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(−)-Carvone: Antispasmodic effect and mode of action

Fábia Valéria M. Souza, Marcelly Barbosa da Rocha, Damião P. de Souza, Rosilene Moretti Marçal *

Physiology Department, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil

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ABSTRACT

(−)-Carvone is a monoterpene ketone found in spearmint (*Mentha spicata* var. *crispa*) essential oil that is widely used as an odor and flavor additive. An intestinal antispasmodic effect was recently reported for (−)-carvone, and it has been shown to be more potent than its (+)-antipode. The mechanism of (−)-carvone action in the intestines has not been investigated. To gain a better understanding of the (−)-carvone antispasmodic effect, we investigated its pharmacological effects in the guinea pig ileum. Terminal portions of the ileum were mounted for isotonic contraction recordings. The effect of (−)-carvone was compared with that of the classical calcium channel blocker (CCB) verapamil. In isolated ileal smooth muscle, (−)-carvone did not produce direct contractile or relaxation responses and did not modify electrically elicited contractions or low K^+-evoked contractions. The submaximal contractions induced by histamine ($p<0.001$), $BaCl_2$ ($p<0.05$), and carbachol ($p<0.01$) were significantly reduced by (−)-carvone. The contractile response elicited by high concentrations of carbachol was reduced but not abolished by (−)-carvone. No additive action was detected with co-incubation of (−)-carvone and verapamil on carbachol-induced contraction. (−)-Carvone reduced the contraction induced by high K^+ and was almost 100 times more potent than verapamil. Thus, (−)-carvone showed a typical and potent CCB-like action. Many effects described for both (−)-carvone and spearmint oil can be explained as a CCB-like mode of action.

1. Introduction

Terpenoids are a group of compounds that occur in nearly every natural food and plant. Their main subclasses that may maintain and improve health are monoterpenes, including limonene, carvone, and carveol, diterpenes, including the retinoids, and tetraterpenes, which include carotenes, lycopene, zeaxanthin, and cryptoxanthin [1].

Many terpenes are used therapeutically or as flavor and fragrance materials. Terpenes can also contain one or more asymmetrical carbons that exhibit optical activity. Terpene chirality has been shown to influence their odor property, biological activity, and mode of action [1]. The monoterpene ketone carvone (*p*-mentha-6,8-dien-2-one; Fig. 1) has a chiral carbon and is found in nature as two optical antipodes. The isomer *R*-(−)-carvone is the main component of the essential oil of *Mentha spicata* var. *crispa* (Lamiaceae) and is responsible for the so-called minty odor and flavor [1]. On the other hand, *S*-(+)-carvone is the only antipode found in the essential oil of caraway (*Carum carvi* L. (Apiaceae)) and produces the well-known caraway odor [2].

In addition to their different odors, carvone antipodes have different biological effects [2–4]. For example, (−)-carvone has a relaxation effect in mice [3], whereas (+)-carvone has a stimulant effect [2]. (*S*)-(+)-carvone, but not *R*-(−)-carvone, appears to have an anticonvulsant-like activity [3]. In healthy human volunteers, inhalation of (−)-carvone increases the pulse rate, diastolic blood pressure, and subjective restlessness [4]. In contrast, inhalation of (+)-carvone only increases systolic and diastolic blood pressure in human volunteers [4].

The intestinal antispasmodic effect of carvone has also been described before. In this study, the (−)-carvone enantiomer

* Corresponding author at: Departamento de Fisiologia, Laboratório de Farmacodinâmica e Etnofarmacologia, Universidade Federal de Sergipe, Av. Marechal Rondon, s/n, CEP 49.100-000, São Cristóvão, SE, Brazil. Tel.: +55 79 2105 6345; fax: +55 79 2105 6641.
E-mail address: rosilenemoretti@gmail.com (R.M. Marçal).

