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Am J Physiol Regul Integr Comp Physiol 307: R1358–R1367, 2014.
First published October 1, 2014; doi:10.1152/ajpregu.00237.2014.

Inhibitory control of the cough reflex by galanin receptors in the caudal nucleus tractus solitarii of the rabbit

Donatella Mutolo, Elenia Cinelli, Fulvia Bongianni, and Tito Pantaleo

Dipartimento di Medicina Sperimentale e Clinica, Sezione Scienze Fisiologiche, Università degli Studi di Firenze, Firenze, Italy

Submitted 5 June 2014; accepted in final form 28 September 2014

Mutolo D, Cinelli E, Bongianni F, Pantaleo T. Inhibitory control of the cough reflex by galanin receptors in the caudal nucleus tractus solitarii of the rabbit. *Am J Physiol Regul Integr Comp Physiol* 307: R1358–R1367, 2014. First published October 1, 2014; doi:10.1152/ajpregu.00237.2014.—The caudal nucleus tractus solitarii (NTS) is the main central station of cough-related afferents and a strategic site for the modulation of the cough reflex. The similarities between the characteristics of central processing of nociceptive and cough-related inputs led us to hypothesize that galanin, a neuropeptide implicated in the control of pain, could also be involved in the regulation of the cough reflex at the level of the NTS, where galanin receptors have been found. We investigated the effects of galanin and galnon, a nonpeptide agonist at galanin receptors, on cough responses to mechanical and chemical (citric acid) stimulation of the tracheobronchial tree. Drugs were microinjected (30–50 nl) into the caudal NTS of pentobarbital sodium-anesthetized, spontaneously breathing rabbits. Galnon antitussive effects on cough responses to the mechanical stimulation of the airway mucosa via a custom-built device were also investigated. Bilateral microinjections of 1 mM galanin markedly decreased cough number, peak abdominal activity, and increased cough-related total cycle duration. Bilateral microinjections of 1 mM galnon induced mild depressant effects on cough, whereas bilateral microinjections of 10 mM galnon caused marked antitussive effects consistent with those produced by galanin. Galnon effects were confirmed by using the cough-inducing device. The results indicate that galanin receptors play a role in the inhibitory control of the cough reflex at the level of the caudal NTS and provide hints for the development of novel antitussive strategies.

airway defensive reflexes; galanin; galanin receptors; galnon; nucleus tractus solitarii

COUGH IS A VERY IMPORTANT airway protective reflex and is one of the most common reason why patients seek medical attention. Cough is purposeful and useful under many circumstances, but in case of persistent or chronic cough (e.g., 15) is without an apparent aim and benefit and can results in a wide range of physical and psychological complications including urinary incontinence, insomnia, depression, and anxiety. The efficacy of antitussive drugs is far from clear from the available evidence and, in addition, limited by important side effects. Therefore, further research to find better antitussive approaches is necessary (for reviews see Refs. 4 and 15). Studies undertaken to provide insights into the neural mechanisms subserving the cough reflex may also provide useful suggestions for novel antitussive therapeutic strategies. The cough reflex involves several brain stem structures that also underlie respiratory rhythm generation (e.g., 7, 9, 10, 19, 24, 27, 47–49, 55). In particular, the nucleus tractus solitarii (NTS), especially in its caudal aspects, is the main central terminus of cough-related afferents (14, 39–41, 43, 53 also for further references) and, in addition, a possible strategic site of synaptic plasticity, where cough-related sensory inputs can be modulated (e.g., 10, 14, 34, 39, 40, 43).

In recent years, we and other researchers have focused our attention on the similarities between the mechanisms underlying the cough reflex and those involved in nociception and pain sensation. Peripheral and central mechanisms of nociception and cough share similar features, such as the type of afferent fibers (Aδ and C), transient receptor potential vanilloid 1 and transient receptor potential ankyrin 1 channels, central and peripheral sensitization, as well as the involvement of mitogen-activated protein kinase (MAPK) signal transduction pathways (4, 11, 12, 14, 20, 29, 40). This correspondence between nociception and cough has led to investigations on the antitussive effects of agents known to be involved in the central control of pain sensation (4, 15).

Galanin is a 29 amino acid neuropeptide (30 amino acids in humans) widely distributed in the central nervous system (e.g., 21, 28, 36, 37, 63, 65) involved in many body functions such as feeding behavior, learning and memory, neuroendocrine control, mood disorders, antinociception, cardiovascular regulation, and respiration (1, 6, 28, 44). In particular, galanin has been implicated in the descending control of pain at the spinal level as well as in its modulation at supraspinal sites probably mainly via galanin-1 (GAL_1) receptors (e.g., 2, 22, 32, 68; reviewed in Refs. 38, 69). Interestingly, galanin and in particular GAL_1 receptors have also been found (16, 36) in the NTS, while neurons in the nodose ganglion have been reported to express different types of galanin receptors (46, 59).

These findings prompted us to investigate the effects of galanin and galnon, a nonpeptide agonist at galanin receptors (52, 58), microinjected into the caudal NTS on cough responses induced by mechanical and chemical stimulation of the tracheobronchial tree in pentobarbital sodium-anesthetized, spontaneously breathing rabbits. In addition, we tried to validate the mechanical stimulation of the tracheobronchial tree delivered by a custom-built device instead of by the manual procedure usually employed in our laboratory. To this purpose, changes in device-induced cough responses following galnon microinjections were also investigated.

MATERIALS AND METHODS

Ethical approval. 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 and 2010/63/UE). The study was approved by the Animal Care and Use Committee of the

Address for reprint requests and other correspondence: D. Mutolo, Dipartimento di Medicina Sperimentale e Clinica, Sezione Scienze Fisiologiche, Viale G.B. Morgagni 63, 50134 Firenze, Italy (e-mail: donatella.mutolo@unifi.it).

University of Florence. All efforts were made to minimize both the number of animals used and their suffering. Experimental procedures and details about the methods employed have previously been described (8, 9, 13, 14, 39–43).

Animal preparation. Experiments were performed on 30 male New Zealand White rabbits (2.8–3.5 kg) anesthetized with pentobarbital sodium (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.

