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Research report

Role of excitatory amino acids in the mediation of tracheobronchial cough induced by citric acid inhalation in the rabbit

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ABSTRACT

We investigated the role of ionotropic glutamate receptors located within the caudal portions of the nucleus tractus solitarii (cNTS) and the caudal ventral respiratory group (cVRG) in the mediation of coughing evoked by citric acid inhalation in spontaneously breathing rabbits under pentobarbitone anaesthesia. Bilateral microinjections (30–50 nl) of 10 mM CNQX and 10 mM D-AP5 were performed to block non-NMDA and NMDA receptors, respectively. An attempt was also made to investigate the effects of ionotropic glutamate receptor blockade within the cVRG on sneezing induced by mechanical stimulation of the nasal mucosa. Blockade of non-NMDA receptors within the cNTS abolished coughing and associated tachypneic responses, while blockade of NMDA receptors only reduced cough responses. Blockade of non-NMDA receptors within the cVRG always abolished spontaneous rhythmic abdominal activity as well as coughing and associated tachypneic responses; blockade of NMDA receptors only reduced spontaneous rhythmic abdominal activity and coughing. As to sneezing, blockade of non-NMDA receptors within the cVRG suppressed the expiratory thrusts without affecting the inspiratory preparatory bursts, while blockade of NMDA receptors only strongly attenuated the expiratory thrusts. This study is the first to provide evidence that ionotropic glutamate receptors, and especially non-NMDA receptors, are involved in the mediation of coughing induced by citric acid inhalation and to suggest that citric acid-activated cough-related afferents terminate within the cNTS. Present data also corroborate the notion that the cVRG is involved in the generation of the whole cough motor pattern, but seems to represent merely an expiratory output system for sneezing.

1. Introduction

Cough is one of the most important airway defensive reflex [22] involving several brainstem structures (e.g., Refs. [7,8,23,24,33,38,46] also for further references) including the first and the last relay medullary station of the reflex pathway, i.e. the second-order neurons within the caudal aspect of the nucleus tractus solitarii (cNTS) and the expiratory premotor neurons of the caudal ventral respiratory group (cVRG). While it is widely agreed that tracheobronchial rapidly adapting receptors (RARs) are primarily involved in cough mediation, the role of bronchopulmonary C-fibers and Aδ-nociceptive afferents in this reflex is still controversial (see, e.g., Refs. [27,33,43,60,62] also for further references). Putative selective stimulants of C-fibers, such as bradykinin and capsaicin, are effective at evoking cough in conscious animals and human subjects [19]. However, these stimuli have consistently failed to produce coughing in anaesthetized cats, dogs and guinea pigs [10,11,20,51,52]. Rather, bronchopulmonary C-fiber activation may inhibit coughing in anaesthetized animals [11,15,51,52,60], but this does not seem to be true for all animal species [31].

Inhalation challenge with low pH solutions, particularly citric acid, is a common tool used to induce coughing in humans and laboratory animals ([10,11,19,21,27,29,43,51] also for further references). In guinea pigs, acid-induced coughing has a C-fiber dependent component sensitive to general anaesthesia and a C-fiber independent component essential during anaesthesia [10]. However, anaesthesia does not prevent acid-induced coughing, C-fiber activation or all C-fiber-mediated reflexes [10,20,26,31,33,51,52]. Evidence has been provided that the inhalation of low pH solutions, including citric acid, stimulates not only C-fiber receptors, but also RARs ([21,27,62] also for further references). Since both types of receptors are activated by a great variety of stimuli, it seems plausible that subgroups of receptors exist within each type according to the specific stimuli to which they are responsive (see Refs. [21,27,43,62]).

Recent studies in anaesthetized guinea pigs have provided evidence suggesting that a receptor subtype found in the larynx and rostral trachea, quite distinct from the well defined slowly adapting receptors (SARs) and RARs, is primarily involved in the mediation of the cough reflex ([11,12] also for further references). These recep-

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tors, termed "cough receptors", are activated by acid and punctuate mechanical stimuli.

From all the above reported findings it appears that both central and peripheral effects induced by citric acid inhalation could be fairly complex even in the anaesthetized preparations and that the mechanisms involved in the mediation of citric acid-induced respiratory responses, and in particular coughing, deserve further investigation. In addition, central terminations of all cough-related afferents have not been clearly defined in any animal species (see, e.g., Refs. [23,24,29]).

Recently, we have provided evidence [33] that ionotropic glutamate receptors located within the cNTS, and especially the commissural subnucleus, are involved in the mediation of coughing evoked by mechanical stimulation of the tracheobronchial tree in the rabbit, with a major role played by non-NMDA receptors. We have also shown [8] that the same receptors mediate the excitatory drive to the cVRG bulbospinal expiratory neurons during eupneic breathing and coughing induced by tracheobronchial mechanical stimuli. Our results also demonstrated that the activation of neurons located in the cVRG is essential for the generation of both the inspiratory and expiratory components of the cough motor pattern. This important finding on the role of the cVRG in cough production needs to be corroborated by further studies.