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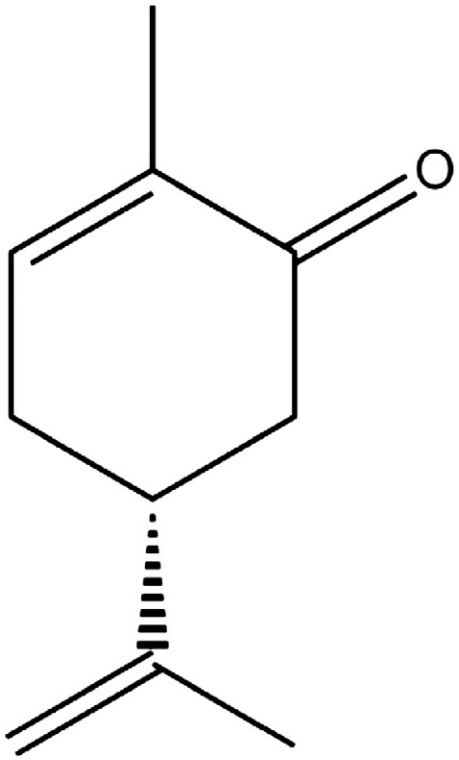

Fig. 1. Chemical structure of (−)-carvone.

showed to be almost 10 times more potent than its (+)-antipode [5]. Carvone intestinal antispasmodic activity indicated that this terpene can be used to treat smooth muscle spasms. To be properly used in therapeutics the mechanisms underlying carvone action should be understood. The most potent isomer is a priori more suitable for therapeutic purposes. Taking these into account, the aim of the present work was to characterize the intestinal response and the mechanisms underlying the action of the most potent carvone enantiomer, i.e. (−)-carvone.

Antispasmodic medicines are used to treat symptoms such as pain and spasm in the gastrointestinal tract. Intestinal spasms can be related to exacerbate neuronal release of the neurotransmitter acetylcholine that acts as muscarinic receptors in intestinal smooth muscle. Muscarinic receptors, after activation by acetylcholine, induce G protein-mediated signal transduction that increases cytosolic calcium ion concentration, and triggers the intestinal smooth muscle contractile process. Therefore, medicines that are able to reduce acetylcholine neuronal release, or antagonize acetylcholine at muscarinic receptor, or even block calcium channels, are also able to reduce intestinal spasms. In addition, $K+$ channel activation, caused by potassium channel openers, or histamine receptor blockade, in the presence of a histamine antagonist, reduces intestinal spasm [6–10].

In the present work, we hypothesized that all the above described modes of action could contribute to the (−)-carvone intestinal antispasmodic effect. Classical protocols have been used in order to test the hypotheses. Aiming to evaluate whether or not (−)-carvone acts in a specific receptor pathway, the effect of (−)-carvone was evaluated in the presence of three different stimuli, i.e. two agonists, histamine and carbachol, that act in different receptors, and $BaCl_2$, an electromechanical stimuli. Another question investigated was whether (−)-carvone acts by activating potassium channels or by blocking calcium channels. To examine this issue, the (−)-carvone effect was evaluated on KCl 20 mM and KCl 60 mM-induced contractions, respectively. Finally, in order to investigate whether (−)-carvone acts by reducing neurotransmitter release, (−)-carvone response was evaluated on the electrically-evoked contractions. In brief, the (−)-carvone mechanism of action was delineated and its potency was estimated.

2. Materials and methods

2.1. Chemicals

(−)-Carvone (optical purity of 98%), verapamil (≥99.0%), carbachol (≥98%), histamine (≥99%), minoxidil (≥99%), and $BaCl_2$ (≥99.9%) were purchased from Sigma-Aldrich (USA). NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, NaH_2PO_4, and glucose (≥98.5% purity) were purchased from VETEC (Rio de Janeiro, Brazil).

2.2. Animals

Male and female guinea pigs (200–300 g) from our own colony were used in the present study. Animals were randomly housed in appropriate cages at 21 ± 1 °C, with a 12/12 h light/dark cycle (06:00–18:00 h) with free access to food (Purina, Brazil) and tap water. Experiments were performed in strict accordance with the guidelines for animal experimentation of the International Guiding Principles for Biomedical Research Involving Animals [11]. The protocols were approved by the Scientific and Bioethics Committee of our institute.

