_T$ augmented due to increases in both $T_I$ and $T_E$. On the other hand, 1 mM baclofen (30-50 nl; 30-50 pmol) suppressed cough responses (Table 2 and Fig. 2). The suppression occurred already within 5 min after the injections and lasted for 15-20 min. Microinjections of 25 mM CGP-35348 (30-50 nl; 750-1250 pmol), a GABA$_B$ receptor
antagonist, were performed at the selected NTS sites in 4 additional rabbits. Neither the pattern of ongoing respiratory activity nor the intensity of cough responses were affected. However, these microinjections prevented all the effects of 1 mM baclofen microinjected at the same sites after an interval of about 10 min (data not shown).

Bilateral microinjections \((n = 5)\) of 10 mM CP-99,994 (30-50 nl; 300-500 pmol), a NK\(_1\) receptor antagonist, into the caudal NTS abolished both the inspiratory and expiratory components of the cough reflex within about 5 min. This effect lasted for about 20 min. On the contrary, no effects on the cough reflex were seen after the application \((n = 5)\) of 5 mM MEN 10376 (30-50 nl; 150-250 pmol), a NK\(_2\) receptor antagonist (Table 2).

In all cases, when the cough reflex was abolished by drug application, mechanical stimulation of the tracheobronchial tree at higher intensity or duration failed to evoke any cough response. Nor any sign of expiration reflex was seen on these occasions. Cough reflex responses returned progressively to control levels. With the lower concentrations of DAMGO and baclofen, a complete recovery was seen within 40 min. Following drug-induced suppression of the cough reflex, the time course of recovery was slower, and complete recovery was observed in most cases within 60-70 min.

**Controls.** The localization of the injection sites was confirmed by histological control. The localization of injection sites and examples of typical placements of the micropipette tips within the NTS have been fully illustrated in a previous report (43). Control injections of equal volumes of the vehicle solution at the responsive sites (3 trials performed in 3 different preparations before drug administration) were ineffective. In 3 additional preparations, bilateral control microinjections of each drug were performed at medullary locations sufficiently far from the responsive sites (see e.g. Refs. 42, 43, 46). They were performed (for comparisons see the atlas of Meessen and Olszewski, Ref. 41) lateral to the responsive sites into the nucleus cuneatus or the nucleus tractus spinalis nervi trigemini (4 trials), as well as > 0.8 mm caudal to the responsive
sites into the nucleus gracilis or the adjacent reticular formation (3 trials). These injections caused no appreciable change in the pattern of breathing and cough responses.
DISCUSSION

The main results of this study can be summarized as follows. DAMGO and baclofen at the lower concentrations consistently decreased cough number, peak abdominal activity and peak tracheal pressure while increased cough-related T_T. At the higher concentrations, these drugs completely suppressed cough responses, and decreased respiratory frequency during eupneic breathing due to increases in T_T. The NK\textsubscript{1} receptor antagonist CP-99,994 abolished cough without influencing baseline respiratory activity. No effects on the cough reflex or the eupneic pattern of breathing were caused by the NK\textsubscript{2} receptor antagonist MEN 10376. This study is the first to provide evidence that the caudal portion of NTS, especially the comNTS, is a site of action of some well-known centrally active antitussive drugs and to suggest that this medullary region is a component of a neural system involved in the central regulation of cough. The results also support the notion that substance P release plays a crucial role in the activation of cough relay NTS neurons.