The present study was carried out on pentobarbitone anaesthetized, spontaneously breathing rabbits with two main purposes. First, to investigate the role of ionotropic glutamate receptors located within the cNTS in the mediation of coughing induced by citric acid inhalation, and possibly to draw some inferences on the nature of afferent fibers implicated. Second, to ascertain whether ionotropic glutamate receptors mediate the excitatory drive to cVRG expiratory neurons also during citric acid-evoked coughing, and especially to corroborate the finding that the activation of neurons located in the cVRG is crucial for the generation of both the inspiratory and expiratory components of the cough response. In this connection, an attempt was also made to investigate whether cVRG neurons play a similar role in the production of both the inspiratory and expiratory components of the sneeze reflex, a defensive motor act that shares many common features with the cough reflex [22,47,52,56]. In fact, the cVRG is also the last medullary station of the sneeze reflex [2,3,17,22,41,47]. If ionotropic glutamate receptor blockade within the cVRG suppressed both the components of citric acid-induced coughing while affecting only the expiratory components of sneezing, the conclusion could be drawn that this medullary region is an important coordinating system for the whole cough motor pattern, but merely an expiratory output system for the sneeze reflex.

2. Methods

2.1. Animal preparation

Experiments were carried out on 16 male New Zealand white rabbits (2.6–3.3 kg) anaesthetized with sodium pentobarbitone (40 mg kg^{-1} i.v., supplemented by 2–4 mg kg^{-1} every 30 min; Sigma–Aldrich, St. Louis, MO, USA). Atropine (0.15 mg kg^{-1} i.m.) and dexamethasone (2 mg kg^{-1} i.m.) were administered to reduce mucosal secretion in the airways and to minimize brain oedema, respectively. The adequacy of anaesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw. Additional criteria were the absence of fluctuations in arterial blood pressure or phrenic nerve activity whether spontaneous or in response to somatic nociceptive stimuli. 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. 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 (see, e.g. Refs. [7,8,32–34]). 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 C_3 or C_5 phrenic root either on the right or the 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 medullary surface by occipital craniotomy. Body temperature was maintained at 38.5–39 °C by a heating blanket controlled by a rectal thermistor probe.

2.2. Recording procedures

Phrenic nerve activity was recorded by means of bipolar platinum electrodes from the central stump of the cut and desheathed C_3 or C_5 phrenic root. The electromyographic (EMG) activity of abdominal muscles was recorded using 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, band pass filtered, full-wave rectified, and "integrated" (low-pass RC filter, time constant 100 ms). Extracellular recordings from medullary neurons were performed with tungsten microelectrodes (5–10 MΩ impedance at 1 kHz). The most rostral extent of the area postrema on the midline was defined as the obex and used as a reference point. Neuronal activity was recorded from expiratory neurons of the cVRG (1.6–3.0 mm caudal to the obex, 2.0–2.5 mm lateral to the midline, and 2.0–2.6 mm below the dorsal medullary surface). Arterial blood pressure was recorded by a strain-gauge manometer. End-tidal CO_2 partial pressure was measured by an infrared CO_2 analyzer (Datex, CD-102; Normocap, Helsinki, Finland). All recorded variables were fed to 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 1200, Axon Instruments, Union City, CA, USA) and appropriate software (Axoscope, Axon Instruments).

2.3. Microinjection procedures

Bilateral microinjections were performed into the cNTS, corresponding to a large extent to the lateral commissural subnucleus, at two different sites along its rostrocaudal extent. The first was approximately 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, 0.4–0.6 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 [30]. Bilateral microinjections were also performed into the cVRG at sites defined by prior extracellular recordings. They were made at three different sites, starting from approximately 0.5 mm caudal to the transitional area where a mix of expiratory and inspiratory neurons is encountered, and continuing along the rostrocaudal extent of the cVRG at intervals of 0.5 mm. These procedures were followed to affect as much as possible the entire population of either cough-related second-order neurons of the cNTS [23,24] or expiration-related neurons of the cVRG (for more details see Refs. [8,33]). The following drugs (Tocris Cookson, Bristol, UK) were used: D(−)-2-amino-5-phosphonopentanoic acid (D-AP5, 10 mM), a NMDA receptor antagonist, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 mM), a non-NMDA receptor antagonist. Only one ionotropic glutamate receptor antagonist was tested in each preparation. Drug concentrations were in the same range as those previously used in in vivo preparations ([8,33,34] also for further references). We have determined the effectiveness and selectivity of ionotropic glutamate receptor blockade in a previous study [8]. All drugs were dissolved in 0.9% NaCl solution. The pH of all drug solutions was adjusted to 7.4 using 0.1N NaOH. Control injections of equal volumes of the vehicle solution were also performed. Microinjections (30–50 nl) were made via a glass micropipette (tip diameter 10–25 μm) according to procedures previously described (e.g., Refs. [8,33]). The time taken to perform one injection ranged from 5 to 10 s. The time taken to perform all the microinjections was 4–5 min for the cNTS and 6–8 min for the cVRG. The localization of injection sites, diagrammatically illustrated in Fig. 1, was confirmed by the histological control.

2.4. Stimulation procedures

Cough was induced by chemical stimulation of the tracheobronchial tree by means of citric acid inhalation. Stimulation parameters adequate to obtain consistent cough responses were selected in preliminary trials performed on rabbits ($n = 6$) employed in previous studies [8,32,33]. 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^{-1}) 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 proved to be adequate to avoid as much as possible tachyphylaxis [10,21,25,49,51]. All the animals coughed in response to such inhalation challenges. The amount of citric acid inhaled depended entirely on the number and depth of inspirations. However, only slight variations in the breathing pattern could be observed in the investigated animals. The interval between chemical challenges was >10 min (usually ≈15 min) since similar cough reflexes could be reliably obtained at minimal intervals of 7 min in the preliminary trials. The respiratory responses presented rapid habituation when chemical stimulation was repeated at shorter intervals (tachyphylaxis). However, when repeated chemical stimulation failed to produce cough, mechanically induced cough (e.g., Refs. [8,32,33]) was still present (D. Mutolo, F. Bongianni, E. Cinelli, T. Pantaleo, unpub-