The trachea was cannulated and polyethylene catheters were inserted into a femoral artery and vein for monitoring arterial blood pressure and for drug delivery, respectively. The C_3 or C_5 phrenic root on one side was 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. Bipolar platinum electrodes were used to record efferent phrenic nerve activity from the central stump of one cut and desheathed C_3 or C_5 phrenic root. Wire electrodes (Nichrome wires, insulated except for 1 mm at the tips, diameter 0.1 mm) were inserted into the external or the internal oblique abdominal muscles to record electromyographic (EMG) activity. Phrenic and abdominal activities were amplified, full-wave rectified, and "integrated" (low-pass RC filter, time constant 100 ms). Arterial blood pressure was recorded by a strain-gauge manometer. End-tidal CO_2 partial pressure was measured by an infrared CO_2 analyzer (Capnocheck Plus, Smiths Medical PM, Waukesha, WI). Integrated phrenic and abdominal activities as well as the signals of the other variables were recorded on an eight-channel rectilinearly writing chart recorder (model 8K20; NEC San-ei, Tokyo, Japan). Cardiorespiratory variables were also acquired and analyzed using a personal computer, equipped with an analog-to-digital interface (Digidata 1440, Molecular Devices, Sunnyvale, CA) and appropriate software (Axoscope, Molecular Devices).

Microinjection procedures. As fully illustrated in our previous studies (see e.g., 14, 40, 41, 43), bilateral microinjections were performed at two different sites along the rostrocaudal extent of the caudal NTS and particularly into the lateral commissural NTS. The first was at the level of the caudal-most end of the area postrema, 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. The stereotaxic coordinates were selected according to the atlas of Meessen and Olszewski (35). These procedures were followed to affect as much as possible areas known to contain cough-related second-order neurons. For details on the localization of these neurons in the caudal NTS see Mutolo et al. (40, 41, 43) and Cinelli et al. (14).

Microinjections were performed as described in our previous reports 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 injected volume by each microinjection was 30–50 nl. The drugs used were the following: galanin (1 mM; Tocris Bioscience, Bristol, UK) and galnon (1 and 10 mM; Tocris Bioscience), a nonpeptide agonist at galanin receptors. Galnon was also employed at 10 mM concentration because of its much lower affinity for galanin receptors shown by binding studies and inhibition of adenylate cyclase activity (51, 52). Only one of these drugs was tested in each preparation unless otherwise stated. Microinjections of galanin and galnon with similar concentrations have been performed in previous studies in in vivo preparations (1, 25, 52, 62, 68). Galanin was dissolved in 0.9% NaCl solution. Galnon was initially dissolved in 100% DMSO (Sigma-Aldrich) and then diluted in 0.9% NaCl solution at the desired concentrations. In the final solution, the concentration of DMSO was no more than 20%. Control injections of equal volumes of both types of vehicle solution at the responsive sites were also made. The localization of injection sites is diagrammatically illustrated in Fig. 1.

Stimulation procedures. Mechanical stimulation was performed by means of a 0.5-mm diameter nylon fiber with a smoothed tip inserted through a lateral port of the tracheal cannula until the tip was judged to be near the carina and main bronchi (for further details see Refs. 9, 13, 14, 39, 40, 42, 43). Forth and back movements of the fiber tip (~3 cm from the lower edge of the tracheal cannula) aimed at touching repeatedly (~1 time every second) the carina or nearby airway walls were made over periods of 4–5 s. The maneuver usually produced a

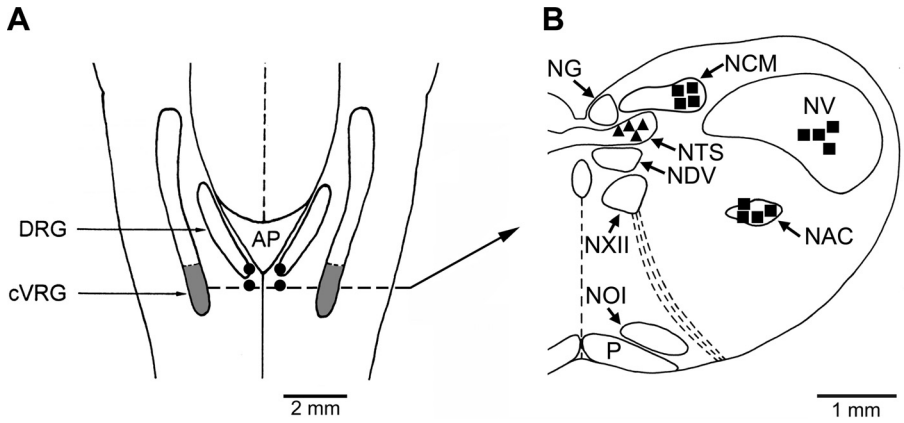

Fig. 1. Localization of injection sites. *A*: a diagrammatic representation of a dorsal view of the medulla oblongata of the rabbit showing where multiple bilateral microinjections (●) of galanin or galnon have been performed into the caudal part of the nucleus tractus solitarii (NTS). The region of drug injections into the caudal ventral respiratory group (cVRG) has been also indicated (shaded area). AP, area postrema; DRG, dorsal respiratory group. *B*: diagram of a coronal section of the medulla oblongata at the level indicated in *A* (dashed line) showing the location of representative sites where the microinjections have been performed. To simplify the presentation of results, only the distribution of sites where 1 mM galanin (▲) was injected has been reported. The diagram also shows the location of injection sites (■) into some control regions where galanin or galnon did not cause appreciable changes in the pattern of breathing and cough responses. Representative sites of control drug microinjections at a location >0.8 mm caudal to the responsive sites of the cNTS have not been illustrated. NAC, nucleus ambiguus caudalis; NCM, nucleus cuneatus medialis; NDV, nucleus dorsalis nervi vagi; NG, nucleus gracilis; NOI, nucleus olivaris inferior; NV, nucleus tractus spinalis nervi trigemini; NXII; nucleus nervi hypoglossi; P, tractus piramidalis.

bout of 2–5 coughs and was always conducted by the same experimenter to ensure consistency of stimulation intensity and frequency between trials. The stimulation protocol comprised three stimulation trials performed in succession before drug administration, repeated ~5 min after the completion of all the microinjections, and at appropriate intervals (at least 5 min) until complete recovery was observed. An interval of ~1 min was scheduled between cough stimulations.