General remarks. We have already provided a detailed description of the microinjection techniques used, along with a discussion on their reliability, the concentration of injected drugs, and the spread of the injectate (see e.g. Refs. 11, 42-44). Injection sites were selected by using stereotaxic coordinates according to the atlas of Meessen and Olszewski (41). The histological control confirmed their localization (43). Our previous observations on the spread of the injectate ≤ 50 nl (42, 44) are in agreement with theoretical calculations by Nicholson (46) suggesting that a volume of 50 nl should spread less than 385 \( \mu \text{m} \) in any direction from the injection site. Accordingly, microinjections of the antitussive drugs into regions sufficiently away from the responsive sites did not affect the cough reflex. The specificity of drug-induced effects is also supported by the absence of changes in the cough reflex following control bilateral microinjections of the vehicle solution. On the other hand, similar drug concentrations have previously been shown to be selective in in vivo preparations (4, 13, 19,
Evidence on the specific receptor activation by DAMGO and baclofen is also provided by the antagonistic effects displayed by naloxone and CGP-35348, respectively. Changes in the eupneic pattern of breathing. Our results on baclofen-induced effects are in agreement with previous findings obtained in rats by using microinjection techniques at the level of the caudal NTS (e.g. Ref. 50) as well as in cats by using systemic administration or local application to the NTS by microelectrophoresis (e.g. Ref. 34). On the contrary, Bolser et al. (8) did not observe any effect on the eupneic pattern of breathing induced by either baclofen or morphine administered through the intravertebral arterial route in the cat.

As to the increases in T₁ in response to DAMGO and baclofen at the higher concentrations, it seems relevant to recall that ionotropic glutamate receptor blockades in the same NTS regions (43) do not affect the breathing pattern, the Breuer-Hering inflation reflex and the pulmonary chemoreflex, thus providing evidence of a specific involvement of the caudal NTS in the cough reflex. In addition, the input arising from RARs and possibly C-fiber receptors activates central mechanisms that induce decreases in Tₑ (for review see Ref. 49). Taking into account the above reported considerations, we propose that DAMGO and baclofen very likely affect neurons unrelated to vagal inputs that are embedded in pontomedullary circuits implicated in the control of respiratory timing, particularly, in the off-switch mechanisms (6, 15, 60). Both pre- and postsynaptic receptors may be involved (e.g. Refs. 6, 25; see also Refs. 5, 14, 30, 38, 50). On the other hand, it is well-known that µ-opioid receptor and GABA₉ receptor agonists may have strong depressant effects on both the intensity and timing of inspiratory activity.

In our study, the absence of respiratory effects in response to CP-99,994 microinjected into the caudal NTS of the rabbit is not surprising since substance P microinjections into the same region caused no significant respiratory changes (43). However, varied results have been obtained in previous studies on the central effects of NK₁ receptor agonists or antagonists in cats, guinea pigs, rats and dogs (8, 17, 39, 40). We do not know the reasons of
these discrepancies. The route of drug administration and the actual concentration reached at neuronal level could have played a role; drug microinjections may have produced extracellular concentrations higher than those attained via other administration routes. Other explanations probably involve differences in the animal species and, possibly, the presence of different NK₁ receptor subtypes (for review see Ref. 52).

Changes in cough-related variables. Changes in cough-related variables induced by the lower DAMGO or baclofen concentrations (Table 2) were similar to those previously observed after N-methyl-D-aspartate (NMDA) receptor blockades within the caudal NTS, except for the increases in T₁ (see Table 2 in Ref. 43). The effects induced by the lower DAMGO and baclofen concentrations on cough variables were largely similar to those previously described by Bolser et al. (8) for different antitussive drugs. However, in the present study DAMGO and baclofen also increased cough-related T₁ and Tₑ and, hence, Tₚ. Since peak phrenic amplitude did not significantly change, the increases in T₁ imply a reduced rate of rise of inspiratory activity (inspiratory drive) and, therefore, an inspiratory depressant effect (see e.g. Ref. 60). Of note, in the study by Bolser et al. (8) only high morphine doses were able to reduce the diaphragm EMG amplitude and lengthen cough-related Tₚ. The increases in cough-related Tₑ may be due, at least in part, to reductions in glutamate release by presynaptic inhibition at the level of the central terminals of cough-related vagal afferents (5, 6, 14, 25, 29, 38, 50).

