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Tetrodotoxin-dependent effects of menthol on mouse gastric motor function

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ABSTRACT

Menthol, the main active constituent of peppermint oil, exerts gut spasmolytic effects, although its mechanism of action remains unclear. We investigated the effects of menthol on gastric emptying and spontaneous- or evoked- mechanical activity of whole murine stomach. Gastric emptying was calculated after i.p. administration of menthol (50 mg/Kg). Responses induced by menthol on gastric intraluminal pressure and evoked-cholinergic contractions were analyzed *in vitro*. Menthol decreased the gastric emptying rate. *In vitro*, menthol (0.3–30 mM) produced a concentration-dependent relaxation of whole stomach, that was significantly reduced by tetrodotoxin or ω-conotoxin GVIA. The gastric relaxant responses were not affected by N_{ω}-nitro-L-arginine methyl ester, inhibitor of nitric oxide-synthase, apamin or [Lys1,Pro2,5,Arg3,4,Tyr6] vasoactive intestinal peptide (VIP)$^{7-28}$, a VIP receptor antagonist, but they were significantly antagonized by atropine or guanethidine, a blocker of adrenergic neurotransmission. The joint application of atropine and guanethidine did not produce any additive effects on menthol effects. Phentolamine, an α-adrenoceptor antagonist, but not propranolol, a β-adrenoceptor antagonist, significantly reduced menthol responses and the contemporary administration of both adrenergic antagonists did not produce additive effects. Menthol (1–100 μM) produced a reduction of the electrically-evoked cholinergic contractions, which was prevented by guanethidine. Menthol did not affect the contractions induced by carbachol. In conclusion, menthol in mouse, is able to reduce the rate of gastric emptying and to relax the stomach *in vitro*. The latter effect appears due, almost in part, to neural mechanisms, with involvement of α-adrenoceptors leading to reduction of tonic ongoing release of acetylcholine.

1. Introduction

Peppermint oil, obtained from the leaves and flowers of *Mentha piperita*, has a long history of safe use in traditional medicine to treat symptoms of different gastrointestinal disorders such as non-ulcera dyspepsia (McKay and Blumberg, 2006; Westfall, 2004), irritable bowel syndrome (IBS) (Ford et al., 2008). In addition, peppermint oil is used to reduce painful muscle spasms in patients undergoing endoscopy of the upper (Hiki et al., 2003) and lower (Asao et al., 2001) gastrointestinal tract. Nevertheless, it remains yet unclear how it exerts its beneficial action.

The antispasmodic effects of peppermint oil have been directly demonstrated *in vitro* in both human and animal tissues (Hawthorn et al., 1988; Hills and Aaronson, 1991; Taylor et al., 1984, 1985; Grigoleit and Grigoleit, 2005) and they have been attributed to menthol, one of the main active components of the oil, due to its ability of interfering with the movement of calcium across the cell membrane (Grigoleit and Grigoleit, 2005). However, Ca^{2+} channel antagonism seems not to be the unique pharmacological effect as recent data have shown that menthol has neuroactive properties. In fact, it is able to modulate a variety of different membrane receptors such as 5-hydroxytryptamine 3 ($5\text{-}HT_3$)- (Heimes et al., 2011), nicotine- (Hans et al., 2012) and γ-aminobutyric acid A- ($GABA_A$) receptors (Hall et al., 2004) and transient receptor potential (TRP) cation channels such as TRP-melastatin8 (TRPM8) and TRP-ankirin1 (TRPA1) (Peier et al., 2002; Karashima et al., 2007). TRPM8 and TRPA1 are mainly expressed in afferent neurons (Zhang et al., 2004) and/or enteric neurons (Poole et al., 2011), respectively.

In addition, the effects of peppermint oil and menthol show marked species- and region-related variability. Accordingly, few studies have examined the influence of menthol on gastric mechanical activity and the results often are conflicting. In rats, activation of TRPM8 induces gastric fundus contractions (Mustafa and Oriowo, 2005). In humans, peppermint oil reduces the contractions in gastric corpus (Micklefield et al., 2003) and the emptying rate (Dalvi et al., 1991), although other reports show no

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influence on gastric emptying (Goerg and Spilker, 2003). Recently, peppermint oil has been shown to reduce intragastric pressure and proximal phasic contractility, with negligible effects on accommodation, in humans (Papathanasopoulos et al., 2013).

Therefore, using the mouse as a animal model, the objectives of the present investigation were (1) to verify whether menthol is able to affect the rate of gastric emptying; (2) to study the menthol effects on the gastric mechanical activity, *in vitro* and (3) to examine if the effects of menthol are mediated by neural mechanisms. The whole stomach was chosen because it is able to relax in the absence of contractile agents, due to the development of spontaneous tone (Mulè and Serio, 2002; Rotondo et al., 2011).

2. Materials and methods

2.1. Experimental animals

Experimental protocols were in conformity with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC). Male mice of the C57BL/6J (B6) strain were purchased from Harlan (Harlan Laboratories, San Pietro al Natisone Udine, Italy). Animals were housed under standard conditions of light (12 h light, 12 h dark cycle) and temperature (22–24 °C), with free access to water and food.