To standardize mechanical stimulation of the tracheobronchial tree and to render it objective and therefore more reliable, in nine experiments a custom-built device was used following the same stimulation protocol and an attempt was made to validate this new cough-inducing procedure. We used a nylon fiber as described above. The external end of the fiber was connected to the noncaptive shaft of a small stepper motor 36000 series linear actuator (Haydon Kerk Motion Solutions, Waterbury, CT). A personal computer connected to a data acquisition board (NI USB-6009, National Instruments Italy Srl, Assago, MI, Italy) and equipped with an appropriate software (LabVIEW 8, National Instruments Italy Srl) was used to drive the stepper motor actuator. When actuated, the motor moved the fiber backward and forward rubbing the smoothed tip onto the airway mucosa. The tip of the fiber was positioned into the trachea just behind the point where its further movement forward caused a cough. The characteristics of the software allowed to set the number of forth and back movements or cycles (1–3), shaft velocity (10–20 mm/s), and shaft displacement (10–20 mm). The relatively slow speed of the fiber tip due to the characteristics of the stepper motor probably implied a more lasting contact of the fiber with the mucosa compared with manual stimulation, and therefore a larger recruitment of cough receptors. After preliminary trials, the mechanical stimulation was adjusted to the following parameters: 1 cycle, 15 mm/s velocity, and 15 mm displacement. These parameters proved to produce a bout of 2–4 coughs. An electrical signal from the device allowed identification of the stimulus onset.

Chemical stimulation of the tracheobronchial tree was performed by means of citric acid inhalation (13, 14, 40, 41). Citric acid (1 M, Sigma-Aldrich) was freshly dissolved in 0.9% NaCl solution and nebulized (particle diameter 80% from 0.5 to 8 μm; nebulization rate 0.5 ml/min) via an ultrasonic nebulizer (Projet, Artsana, Grandate, CO, Italy). The opening of the tracheal cannula, through which the rabbits were spontaneously breathing, was exposed to a steady stream of the nebulized citric acid solution for ~3 s. This short period as well as time intervals between chemical challenges >10 min (usually ~15 min) proved to be adequate to avoid tachyphylaxis (41). Chemical stimulation was always applied 2–3 min after mechanically induced cough and caused a bout of several coughs usually immediately followed by a tachypneic response (41). As a rule, chemical stimulation was performed both before and ~15 min after the completion of the injections and repeated at appropriate intervals to follow the time course of the recovery process. All stimulation procedures were performed at least 5–6 min after each supplemental dose of pentobarbital to avoid the possible immediate influence of the injected bolus on both the breathing pattern and reflex responses.

Histology. At the end of each experiment, the brain was perfused via a carotid artery with 0.9% NaCl solution and subsequently with 10% Formalin solution. 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. The atlas of Meessen and Olszewski (35) and the more recent atlas of Shek et al. (56) were used for comparison. Details on the localization of injection sites and pipette tracks have already been described in several previous reports (14, 40, 41, 43).

Data collection and analysis. Respiratory variables were measured during eupneic breathing and reflex responses. The inspiratory (T_I) and expiratory (T_E) times, as well as the total duration of the respiratory or cough cycle (T_T), were measured on recordings of raw phrenic nerve activity. The respiratory frequency was subsequently calculated (breaths/min). T_T was measured from the onset of phrenic nerve activity to the onset of the next phrenic burst. T_I was defined as the period from the onset of phrenic nerve activity until its maximum, while T_E was defined as the interval from the peak of phrenic nerve activity to the onset of the next phrenic burst (e.g., 49). 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 observed during coughing. Therefore, all amplitudes have been expressed as in our previous reports in relative units (RU). Breathing pattern variables were measured for an average of five consecutive breaths before and after drug bilateral microinjections into the caudal NTS. Furthermore, systolic and diastolic blood pressures were measured at 2-s intervals, and mean arterial pressure was calculated as the diastolic pressure plus one-third of the pulse pressure. The measurement periods were the same selected for cough-related variables (see below). 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.

The cough motor pattern in response to mechanical or chemical stimulation of the tracheobronchial tree is characterized by repeated coughs. Each of them consists of an augmented phrenic burst (preparatory inspiration) immediately followed by a burst of expiratory abdominal activity (9, 13, 14, 39–43). In agreement with our previous results, repeated coughs usually started during stimulation and continued shortly after stimulus cessation. Respiratory variables of coughs (cough-related variables) included the cough-related T_T, T_I, and T_E, peak phrenic amplitude (RU), peak abdominal activity (RU), and the cough number, i.e., the number of coughs following each stimulation. Cough-related variables were measured and averaged before and after drug administration at the time when the maximum response occurred as well as at the time when a complete recovery was observed (three trials for mechanical stimulation and a single trial for citric acid inhalation). The average values of cough-related variables were taken as single measurements for subsequent statistical analysis (Sigma Stat, SPSS, Chicago, IL). 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. This pattern could fit more appropriately the definition of expiration reflex that is typically evoked by mechanical stimulation of the vocal folds, but that can be also produced by mechanical stimulation of the tracheobronchial tree (26, 64). For further details see Mutolo et al. (39–43) and Cinelli et al. (13, 14). 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.

Average values of cough-related variables observed under control conditions at the time when the maximum response to drug microinjections occurred and after complete recovery were used for statistical analysis. Average values of baseline cardiorespiratory variables during the same periods were also considered. Comparisons were performed by means of the one-way repeated-measures ANOVA followed by Student-Newman-Keuls tests. To validate the reliability of the cough-inducing device, cough responses at 30-min intervals were compared by the same statistical analysis. All reported values are means ± SE; $P < 0.05$ was taken as significant.

