Downregulation of the cough reflex by aclidinium and tiotropium in awake and anesthetized rabbits

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Abstract

Long-acting muscarinic receptor antagonists (LAMAs) have been reported to attenuate cough in preclinical and clinical studies. The present study was performed on rabbits to compare aclidinium and tiotropium efficacy in the downregulation of the cough reflex. This reflex was evoked by citric acid inhalation in unanesthetized animals and by both citric acid inhalation and mechanical stimulation of the tracheobronchial tree in anesthetized animals 90 min following the inhalation of each drug (nebulizer output always at 1 mL/min). Aclidinium 4 mg/mL and tiotropium 200 μg/mL inhaled in 1 min proved to have similar protective effect on methacholine-induced bronchoconstriction in anesthetized animals. The total dosage employed for aclidinium and tiotropium was 4 mg and 200 μg, respectively. In awake animals, similar reductions in the cough number were observed following 10-min inhalation of each drug with a slight, not significant tendency to higher antitussive effects for aclidinium. In anesthetized animals, 1-min inhalation of each drug caused similar depressant effects on cough responses induced by both mechanical and chemical stimulation. A complete suppression of cough responses to mechanical stimuli was seen in some preparations. The results strongly suggest that the LAMA-induced downregulation of cough may be mediated not only by transient receptor potential vanilloid type 1 channels, as already reported, but also by acid-sensing ion channels and mechanoreceptors. The route of administration along with the more rapid hydrolysis of aclidinium into inactive metabolites minimize potential systemic side effects and give to this drug a very favorable safety profile.

Keywords: Tiotropium; Aclidinium; The cough reflex; Long-acting muscarinic receptor antagonists; ASICs; Airway mechanoreceptors

Abbreviations: ASICs, acid-sensing ion channels; COPD, chronic obstructive pulmonary disease; DMSO, dimethyl sulfoxide; LAMAs, long-acting muscarinic receptor antagonists; MCh, methacholine; P_{ao}, alveolar pressure; P_{es}, esophageal pressure; P_{TP}, transpulmonary pressure; R_L, lung resistance; TRPV1, transient receptor potential vanilloid type 1

1 Introduction

Cough is a very important airway defensive reflex [1–4] and is the most common symptom for which patients seek medical advice. Despite considerable efforts in the last decades to find appropriate therapies, a safe and effective cough remedy is still lacking [5–11].

Inhaled bronchodilator therapies with long-acting muscarinic receptor antagonists (LAMAs) are of crucial importance for chronic obstructive pulmonary disease (COPD) management and cause an improvement in symptoms including cough ([12–16] also for further Refs.). Tiotropium was the first LAMA, reaching the market in 2002 [17]. Dicpinigaitis et al. [18] reported that tiotropium (1 h after its inhalation) inhibits cough induced by capsaicin, a transient receptor potential vanilloid type 1 (TRPV1) agonist, in patients with upper respiratory tract infections. Furthermore, tiotropium inhalation has been shown to improve cough and other symptoms in patients with chronic pulmonary disease due to sulphur mustard lung injury [19]. More recently, it has been shown that inhaled tiotropium attenuates after 1 h cough induced by capsaicin in the guinea pig and that this effect is mediated by TRPV1 receptors through a mechanism unrelated to its anticholinergic activity [17]. However, Clay et al. [7] have reported that, in contrast to the guinea pig, the ozone-induced hypertussive responses to citric acid are not inhibited by tiotropium in the rabbit.

Aclidinium bromide is a LAMA that has recently been approved as a maintenance bronchodilator treatment for patients with COPD and asthma [14,20,21]. In clinical studies, aclidinium provides greater improvements in COPD

symptoms, including cough, than tiotropium and is well tolerated, with a similar safety profile [12,13]. A recent study in the guinea pig chronically exposed to cigarette smoke (an experimental model of COPD) indicates that aclidinium, in addition to beneficial effects on lung structure and function, shows a trend toward fewer cough episodes [22]. A comparative study on the antitussive effects of aclidinium and tiotropium in animal models is lacking.

The present study was undertaken to compare aclidinium and tiotropium efficacy in the downregulation of the cough reflex in the rabbit. The two drugs were administered by inhalation, whilst the cough reflex was evoked by citric acid inhalation in awake animals and by both citric acid inhalation and mechanical stimulation of the tracheobronchial tree in anesthetized animals [8–11,23–28].

2 Materials and methods

2.1 Preliminary remarks

A total of 48 rabbits were enrolled in this study, including 2 rabbits used in preliminary trials and 6 rabbits employed to investigate the LAMA protective action on cholinergic-induced bronchoconstriction (see below). 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 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 [8–11,23–28].

