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A Rho-kinase inhibitor, Y-27632, reduces cholinergic contraction but not neurotransmitter release

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Abstract

This study examined the effects of the selective Rho-kinase inhibitor Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-(4-pyridyl)cyclohexanecarboxamide dihydrochloride]) on cholinergic nerve-mediated contraction and neurotransmitter release in murine and guinea-pig isolated tracheal preparations. In tracheal preparations obtained from both species, Y-27632 shifted carbachol concentration–effect curves to the right and reduced the maximal contractile response. Repeated electrical field stimulation (EFS) evoked transient, consistent and reproducible contractions in murine and guinea-pig tracheal preparations. Y-27632 inhibited these cholinergic nerve-mediated contractions in a concentration-dependent manner. EFS (0.1–30 Hz) elicited frequency-dependent cholinergic nerve-mediated contractile responses. In murine tracheal preparations, Y-27632 (3 μM and 10 μM) shifted frequency–response curves to EFS to the right by 5.5 and 13.0 fold respectively and markedly reduced the maximal contractile response. In murine and guinea-pig tracheal preparations loaded with [^{3}H]-choline, Y-27632 (10 μM) significantly increased the EFS-induced outflow of radioactivity from airway cholinergic nerves by 27% and 54% respectively. Thus, Y-27632 inhibited both carbachol-induced and cholinergic nerve-mediated contractile responses. Conversely, Y-27632 increased neurotransmitter release from airway cholinergic nerves. However, since antagonism of acetylcholine-induced contraction by Y-27632 overwhelmed the increased neurotransmitter release, the overall effect of this Rho-kinase inhibitor was to inhibit cholinergic nerve-mediated contraction.

Keywords: Rho-kinase; Trachea; Smooth muscle contraction; Cholinergic nerve; Acetylcholine release

1. Introduction

It has been suggested that cholinergic nerve function may be upregulated in airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Jacoby and Fryer, 2001; Jartti, 2001). Anti-cholinergic bronchodilators such as ipratropium and tiotropium are effective bronchodilators in the management of both asthma and COPD (Barnes, 2004; Rodrigo and Rodrigo, 2002; Westby et al., 2004). Indeed, some asthmatic patients show a greater bronchodilator response to ipratropium as compared with salbutamol (Chhabra and Pandey, 2002), suggesting increased cholinergic bronchoconstriction in this sub-group of individuals. Some common triggers of asthma exacerbations include respiratory tract viral infection, inhalation of allergens or exposure to irritant air pollutants. These triggers have been shown to increase cholinergic nerve function in the airways and may explain the bronchodilator effect of anti-cholinergic agents in acute asthma (Jacoby and Fryer, 2001). Furthermore, since cholinergic tone is the main reversible component of COPD, anti-cholinergic drugs play an important role in the treatment of bronchial obstruction in COPD (Barnes, 2004).

Stimulation of muscarinic acetylcholine G-protein-coupled receptors is now known to be linked to a signal transduction pathway involving RhoA and its downstream modulator Rho-kinase. Activation of the RhoA/Rho-kinase pathway by agonists such as acetylcholine can promote phosphorylation of myosin light chain kinase and increase $[Ca^{2+}]_i$, resulting in contraction of airway smooth muscle.

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The Rho-kinase inhibitor Y-27632 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] has been shown to induce relaxation in airway preparations obtained from various animal species as well as human airways. Indeed, Y-27632 has been shown to reverse airway tone caused by a range of spasmogens in vitro (Burdyga et al., 2003; Chiba et al., 2001; Gosens et al., 2004; Nakahara et al., 2000; Setoguchi et al., 2001; Yoshii et al., 1999) and in vivo (Hashimoto et al., 2002a,b; Iizuka et al., 2000). The Rho-kinase inhibitor Y-27632 has also been shown to inhibit cholinergic, noradrenergic and purinergic nerve-mediated contractile responses in isolated smooth muscle preparations. For example, Y-27632 reversed electrical field stimulation (EFS)-induced nerve-mediated contractions in mouse (Büyükafşar and Levent, 2003), rabbit and human penile corpus cavernosum tissue (Rees et al., 2001), in rat and human prostatic smooth muscle (Rees et al., 2003) as well as in mouse vas deferens (Büyükafşar and Levent, 2003). In addition, Y-27632 attenuated electrically-evoked contractions in rat urinary bladder (Wibberley et al., 2003) and sheep ureter (Levent and Büyükafşar, 2004). Consistent with a direct inhibitory influence on cholinergic nerve function, Y-27632 has been shown to cause both decreased neurotransmitter release from cholinergic nerves in the mouse gastric fundus (Büyükafşar and Levent, 2003) and relaxation of these preparations. These data suggest that Y-27632 acts pre-synaptically to reduce excitatory neurotransmitter release. However, Y-27632 may also act post-synaptically to directly reduce smooth muscle tone. In the airways, the effect of Y-27632 to modulate cholinergic nerve-mediated contraction as well as neurotransmitter release has not been examined.

