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Effects of menthol on circular smooth muscle of human colon: Analysis of the mechanism of action

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ABSTRACT

Menthol is the major constituent of peppermint oil, an herbal preparation commonly used to treat nausea, spasms during colonoscopy and irritable bowel disease. The mechanism responsible for its spasmolytic action remains unclear. The aims of this study were to investigate the effects induced by menthol on the human distal colon mechanical activity *in vitro* and to analyze the mechanism of action. The spontaneous or evoked-contractions of the circular smooth muscle were recorded using vertical organ bath. Menthol (0.1 mM–30 mM) reduced, in a concentration-dependent manner, the amplitude of the spontaneous contractions without affecting the frequency and the resting basal tone. The inhibitory effect was not affected by 5-benzyloxytryptamine (1 μM), a transient receptor potential-melastatin8 channel antagonist, or tetrodotoxin (1 μM), a neural blocker, or 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (10 μM), inhibitor of nitric oxide (NO)-sensitive soluble guanylyl cyclase, or tetraethylammonium (10 mM), a blocker of potassium (K^+)-channels. On the contrary, nifedipine (3 nM), a voltage-activated L-type Ca^{2+} channel blocker, significantly reduced the inhibitory menthol actions. Menthol also reduced in a concentration-dependent manner the contractile responses caused by exogenous application of Ca^{2+} (75–375 μM) in a Ca^{2+}-free solution, or induced by potassium chloride (KCl; 40 mM). Moreover menthol (1–3 mM) strongly reduced the electrical field stimulation (EFS)-evoked atropine-sensitive contractions and the carbachol-contractile responses. The present results suggest that menthol induces spasmolytic effects in human colon circular muscle inhibiting directly the gastrointestinal smooth muscle contractility, through the block of Ca^{2+} influx through sarcolemma L-type Ca^{2+} channels.

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1. Introduction

Menthol is a cyclic monoterpene alcohol which is found as a major constituent of peppermint (*Mentha piperita* L) oil, an herbal preparation commonly used in traditional medicine to treat various gastrointestinal disorders, such as nausea and vomiting (Haniadka et al., 2012), pain caused by endoscopy (Asao et al., 2001) and irritable bowel disease (Ford et al., 2008). *In vitro* and *in vivo* animal studies have documented the inhibitory effects of menthol on gastrointestinal motility (Hawthorn et al., 1988; Grigoleit and Grigoleit, 2005; Amato et al., 2013), justifying its spasmolytic effects. However, previous studies about the effect of peppermint oil on the human colonic contractility seem to be contradictory. Using similar techniques and doses of peppermint oil, some workers have shown decreased motility (Duthie, 1981) whereas others report increased colonic contraction with spasm (Rogers et al., 1988).

To date, the site of action and pathways mediating the effects of peppermint oil and other menthol-containing products remain controversial (Hawthorn et al., 1988; Hills and Aaronson, 1991; Mahmood et al., 2003; De Araujo et al., 2011). Despite the Ca^{2+}-channel antagonism seems to be the most likely pharmacological effect (Grigoleit and Grigoleit, 2005), it may not to be the unique mechanism of action underlying the antispasmodic effect of peppermint oil in intestinal tract. In fact, the gastrointestinal effect of menthol could be, at least in part, mediated by the transient receptor potential-melastatin8 (TRPM8) channel, a nonselective cation channel, gated by cold stimuli and menthol. The activation of TRPM8 by menthol results in an increase in intracellular Ca^{2+} concentration (Peier et al., 2002, Reid et al., 2002) and menthol-induced release of Ca^{2+} from intracellular stores enhances neurotransmission at sensory sinapses (Tsuzuki et al., 2004). The TRPM8 channels are widely expressed in sensory neurons in dorsal root and trigeminal ganglia (McKemy et al., 2002). Concerning the

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gastrointestinal tract, TRPM8 channels have been localized in rat gastric and colonic muscle (Mustafa and Oriowo, 2005), in mouse small and large intestine mucosa and myenteric plexus (Zhang et al., 2004; Ramachandran et al., 2013) and in human colon (Ramachandran et al., 2013). Moreover, menthol has been reported to be able to modulate other membrane proteins such as TRP-ankyrin 1 (TRPA1) ion channels (Karashima et al., 2007), mainly expressed in enteric neurons (Zhang et al., 2004), 5-hydroxytryptamine 3 receptors (Ashoor et al., 2013) and α7-nicotine receptors (Kabbani, 2013). Menthol is also able to block the voltage-gated neuronal and skeletal muscle Na^+-channels in resting and inactivated states (Haeseler et al., 2002).

Therefore, the aims of the present study were (a) to assess the effects of menthol on the mechanical activity of human distal colon circular smooth muscle *in vitro* and (b) to characterize the mechanism of action involved in the responses observed.

2. Materials and methods

2.1. Human tissue specimens

The experimental protocols were approved by the Institutional Ethics Committee of the Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT), in Palermo, Italy. Informed consent was obtained from all patients before the use of their tissues. Samples of human distal colon were obtained from 32 patients with no symptoms of major clinical motility disorders who underwent left hemicolectomy for sigmoid cancer (average age 71; range 55–86, 35% female). All samples were obtained from macroscopically normal regions. The colonic specimens were immediately placed in preoxygenated Krebs solution and stored overnight at 4 °C.