2.2 Effects of aclidinium or tiotropium on methacholine-induced bronchoconstriction

The first step was to assess the concentrations of the two drugs effective in counteracting the bronchoconstriction induced by methacholine (MCh; Sigma-Aldrich, St. Louis, MO, USA). Experiments were performed in New Zealand white rabbits (3.1–3.6 kg) anesthetized with pentobarbital sodium (40 mg/kg i.v., supplemented by 2–4 mg/kg every 30 min; Sigma-Aldrich) and spontaneously breathing. The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw. 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 animal was placed in a prone position and fixed by a stereotaxic head holder and vertebral clamps. Body temperature was maintained at 38.5–39 °C by a heating blanket controlled by a rectal thermistor probe.

Esophageal pressure (P_{es}) was measured with a thin-walled latex balloon (5-cm length) sealed over a polyethylene catheter (100-cm length, 1.7 mm ID) with several holes in the section covered by the balloon, positioned in the midesophagus and connected to a pressure transducer. This corresponds to the transpulmonary pressure (P_{TP}) under static conditions, i.e. $P_{TP} = P_{es} - P_{ao}$, where P_{ao} is the alveolar pressure at end inspiration or expiration, equal to the atmospheric pressure (see e.g. Ref. [29]). The flow signal was recorded by means of a pneumotachograph (Fleish no. 00) and a differential pressure transducer. The quotient of the maximum inspiratory value of P_{es} and the maximum inspiratory value of tidal flow (Fig. 1A) was considered a reliable index of lung resistance (R_L; see e.g. Refs. [30,31]). All recorded variables were acquired and analyzed using a personal computer, equipped with an analog-to-digital interface (Digidata 1440, Molecular Devices, Sunnyvale, CA, USA) and appropriate software (Axoscope, Molecular Devices). Obviously, the pneumotachograph was disconnected during drug inhalation and connected again for R_L assessment.

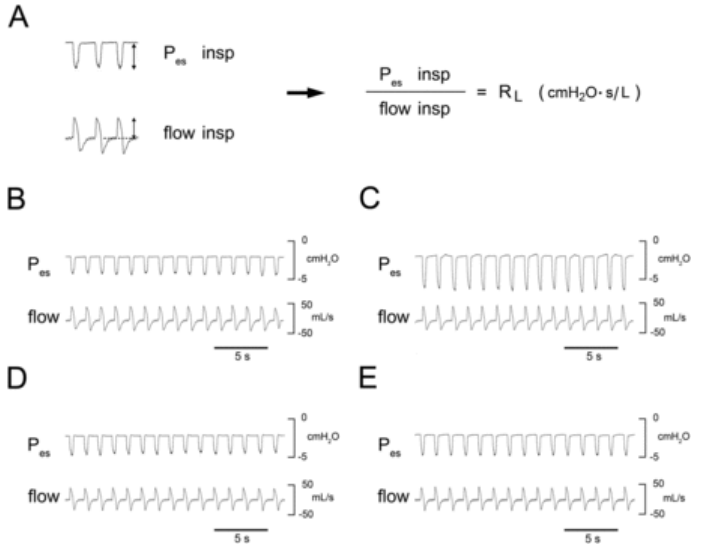

Fig. 1 Evaluation of lung resistance (R_L) and protective effects exerted by aclidinium on bronchoconstriction in one rabbit. A, esophageal pressure (P_{es}) and flow recordings at end inspiration (insp) were used to evaluate R_L through their ratio. B, control conditions (R_L = 49 cmH_2O s/L). C, bronchoconstriction induced by aerosolized methacholine (MCh) at 16 mg/mL. Note the clear increase in end-inspiration P_{es}; R_L increased by 85% (R_L = 91 cmH_2O s/L). D, recordings following aclidinium inhalation (total dosage 4 mg/mL; nebulizer output at 1 mL/min) before MCh challenge

(R_L = 51 cmH_2O s/L). E, challenge with aerosolized MCh at 16 mg/mL 90 min after aclidinium inhalation. Note the virtually absence of changes in both recorded variables and thus in R_L (R_L = 56 cmH_2O s/L; decrease in R_L > 90%), revealing the protective effect on MCh-induced bronchoconstriction.