As recently discussed by Bolser et al. (8, 9), the evidence supporting the presence of a cough gating mechanism derives in part from studies of the effects of antitussive drugs on the cough and breathing pattern (8, 37). These Authors propose that a cough gating mechanism accounts for the fact that antitussive drugs do not suppress breathing at doses that inhibit cough, suggesting the presence of a neural component important for cough that does not participate in breathing pattern generation. In addition, they support this hypothesis with the finding that antitussive drugs do not exert a generalized suppression on the entire central pattern generator, rather they have very specific effects on various components of this
mechanism. Although our results may imply different speculative interpretations, it seems rather obvious that they agree, at least in part, with the assumptions by Bolser et al. (8, 9). However, at variance with the hypothesis advanced by Bolser et al. (8, 9), present results show that some antitussive drugs may influence inspiratory timing and drive (Table 2). Previous studies (e.g. Refs. 10, 45, 47, 53, 54) have provided evidence that the neuronal network implicated in the generation of the eupneic pattern of breathing is also involved in the production of the cough motor pattern. Cough-related second order neurons probably provide excitatory inputs to virtually all the components of the respiratory network, including neurons that participate in the control of cough phase durations (53; see also Ref. 9). Decreased excitability of this group of neurons may result in prolongation of cough phase durations. Accordingly, this effect has been reported to be induced by NMDA receptor blockades within the caudal NTS (43) or by unilateral vagotomy (26). As already mentioned, it may be that some antitussive drugs have affected neurons unrelated to vagal tussigenic inputs, but implicated in the control of the respiratory timing (6, 15, 60). Remote effects due to disinhibition phenomena cannot be excluded (see also Ref. 9).

In agreement with previous results (8, 9), microinjections of antitussive drugs into the caudal NTS selectively decrease expiratory motor activation during cough at doses that do not significantly reduce the intensity of the inspiratory bursts. This does not appear to be consistent with inhibition of cough relay NTS neurons that send excitatory inputs to both inspiratory and expiratory premotor neurons and could suggest the existence of a control system located outside the cough generating network. However, our results also strongly support the notion that cough-related second order neurons in the caudal NTS are responsive to some antitussive agents and may have an important role in the central control of cough. It seems plausible to propose that several neural substrates are responsive to antitussive drugs and that the effects on cough-related variables may be different when drugs are applied simultaneously to all the responsive sites at low concentration or when drugs are applied to a
single site at high concentration. Therefore, drug-induced effects may depend upon the route of administration (local, intracerebroventricular, intravertebral artery and systemic) and the actual drug concentration attained at the neuronal level. This hypothesis remains to be verified. Several brainstem neural substrates that have been proved to influence cough (9-11, 27, 45, 47, 53, 54; see also Ref. 43) may also be sites of action of antitussive drugs. Preliminary results from our laboratory indicate that also the caudal ventral respiratory group is a site of action of some antitussive agents (unpublished observations).

Varied results have been obtained with CP-99,994 or different NK₁ receptor antagonists in anesthetized and unanesthetized animals of different species as well as in asthmatic patients (see e.g. Refs. 1, 7, 8, 17, 28, 40 also for further Refs.). In particular, our results on the effects of CP-99,994 on cough are consistent with those previously described by Bolser et al. (7, 8) and with those of a recent study by Chapman et al. (17) in dogs orally dosed with this antagonist. On the contrary, they are at variance with those obtained by Mazzone et al. (40). The reasons of these discrepancies are unclear. However, differences in the animal species and in the type of preparation may have played a role. For instance, guinea pigs were awake in the study by Bolser et al. (7) and anesthetized in that by Mazzone et al. (40). The absence of substance P and CP-99,994 effects on the eupneic pattern of breathing as opposed to their marked influence on the cough reflex (see also Ref. 43) indicates that neurokinins have a crucial role in the neurotransmission of central inputs triggering cough. Present results, along with our previous findings on the effects of ionotropic glutamate receptor blockades (43), lead us to tentatively suggest that the simultaneous release of glutamate and substance P within the caudal NTS underlies the genesis of cough responses to mechanical stimulation of the tracheobronchial tree. Neurokinins display excitatory effects on neurons located in the caudal NTS and may originate from different sources such as bronchopulmonary C-fiber afferents, NTS interneurons, oesophageal vagal afferents, trigeminal afferents and higher brain centres, in particular, from medullary raphe nuclei (see
e.g. Refs. 18, 40, 51). These latter contain neurons whose activity is altered during cough (2). Interestingly, other mechanisms leading to SP release from axon terminals within the NTS have been suggested; for instance, glutamate may contribute to neurokinin release in the NTS by excitation of postsynaptic ionotropic glutamate receptors localized on NK-containing neurons (Ref. 18 also for further Refs.) The modulatory role of substance P within the caudal NTS may be relevant to central mechanisms involved in the regulation of the cough reflex, including central sensitization and plasticity phenomena induced by some pathophysiological conditions such as gastroesophageal reflux and airway diseases (12, 28, 40, 43, 63).