RESULTS

Galanin-induced effects. Bilateral microinjections of 1 mM galanin (30–50 nl; 30–50 pmol) into the caudal NTS were performed in four animals. They induced within ~15 min

(maximum effect) consistent and marked reductions in the cough response to both mechanical and chemical stimulation of the tracheobronchial tree (Table 1 and Fig. 2*A*). The cough number and peak abdominal activity decreased, whereas the cough-related T_T increased due to a rise in both T_I and T_E. Cough-depressant effects developed progressively, but they were already clearly appreciable on mechanically induced cough within ~5 min after the microinjections. The complete recovery of the cough reflex was seen after 60–90 min from the completion of the microinjections. The time course of galanin effects on the cough number in cough bouts induced by both mechanical and chemical stimulations of the tracheobronchial tree is reported in Fig. 2*B*.

Galnon-induced effects. Bilateral microinjections of 1 mM galnon (30–50 nl; 30–50 pmol) into the caudal NTS performed in four animals induced mild suppressant effects on the cough reflex. About 10 min after the injections (maximum effect), decreases only in the cough number and in the peak abdominal activity were observed. The cough number decreased from 3.7 ± 0.14 to 2.7 ± 0.25 ($P < 0.05$) during mechanical stimulation and from 5.0 ± 0.91 to 4.3 ± 0.81 ($P < 0.05$) during chemical stimulation. Peak abdominal activity changed from 0.60 ± 0.03 to 0.42 ± 0.02 RU ($P < 0.05$) and from 0.54 ± 0.01 to 0.44 ± 0.02 RU ($P < 0.05$) during mechanical and chemical stimulation, respectively. The recovery of these variables occurred within ~40 min.

Bilateral microinjections of 10 mM galnon (30–50 nl; 300–500 pmol) into the caudal NTS of five rabbits caused within ~15 min (maximum effect) marked decreases in cough responses (Table 1 and Fig. 3). The cough number and peak abdominal activity decreased, whereas the cough-related T_T increased as a consequence of increases in both T_I and T_E. Galnon turned out to induce changes in cough-related variables consistent with those caused by galanin. Also galnon-induced effects developed gradually, but they were already evident within 5 min after the microinjections. The recovery was complete within 60–90 min.

Galnon-induced effects on device-induced cough responses. In a series of nine experiments, cough reflex responses were induced by the custom-built device. In four animals, cough-related variables were measured at different time intervals, i.e., at the beginning of the experiment after animal stabilization (control) and at intervals of about 30 min. They remained very accurately repeatable at least for 2 h. For simplicity only control values and those recorded after 2 h have been reported in Table 2. Thereafter, within a variable time period ranging from 30 to 60 min, cough responses resulted altered in three of four animals, possibly due to mucus production and to some time-dependent deterioration of the airway mucosa.

In five preparations, changes in cough responses following microinjections of 10 mM galnon into the caudal NTS were investigated. Galnon-induced effects (Table 2 and Fig. 4) consisted of decreases in the cough number and peak abdominal activity associated with increases in the cough-related T_T due to a rise in both T_I and T_E. The recovery was complete within 60–90 min. Noticeably, these effects were consistent with those induced by 10 mM galnon on cough responses to mechanical stimulation executed manually (see Table 1 and 2).

Drug treatment and cardiorespiratory variables during eupneic breathing. Bilateral microinjections of galanin or galnon into the caudal NTS did not affect cardiorespiratory variables during eupneic control breathing (see e.g., control recordings before reflex responses in Figs. 2, 3, and 4; statistical data not shown). However, we observed that following microinjections of both galanin and galnon, basal abdominal activity was greatly reduced without concomitant changes in phrenic bursts. Abdominal activity decreased from 0.06 ± 0.005 to 0.02 ± 0.01 RU ($P < 0.05$) after 1 mM galanin ($n = 4$) and from 0.05 ± 0.006 to 0.03 ± 0.002 RU ($P < 0.05$) after 10 mM galnon ($n = 5$). The recovery of abdominal activity paralleled the recovery of cough-related variables. Mean arterial blood pressure was maintained between 96 and 104 mmHg. For a general evaluation of cardiorespiratory variables in the rabbit under control conditions, see also previous reports (39, 41, 43).

Table 1. *Cough-related variables before and 15 min after bilateral microinjections of galanin and galnon into the caudal NTS*

	CN	T_T, s	T_I, s	T_E, s	PPA, RU	PAA, RU
			Galanin ($n = 4$)			
Mechanical stimulation						
Control	3.5 ± 0.28	0.59 ± 0.04	0.36 ± 0.02	0.22 ± 0.03	0.63 ± 0.02	0.58 ± 0.03
1 mM	1.7 ± 0.32*	0.77 ± 0.05*	0.44 ± 0.02*	0.32 ± 0.04*	0.55 ± 0.05	0.37 ± 0.06*
Recovery	3.4 ± 0.26	0.60 ± 0.05	0.37 ± 0.03	0.22 ± 0.04	0.64 ± 0.03	0.57 ± 0.03
Citric acid inhalation						
Control	5.7 ± 0.94	0.49 ± 0.04	0.31 ± 0.02	0.17 ± 0.02	0.61 ± 0.02	0.58 ± 0.03
1 mM	4.0 ± 0.70*	0.65 ± 0.03*	0.37 ± 0.01*	0.27 ± 0.03*	0.58 ± 0.02	0.29 ± 0.05*
Recovery	5.6 ± 0.92	0.48 ± 0.03	0.31 ± 0.03	0.16 ± 0.02	0.60 ± 0.01	0.58 ± 0.04
			Galnon ($n = 5$)			
Mechanical stimulation						
Control	3.7 ± 0.27	0.58 ± 0.03	0.34 ± 0.04	0.23 ± 0.03	0.64 ± 0.02	0.59 ± 0.06
10 mM	1.8 ± 0.24*	0.78 ± 0.06*	0.42 ± 0.02*	0.35 ± 0.04*	0.55 ± 0.06	0.40 ± 0.07*
Recovery	3.5 ± 0.25	0.59 ± 0.03	0.34 ± 0.03	0.24 ± 0.04	0.63 ± 0.02	0.57 ± 0.06
Citric acid inhalation						
Control	5.4 ± 0.40	0.50 ± 0.01	0.32 ± 0.01	0.17 ± 0.01	0.69 ± 0.03	0.56 ± 0.02
10 mM	3.3 ± 0.37*	0.63 ± 0.03*	0.37 ± 0.02*	0.26 ± 0.03*	0.60 ± 0.06	0.38 ± 0.07*
Recovery	5.3 ± 0.39	0.49 ± 0.01	0.31 ± 0.01	0.18 ± 0.02	0.67 ± 0.03	0.54 ± 0.02

Values are means ± SE; n, number of animals. CN, cough number; T_T, cycle duration; T_I, inspiratory time; T_E, expiratory time; PAA, peak phrenic activity in relative units (RU); PPA, peak abdominal activity in relative units (RU). *$P < 0.05$ compared with control cough as well as with recovery.

