European Journal of Pharmacology 745 (2014) 129–134

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, gastrointestinal and urogenital pharmacology

Involvement of cholinergic nicotinic receptors in the menthol-induced gastric relaxation

Antonella Amato *, Rosa Serio, Flavia Mulè

Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università di Palermo, 90128 Palermo, Italy

ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form
16 September 2014
Accepted 5 October 2014
Available online 16 October 2014

Chemical compounds studied in this article:
Menthol (PubChem CID: 16666)

Keywords:
Menthol
Gastric relaxation
TRPM8
TRPA1
Nicotinic receptors

ABSTRACT

We have previously demonstrated that menthol reduces murine gastric tone in part through a neural mechanism, involving adrenergic pathways and reduction of ongoing release of acetylcholine from enteric nerves. In the present study we aimed to verify whether the gastric relaxation to menthol may be triggered by interaction with neural receptors or ionic channels proteins, such as transient receptor potential (TRP)-melastatin8 (TRPM8), TRP-ankyrin 1 (TRPA1), 5-hydroxytriptamine 3 (5-HT_3) receptor or cholinergic nicotinic receptors. Spontaneous mechanical activity was detected *in vitro* as changes in intraluminal pressure from isolated mouse stomach. Menthol (0.3 – 30 mM) induced gastric relaxation which was not affected by 5-benzyloxytryptamine, a TRPM8 receptor antagonist, HC030031, a TRPA1 channel blocker. In addition, allylisothiocyanate, a TRPA1 agonist, but not (2S,5R)-2-Isopropyl-N-(4-methoxyphenyl)-5-methyl-cyclohexanecarboximide, a selective TRPM8 agonist, induced gastric relaxation. Genic expression of TRPA1, but not of TRPM8, was revealed in mouse stomach. Indeed, menthol-induced gastric relaxation was significantly reduced by hexamethonium, cholinergic nicotinic receptor antagonist. Menthol, at concentrations that failed to affect gastric tone, reduced the contraction induced by dimethylphenylpiperazinium, nicotinic receptor agonist. The joint application of hexamethonium and atropine, muscarinc receptor antagonist, or hexamethonium and phentholamine, α-adrenergic receptor antagonist, did not produce any additive reduction of the relaxant response to menthol. Lastly, ondansetron, a 5-HT_3 receptor antagonist, was ineffective. In conclusion, our study suggests that nicotinic receptors, but not TRP and 5-HT_3 receptors, are molecular targets for menthol inducing murine gastric relaxation, ultimately due to the reduction of acetylcholine release from enteric nerves.

1. Introduction

Menthol is a cyclic terpene alcohol that occurs naturally in most essential oils, including peppermint and is responsible for the antispasmodic activity. Although, enteric-coated peppermint formulations are used to treat nausea (Westfall, 2004; Haniadka et al., 2012), non-ulcera dyspepsia and irritable bowel syndrome (Grigoleit and Grigoleit, 2005; McKay and Blumberg, 2006), the cellular and molecular targets mediating the effects of peppermint and menthol on gastrointestinal disorders are currently unknown.

In humans and rodents, the essential oil and menthol reduce intestinal motility through a direct action on smooth muscle cells by blocking calcium-channel activity (Hawthorn et al., 1988; Penuelas et al., 2007; Amato et al., 2014). However, a recent study in mouse stomach showed that menthol induces gastric relaxation also through neural mechanisms. In particular, the menthol is able to decrease mouse gastric tone through reduction of ongoing release of acetylcholine involving adrenergic pathways (Amato et al., 2013). This evidence well fits with the neuroactive properties of menthol that is able to modulate different neural membrane receptors and ionic channels (Boesmans et al., 2011; Kamatou et al., 2013). Indeed, menthol is a primary activator of the cold and menthol-sensitive transient receptor potential-melastatin8 (TRPM8) channel. TRPM8 channels have been detected in the dorsal root ganglia, vagal afferent neurons and in the gut (McKemy et al., 2002; Zhang et al., 2004; Mustafa and Oriowo, 2005; Ramachandran et al., 2013). Another member of the transient receptor potential (TRP) family, TRP-ankyrin 1 (TRPA1) ion channel, activated by irritant and inflammatory signaling (Bandell et al., 2004; Bautista et al., 2006), can be modulated by menthol (Karashima et al., 2007). Apart from visceral afferents, expression of TRPA1 was found in the enteric neurons and enteroendocrine cells (Holzer, 2011; Poole et al., 2011). In these cells TRPA1 agonists cause Ca^{2+} influx and 5-HT release (Nozawa et al., 2009). The 5-hydroxytriptamine 3 (5-HT_3) receptor could contribute to the pharmacological actions of menthol, as it has been shown to be inhibited by menthol (Ashoor et al., 2013b). The 5-HT_3 receptors are expressed by enteric sensory neurons in the

* Correspondence to: Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche, Laboratorio di Fisiologia generale, Università di Palermo, Viale delle Scienze, 90128 Palermo Italia.
Tel.: +39 091 23897506; fax: +39 091 6577501.
E-mail address: antonella.amato@unipa.it (A. Amato).