2.3. Guinea pig ileum

Guinea pigs ($n = 6$) fasted overnight and were sacrificed by a blow to the base of the skull and cervical dislocation. Pieces of the ileum of 1 cm in length were dissected from the ileum segment 5–10 cm proximal to the ileocecal valve [12]. The material was mounted for tension recording and allowed to equilibrate for 1 h in 10-ml chambers containing aerated normal Tyrode's solution (mM: NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2 \cdot 6H_2O$ 0.5; $NaHCO_3$ 12; NaH_2PO_4 0.4, and glucose 5.5; pH 7.4), maintained at 37 °C, and bubbled with air. The mechanical response of the ileum was recorded with an isometric force transducer (UC2 model; Gould) linked to a pre-amplifier and computerized data acquisition system (DASA 6600). To characterize the pharmacological response to (−)-carvone in the guinea pig ileum, the following protocols were used.

For all concentration–response curves, each concentration of (−)-carvone was added 30 min after the preceding one.

1) Cumulative concentration–response curves for (−)-carvone (0.1 nM–10 μM) were obtained using isolated guinea pig ileum in normal Tyrode's solution.

2) (−)-Carvone (0.1 nM–10 μM; cumulative application) concentration–response curves were obtained during the tonic phase of the 20 mM KCl solution (K20). The potassium solution was an equimolar replacement of NaCl by KCl in the Tyrode's solution. Relaxation of the ileum was expressed as a percent of the control K20-induced contraction. Minoxidil (1 nM–10 μM) was used as a positive control for K20-induced contraction.
3) The submaximal contractions induced by histamine (2 μM), carbachol (100 μM), and $BaCl_2$ (0.03 M) were obtained in the presence or absence of (−)-carvone (30 μM) in normal Tyrode's solution. Ileum pieces were incubated with each drug for 20 min before the second contractile response was recorded.
4) To verify whether carvone and verapamil have additive responses, submaximal carbachol (100 μM) responses were also obtained in the presence and absence of (−)-carvone (30 μM) plus verapamil (10 nM). Ileum pieces were incubated with (−)-carvone and verapamil for 20 min before the second carbachol contractile response was recorded. Normal Tyrode's solution was used in this set of experiments.
5) (−)-Carvone (0.1 nM–10 μM; cumulative application) concentration–response curves were obtained during the tonic phase of the 60 mM KCl solution (K60). The potassium solution was an equimolar replacement of NaCl by KCl in the Tyrode's solution. Relaxation of the ileum was expressed as a percent of the control K60-induced contraction. Verapamil (10 nM–10 μM) was used as a positive control for K60-induced contractions.
6) In each preparation, (−)-carvone cumulative concentration–response curves (0.1 nM–10 μM) were recorded in the presence of electrically induced contractions (40 V, 0.5 ms, and 0.1 Hz). Morphine (1 μM) was used as a positive control.

2.4. Statistical analysis

Data are expressed as the mean ± S.E.M. or mean [confidence interval] of at least six experiments. The responses obtained are expressed as a percent of the maximum height attained in the control. The IC_{50} was determined with nonlinear regression using GraphPad Prism version 4.0 software. Values were compared using Student's *t*-test for paired data or analysis of variance, followed by Bonferroni's test, when appropriate. A significance level of 5% was set for all analyses.

3. Results and discussion

In a previous study that screened for natural antispasmodic medicines, several terpenes were found to reduce the contraction induced by high potassium concentration in the guinea pig ileum. Among them, the monoterpene ketone (−)-carvone was the most potent antispasmodic compound [5]. In the present study, we further characterized intestinal response to (−)-carvone and its mode of action as an antispasmodic compound.