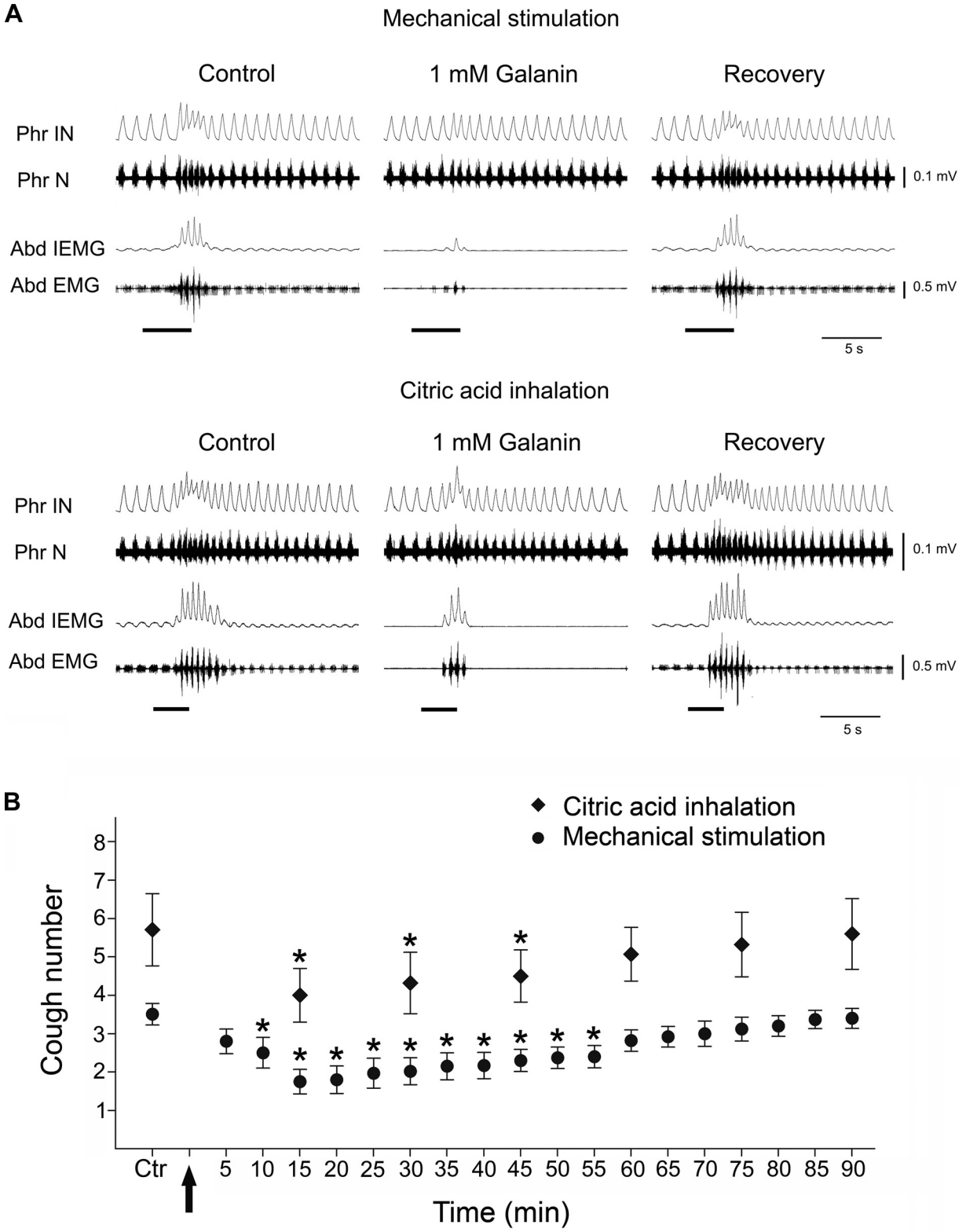

Fig. 2. Depressant effects of 1 mM galanin microinjected into the selected sites of the caudal NTS on cough reflex responses elicited by mechanical stimulation of the tracheobronchial tree and by the inhalation of 1 M citric acid in one anesthetized spontaneously breathing rabbit. *A*: original recordings illustrating the reduction of cough responses ~15 min after bilateral microinjections of galanin when the maximum effect occurred. Recovery of cough responses was taken ~90 min after the injections. Stimulation periods marked by filled bars. Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity. *B*: time courses of galanin effects on the cough number. Galanin microinjections indicated by the arrow. Ctr, control. $^*P < 0.05$ compared with control.

