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Inhibitory modulation of the cough reflex by acetylcholine in the caudal nucleus tractus solitarii of the rabbit

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ABSTRACT

A cholinergic system has been described in the nucleus tractus solitarii (NTS). However, no information is available on the role played by acetylcholine (ACh) in the modulation of the cough reflex within the caudal NTS that has an important function in cough regulation. We addressed this issue making use of bilateral microinjections (30–50 nl) of 10 mM ACh combined with 5 mM physostigmine as well as of 10 mM mecamylamine or 10 mM scopolamine into the caudal NTS of pentobarbital sodium-anesthetized, spontaneously breathing rabbits. Microinjections of ACh/physostigmine caused depressant effects on the cough reflex induced by mechanical and chemical stimulation of the tracheobronchial tree. They also elicited transient increases in respiratory frequency and decreases in abdominal activity. These effects were prevented by scopolamine, but not by mecamylamine. The results show for the first time that ACh exerts an inhibitory modulation of the cough reflex through muscarinic receptors within the caudal NTS. They also may provide hints for novel antitussive approaches.

1. Introduction

Cough is one of the most important defensive reflex brought into action by actually or potentially damaging events applied to the airways (Korpáš and Tomori, 1979). Peripheral and central mechanisms underlying nociception and cough share similar features; neuroactive agents involved in the central control of pain sensation (Millan, 2002; Yan et al., 2017) and concomitant reflex responses play a role also in the downregulation of the cough reflex (see Mutolo, 2017 also for further Refs. Mutolo et al., 2008, 2012, 2014; Cinelli et al., 2013, 2016).

Several brainstem areas appear to contribute to the generation and regulation of cough responses in mammals (*e.g.* Gestreau et al., 1997; Bongianni et al., 1998; Jakus et al., 2008; Mutolo et al., 2002b; Poliacek et al., 2004, 2005, 2014; Shannon et al., 2004; Simera et al., 2013; see also Mutolo, 2017). Recent findings obtained mainly in the rabbit (for review see Mutolo, 2017) have led to the proposal that two medullary structures play a prominent role in the control of cough reflex responses and are sites of action of antitussive or protussive drugs, *i.e.* the caudal nucleus tractus solitarii (NTS), the first relay medullary station of the cough reflex pathway, and the caudal ventral respiratory group (cVRG), where neurons responsible for the expiratory component of the reflex are located (for the role of the cVRG see also Poliacek et al., 2007, 2010, 2015). Of note, afferent inputs from peripheral chemoreceptors and pulmonary rapidly adapting receptors converge on neurons of the caudal NTS (Mifflin et al., 1988; Mifflin, 1992; Machado, 2001; Kubin et al., 2006). However, recently evidence has been provided of the existence in the cat of important control mechanisms within the rostral NTS (Poliacek et al., 2017a,b; for review see Mutolo, 2017) very similar to those described for the caudal NTS in the rabbit. This could be possibly related to marked differences in the animal species.

Besides its modulatory quality, acetylcholine (ACh) also acts as one of the most prominent neurotransmitters in the peripheral and central nervous system. Interestingly, cholinergic transmission profoundly affects the perception of pain *via* both nicotinic (nAChRs) and muscarinic (mAChRs) receptors (for review see Naser and Kuner, 2017). There is considerable direct and indirect evidence that ACh is widely distributed in the region of NTS (Kobayashi et al., 1978; Criscione et al., 1983; Ernsberger et al., 1988; Ruggiero et al., 1990; Zoccal et al., 2014) where both mAChRs and nAChRs are present. Muscarinic receptors are located in more caudal regions, including the commissural subnucleus, while nicotinic receptors are predominantly located at more rostral levels, *i.e.* in the medial, ventrolateral and ventral subnuclei (Maley, 1996; see also Furuya et al., 2014).

ACh contributes to autonomic regulation. In particular, it is involved in the regulation of both cardiovascular (*e.g.* Shihara et al., 1999; Furuya et al., 2014, 2017; Zoccal et al., 2014) and respiratory activity (*e.g.* Haxhiu et al., 1984; Bianchi et al., 1995; Shao and

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Feldman, 2000, 2001, 2002, 2009; Shao et al., 2008; Boutin et al., 2017) through both nAChRs and mAChRs. However, only scanty knowledge is available on its contribution to the modulation of the cough motor pattern. Recently, it has been shown that (−)-nicotine administered *via* brainstem circulation or directly applied to the cVRG causes mecamylamine-insensitive inhibitory effects on mechanically-induced cough (Poliacek et al., 2015). In the present research, the possible role of ACh in the modulation of the cough reflex at the level of the caudal NTS of pentobarbital sodium-anesthetized, spontaneously breathing rabbits was investigated making use of microinjection techniques.

