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Inhibition by the cold receptor agonists menthol and icilin of airway smooth muscle contraction

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ABSTRACT

Menthol, known as a cold receptor agonist, has widely been used in the relief of respiratory symptoms such as coughing and chest congestion. Previous studies have demonstrated that menthol reduces bronchoconstriction and airway hyperresponsiveness. The aim of this study was to examine the effects of menthol and icilin, another cold receptor agonist, on airway smooth muscle contraction. Isometric force was monitored using epithelium-denuded tracheal smooth muscle tissues isolated from guinea pigs. Intracellular Ca^{2+} concentrations were assessed by fura-2 fluorescence. (−)Menthol (0.01–1 mM) inhibited contraction induced by methacholine (MCh, 0.01–10 μM) and high extracellular K^+ concentrations (20–60 mM) in a concentration-dependent manner. Moreover, the increases of intracellular Ca^{2+} concentrations induced by MCh or high K^+ were significantly reduced by (−)menthol. Icilin (100 μM) also significantly attenuated contraction induced by MCh or high K^+. The inhibitory effect of 1 mM (−)menthol on MCh-induced contraction was significantly higher at cool temperature (24–26 °C) than at 37 °C. The present results demonstrate that inhibition of Ca^{2+} influx plays an important role in the menthol-mediated inhibition of contraction in airway smooth muscle. Furthermore, our findings indicate that stimulation of unknown cold receptors may be involved in these mechanisms. These findings suggest that the use of menthol is beneficial for reducing respiratory symptoms because of its inhibitory effects on airway smooth muscle contraction.

1. Introduction

Menthol has widely been used in the relief of respiratory symptoms including cough, wheezing and chest congestion [1,2]. It was shown that menthol reduces airway hyperresponsiveness in patients with asthma [3]. A previous study demonstrated that inhaled menthol reduces bronchoconstriction and airway resistance in guinea pigs *in vivo* [4]. Moreover, menthol directly reduced the contractile force due to acetylcholine and high extracellular K^+ concentrations (high K^+) in guinea pig bronchial smooth muscle *in vitro* [4]. Therefore, one of the reasons menthol reduces respiratory symptoms is that it relaxes airway smooth muscle (ASM) tone. However, the mechanisms by which menthol inhibits ASM contraction are still unclear.

It has been recognized that menthol causes a sensation of coolness by stimulating cold receptors in sensory neurons [1,5,6]. The activation of cold receptors by menthol is associated with an increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in sensory neurons [5,7]. In non-neuronal systems, menthol stimulated Cl^- secretion via $[Ca^{2+}]_i$ elevation in canine airway epithelial cells [8], and menthol caused contraction with an increase in $[Ca^{2+}]_i$ in rat pulmonary arterial and aortic smooth muscle [6]. In contrast, menthol inhibited contraction by blocking Ca^{2+} channels in smooth muscle isolated from the guinea pig gastrointestinal tract [9]. Additionally, menthol blocked Ca^{2+} currents in cultured sensory neurons from chick and rat embryos [10]. Although the effects of menthol on $[Ca^{2+}]_i$ are opposite in different tissues and species, these findings reveal that Ca^{2+} homeostasis plays an important role in determining the effects of menthol on various cell properties.

The aim of this study was to explore the mechanisms underlying the inhibitory effects of menthol on ASM contraction. We hypothesized that menthol attenuates ASM contraction by inhibiting Ca^{2+} influx pathways. In addition, we examined the possible involvement of cold receptor stimulation in the menthol-induced inhibition of ASM contraction. In order to test the hypothesis, we investigated the effects of menthol and icilin, another cold receptor agonist, on contractile force in epithelium-denuded tracheal smooth muscle tissues isolated from guinea pigs.

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2. Materials and methods

2.1. Isolation of tracheal smooth muscle tissues

The tracheae were excised from male Hartley guinea pigs (250–350 g) after intraperitoneal injection of pentobarbital (50 mg/animal). The tracheal rings were opened by cutting them longitudinally in the cartilaginous region. The airway epithelium was dissected out in order to exclude the effect of endogenous release of contractile and/or relaxing factors such as nitric oxide from the epithelial cells. All animal procedures were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine.