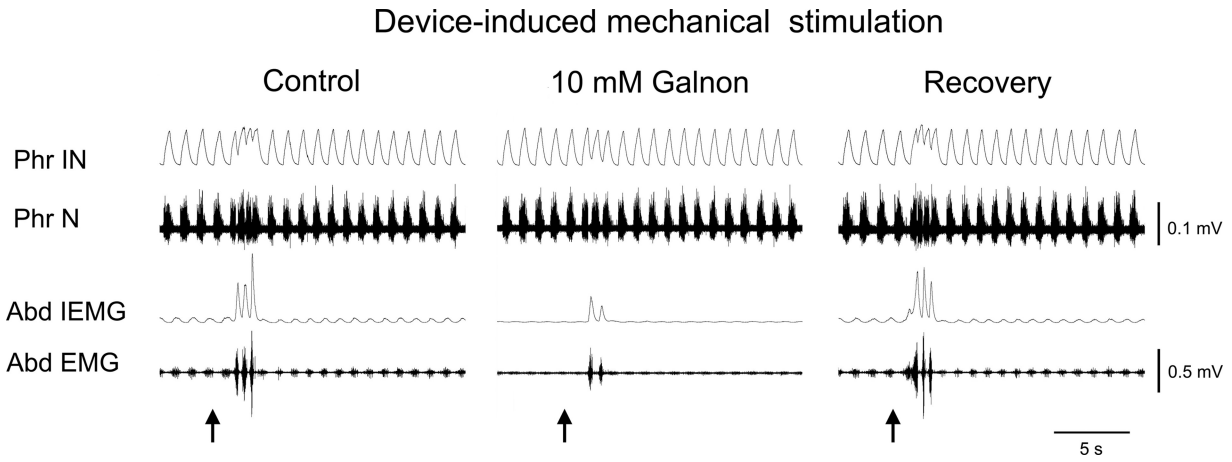

Fig. 3. Depressant effects of 10 mM galnon microinjected into the caudal NTS on cough reflex responses induced by mechanical stimulation of the tracheobronchial tree and by the inhalation of 1 M citric acid in one anesthetized spontaneously breathing rabbit. Depression of cough responses ~15 min after bilateral microinjections when the maximum effect occurred. Recovery of cough responses was taken ~90 min after the injections. Stimulation periods marked by filled bars.

Controls. In eight additional preparations, bilateral control microinjections of 1 mM galanin (4 animals) and 10 mM galnon (4 animals) were performed at different medullary locations sufficiently far from the responsive sites (14, 39–41, 43, 45, 60). Four microinjections of each drug were executed [for comparison, see Meessen and Olszewski (35) and Shek et al. (56)] into the nucleus cuneatus medialis, the nucleus tractus spinalis nervi trigemini, and at a location >0.8 mm caudal to the responsive sites of the caudal NTS. Similarly, in all these additional preparations control microinjections of galanin (4 animals) and galnon (4 animals) were performed into the caudal ventral respiratory group at sites defined in previous reports by stereotaxic coordinates derived from prior extracellular recordings from expiratory neurons (13, 14, 41, 42). In all instances, no appreciable changes in the pattern of breathing and cough responses were observed. In particular, it is worth noting that galanin and galnon did not cause any significant effect on the cough reflex when microinjected into the caudal ventral respiratory group, a widely recognized site of action of centrally acting antitussive drugs and a neural structure with a prominent role in the regulation of the cough reflex (see e.g., 9, 13, 14, 41, 42, 48, 50).

Control bilateral microinjections of each type of vehicle solution at the responsive sites were performed in some preparations before the administration of galanin (3 trials) or galnon (3 trials). In addition, control bilateral microinjections of either saline or DMSO solution at the responsive sites were also performed in two of the eight additional preparations. In accordance with the results of our previous studies (39, 40, 43), these control injections did not significantly affect cough reflex responses. The localization of the injection sites was confirmed by the histological control (for details see Refs. 14, 40, 41, 43). Figure 1 illustrates the localization of injection sites on a dorsal view of the medulla oblongata as well as the distribution of injection sites within the caudal NTS and within some of the control regions. For simplicity, only the distribution of sites where 1 mM galanin was injected has been reported.

DISCUSSION

In this study we provide for the first time evidence that galanin and the nonpeptide galanin receptor agonist galnon microinjected into the caudal NTS cause depressant effects on cough responses induced either by mechanical stimulation of the tracheobronchial tree or by citric acid inhalation in the anesthetized rabbit. Present findings support the notion that the caudal NTS is an important site for the inhibitory modulation of the cough reflex and indicate that galanin receptors play an important role in this function. At variance with other antitussive agents (9, 13, 14, 41, 42, 50), galanin does not appear to be involved in the central regulation of cough at the level of the caudal ventral respiratory group. These results provide further evidence supporting the similarities between the characteristics of central processing of nociceptive and cough-related inputs. Device-induced cough responses appear to be fairly repeatable

Table 2. *Cough-related variables during device-induced mechanical stimulation of the tracheobronchial tree in control conditions as well as before and 15 min after 10 mM galnon bilateral microinjections into the caudal NTS*

	CN	T_T, s	T_I, s	T_E, s	PPA, RU	PAA, RU
Control conditions (n = 4)						
Control	3.0 ± 0.24	0.51 ± 0.03	0.35 ± 0.01	0.16 ± 0.01	0.62 ± 0.01	0.52 ± 0.03
2 h later	2.9 ± 0.25	0.52 ± 0.02	0.36 ± 0.01	0.17 ± 0.02	0.61 ± 0.02	0.51 ± 0.04
Galnon (n = 5)						
Control	3.0 ± 0.32	0.54 ± 0.02	0.34 ± 0.01	0.19 ± 0.03	0.62 ± 0.03	0.52 ± 0.04
10 mM	1.7 ± 0.13*	0.77 ± 0.06*	0.44 ± 0.03*	0.33 ± 0.05*	0.58 ± 0.02	0.30 ± 0.05*
Recovery	3.1 ± 0.34	0.53 ± 0.02	0.34 ± 0.02	0.18 ± 0.04	0.61 ± 0.03	0.51 ± 0.03

Values are means ± SE; n, number of animals. *$P < 0.05$ compared with control cough as well as with recovery.