MCh, dissolved in 0.9% NaCl solution, was delivered by an ultrasonic nebulizer (Projet, Artsana, Grandate, CO, Italy). The diameter of the droplets ranged from 0.5 to 8 μm and the nebulizer output was always set at 1 mL/min. The opening of the tracheal cannula, through which the rabbits were spontaneously breathing, was exposed to a steady stream of the nebulized MCh solution for ~30 s. In preliminary trials (two rabbits), MCh was delivered at increasing concentrations (8, 16 and 32 mg/mL) as previously reported [30] to select the dose able to induce a submaximal increase of R_L. The selected dose was 16 mg/mL. MCh challenges and induced bronchoconstriction were evaluated before and after aclidinium (1, 2 and 4 mg/mL for 1 min) and tiotropium (50, 100 and 200 μg/mL for 1 min) to counteract bronchoconstriction and cause a fall in R_L > 90%. R_L was measured under basal conditions and after aerosolized MCh at 16 mg/mL. A maximum bronchoconstriction occurred within about 1 min and was maintained during the following 2–3 min. A rapid recovery followed. Nevertheless, a time interval of at least 30 min was allowed before 1-min inhalation of aclidinium or tiotropium. Ninety min after drug inhalation, the MCh challenge was repeated and R_L re-evaluated. The interval of 90 min was chosen to ensure LAMA maximum effects; both aclidinium and tiotropium (as other LAMAs) have been proved to fully express their protective action on cholinergic-induced bronchoconstriction at least 1 h post-administration [32]. As illustrated in Fig. 1, we found that the adequate concentration of each drug to produce similar decreases in R_L (range 90–95%) was 4 mg/mL of aclidinium and 200 μg/mL of tiotropium. Since these muscarinic receptor antagonists are long-acting [33], it was necessary to use one animal for each drug concentration, i.e. three rabbits for each drug.

Aclidinium bromide (ShanHai Biochempartner Co., Ltd, China) was initially dissolved in a 0.1 N hydrochloric acid/DMSO (10:90, v/v) mixture and then diluted in 0.9% NaCl solution at the desired concentrations [34]. In the final solution, the concentration of DMSO was less than 5%. Tiotropium bromide monohydrate (Sigma-Aldrich) was dissolved in 0.9% NaCl solution at the desired concentrations [7,33]. All drugs were freshly prepared on the day of administration. Vehicle solutions administered in the same preparations proved to be without effects on MCh-induced bronchoconstriction.

We roughly estimate that over the 1-min challenge, the lungs will be exposed to 160–200 μg of aclidinium. This was based on a normal breathing rate of 40–50 breaths/min and a tidal volume of ~30 mL as well as a deposition rate of 10% with the aerosolized 4 mg/mL solution, which is “diluted” ~3000-fold with room air from the nebulizer system (airflow of the nebulizer set at ~3 l/min). Taking into consideration an aerosolized 200 μg/mL solution of tiotropium, similar calculations led to the estimation that over the 1 min of challenge the lungs will be exposed to 8.4–10.5 μg of tiotropium. For the total dose calculation see also Birrell et al. [17]. The same total dosage (aclidinium 4 mg and tiotropium 200 μg) was employed to investigate drug effects on the cough reflex in both unanesthetized and anesthetized animals. In particular, for an evaluation of total lung exposition to aclidinium and tiotropium in awake animals a similar calculation was performed, taking into account in addition the 10-min duration of the inhalation period in the Perspex chamber and accordingly reducing the concentration of drug solutions to be aerosolized (see below).

2.3 Experiments on unanesthetized animals

Experiments were performed on 20 unanesthetized male New Zealand white rabbits (3.0–3.6 kg). In each cough induction test [23,24], the rabbits were placed individually into a transparent Perspex inhalation chamber (approximately 0.050 m^3) and exposed for 5 min to 1 M nebulized citric acid monohydrate (Sigma-Aldrich). Chemical stimulation was performed by using aerosolized citric acid, that exerts its action on airway receptors innervated by Aδ- and C-fibres [3], since the rabbit does not cough in response to capsaicin [35,36]. Accordingly, TRPV1 receptors are scarcely expressed in the rabbit respiratory system [29]. In our experience, rabbits do not cough at all in response to capsaicin, but about 40% of them respond to citric acid with variable intensity (see also [35] and the Discussion for further comments on the variability in cough sensitivity). Citric acid was freshly dissolved in 0.9% NaCl solution and nebulized via an ultrasonic nebulizer (Projet, Artsana, Grandate, CO, Italy). The number of coughs during the 5-min citric acid challenge was evaluated by two trained observers unaware of the treatment used and the mean of the two observer's counts was taken as the final count. As already reported [23,24], coughs were recognized mainly from the characteristic behaviour of the animal and the sound produced, and readily distinguished from sneezing. In addition, cough sounds were recorded by a microphone placed in the box and monitored both visually and acoustically, making use of a personal computer equipped with an analog-to-digital interface (Digidata 1440, Molecular Devices) and appropriate software (Axoscope, Molecular Devices). Cough sounds displayed characteristics similar to those observed during cough challenges in anesthetized animals. All studies were carried out at the same time of day. The animals were randomly assigned to two groups (10 for each group) to undergo the following inhalation treatments (nebulizer output set at 1 mL/min) for 10 min: 1) aclidinium (aerosolized 400 μg/mL; total dosage 4 mg); 2) tiotropium (aerosolized 20 μg/mL; total dosage 200 μg). The experimental protocol was as follows: a) control trials (first citric acid challenge) were performed on each group by exposing the rabbits for 10 min to an aerosol of each vehicle solution and after a ~90-min delay to a citric acid aerosol. The number of coughs was counted; b) after an interval of 5–7 days scheduled to avoid tachyphylaxis [23,24], the animals of each group were treated either with aerosolized aclidinium or tiotropium for 10 min and after a ~90-min lag were exposed to a citric acid aerosol (second citric acid challenge). The number of coughs was counted again. To reduce as much as possible the number of animals employed, we compared vehicle-treated vs. LAMA-treated rabbits. On the other hand, we were aware of the absence of vehicle-induced effects on cough responses to citric acid inhalation (Student's paired t-tests) in awake animals from previous trials using 0.9% NaCl solution (n = 7; P > 0.05) or low (≤5%) DMSO concentrations (n = 6; P > 0.05) in rabbits devoted to other studies (unpublished observations). Rabbits that did not cough in response to citric acid inhalation were not included in the study.