The results obtained with the NK2 receptor antagonist MEN 10376 show that in the caudal NTS these tachykinin receptors do not have a role in the control of both the eupneic pattern of breathing and cough reflex. These findings are at variance with those obtained by Bolser et al. (7, 8) using the NK2 receptor antagonist SR48168 administered either by intracerebroventricular or intravertebral arterial route. The discrepancy may be related to the existence of other central sites of action of NK2 receptor antagonists, but it may also be that NK2 receptor subtypes with different binding properties are present in the caudal NTS of different animal species: it is well-known that each type of NK receptor is an heterogeneous entity presenting different active conformations (for review see Ref. 52).

**Perspectives and Significance**

The results of the present study provide insights into the central organization of the cough reflex in the rabbit, an animal species suitable for studies on the neurogenesis of cough and its pharmacological control. We propose that the caudal NTS is probably only one of the different neural structures responsive to centrally acting antitussive drugs. Other brainstem regions implicated in the regulation of cough may also be sites of action of these drugs (see Refs. 9, 10, 27, 45, 47, 53, 54; see also Ref. 43). As already mentioned, these responsive structures may comprise, in particular, the caudal ventral respiratory group, in agreement with our previous suggestions (11, 43) and those by Bolser et al. (9, 48). Present results do not
allow us to support or to refute the notion of the existence of a cough gating mechanism or any particular pool of neurons specifically relevant to this function. Nevertheless, we suggest that the caudal NTS is a drug-sensitive neural substrate subserving the central regulation of cough. Noticeably, high counts of cough-related Fos-like immunoreactivity were detected in the comNTS of the cat, but they were not increased as compared with controls (27). We do not know the meaning of this finding, however the relatively high levels of basal staining in this region may have interfered with the level of recruitment of cough-related Fos-like immunoreactivity (Ref. 27 also for further Refs.).

Future studies need to investigate the clinical relevance of the employed antitussive drugs by their systemic administration combined with microinjections of appropriate agonists and antagonists into putative responsive neural structures. The outcomes of these studies will provide further information concerning the sites of action and role of neurotransmitters in the central control of the cough reflex.

ACKNOWLEDGEMENTS

This study was supported by grants from the Ministero dell’Università e della Ricerca of Italy.
FIGURE LEGENDS

Fig. 1. Effects of DAMGO microinjections into the caudal NTS on cough reflex responses evoked by mechanical stimulation of the tracheobronchial tree in one anesthetized spontaneously breathing rabbit. Reduction and suppression of cough responses approximately 10 min after bilateral microinjections of 0.5 and 5 mM DAMGO, respectively. The recovery of cough responses was taken about 70 min after 5 mM DAMGO injections. Traces are: Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity; TP, tracheal pressure. Stimulation period marked by filled bars.

Fig. 2. Effects of baclofen microinjected into the selected sites of the caudal NTS on the cough reflex elicited by mechanical stimulation of the tracheobronchial tree in one anesthetized spontaneously breathing rabbit. Reduction and suppression of the cough reflex about 10 min after bilateral microinjections of 0.1 and 1 mM baclofen, respectively. The recovery of cough responses was taken about 70 min after 1 mM baclofen injections. Traces arranged as in Fig. 1. Stimulation period marked by filled bars.
REFERENCES


40. **Mazzone SB, Mori N and Canning BJ.** Synergistic interactions between airway afferent nerve subtypes regulating the cough reflex in guinea-pigs. *J Physiol* 569: 559-573, 2005.