Antispasmodic compounds can also reduce smooth muscle spasms without modifying normal intestinal tonus. To determine whether (−)-carvone modified the basal intestinal tonus, its response on basal contraction of the isolated ileum was directly evaluated. After 1 h under 1 g of tension suspended in Tyrode's solution, guinea pig ileum showed a stable tension and spontaneous contractile activity. In this spontaneous tonus, (−)-carvone did not exert any direct contractile or relaxation response in the smooth muscle at any concentration tested (data not shown).

Potassium channel activators, such as minoxidil, pinacidil and cromakalim, act by opening potassium channels that leads to K+ efflux, membrane hyperpolarization, and smooth muscle relaxation. In isolated smooth muscles, it is well-known that potassium channel openers reduce contractile response evoked by low potassium concentration (20 mM KCl solution) [6]. In the present work (Fig. 2), we found that the potassium channel opener minoxidil (1 nM–10 μM), which was used as a positive control, reduced the tonic phase of the K20-elicited response in a concentration-dependent manner (IC_{50} = 28.7 μM [10–83 μM]; r^2 = 0.97). In contrast, (−)-carvone (0.1 nM–10 μM) did not modify the K20-induced contraction. Thus, a mode of action as a potassium channel opener seems unlikely for (−)-carvone.

Antispasmodic agents can act by blocking specific receptors present in smooth muscles, such as muscarinic cholinergic or histamine receptors. In contractions, antispasmodics can act in a non-specific way, and reduce the contractile response from several patterns of stimuli [7]. In the present work, we found that (−)-carvone significantly reduced the spasmodic response induced by three different stimuli, e.g., histamine, $BaCl_2$, and carbachol, which act on different receptors and/or in different signal transduction pathways [9]. The percentages of inhibition were 40.3 ± 3.6% ($p<0.001$), 31.5 ± 7.6% ($p<0.01$) and 16.0 ± 5.0% ($p<0.5$) for histamine-, carbachol- and $BaCl_2$-elicited contractions, respectively (Figs. 3, 4). These data indicate that (−)-carvone acts by a mechanism other than via activation of a specific receptor or pathway.

Smooth muscle contractile responses, including ileal contractions, are highly dependent on an increase in free cytoplasmic Ca^{++}, which activates contractile elements. The increase in intracellular calcium is due to either influx via voltage-dependent calcium channels or release from intracellular stores of the sarcoplasmic reticulum [8]. CCBs such as verapamil inhibit smooth muscle contractile response by

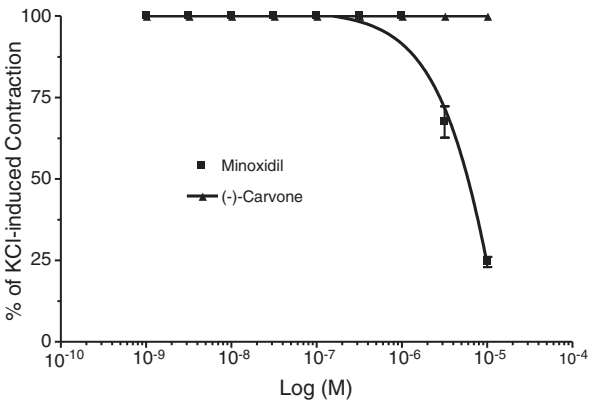

Fig. 2. Evaluation of the effect of (−)-carvone (0.1 nM–10 μM) on the contraction elicited by low KCl (20 mM). Minoxidil (1 nM–10 μM) was used as a positive control. Values are the mean ± S.E.M. (n = 6).