2.4 Experiments on anesthetized animals

Experiments were performed on 20 male New Zealand white rabbits (3.3–3.8 kg) anesthetized with pentobarbital sodium (40 mg/kg i.v., supplemented by 2–4 mg/kg every 30 min). The general characteristics of these preparations were similar to those already described for MCh experiments. The C_3 or C_5 phrenic root on one side was prepared for recordings. Bipolar platinum electrodes were used to record efferent phrenic nerve activity from the central stump of one cut and desheathed phrenic root. Wire electrodes were used to record abdominal muscle 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 and end-tidal CO_2 partial pressure were recorded. Cardiorespiratory variables were acquired and analyzed using a personal computer, equipped with an analog-to-digital interface (Digidata 1440, Molecular Devices) and appropriate software (Axoscope, Molecular Devices).

Both mechanical and chemical stimulation of the tracheobronchial tree were employed to induce cough. All anesthetized animals coughed in response to both types of stimuli. Mechanical stimulation was delivered by a custom-built device recently described and validated [27,28] using a 0.5 mm diameter nylon fibre with a smoothed tip inserted through a lateral port of the tracheal cannula. The device allowed to set the number of forth and back movements or cycles (1–3 cycles), shaft velocity (10–20 mm/s), and shaft displacement (10–20 mm). 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. Chemical stimulation of the tracheobronchial tree was performed by means of 1 M citric acid inhalation. Citric acid was delivered by an ultrasonic nebulizer (Projet, Artsana). 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 proved to be adequate to avoid tachyphylaxis (for details on chemical stimulation see Ref. [25]). Chemical stimulation was always applied 2–3 min after mechanically-induced cough and caused a bout of several coughs that could be followed by a tachypneic response.

The animals were randomly assigned to one of the two experimental groups (10 for each group) to undergo inhalation treatments (nebulizer output set at 1 mL/min) for 1 min with aclidinium (4 mg/mL) or tiotropium (200 μg/mL). Owing to the absence of vehicle effects on cough responses in awake animals (see above) as well as on MCh-induced bronchoconstriction, we reasonably inferred that vehicle solutions were ineffective also in anesthetized preparations. Thus, we preferred to omit control trials with vehicle solutions to minimize as much as possible the number of employed animals (separate experiments) or, alternatively, to avoid a too long interval between control trials and LAMA effects. This time delay could possibly lead to a deterioration of the preparation (trials executed with vehicle solution and LAMA in the same animal). Cough was induced by both mechanical (3 trials performed in succession at ~1-min interval) and chemical (1 trial) stimulation in each animal before (control) and ~90 min after drug administration. The stimulation protocol was repeated at appropriate intervals (~5 min for mechanical stimulation and 10–15 min for citric acid inhalation) for about further 120 min to follow the recovery process. No attempt was made to investigate the recovery process for longer time periods owing to the very long-lasting duration of drug-induced effects [32]. All stimulation procedures were performed at least 5–6 min after each supplemental dose of pentobarbital to avoid its possible immediate influence on the recorded variables.

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 cycle (T_T) were measured on recordings of raw phrenic nerve activity [28,37]. 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 and normalized by expressing them as a fraction (or percentage) of the highest achievable amplitude observed in each animal, i.e., in relative units (RU). Breathing pattern variables were measured for an average of five consecutive breaths prior to and following drug administrations. 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 of cardiorespiratory variables 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. 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 (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. On some occasions, an expiration reflex could occur as the first motor event in a cough epoch [1,36,38]. Expiration reflexes were not considered for data analysis. For a discussion on the expiration reflex, see our previous reports [8,9,11,25,26].

2.5 Statistical analysis

In unanesthetized animals, the number of coughs for each citric acid challenge was counted and the mean value for each group calculated. Changes in the number of coughs induced by each treatment were evaluated by Student's paired t-tests. The changes in the number of coughs due to the different treatments were compared by unpaired t-tests.