The Rho-kinase inhibitor Y-27632 has proven bronchodilator (Hashimoto et al., 2002a,b; Iizuka et al., 2000) and anti-inflammatory (Henry et al., 2005) activity. The ability of Y-27632 to also reduce neurotransmitter release from airway cholinergic nerves would further enhance the therapeutic potential of Rho-kinase inhibitors in the treatment of asthma and COPD, since this anti-spasmogenic, pre-junctional action would be matched by its well recognised post-synaptic inhibitory action against acetylcholine-induced contraction. Accordingly, the present study investigates the effect of the Rho-kinase inhibitor Y-27632 on cholinergic nerve-mediated contraction in murine and guinea-pig isolated trachea and evaluates the influence of Y-27632 on acetylcholine release from airway cholinergic nerves.

2. Methods

2.1. Tissue preparation

All experimental protocols were approved by the Animal Ethics Committee, University of Western Australia and were carried out in accordance with the National Health and Medical Research Council's Australian code of practice for the care and use of animals for scientific purposes. Mice (CBA/CaH, 5–10 weeks old) and guinea-pigs (SR/C Tricolour, 200–300 g) were sacrificed with Nembutal (sodium pentobarbitone, 300 mg/kg i.p.) and exsanguinated via the renal vein. The trachea was dissected free of associated tissues and placed in Krebs Bicarbonate Solution (KBS; composition in mM: NaCl 117, KCl 5.36, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4 \cdot 7H_2O$ 0.57, $CaCl_2 \cdot 2H_2O$ 2.5 and glucose 11.1).

2.2. Carbachol-mediated contractions

Murine and guinea-pig tracheal ring preparations were suspended between 2 stainless steel hooks in 2 ml organ baths containing KBS and connected to an isometric force transducer. The resting tension of tracheal preparations was adjusted to 200–350 mg for murine and 600–700 mg for guinea-pig tracheal preparations. KBS was aerated with 5% CO_2 in oxygen and maintained at 37 °C. KBS contained indomethacin (3 μM) since cyclooxygenase products including prostaglandins have been shown to influence airway tone. Tracheal preparations were equilibrated for 30 min during which time the bathing medium was replaced. The tension in murine and guinea-pig tracheal preparations was readjusted as required to 200–350 mg or 600–700 mg, respectively. Tracheal rings were then exposed to cumulative concentrations of carbachol (0.2 and 10 μM) to confirm tissue viability. This process was repeated to establish the maximum contractile response to carbachol at 10 μM (C_{max}). Isometric contractile responses were measured using an FTO3 force-displacement transducer (Grass Instruments, Quincy, MA) linked to a pre-amplifier and a computer-based data acquisition system.

Concentration–effect curves to carbachol (0.03–30 μM) were constructed in the absence and presence of Y-27632 (0.3–10 μM). In the latter experiments, tracheal preparations were equilibrated with Y-27632 for 15 min prior to the construction of each carbachol concentration–effect curve. Y-27632 failed to alter baseline tone in either murine or guinea-pig tracheal preparations, suggesting the absence of inherent tone in the presence of indomethacin. Changes in carbachol-induced tone in the absence and presence of Y-27632 were measured as a percentage of the response to 10 μM carbachol.

2.3. Cholinergic nerve-mediated contractions

Murine tracheal preparations were suspended in organ baths between two parallel platinum electrodes. Electrical field stimulation (EFS) was delivered by a Grass S44 stimulator connected to a Med-Lab Stimu-Splitter II (Med-Lab Instruments, Loveland, Co) and an automated timing device. Tracheal preparations were exposed to indomethacin (3 μM), propranolol (1 μM) and N^{w}-nitro-L-arginine methyl ester (L-NAME, 100 μM). Cyclooxygenase products have been shown to be released from airway tissue following EFS and have the potential to modulate cholinergic nerve-mediated contractions and neurotransmitter release (Fernandes et al., 1994; Spicuzza et al., 1998. Propranolol and L-NAME were used to inhibit adrenergic and inhibitory non-adrenergic non-cholinergic nerve-mediated responses respectively. The EFS parameters used were: 0.1–30 Hz, 11–15 V, 150–200 mA, 0.5 ms pulse duration, 10 s train with 3 min intervals between successive stimulations. These stimulation parameters have been shown to selectively activate airway cholinergic nerves (Ellis and Undem, 1990). EFS-induced contractions were abolished in the presence of either atropine