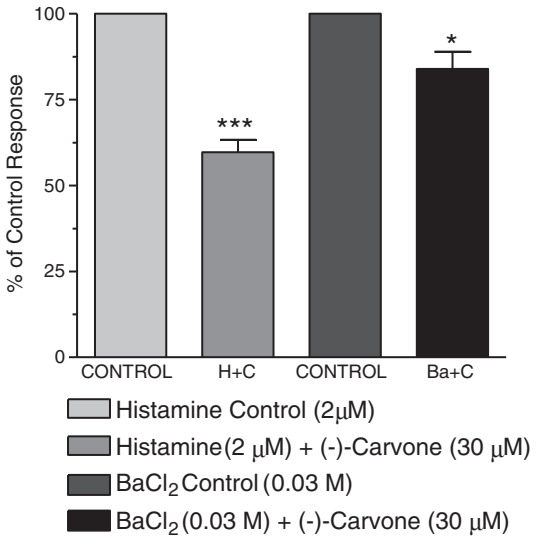

Fig. 3. Effect of (−)-carvone on the contraction elicited by histamine and $BaCl_2$. The responses are expressed as percent inhibition of the control contraction. Values are the mean ± S.E.M. ($n = 6$). *$p<0.05$ vs $BaCl_2$ control, ***$p<0.001$ vs histamine control (*t*-test). C = (−)-carvone; H = histamine; Ba = $BaCl_2$.

blocking calcium entry through voltage-dependent calcium channels [8,9]. For this reason, CCBs can reduce the effects of both electrochemical and pharmacochemical stimuli such as carbachol, histamine, and barium-induced contractions [7]. Therefore, it is reasonable to propose that (−)-carvone may act as a CCB. Two experiments have been performed to test this hypothesis.

In smooth muscles, high carbachol concentration-induced contraction is highly dependent on intracellular calcium stores. For this reason, such contraction is reduced, but not abolished, in the presence of CCBs [7]. In the present work, we found that the CCB verapamil, which was used as a positive control, significantly reduced the contraction induced by high carbachol concentrations. Similarly, the contractile response elicited by high carbachol concentrations was reduced, but not abolished, in the presence of (−)-carvone. Furthermore, no additive action ($p > 0.05$ vs verapamil; 89.8 ± 2.5% of inhibition) was detected by co-incubation of (−)-carvone (30 μM) and the CCB verapamil (10 nM). These findings are in agreement with the idea that (−)-carvone acts as a CCB. These data are shown in Fig. 4.

CCBs can also reduce the contraction induced by high potassium (60 mM KCl solution) concentrations [8,9]. (−)-Carvone was recently reported to reduce the contraction induced by high potassium concentration. To further test the hypothesis that (−)-carvone acts as a CCB, (−)-carvone concentration–response curves were generated during the tonic phase of the K60–induced contraction and compared with the classical CCB verapamil (Fig. 5). In the guinea pig ileum, K60 (Fig. 5) produced a sustained contraction that was maintained during the course of the experiments. During the tonic phase of the contraction induced by K60, the positive control, verapamil (10 nM–10 μM), showed a concentration-dependent relaxing response ($IC_{50} = 100$ nM [57–190 nM]). Similar to verapamil, (−)-carvone (0.1 nM–10 μM) showed a potent relaxation response ($IC_{50} = 3.6$ nM [1.8–7.3 nM]). This result indicates that (−)-carvone acts as a CCB. Mainly, (−)-carvone showed to be almost 100 times more potent than the marketed CCB verapamil. This data strongly suggests that (−)-carvone has a potential use in therapy as a new calcium channel blocker.

Interestingly, (−)-carvone was more potent in this study compared to a previous report [5]. The most probable reason for this discrepancy is the time between the addition of two consecutive concentrations of (−)-carvone. Indeed, we observed that the maximum effect of every concentration was not obtained as fast as usual when classical drugs were evaluated. For all concentration–response curves for (−)-carvone, each concentration of (−)-carvone was added 30 min after the