In anesthetized animals, the effects of each individual treatment on cough-related variables were compared by means of Student's paired t-tests. Changes in the cough number, i.e. the only cough-related variable that resulted to be significantly different from control, were compared by means of unpaired t-tests (i.e. aclidinium vs. tiotropium). Similarly, changes in the cough number in response to citric acid inhalation induced by aclidinium or tiotropium in awake and anesthetized animals were compared by unpaired t-tests. In the anesthetized preparations, the same statistical analysis was used to compare mean changes in the cough number in response to mechanical and chemical stimuli for each employed LAMA. Changes in the cough number were also expressed as percentage variations of control values. All reported values are means ± standard error of the mean (SEM). $P < 0.05$ was considered as significant.

3 Results

3.1 Unanesthetized animals

Significant reductions in the cough number were observed following 10-min inhalation of aclidinium (from 31.90 ± 7.15 to 6.90 ± 3.69, –77.3 ± 9.4%, $n = 10$; $P < 0.01$) or tiotropium (from 35.70 ± 6.20 to 19.01 ± 5.56, –38 ± 19%, $n = 10$; $P < 0.05$) compared with the corresponding control treatment with vehicle solutions. No significant difference between the effects of the two drugs were seen, although aclidinium showed a tendency to cause more marked antitussive effects. It should be recalled that the employed dose of each drug is that proved to fully express its protective action on cholinergic-induced bronchoconstriction. A useful graphic representation of the results is provided in Fig. 2, where individual control values of the cough number and the respective

decreases ~90 min following 10-min inhalation of each drug are reported. A complete suppression of the cough reflex was observed in some preparations with both treatments (aclidinium: $n = 6$; tiotropium: $n = 2$).

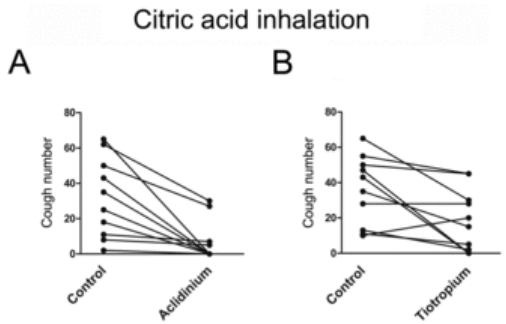

Fig. 2 Effects on the cough number induced by aclidinium or tiotropium in unanesthetized rabbits. Changes in the cough number induced by citric acid in each individual rabbit ~90 min following vehicle inhalation (control) as well as ~90 min after inhalation of aclidinium (A) or tiotropium (B). LAMAs were administered 5–7 days after the vehicle treatment. Note the complete suppression of the cough reflex in some preparations.

3.2 Anesthetized animals

Cough responses induced by mechanical stimulation were completely suppressed in some preparations with both treatments (aclidinium: $n = 5$; tiotropium: $n = 4$). Cough responses induced by chemical stimulation of the tracheobronchial tree were reduced, but not abolished by both treatments. In animals with depressed cough responses, the cough number significantly changed, at variance with the other cough-related variables that did not show any significant variation. The overall data showed that the cough number in response to mechanical stimulation varied from 3.39 ± 0.16 to 0.94 ± 0.35 (–74.3 ± 9.2%, $n = 10$; $P < 0.001$) and from 3.17 ± 0.18 to 0.99 ± 0.33 (–70.9 ± 9.4%, $n = 10$; $P < 0.001$) for aclidinium and tiotropium, respectively. Further, the cough number in response to citric acid inhalation changed for aclidinium from 4.20 ± 0.47 to 3.14 ± 0.37 (–25.1 ± 5.1%, $n = 10$; $P < 0.005$) and for tiotropium from 4.23 ± 0.36 to 3.13 ± 0.24 (–23.8 ± 4.5%, $n = 10$; $P < 0.005$). No significant differences were observed between the effects of aclidinium and tiotropium. These data are illustrated by graphic representations of individual values of the cough number for each animal before and after treatment (Fig. 3). LAMA antitussive effects on cough responses induced by mechanical stimulation were more intense than those evoked by citric acid inhalation ($P < 0.05$). Data concerning cough-related variables that did not change have not been shown. Nevertheless, control values of these variables under control conditions have already been extensively reported [8–11,23–25]. Note that the reductions in the cough number in response to chemical stimulation induced by aclidinium or tiotropium in the anesthetized preparations were significantly lower than those observed in awake animals ($P < 0.001$ and $P < 0.05$, respectively). The effects of aclidinium on the cough reflex induced by mechanical and chemical stimulation of the tracheobronchial tree are shown through examples of original recordings in Figs. 4 and 5, respectively. Since the effects of the two drugs on cough responses induced by mechanical and chemical stimulation were similar, only the effects induced by aclidinium have been reported. No recovery of control cough responses was present for both treatments within the observation period. Both aclidinium and tiotropium did not cause any change either in arterial blood pressure or in baseline respiratory activity. For a general appraisal of cardiorespiratory variables under control conditions see our previous studies [9,10,23–26].