2.2. Isometric force recording and solution

Freshly isolated muscle tissue strips containing two cartilaginous rings were prepared [11,12]. One end of the cartilaginous ring was fixed to the wall of a tissue bath chamber, and the other end was connected to a force-displacement transducer to monitor isometric force in the organ bath (0.6 ml volume). The normal solution was composed of (in mM) NaCl (137), $KHCO_3$ (5.9), $CaCl_2$ (2.4), $MgCl_2$ (1.2) and glucose (11.8), bubbled with a mixture of 99% O_2 and 1% CO_2 (pH 7.4). The solutions containing high concentrations of K^+ (20–60 mM) were prepared by replacing NaCl with KCl. Ca^{2+}-free solution was prepared by replacing 2.4 mM of $CaCl_2$ in the normal solution with 2.2 mM of NaCl and 0.2 mM of EGTA. The bath solution was perfused at a constant flow of 3 ml/min. The temperature of the organ bath was maintained at 37 °C or room temperature (24–26 °C). To obtain the control contraction level, 1 μM methacholine (MCh) was applied to the tissue for 10 min before each measurement. To achieve a complete relaxation, the Ca^{2+}-free solution was applied to the tissues at the end of each measurement. Spontaneous contraction was abolished by addition of 2 μM indomethacin throughout the experiments [13]. The force levels obtained after equilibration in the Ca^{2+}-free solution and 1 μM MCh at 37 °C were defined as 0% and 100% of contraction, respectively. The level of force corresponding to a particular contraction was expressed on a normalized scale between 0% and 100% contraction as defined above. Data of the tissue strips which developed $\geqslant$3 mN contractile force in response to 1 μM MCh were included in the data analysis.

2.3. Measurement of intracellular calcium concentrations

In some experiments, the force and $[Ca^{2+}]_i$ of tracheal smooth muscle tissues were recorded simultaneously at 37 °C as described previously [14,15]. The preparation containing two cartilaginous rings was mounted horizontally in a tissue bath. The tissue strip was treated with 10 μM fura-2/acetoxymethyl (fura-2/AM) and 0.01% pluronic F-127 for 4 h at room temperature (24–26 °C). The mucosal side of the muscle strip was exposed to the excitation light, and the light emitted from the strip was collected in a photomultiplier through a band-pass filter centered at 500 nm. The intensity of fura-2 fluorescence due to excitation at 340 nm (F_{340}) and at 380 nm (F_{380}) was measured after background subtraction using a spectrofluorometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). The absolute amount of $[Ca^{2+}]_i$ was not calculated because the dissociation constant of fura-2 for Ca^{2+} in smooth muscle cytoplasm is different from that obtained *in vitro* [16]. Thus, F_{340}/F_{380} ratio was used as an indicator of the relative level of $[Ca^{2+}]_i$.

2.4. Experimental protocols

To assess the effects of (−)menthol, also known as L-menthol, and icilin, another cold receptor agonist, on contraction, either agent or 0.1% DMSO (control) was applied 10 min prior to cumulative application of MCh (0.01–10 μM) or cumulative increases in extracellular K^+ concentrations (20–60 mM). A previous study demonstrated that the pharmacological efficacy of (−)menthol was higher at temperatures lower than 30 °C [17]. Thus, in order to investigate how temperature influences the effect of (−)menthol on contraction induced by MCh or high K^+, the temperature of the bath solution perfused in the organ bath was maintained either at 37 °C or room temperature (24–26 °C).

2.5. Drugs

Indomethacin, MCh, and pluronic F-127 were obtained from Sigma (St. Louis, MO). Icilin was from Cayman (Ann Arbor, MI). Fura-2/AM was from Dojin (Kumamoto, Japan). (−)Menthol obtained from Wako (Osaka, Japan) was dissolved with DMSO. The solvent DMSO did not affect either contractile force or F_{340}/F_{380} ratio at concentrations used ($\leqslant$0.1%).

2.6. Statistical analysis

All data are expressed as means ± S.D. Student's *t*-test or analysis of variance (ANOVA) followed by the Bonferroni test for post hoc analysis was used to evaluate the statistical significance. $P<0.05$ was considered statistically significant.

3. Results

3.1. Menthol inhibits MCh-induced contraction

A representative recording of contraction induced by cumulative application of MCh (0.01–10 μM) in the absence or presence of 1 mM (−)menthol is shown in Fig. 1A. The temperature of the bath solution was 37 °C. The MCh-induced contraction was attenuated by application of 1 mM (−)menthol (Fig. 1A). The concentration–response curves for MCh-induced contraction were significantly lowered by (−)menthol (0.01–1 mM) in a concentration-dependent manner ($n = 8$) ($P<0.001$) (Fig. 1B).

3.2. Menthol inhibits high potassium-induced contraction

A representative recording of the force induced by high K^+ (20–60 mM) in the absence or presence of (−)menthol (1 mM) at 37 °C is shown in Fig. 2A. Cumulative increases in extracellular K^+ concentrations (20–60 mM) caused contractile response in a concentration-dependent manner. The force was returned to the basal level by applying the normal physiological solution containing 5.9 mM K^+. The high K^+-induced contraction was markedly attenuated by application of 1 mM (−)menthol (Fig. 2A). The concentration–response curves for high K^+-induced contractions were significantly lowered by (−)menthol (0.01–1 mM) in a dose-dependent manner ($n = 6$) ($P<0.001$) (Fig. 2B).

3.3. Menthol reduces intracellular calcium concentrations

The effects of (−)menthol on $[Ca^{2+}]_i$ evoked by MCh or high K^+ were examined. The temperature of the bath solution was 37 °C. Representative simultaneous recordings of F_{340}/F_{380} ratio, a measure of $[Ca^{2+}]_i$, and the tension induced by 0.1 μM MCh in the absence and presence of 1 mM (−)menthol, are shown in