Local Anaesthetic Properties vs. Neurotoxicity for (+)- and (−)-Carvone: An *Ex Vivo* Electrophysiological Study

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Abstract

The two carvone enantiomers were tested for local anaesthetic activity at concentrations of 10 and 20 mM using a nerve preparation based on the isolated sciatic nerve of the frog and were found to induce similar responses. They eliminated the evoked compound action potential within 6–7 min, while there was an instant 83 to 87% recovery when they were washed out from the nerve preparation and replaced with normal saline. Both carvones have the same pattern of action as 10 mM lidocaine, the standard local anaesthetic, but they are 3–4 times less active with respect to the time of response. When the nerves were exposed to the carvones for a period of time longer than 6–7 min, for example 1 h, there was no recovery at all of the evoked compound action potential, indicating a neurotoxic action. In contrast, when nerves were exposed under the same conditions to lidocaine and another natural compound with local anaesthetic properties, 2-heptanone, there was an 80–100% recovery. This unusual neurotoxic effect of (+)- and (−)-carvone can be a disadvantage for their use in clinical practice.

Key words

(+)-carvone · (−)-carvone · local anaesthetic · neurotoxic · sciatic nerve · electrophysiology

Abbreviations

CAP:	compound action potential
IT_{50}:	inhibitory time to 50%
TRPV-1:	transient receptor potential vanilloid type 1
VGNaC:	voltage-gated sodium channel

Carvone (*p*-mentha-6,8-dien-2-one), an active constituent of essential oils, is found in two enantiomeric forms: (−)-carvone is the main constituent of spearmint (*Mentha spicata*), while (+)-carvone is mainly found in caraway (*Carum carvi*) and dill (*Anethum graveolens*) seed oils. The two carvones are found in these oils in concentrations that may exceed 60%. Carvone is used extensively as a fragrance and flavor component, whereas carvone-producing plants find extensive use as spices and condiments and also in medicine. The pharmaceutical properties of the two carvone enantiomers have been studied widely for antifungal action [1], immunomodulatory activity [2], and chemopreventive properties against anticancer drugs [3]. They have also been investigated as local anaesthetics and proven to exhibit antinociceptive activities in both *in vitro* and *ex vivo* assays [4–6], and in *in vivo* tests [4, 7, 8]. *Ex vivo* electrophysiological data indicate that both enantiomers of carvone are able to inhibit the nerve-evoked CAP of the isolated sciatic nerve of Wistar rats and of the frog [4, 5], in a concentration-dependent manner and in an almost reversible way [6]. Recently, (−)-carvone was reported as a novel agonist of TRPV-1 channels [9], and thus a promising target for the development of analgesic drugs [10, 11]. Since carvone-producing plants are used in everyday life and in medicine, information on the mode of their action is crucial and can contribute to the development of new compounds with desired properties. The purpose of this study was to evaluate the local anaesthetic potential of the two carvones using a nerve preparation based on the isolated sciatic nerve of the frog. Given the results taken, it was also to demonstrate an unusual neurotoxic effect that they both have on this nerve.

When the nerve preparation was incubated in normal saline and exposed continuously to supramaximal stimulation (1 Hz), the amplitude of the evoked CAP (example is given in **Fig. 1 A**) remained constant for over 25 h [12–14]. Thus, the vitality of the sciatic nerve fibres was not affected by the recording conditions in the first 24 h of the experiment. In our study, (+)- and (−)-carvone were diluted using 0.1% Tween 80 before application on the nerve preparation. Our results, based on six sciatic nerves, have shown that 0.2% Tween 80, at double the concentration used in the carvone-enriched samples, has no effect on the function and vitality of the sciatic nerve for 24 h. Other studies have shown that the local anaesthetic effect of carvone occurred at an optimum concentration of 10–20 mM [5]. In our study, when 10 mM of (+)-carvone were applied on the desheathed sciatic nerve of the frog, the evoked CAP was eliminated completely within 6–7 min (**Fig. 1 A**). This effect was reversed almost instantly when the (+)-carvone enriched saline was washed out thoroughly and replaced with a normal saline, free of carvone (see CAPs after the second arrow in **Fig. 1 A**). Exactly the same response was obtained with (−)-carvone (data not shown). Using these recordings, we plotted the mean time-response curves for 10 mM of (+)- and (−)-carvone on a per minute sampling rate (**Fig. 1 B** and **D**, curve a). The IT_{50} s, representing the time required for the decrease of CAP to reach 50% of the initial value (before treatment), for (+)- and (−)-carvone were 2.37 ± 0.1 (n = 8) and 2.41 ± 0.1 min (n = 5), respectively, not differing significantly ($p > 0.05$). Also, the IT_{50} values for 20.0 mM of (+)- and (−)-carvone were estimated to be 2.15 ± 0.2 (n = 4) and 1.84 ± 0.1 (n = 4) min, respectively (from **Fig. 1 B** and **D**, curve b); these values showed a significant difference of 14.4% ($p < 0.01$).To compare the local anaesthetic activity of carvones with that of a standard local anaesthetic, similar experiments were conducted using 10 and 20 mM of lidocaine. From the mean time-response curves shown in **Fig. 1 F**, it was possible to estimate that the IT_{50} value for 10.0 mM lidocaine was 0.76 ± 0.11 min (n = 4) and for 20 mM, 0.54 ± 0.9 min (n = 4) (data not shown). The difference between these IT_{50} values was 28.94% ($p < 0.01$). Comparison of the IT_{50} values of carvone and lidocaine indicates that the two carvones are 3–4 times less active than lidocaine, with respect to the time of response.