2.2. Gastric emptying

Mice were food deprived for 18 h with free access to water. At $t=0$ animals were given free access to pre-weighed standard chow for 1 h, and then were injected i.p. with 100 μl of either saline or menthol (50 mg/Kg b.w.). Mice were food deprived for 3 h after i.p. administration and then sacrificed. The amount of food remaining in the stomach after the 3 h postinjection period was determined as previously described (Baldassano et al., 2012). Gastric emptying (%) was calculated as: (1 −(dry weight of food recovered from the stomach/total food intake) × 100). Total food intake during the 1 h feeding period was determined by measuring the difference between the pre-weighed standard chow and the weight of chow and spill.

2.3. In vitro studies

Animals were killed by cervical dislocation. The abdomen was immediately opened, the esophagus was tied just below the lower esophageal sphincter, and the entire stomach was excised and rapidly mounted in a custom designed organ bath (volume = 5 ml), continuously perfused with oxygenated (95% O_2 and 5% CO_2) and heated (37 °C) 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. The pyloric end was cannulated and connected to a standard pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, USA). The stomach mechanical activity was monitored as changes of endoluminal pressure and recorded on ink-writer polygraph (Grass model 7D, Grass Medical Instruments, Quincy, MA, USA) as previously described (Rotondo et al., 2011). Preparations were allowed to equilibrate for about 60 min before starting the experiment. At the beginning of each experiment, the preparation was challenged with isoproterenol (1 μM) until reproducible responses were obtained, to ensure that a stable and acceptable level of sensitivity had been reached before the experimental procedure was begun. Then, the responses to non-cumulative concentrations of menthol (0.3–300 mM) were examined. Menthol was added into the bath at increasing concentrations in volumes of 50 μl after switching off the perfusion at 30 min intervals. A time contact with the tissue of 3 min was selected.

Relaxant responses to the menthol were tested in presence of the following agents, which were added to the perfusing solution at least 30 min before testing menthol: tetrodotoxin (TTX) (1 μM), a voltage-dependent Na^+-channel blocker, ω-conotoxin GVIA (0.3 μM), a blocker of neuronal N-type voltage-operated Ca^{2+} channels, N_ω-nitro-L-arginine methyl ester (L-NAME) (300 μM), an inhibitor of nitric oxide (NO) synthase, apamin (0.1 μM), a blocker of small conductance Ca^{2+}-dependent K^+-channels, [Lys1, Pro2,5,Arg3,4,Tyr6] vasoactive intestinal peptide $(VIP)^{7\text{-}28}$ (300 nM), a VIP receptor antagonist, atropine, a muscarinic cholinergic receptor blocker, guanethidine (1 μM, for 60 min), a blocker of adrenergic transmission, propranolol (1 μM), a non-selective β-adrenoceptor antagonist, phentolamine (100 μM), a non-selective α-adrenoceptor antagonist. Experiments were performed also to test possible additive effects on the responses induced by menthol, induced by the joint perfusion with guanethidine and atropine or with propranolol and phentolamine.

In another set of experiments, the effects of menthol on the electrically evoked cholinergic contractile responses were tested. Electrical field stimulation (EFS) was applied by a S88 stimulator (Grass Medical Instruments, Quincy, MA, USA) in 5-s trains (0.5 ms pulse duration, 100 V, 8 Hz) at intervals of 5 min. As previously described (Mulè et al., 2007) [1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one] (ODQ) (10 μM), inhibitor of NO-dependent soluble guanylate, and neostigmine (1 μM), inhibitor of acetylcholinesterase, were added to the Krebs solution to block nitrergic transmission and to potentiate cholinergic neurotransmission. In these conditions, stable and reproducible responses can be obtained for long time-period. The responses evoked by EFS were analyzed in the presence of menthol, at a range of concentrations (1–100 μM) which *per se* did not affect gastric tone. The contact time for each concentration was 20 min. Moreover, the myogenic gastric contractions produced by carbachol (CCh) (1 μM), were evaluated in the absence or in the presence of 100 μM menthol. The concentration of CCh used has been shown to induce a submaximal response in the same preparation (Mulè and Serio, 2002; Mulè et al., 2007).

2.4. Data and statistical analysis

Relaxant responses to menthol were expressed as a percentage of the response produced by isoproterenol (1 μM), considered 100%. 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). The inhibitory effects of menthol on the EFS-evoked cholinergic contractions were expressed as a percentage of the mean value of 5 contractions induced by EFS in control condition, considered as 100%. All data are expressed as mean values ± S.E.M. The letter *n* indicates the number of experimental animals. Statistical analysis was performed by means of Student's *t*-test or ANOVA followed by Bonferroni *post hoc* test, when appropriate. A probability value of less than 0.05 was regarded as a significant.

2.5. Drugs

The following drugs were used: isoproterenol hydrochloride, menthol, L-NAME, apamin, atropine sulfate, guanethidine monosulfate, CCh, propranolol hydrochloride, phentolamine hydrochloride (Sigma-Aldrich, Milan, Italy), TTX, ω-CNT (Alomone Labs, Jerusalem, Israel), [Lys1,Pro2,5,Arg3,4,Tyr6] VIP7-28 (California Peptide Research, Napa, *CA*, USA.). Each compound was prepared