http://dx.doi.org/10.1016/j.ejphar.2014.10.012
0014-2999/

mucosa layer and in the cell body of interneurons and motor neurons of the enteric nervous system (Galligan, 2002) and their involvement in nausea, vomiting and irritable bowel syndrome has been well established (Riering et al., 2004; Faerber et al., 2007). Moreover, menthol might act as a negative allosteric modulator of cholinergic nicotinic receptors expressed in neural cells (Hans et al., 2012; Ashoor et al., 2013a).The nicotinic receptors are broadly involved as mediating excitatory transmission in myenteric neurons (Galligan, 2002), and they play a major role in the regulation of gut motility (Galligan, 2002; Xue et al., 2006).

Since the receptor and channel proteins, reported in the literature to be able to interact with menthol, are present in the neural pathways regulating gastrointestinal motility, the present study was designed to investigate which molecular target(s) can be responsible for the menthol-induced relaxant effects in mouse stomach.

2. Materials and methods

2.1. Experimental animals

All of the animal procedures for the care and use of laboratory animals were in conformity with the Italian D.L. no. 116 of 27 January 1992 and subsequent variations and the recommendations of the European Economic Community (86/609/ECC). Adult male mice (C57BL/6J, weighing 25.5 ± 0.5 g), purchased from Harlan (Harlan Laboratories, San Pietro al Natisone, Udine, Italy), were used for the study. Animals were housed under standard conditions of light–dark cycle (12 h light, 12 h dark), temperature (22 ± 1 °C) and humidity (55 ± 5%), with free access to food and water.

2.2. Functional 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 tied around the mouth of a tube, which was connected to a standard pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, USA).

The mechanical activity, monitored as changes of endoluminal pressure, was recorded on an ink-writer polygraph (Grass model 7D, Grass Medical Instruments) as previously described (Rotondo et al., 2011b). 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.

The relaxant responses were tested by adding into the bath menthol at non-cumulative increasing concentrations (0.3–30 mM) 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. In this study, the relaxant responses to the menthol were tested in the presence of 5-benzyloxytryptamine (5-BT), antagonist of TRPM8 receptor, HC-030031, a TRPA1 channel blocker, ondansetron, a 5-HT_3 receptor antagonist, and hexamethonium, antagonist of nicotinic receptors. The gastric preparations were incubated with the antagonists at least 30 min before testing menthol. Moreover, the effects of (2S,5R)-2-Isopropyl-N-(4-methoxyphenyl)-5-methylcyclohexanecarboximide (WS-12) (100 μM), a high-affinity selective TRPM8 agonist, allylisothiocyanate (AITC) (100 μM), a TRPA1 agonist and dimethylphenylpiperazinium (DMPP) (10 μM), a nicotinic receptor agonist, and 5-HT on the spontaneous mechanical activity of the mouse stomach were analyzed in order to verify the efficacy of the antagonist used. The responses evoked by DMPP (10 μM) were also analyzed in the presence of menthol, at a concentration that *per se* did not affect gastric tone. Lastly, experiments were performed to test additive effects on the responses induced by menthol obtained by the joint perfusion with hexamethonium and atropine (1 μM), a cholinergic muscarinic receptor blocker, or hexamethonium and phentolamine (100 μM), a non-selective α-adrenoceptor antagonist.

2.3. Data analysis and statistical tests

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 (Prism 4.0, GraphPad Software San Diego, CA, USA).

The inhibitory effects of menthol (100 μM) on the DMPP response were expressed as a percentage of the response obtained under control condition. 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 significant.

2.4. Drugs

The following drugs were used: isoproterenol hydrochloride, menthol, WS-12, AITC, DMPP iodide, 5-BT hydrochloride, ondansetron hydrochloride dehydrate, hexamethonium bromide, atropine sulfate, phentolamine hydrochloride (Sigma-Aldrich, Milan, Italy), and HC030031 (Tocris Bioscience, Bristol, UK). Each compound was prepared as a stock solution in distilled water, except for WS-12, AITC and HC030031 dissolved in dimethyl sulphoxide. The final solvent concentration (0.1%) had no effect on the preparations. The working solutions were prepared fresh the day of the experiments by diluting the stock solutions in Krebs.

2.5. RT-PCR analysis

The TRPM8 and TRPA1 mRNA expression was assessed by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) in whole

Table 1
Primers sequences used for PCR experiments.

Gene	Sequences	TM (°C)	Fragment size (bp)
TRPM8	Forward ATC TAT GAG CCC TAC CTG Reverse CAG CCT TAC TTG ATG TTA TT	51	611
TRPA1	Forward GAA GCA TGC GTC ATT GAA GAG GAT Reverse TCC ATT TCC AAG CAT GTG TCA ATG	50	227
β-actin	Forward CGG GAT CCC CGC CCT AGG CAC CAG GGT Reverse GGA ATT CGG CTG GGG TGT TGA AGG TCT CAAA	60	286