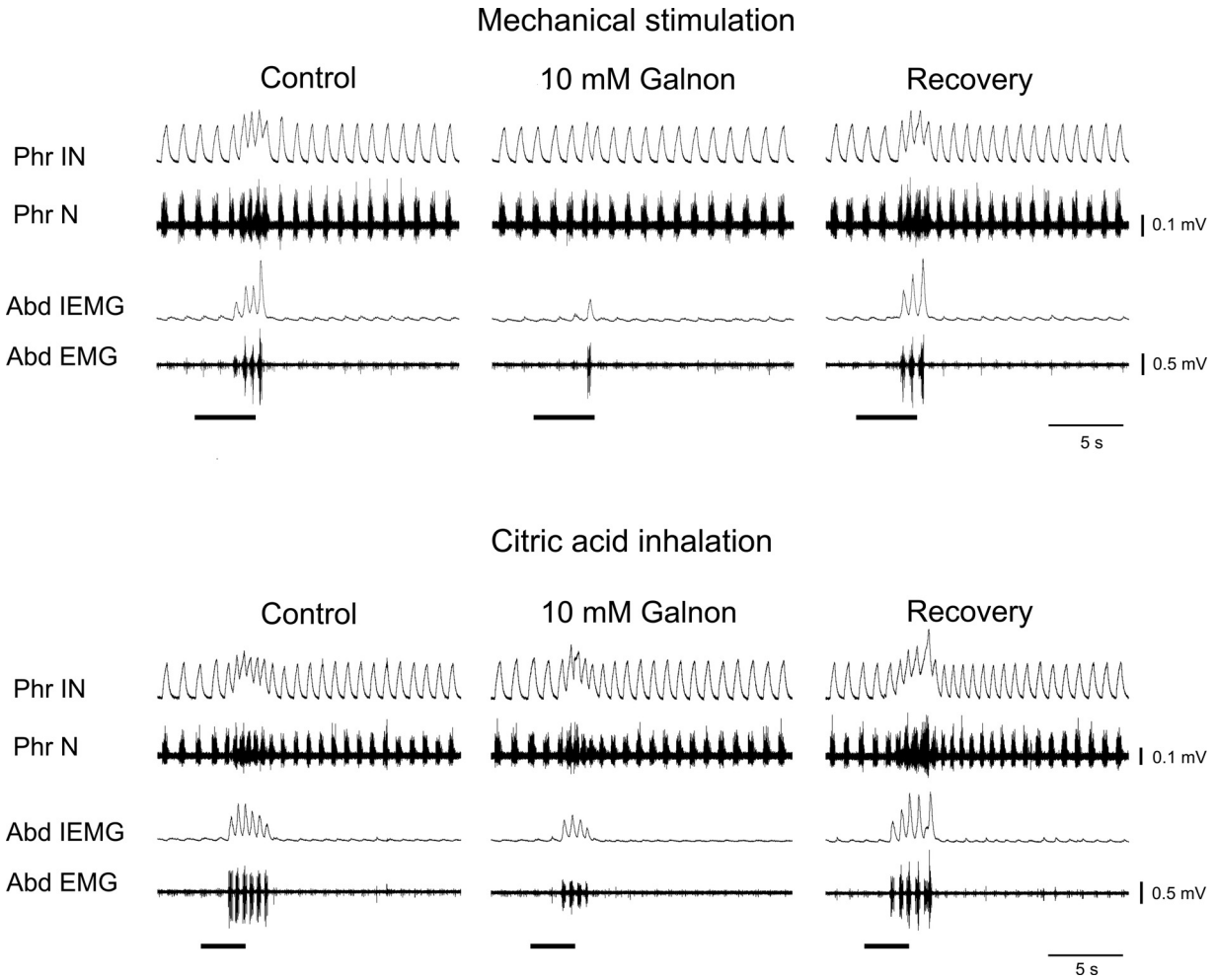

Fig. 4. Downregulation of the cough reflex responses to device-induced mechanical stimulation of the tracheobronchial tree following bilateral microinjections of 10 mM galnon into the caudal NTS in one anesthetized spontaneously breathing rabbit. Reduction of the cough reflex ~15 min after the completion of the injections. The complete recovery of cough reflex was obtained ~90 min after the injections. The onset of mechanical stimulation is indicated by arrows.

for at least 2 h and suitable to disclose drug-induced effects on the cough reflex.

General remarks. Unfortunately, we were not able to employ galanin receptor antagonists (M40 and M871, Tocris Bioscience) and selective agonists (M617 and ARM1896, Tocris Bioscience) owing to their poor aqueous solubility that did not allow us to obtain stable and reliable solutions at final millimolar concentrations and, as far as the antagonists are concerned, even at concentrations <0.5 mM. Relatively high drug concentrations (mM) have been shown to be as a rule necessary for microinjection techniques, as fully discussed in a previous report (8). The reliability of microinjection techniques and the characteristics of the spread of the injectate have been discussed in detail in previous studies (9, 13, 14, 39–43). However, for a more exhaustive appraisal and discussion on drug diffusion following microinjections into the central nervous system as well as on their reliability see Refs 18, 31, 45, and 60. The specificity of drug-induced effects is supported by the absence of changes in cough-reflex responses following bilateral microinjections of vehicle solutions into the caudal NTS or drug microinjections into regions sufficiently away from the responsive sites. In the present study, no significant effects on the eupneic pattern of breathing were induced by galanin or galnon microinjections into the caudal NTS except for a selective depression in basal abdominal activity. Interestingly, changes in mean arterial blood pressure that may affect the intensity of the cough reflex (49) were not seen. These findings reveal decreased motoneuron excitability, a phenomenon that could underlie changes in cough-related variables and especially decreases in peak abdominal activity (for a review, see Ref. 53) and imply a specific action of the employed drugs on central cough-related pathways within the NTS (see also below).

Changes in cough-related variables. Interestingly, changes in cough-related variables induced by galanin or galnon microinjections into the caudal NTS (Tables 1 and 2) recall those previously observed following microinjections of low concentrations of [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO), a μ-opioid agonist, baclofen, a $GABA_B$ receptor agonist, and clonidine, an α_2-adrenergic receptor agonist, into the same medullary region (14, 39). As in these previous reports, both galanin and galnon increased the cough-related T_T because of increases in T_I and T_E. In addition, the absence of changes in peak phrenic amplitude associated with an increase in T_I indicates depressant effects on inspiratory activity and inspiratory drive revealed by the reduced rate of rise of inspiration

(61). Noticeably, increases in the cough-related T_E were also observed after *N*-methyl-D-aspartate (NMDA) receptor blockades within the caudal NTS (41, 43). On the basis of the differential effects of antitussive drugs on the cough and breathing pattern, Bolser and colleagues (7, 50) have proposed the presence of a cough-gating mechanism controlling inspiratory and expiratory burst amplitude as well as cough number, but not phase duration. Present and previous results from our laboratory (e.g., 13, 14, 39–43) are, at least in part, consistent with this view. However, as already mentioned, we have observed not only changes in the cough number, but also in the time components of the cough reflex. In agreement with our previous interpretation, this discrepancy can be tentatively attributed to differences in the animal species, experimental conditions, and characteristics of the employed drugs.