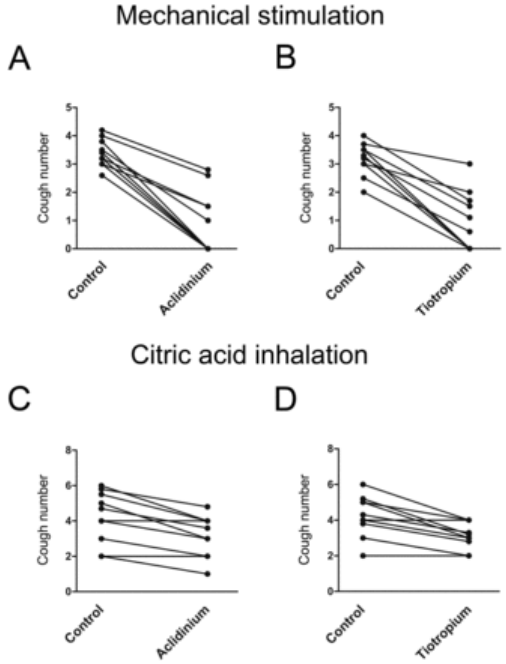

Fig. 3 Cough number induced by mechanical or chemical stimulation of the tracheobronchial tree before (control conditions) and ~90 min following inhalation of aerosolized aclidinium or tiotropium in anesthetized rabbits. Values of the cough number evoked in each rabbit by mechanical stimulation before and after inhalation of aclidinium (A) or tiotropium (B) are reported. Individual values of the cough number in response to chemical stimulation before and after drug inhalation are reported in C and D. Note the complete absence of the cough response to mechanical stimulation observed in some preparations following both treatments.

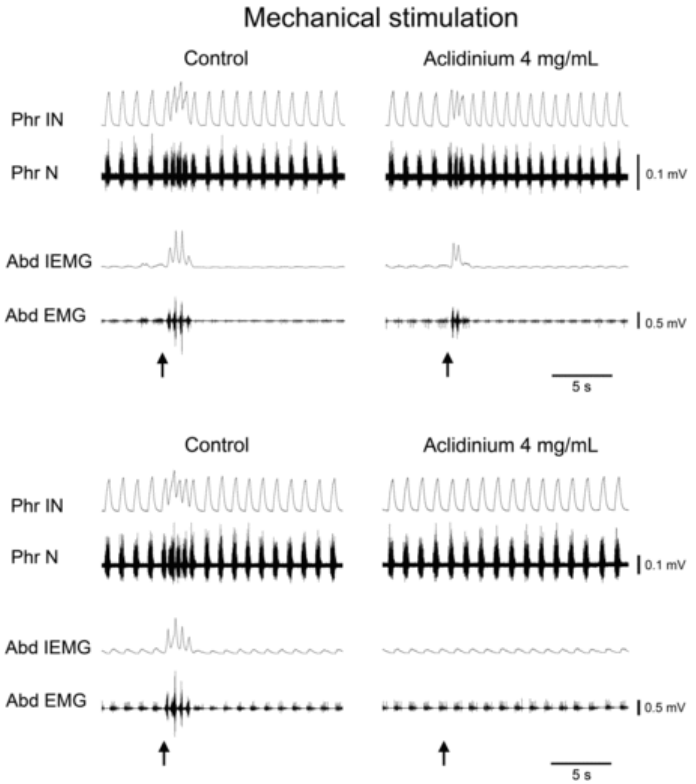

Fig. 4 Examples of cough responses induced in anesthetized rabbits by mechanical stimulation under control conditions and ~90 min after aclidinium inhalation. After anticholinergic treatment, cough responses could be either only depressed or completely abolished. Traces are: Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity. The onset of mechanical stimulation is indicated by arrows.

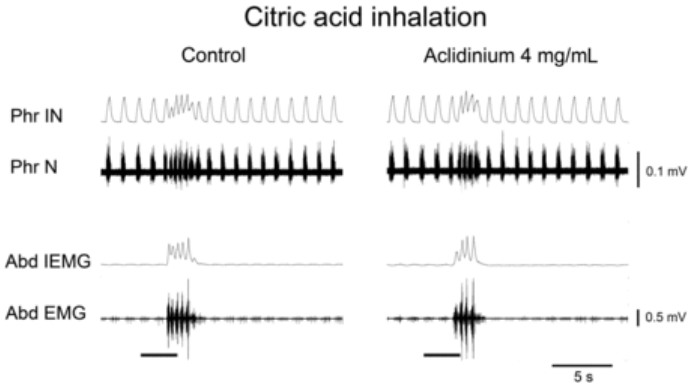

Fig. 5 Examples of cough responses induced in one anesthetized rabbit ~~induced~~ by citric acid inhalation under control conditions and ~90 min after aclidinium inhalation. After anticholinergic treatment, cough responses were depressed, but in no case completely abolished. Traces are: Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity. Stimulation periods are indicated by filled bars.