60. **Von Euler C.** Brain stem mechanisms for generation and control of breathing pattern. 
   
   In: *Handbook of Physiology. The Respiratory System. Control of Breathing.* Bethesda, 
   


Table 1. Cardiorespiratory variables during eupneic breathing before and 10 min after bilateral microinjections of different putative antitussive drugs into the caudal NTS

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>T (s)</th>
<th>T (s)</th>
<th>T (s)</th>
<th>PPA (RU)</th>
<th>PAA (RU)</th>
<th>MAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T₁ (s)</td>
<td>T₂ (s)</td>
<td>T₃ (s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DAMGO</strong></td>
<td>6</td>
<td>1.20 ± 0.12</td>
<td>0.35 ± 0.02</td>
<td>0.84 ± 0.12</td>
<td>0.58 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>99.6 ± 5.3</td>
</tr>
<tr>
<td>0.5 mM</td>
<td></td>
<td>1.40 ± 0.19</td>
<td>0.42 ± 0.05</td>
<td>0.98 ± 0.13</td>
<td>0.60 ± 0.06</td>
<td>0.05 ± 0.04</td>
<td>98.0 ± 5.1</td>
</tr>
<tr>
<td>5 mM</td>
<td></td>
<td>1.65 ± 0.16*</td>
<td>0.64 ± 0.03*</td>
<td>1.00 ± 0.15</td>
<td>0.61 ± 0.07</td>
<td>0.04 ± 0.04</td>
<td>98.2 ± 5.3</td>
</tr>
<tr>
<td><strong>Baclofen</strong></td>
<td>6</td>
<td>1.30 ± 0.19</td>
<td>0.38 ± 0.03</td>
<td>0.93 ± 0.14</td>
<td>0.55 ± 0.03</td>
<td>0.07 ± 0.01</td>
<td>98.7 ± 5.6</td>
</tr>
<tr>
<td>0.1 mM</td>
<td></td>
<td>1.36 ± 0.20</td>
<td>0.38 ± 0.03</td>
<td>0.98 ± 0.13</td>
<td>0.45 ± 0.07</td>
<td>0.06 ± 0.04</td>
<td>98.7 ± 6.0</td>
</tr>
<tr>
<td>1 mM</td>
<td></td>
<td>1.51 ± 0.14*</td>
<td>0.43 ± 0.02*</td>
<td>1.07 ± 0.10</td>
<td>0.53 ± 0.03</td>
<td>0.08 ± 0.01</td>
<td>98.8 ± 6.4</td>
</tr>
<tr>
<td><strong>CP-99,994</strong></td>
<td>5</td>
<td>1.10 ± 0.09</td>
<td>0.39 ± 0.03</td>
<td>0.70 ± 0.06</td>
<td>0.47 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>98.8 ± 5.1</td>
</tr>
<tr>
<td>10 mM</td>
<td></td>
<td>1.12 ± 0.10</td>
<td>0.40 ± 0.03</td>
<td>0.72 ± 0.07</td>
<td>0.51 ± 0.03</td>
<td>0.04 ± 0.01</td>
<td>97.6 ± 7.1</td>
</tr>
<tr>
<td><strong>MEN 10376</strong></td>
<td>5</td>
<td>1.25 ± 0.11</td>
<td>0.40 ± 0.03</td>
<td>0.85 ± 0.13</td>
<td>0.57 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>101.2 ± 4.8</td>
</tr>
<tr>
<td>5 mM</td>
<td></td>
<td>1.30 ± 0.12</td>
<td>0.39 ± 0.03</td>
<td>0.91 ± 0.11</td>
<td>0.58 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>100.0 ± 7.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of animals; T₁, cycle duration; T₂, inspiratory time; T₃, expiratory time; PPA, peak phrenic activity in relative units (RU); PAA, peak abdominal activity; MAP, mean arterial pressure. *P < 0.05 compared with controls.
Table 2. Changes in cough-related variables 10 min following bilateral microinjections of different putative antitussive drugs into the caudal NTS