2. Materials and methods

2.1. Ethical approval

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 (Decreto Legislativo 4/3/2014 no. 26 and Directive 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. Details about the methods employed have been described in our previous studies on the NTS region and will be concisely reported here (Cinelli et al., 2013, 2016; Mutolo et al., 2007, 2008, 2009, 2012, 2014; Mutolo, 2017).

2.2. Animal preparation and recording procedures

Experiments were carried out on 15 male New Zealand White rabbits (2.7–3.3 kg) anesthetized with pentobarbital sodium (40 mg/kg i.v., supplemented by 2–4 mg/kg every 30 min; Sigma–Aldrich, St. Louis, MO, USA). Atropine (0.15 mg/kg i.m.) 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. The trachea was cannulated and polyethylene catheters were inserted into a femoral artery and vein for monitoring arterial blood pressure and 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.

Efferent phrenic nerve activity was recorded with bipolar platinum electrodes. Abdominal muscle electromyographic (EMG) activity was recorded by wire electrodes. 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 and end-tidal CO_2 partial pressure by an infrared CO_2 analyzer (Capnocheck Plus, Smiths Medical PM, Waukesha, WI, USA). Cardiorespiratory variables were analyzed using a personal computer, supplied with an appropriate interface (Digidata 1440, Molecular Devices, Sunnyvale, CA, USA) and software (Axoscope, Molecular Devices).

2.3. Microinjection procedures

Bilateral microinjections were performed at two different sites along the rostrocaudal extent of the caudal NTS. The first was at the level of the caudal-most end of the area postrema that approximately corresponds to the opening of the central canal of the IV ventricle, 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. Owing to the spread of the 50 nl-injectate, < 400 µm in any direction (for the spread of the injectate see also Results and Discussion), injections at the first sites affected a NTS area probably including the most caudal extent of the medial subnucleus (see Mutolo et al., 2007). The stereotaxic coordinates were selected according to the atlas of Meessen and Olszewski (1949).

Microinjections (30–50 nl) were performed as described in our previous reports *via* a single-barrel glass micropipette (tip diameter 10–25 µm). The volume of the injectate was measured directly by monitoring the movement of the fluid meniscus in the pipette barrel with a dissecting microscope equipped with a fine reticule. The following drugs were used: 10 mM ACh chloride (endogenous neurotransmitter at cholinergic synapse; Sigma–Aldrich), 5 mM physostigmine salycilate (an acetylcholinesterase inhibitor; Sigma–Aldrich), 10 mM mecamylamine hydrochloride (a noncompetitive nAChR antagonist; Sigma–Aldrich), 10 mM (−)-scopolamine hydrobromide trihydrate (a nonselective mAChR antagonist; Sigma–Aldrich). Each drug was dissolved in 0.9% NaCl solution. Drug concentrations were in the same range as those previously used in *in vivo* preparations (*e.g.* Furuya et al., 2014; Zhang et al., 2016; Boutin et al., 2017). ACh at 10 mM was injected in combination with 5 mM physostigmine to obtain relative more pronounced and lasting effects. Control injections of equal volumes of the vehicle solution at the responsive sites were also performed. Fig. 1 illustrates the localization of injection sites that was confirmed in some preparations by injecting green fluorescent latex microspheres (LumaFluor, New City, NY, USA) added to the drug solution (three for ACh/physostigmine and two for scopolamine).

2.4. Stimulation procedures

Mechanical stimulation was delivered by a custom-built device recently described and validated (Mutolo et al., 2014) 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 set at 1 cycle, 15 mm/s velocity, and 15 mm displacement to produce a bout of 2–4 coughs. The stimulation protocol comprised three stimulation trials performed in succession (at 1–2 min interval) before drug administration, repeated ~5 min after the completion of all the microinjections and at appropriate intervals (at least 5 min) to follow the recovery process for a maximum of 90 min.

Chemical stimulation of the tracheobronchial tree was performed by means of citric acid inhalation (for details see Mutolo et al., 2009). Citric acid (1 M, Sigma–Aldrich) was freshly dissolved in 0.9% NaCl solution and nebulized. 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. 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. As a rule, chemical stimulation was performed both before and ~10 min after the completion of the injections and repeated at appropriate intervals (≥10 min) to follow the recovery process.

2.5. Histology

The histological control of pipette tracks and injection sites was performed as previously described (for details, see Mutolo et al., 2007, 2012; Cinelli et al., 2016). Frozen 20-µm coronal sections stained with Cresyl Violet were used. Coronal sections of the medulla in which injection sites were marked by fluorescent microspheres were examined in a light and epifluorescence microscopy (Eclipse E400, Nikon, Japan) equipped with the Nikon Intensilight C-HGFI mercury-fibre illuminator. A Nikon DS-Fi1digital camera was used to take photomicrographs.