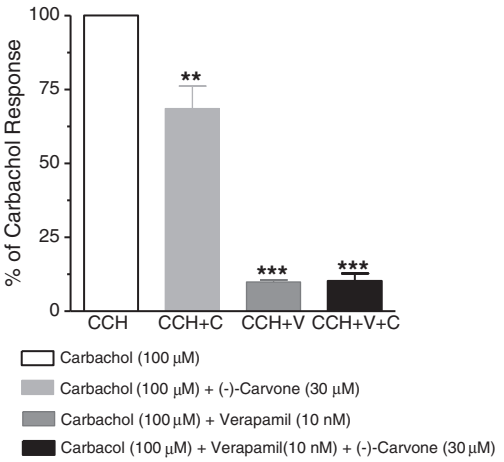

Fig. 4. Effect of (−)-carvone on the contraction elicited by carbachol. In a set of paired experiments, carbachol-elicited contractions were achieved in the absence or presence of (−)-carvone, verapamil, or (−)-carvone and verapamil for 20 min. The responses are expressed as percent inhibition of the contraction elicited by the control carbachol. Values are the mean ± S.E.M. ($n = 6$). **$p<0.01$; ***$p<0.001$ vs the carbachol control (one-way analysis of variance followed by Bonferroni's test). CCH = carbachol; V = verapamil; C = (−)-carvone.

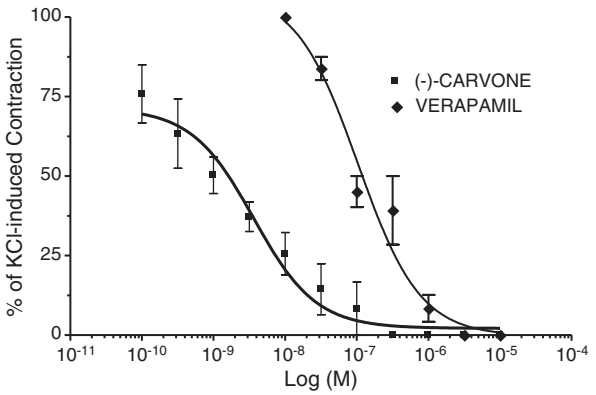

Fig. 5. Effect of (−)-carvone (0.1 nM–10 μM) on the contraction elicited by high KCl (60 mM). Verapamil (10 nM–10 μM) was used as a positive control. The responses are expressed as percent inhibition of the maximum contraction (plateau) induced by K60. Values are the mean ± S.E.M. ($n = 6$).

preceding one. During this time, the initial antispasmodic response changed for the effective concentrations.

Opiate agonists such as morphine and papaverine are well known for their antispasmodic effect. Indeed, opiate agonists activate neuronal opiate receptors that reduce neural acetylcholine release, and consequently they reduce neurogenic spasmodic contractions [10]. In the present work, we evaluated the effect of (−)-carvone on neurogenic (electrically elicited) contraction and compared it with that of morphine. As expected, the positive control morphine (1 μM) reduced (64.7 ± 4.1% of inhibition; $p<0.001$) electrically induced contractions (0.1 Hz; 0.5 ms; 40 V) in the guinea pig ileum. On the other hand, (−)-carvone (0.1 nM–10 μM) did not modify the electrically elicited ileal contractions (data not shown).

These results indicate that a neuronal opiate-like action is unlikely for (−)-carvone.

(−)-Carvone was also previously suggested to act by blocking voltage-gated neuronal Na^+ channels [3]. However, the absence of a carvone response on the electrically elicited contractions indicates that the primary action of (−)-carvone does not involve a neuronal effect in the guinea pig ileum.

Finally, an analgesic effect has been previously reported for (−)-carvone [13]. However, the analgesic effect reported for (−)-carvone is not reversed by the opiate antagonist naloxone [15]. This finding is in agreement with our present data because neurogenic contractions were not reduced by (−)-carvone in the guinea pig ileum. Also, CCBs are well known to show an antinociceptive effect that is not reversed by pre-treatment with opiate antagonists [14,15]. Therefore, the mode of action described here for (−)-carvone can explain its analgesic effect.

In brief, the results of the present study support the conclusions that (−)-carvone does not act in the absence of intestinal spasm, does not reduce neuronal-induced spasm, and does not act as a K+ channel activator. Moreover, the present results support the conclusion that (−)-carvone acts beyond a specific receptor activation. Mainly, (−)-carvone acts as a potent calcium channel blocker, being almost 100 times more potent than the marketed calcium channel blocker verapamil. Its potent effect as calcium channel blocker strongly suggest that (−)-carvone can be used in therapeutics.

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