<table>
<thead>
<tr>
<th></th>
<th>CN</th>
<th>T_T (s)</th>
<th>T_I (s)</th>
<th>T_E (s)</th>
<th>PPA (RU)</th>
<th>PAA (RU)</th>
<th>TP (cmH₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAMGO</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.9 ± 0.33</td>
<td>0.61 ± 0.05</td>
<td>0.46 ± 0.03</td>
<td>0.14 ± 0.02</td>
<td>0.62 ±0.02</td>
<td>0.62 ± 0.02</td>
<td>7.47 ± 0.32</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>1.1 ± 0.10*</td>
<td>1.32 ± 0.25*</td>
<td>0.72 ± 0.08*</td>
<td>0.59 ± 0.20*</td>
<td>0.74 ± 0.04</td>
<td>0.42 ±0.05*</td>
<td>4.48 ± 0.20*</td>
</tr>
<tr>
<td>5 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Baclofen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.2 ± 0.20</td>
<td>0.58 ± 0.05</td>
<td>0.40 ±0.03</td>
<td>0.18±0.02</td>
<td>0.70 ± 0.07</td>
<td>0.56 ± 0.04</td>
<td>7.52 ± 0.31</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>1.1 ± 0.11*</td>
<td>0.71 ± 0.04*</td>
<td>0.47±0.03*</td>
<td>0.24±0.03*</td>
<td>0.68 ± 0.03</td>
<td>0.33 ±0.03*</td>
<td>4.12 ± 0.21*</td>
</tr>
<tr>
<td>1 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>CP-99,994</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.7 ± 0.27</td>
<td>0.60 ± 0.07</td>
<td>0.40 ±0.03</td>
<td>0.20 ± 0.06</td>
<td>0.63 ± 0.03</td>
<td>0.50 ± 0.02</td>
<td>7.03 ± 0.29</td>
</tr>
<tr>
<td>10 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>MEN 10376</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.1 ± 0.32</td>
<td>0.62 ± 0.06</td>
<td>0.40 ±0.03</td>
<td>0.22 ± 0.05</td>
<td>0.60 ± 0.03</td>
<td>0.53 ± 0.03</td>
<td>7.30 ± 0.41</td>
</tr>
<tr>
<td>5 mM</td>
<td>3.0 ± 0.29</td>
<td>0.64 ± 0.06</td>
<td>0.39 ±0.03</td>
<td>0.25 ± 0.03</td>
<td>0.61 ± 0.03</td>
<td>0.56 ± 0.03</td>
<td>7.48 ± 0.34</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of animals; CN, cough number; T_T, cycle duration; T_I, inspiratory time; T_E, expiratory time; PPA, peak phrenic activity in relative units (RU); PAA, peak abdominal activity; TP, tracheal pressure (cmH₂O). Negative marks indicate the absence of cough responses. *P < 0.05 compared with controls.
Fig. 1. Effects of DAMGO microinjections into the caudal NTS on cough reflex responses evoked by mechanical stimulation of the tracheobronchial tree in one anesthetized spontaneously breathing rabbit. Reduction and suppression of cough responses approximately 10 min after bilateral microinjections of 0.5 and 5 mM DAMGO, respectively. The recovery of cough responses was taken about 70 min after 5 mM DAMGO injections. Traces are: Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity; TP, tracheal pressure. Stimulation period marked by filled bars.
Fig. 2. Effects of baclofen microinjected into the selected sites of the caudal NTS on the cough reflex elicited by mechanical stimulation of the tracheobronchial tree in one anesthetized spontaneously breathing rabbit. Reduction and suppression of the cough reflex about 10 min after bilateral microinjections of 0.1 and 1 mM baclofen, respectively. The recovery of cough responses was taken about 70 min after 1 mM baclofen injections. Traces arranged as in Fig. 1. Stimulation period marked by filled bars.