2.2. Preparation of circular muscle strips

Methods used in the present study were unchanged from those previously reported (Amato et al., 2014). Briefly, circular muscular strips (4 mm wide by 10 mm long), free of mucosal layer, were cut and suspended in a four-channel organ bath. One end of each strip was tied to organ holders, while the other end was secured with a silk thread to an isometric force transducer (FORT 25, Ugo Basile, Biological Research Apparatus, Comerio, VA, Italy). The organ bath contained 8 ml of heated (37 °C) and oxygenated (95% O_2 and 5% CO_2) Krebs solution with the following composition (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 11.1. Mechanical activity was digitized on an analog-to-digital converter, visualized, recorded and analyzed on a personal computer using the PowerLab/400 system (Ugo Basile). A tension of 1 g was applied, and the tissues were allowed to equilibrate for 1 h. During this period, the strips developed spontaneous phasic contractions. In each experiment, up to six strips from the same specimen were studied.

2.3. Experimental protocol

2.3.1. Menthol on the spontaneous mechanical activity

After the equilibration period, the effects induced by cumulative concentrations of menthol (0.1 mM–30 mM) on the spontaneous mechanical activity were examined. Menthol was added to the bath in volumes of 80 μl, and each doses was left in contact with the preparation for 4 min because within this time the effect was stabilized. The effect of menthol was also evaluated in the presence of the following agents, which were added into the bath at least 15 min before testing menthol: 5-benzyloxytryptamine (5-BT) (1 μM), antagonist of TRPM8 receptor, tetrodotoxin (TTX) (1 μM), a voltage-dependent Na^+-channel blocker, 1H-[1,2,4] oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) (10 μM), an inhibitor of nitric oxide (NO)-sensitive soluble guanylyl cyclase, tetraethylammonium chloride (TEA) (10 mM), a blocker of K^+ channels, nifedipine (3 nM), a voltage-activated L-type Ca^{2+} channel blocker. The concentrations of the drugs used were determined from the literature. Moreover, the effects of (2S,5R)-2-Isopropyl-N-(4-methoxyphenyl)-5-methylcyclohexanecarboximide (WS-12) (0.1 mM), a high-affinity selective TRPM8 agonist, on the spontaneous mechanical activity of human colon were analyzed.

In separate experiments, menthol was tested as Ca^{2+} antagonist by examining its ability to antagonize the contraction elicited by Ca^{2+} addition in a Krebs solution prepared by omitting $CaCl_2$ or the contractions induced by potassium chloride (KCl). Although the Krebs solution without $CaCl_2$ has a very low Ca^{2+} concentration because it was prepared without adding ethylene-glycol-bis (β-amino-ethylether)-N,N,N′,N′-tetraacetic acid (EGTA) to chelate any free Ca^{2+}, it will be indicated as "calcium-free" solution. In Ca^{2+}-free solution, $CaCl_2$ from 75 μM to 375 μM was cumulatively added to the organ bath in the absence and in the presence of menthol (1–10 mM). In preliminary experiments 375 μM $CaCl_2$ caused a maximal contractile response. The contractions induced by KCl (40 mM) were tested in the absence or in the presence of menthol (30 mM).

2.3.2. Menthol on the EFS- or CCh evoked contractions

Effects of increasing concentrations of menthol (0.3–10 mM) were also tested on the contractions evoked by electrical field stimulation (EFS). EFS was delivered by an S88 square-wave pulse generator (Grass Medical Instruments, Quincy, MA, USA) coupled *via* a stimulus isolation unit (Grass SIU5) to a pair of platinum electrodes placed in parallel on either side of the strips. EFS was applied before and after treatment with different concentrations of menthol, which were added to the bath cumulatively at least 4 min before testing evoked-responses. Stimuli were applied as 10-s single trains (pulse duration 0.5 ms, amplitude 100 V, frequency 8 Hz) and were performed at 8 min intervals until to stable and reproducible contractile responses were observed. The evoked contractions were significantly reduced by the muscarinic receptor antagonist atropine (1 μM) or blocked by the neuronal blocker TTX (1 μM), suggesting that they were due to activation of cholinergic nerves.

The influence of menthol on the contractions induced by the muscarinic receptor agonist carbachol (CCh; 1 μM) was also evaluated. The tissue was allowed to stabilize in normal Krebs and after that at least two comparable responses to CCh were recorded; the colonic strips were pre-incubated with the menthol (1 mM).

2.4. Data and statistical analysis

The inhibitory effects of menthol were evaluated by measuring the mean amplitude of spontaneous contractions prior to and following drug administration. The results are expressed as the changes in mean amplitude of the phasic contractions and reported as percentages of the values obtained in the control (*e.g.* 100% corresponds to the abolition of spontaneous activity). Concentration–response curves were computer fitted to a sigmoidal curve using non-linear regression and the concentration (EC_{50}), with 95% confidence limits (Cls), producing half-maximum response, was calculated using Prism 4.0, GraphPad Software (San Diego, CA, USA).

Changes in the basal tone induced by the stepwise increase in the concentration of Ca^{2+} into the bath, in presence of menthol (1–10 mM), were expressed as a percentage of the maximum contraction obtained in the control curve, obtained in the absence of menthol. Effects on the contraction evoked by KCl were expressed as a percentage of the responses to the contractile agent obtained in

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