Role of galanin receptors. There are three types of galanin receptors, GAL_1, GAL_2, and GAL_3, and all these are G protein-coupled receptors (28, 63). GAL_1 receptors are the most abundant and widely expressed at least in the central nervous system of adult rats (28, 30, 63 also for further references). In particular, GAL_1 receptors are strongly represented in the pons-medulla oblongata and in dorsal spinal cord regions. GAL_2 receptors are also broadly expressed in the central nervous system, but in regions less relevant to cough regulation such as the hippocampus, the hypothalamus, and the spinal trigeminal tract, whereas GAL_3 receptors are mainly expressed in peripheral tissues (for a review, see Ref. 28). Interestingly, GAL_1 receptors have also been found in the NTS (16, 36), a neural structure, as already mentioned, essential for the genesis of cough reflex responses. Owing to the similarities between nociception and cough-related mechanisms, a major involvement of GAL_1 receptors in the downregulation of the cough reflex can be hypothesized. In fact, it has been reported that GAL_1 receptors probably mediate analgesic effects, whereas GAL_2 receptors are mainly involved in pro-nociceptive effects (e.g. 2, 22, 32, 68; reviewed in Refs. 38, 69). Galanin may exert its action presynaptically through both GAL_1 and GAL_2 receptors as well as postsynaptically predominantly via GAL_1 receptors (2, 28). In this context, it should be also recalled that galanin antagonizes substance P-evoked hyperalgesia (23, 70; see Ref. 69 also for further references) and that its antinociceptive effects involve opioid receptors and display positive interactions with NMDA receptor antagonists (22, 25, 62, 66; see Ref. 69 also for further references). These findings are relevant to the galanin antitussive effects since both a protussive role of substance P (34, 43) and an antitussive action of opioid receptor agonists and NMDA receptor antagonists (39, 41, 43) have been demonstrated within the caudal NTS.

Projections of galaninergic neurons to caudal NTS neurons may derive from several sites of the central nervous system (see e.g., 28, 36, 37, 63). However, their antitussive role is, at present, only matter of speculation. Galaninergic neurons possibly involved in this function could be localized in the NTS itself or in neural structures already proved to be engaged in the antinociceptive control, such as the medullary midline raphe nuclei or the periaqueductal grey (e.g., 38, 62). To further support the parallel between nociception and cough, we can also mention that medullary raphe nuclei have a complex role in the generation of airway defensive reflexes including cough (e.g., 3, 24, 57) and that, in addition, suppressive influences from the periaqueductal grey and the nucleus raphe magnus on the cough reflex have been demonstrated (54). Interestingly, similarly to galanin-induced antinociception, these influences are in part mediated by opioids.

Galnon has been involved in several physiological functions including nociception, that has been suggested to engage mainly galanin receptors (52, 58, 67). Galnon has been reported to display higher affinity for GAL_1 receptors and exerts its effects mainly through this receptor subtype (5, 33, 52). Although galnon has also been shown to be a ligand to other G protein-coupled receptors in addition to $GAL_{1–3}$ receptors and to activate intracellular G proteins directly (17, 51), we are confident that its antitussive effects are mainly due to an action on galanin receptors owing to their similarities with those caused by galanin. In fact, as we have suggested above for galanin-induced downregulation of the cough reflex, it seems reasonable to propose that GAL_1 receptors have a prominent role. On the other hand, GAL_1 receptors have been implicated in the mediation of the antinociceptive and anticonvulsant functions of galnon (52, 67). Galnon depressant effects on the cough reflex induced by mechanical stimulation of the tracheobronchial tree as well as the subsequent complete recovery of cough responses were confirmed by using the cough-inducing device. These outcomes appear to validate this new stimulation procedure. In particular, the repeatability of device-induced cough responses and the complete recovery after drug administration ensure reliability. Device-induced cough responses, as already mentioned, were accurately repeatable for at least 2 h in all tested preparations. After this period, despite cleaning and repositioning of the nylon fiber, cough responses were no more reliably comparable with those obtained before because of consistent decreases especially in the cough number and peak abdominal activity. Thus we took as appropriate for cough studies a 2-h working period of the device. Post mortem analysis of the trachea and main bronchi revealed the presence of mucus, but no evidence of obvious mucosal lesions that, however, could be present at the histological level given the relatively high number of stimulation trials performed (histological analysis not performed).

Perspectives and Significance

Present results contribute to get further insights into the central neural mechanisms involved in the control of the cough motor pattern and especially of those operating within the caudal NTS. They confirm that the caudal NTS is an important neural structure where cough-related sensory inputs can be modulated and a prominent site of action of centrally acting antitussive drugs (e.g., 10, 14, 34, 39, 40, 43). We believe that the present findings are of particular interest since they are the first to provide evidence that galanin receptors are involved in the downregulation of the cough reflex at the level of the NTS, where galanin receptors have been found (16, 36).

In addition, present results corroborate the correlation between nociception and cough (4, 11, 12, 14, 20, 29, 40) and support the advantage of treating chronic cough with drugs already employed in neuropathic pain. They also encourage further studies on galanin receptor agonists to develop novel antitussive compounds with proved efficacy and devoid of important side effects.

ACKNOWLEDGMENTS

The authors thank Alessandro Aiazzi and Claudio Pregno for their technical assistance and in particular for the implementation of the cough-inducing device.

GRANTS

This study was supported by grants from the Ministry of Education, University, and Research of Italy and from the A. Menarini United Pharmaceutical Industries. E. Cinelli is supported by a Postdoctoral Fellowship from Regione Toscana and Menarini United Pharmaceutical Industries.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.M., E.C., F.B., and T.P. conception and design of research; D.M., E.C., F.B., and T.P. performed experiments; D.M., E.C., F.B., and T.P. analyzed data; D.M., E.C., F.B., and T.P. interpreted results of experiments; D.M. drafted manuscript; D.M., F.B., and T.P. edited and revised manuscript; D.M., E.C., F.B., and T.P. approved final version of manuscript; E.C. prepared figures.

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