4 Discussion

This is the first preclinical study comparing the effects of aclidinium and tiotropium on the cough reflex induced by mechanical and chemical stimulation of the tracheobronchial tree in healthy animals. These two LAMAs displayed

antitussive effects in unanesthetized rabbits in response to citric acid inhalation. In anesthetized animals, aclidinium and tiotropium caused similar antitussive effects that were less intense for cough responses induced by chemical stimulation. Interestingly, the results strongly suggest that inhaled LAMAs have a possible novel antitussive mechanism of action, probably unrelated to their anticholinergic activity and to their action on TRPV1 receptors, and involving acid-sensing ion channels (ASICs) and mechanoreceptors of cough-related airway sensory afferents.

4.1 Methodological considerations and general remarks

Despite some rabbits could not present a prompt cough reflex [1], this animal species is a very suitable model for studies on the physiology and pharmacology of cough [7–11,23–26,35,39–43]. Admittedly, suggestions for new antitussive agents could stem more directly from studies on animal models of human lung diseases characterized by cough hypersensitivity, although the results of studies carried out on animal models could not be predictive for new antitussive agents [6,7]. Nevertheless, we are confident that investigations performed on healthy preparations can provide useful insights into the regulation of the cough reflex as well as hints for further studies on this reflex and possibly for the development of novel antitussive strategies. Preclinical studies have indicated that aclidinium may have a faster onset of action than tiotropium, but comparable protective effects on bronchoconstriction [32,33]. In addition, since it has been reported that the clinical dose of tiotropium 18 μg is very low with respect to the clinical dose of aclidinium 400 μg [14], we made an attempt to find the isoeffective dose of the two drugs as far as bronchodilation is concerned. The potency of these two LAMAs has been previously assessed in guinea pigs [32,33]. Aclidinium 100 μg/mL and tiotropium 10 μg/mL produced 1 h post-administration equi-effective (97–98%) inhibition of acetylcholine-induced bronchoconstriction that lasted several hours. In the present study, the isoeffective single aerosolized doses resulted to be 4 mg/mL and 200 μg/mL for aclidinium and tiotropium, respectively. The discrepancy with these previous findings may be related to the differences in the animal species and the bronchoconstrictor agent employed.

The time interval of 90 min between drug administration and cough challenge was higher than that of 1 h used by Birrell et al. [17]. It was scheduled to ensure the complete development of bronchodilator effects (see Methods). It is noteworthy that changes in the ongoing respiratory activity or in arterial blood pressure, that have been proved to affect the timing and intensity of cough responses [8,24,37], were not induced by the two employed LAMAs.

4.2 Effects of treatments

Present results on aclidinium are consistent with previous findings obtained in cigarette smoke-exposed guinea pigs [22] as well as in COPD patients [12,13] in whom aclidinium proved to have antitussive effects more intense than tiotropium. Apart from the study by Birrell et al. [17] on guinea pigs, there are a few other investigations on the antitussive effects of tiotropium both in animal species [44] and patients [12,13,16,18,19]. Recently, at variance with previous and present results, a lack of tiotropium antitussive effects on ozone-induced hypertussive responses in rabbits has been reported [7]. The reasons of the discrepancy with our results are not clear, but could be tentatively attributed to differences in the experimental conditions. In more detail, our animals were selected on the basis of their responsiveness to citric acid inhalation and most of them presented control cough responses more intense than those of the rabbits employed by Clay et al. [7]. Furthermore, we cannot exclude that ozone-induced sensitization, i.e. a treatment that probably involves a complex series of phenomena within the airways, could have affected the responsiveness of pulmonary cough-related receptors to the employed drugs. In this context, it should be mentioned that differences in cough sensitivity and regulation do exist not only between different animal species [45], but also within the same species and, according to our experience, in New Zealand white rabbits too (see also [35] and [40]). Finally, our results are corroborated by the observations on anesthetized animals obtained under controlled and less variable conditions.

According to previous results [17,18], bronchodilation does not seem to be the mechanism by which LAMAs downregulate cough responses. This conclusion is also supported by the results of several studies showing the inability of bronchodilation or bronchoconstriction to alter cough receptor sensitivity and produce cough ([46–50], see also [18] for further Refs.). The main difference reported in the literature between unanesthetized and anesthetized animals is that the C-fibre stimulation has consistently failed to evoke coughing in the latter [2,4,48,49,51–55]. It is well known that citric acid activates both Aδ- and C-fibres in most animal species including the rabbit, likely owing to the contribution of both ASICs and, to some extent, C-fibre TRPV1 receptors ([2,48,56–58] also for further Refs.). Thus, while in the absence of anesthesia both types of afferents are engaged along with the related reflex pathways, only Aδ-fibres and their pathways are probably involved in cough responses obtained in anesthetized animals. The effects we observed in awake rabbits strongly suggest that the mechanism of action of the employed LAMAs on cough receptors cannot be the same suggested by Birrell et al. [17], since TRPV1 receptors are very scarcely expressed in the rabbit respiratory system [59]. Accordingly, the rabbit, as already stated in the Methods, does not cough in response to inhaled capsaicin and, in addition, does not develop the pulmonary chemoreflex in response to capsaicin injected into the pulmonary circulation [35,36,60,61]. Thus, other receptors in addition to TRPV1 should be taken into consideration in the mediation of LAMA antitussive effects. It seems obvious to suggest that ASICs on Aδ- and C-fibre endings are involved in cough production by 1 M citric acid in awake rabbits and that LAMAs probably act especially on C-fibre ASICs without an important involvement of TRPV1 receptors. Consistently with this interpretation, significantly less pronounced effects of LAMAs on citric acid-induced cough were observed in anesthetized animals where C-fibres are not active in cough production. In addition, these LAMA effects were lower than those observed in cough responses induced by mechanical stimulation. In this context, it should be recalled that receptors involved in cough production are polymodal and may express either a single type of channel sensitive to both acid and punctate stimuli or two different channel types, i.e. one for each modality. Evidence has been provided that ASICs are involved in mechanosensory function (e.g. Refs. [62,63]), but also that separate mechano-sensitive channels may exist in the tracheobronchial tree (e.g. Refs. [64–66]) or elsewhere (e.g. Refs. [67,68]). Present results may suggest two separate types of channels. Furthermore, the possibility should be also considered that the two channel types are located on different sensory Aδ-fibres. However, the exact mechanism involved in the activation of mechano-sensitive channels of tracheobronchial tree receptors is, at present, unknown. Nevertheless, our results in anesthetized animals could imply that these drugs also affect, to some extent, ASICs on Aδ-fibres. Interestingly, the results in anesthetized animals strongly suggest that LAMAs have an important role in cough downregulation through an action on Aδ-mechanoreceptors of the tracheobronchial tree and that they could act on airway mechanoreceptors also in awake animals, even if a direct evidence for this is not available. In this context, it is worth recalling that both C-fibre endings and Aδ-receptors are responsive to mechanical stimuli [3].

A subtype of airway receptors has been described in the anesthetized guinea pigs and called “cough receptors” [48–50]. These receptors, innervated by slowly conducting Aδ-fibres, have been suggested to play an essential role in regulating the cough reflex. They are sensitive to punctate mechanical stimuli and acid, but unresponsive to changes in luminal pressure, to capsaicin, bradykinin or hypertonic saline and do not express TRPV1 channels. Our results are consistent with the possible role of this type of receptors in cough production both in awake and anesthetized rabbits, although, to our knowledge, there is no evidence of their presence in the tracheobronchial tree of this animal species.

As proposed in our previous reports ([8,11,28] also for further Refs), cough and nociception share similar central and peripheral features. This led to the proposal that agents involved in the control of pain sensation could be also effective in the cough regulation and viceversa (see e.g. Refs. [6,69]). In particular, it is well known that ASICs are involved not only in cough production, but also in pain sensation [70–72]. Here we provide for the first time evidence that LAMAs display antitussive effects through an action on ASICs and mechanoreceptors in the rabbit tracheobronchial tree. It should also be acknowledged that aclidinium is hydrolyzed into inactive metabolites more rapidly than tiotropium or other LAMAs [32,73], thus minimizing its potential for systemic side effects. The route of administration, i.e. via inhalation, that avoids central effects characteristic of many systemically administered antitussive drugs, contributes to its favorable safety profile. Further studies on the role of ASIC blockers or modulators could provide insights into the neural mechanisms underlying the cough reflex and possibly lead to novel therapeutic strategies for both cough and pain.

Authorship contribution

D Mutolo, E Cinelli, L Iovino, T Pantaleo, and F Bongianni performed the research.

D Mutolo, E Cinelli, T Pantaleo, and F Bongianni designed the research study.

D Mutolo, E Cinelli, L Iovino, T Pantaleo, and F Bongianni analyzed the data.

D Mutolo, T Pantaleo, and F Bongianni wrote the paper.

Conflict of interest

No conflict of interest exists for all Authors.

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