The effects of (+)-carvone at 10.0 mM were immediately reversible. An instant recovery in the amplitude of CAP (**Fig. 1 A**, second arrow) was achieved when the part of the nerve that was exposed to carvone was washed out 5–6 times and left in normal saline, free of carvone, afterwards (second arrow). From such experiments, the mean time-response curve for the 24-h recovery period of the nerves exposed to 10.0 mM (+)-carvone was plotted, indicating a 87.0% (n = 7) recovery (**Fig. 1 C**). Also, 10 mM

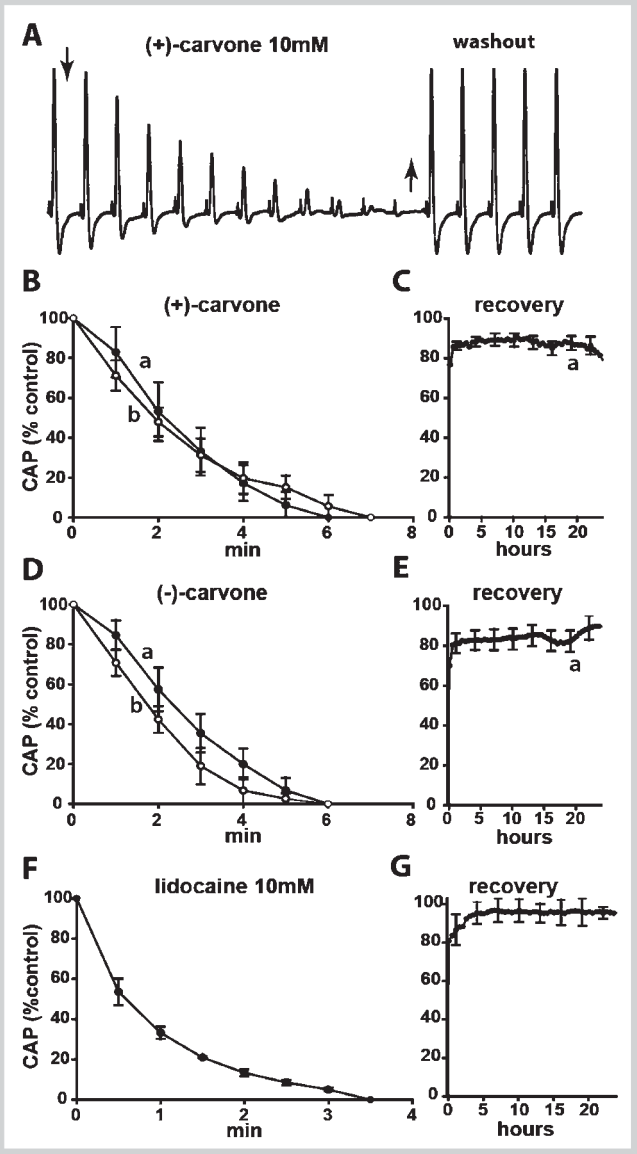

Fig. 1 **A** Decrease in the amplitude of the evoked CAP with exposure of the sciatic nerve to 10.0 mM (+)-carvone; the first arrow indicates the beginning of the application of carvone, whereas the second arrow indicates the time when the nerve was washed and bathed in normal saline. CAP records were taken at a rate of 1 CAP per min. After replacement of the (+)-carvone enriched saline with normal saline, records were taken every 1 h. **B** Time-response curves representing the decrease in the amplitude of the evoked CAP of the sciatic nerve fibres of the frog as a function of incubation time in (+)-carvone-enriched saline at 10.0 mM (curve a, n = 8) and 20 mM (curve b, n = 4). The amplitude at time t = 0 was considered to be 100%; values are means ± SEM. **C** Time-response curve for the recovery of the amplitude of the evoked CAP after replacing the (+)-carvone-enriched saline, at 10 mM (n = 7), with normal saline as a function of incubation time. Records were obtained every 1 h and the CAP was monitored for 24 h. Before the recovery process, the incubation time was 7 min. The amplitude at time t = 0 was considered to be 100%; values are means ± SEM. **D** As in **B**, but with the nerve incubated in (−)-carvone at 10 mM (curve a, n = 5) and 20 mM (curve b, n = 4). **E** As in **C**, the recovery of the CAP after 10 mM (−)-carvone was washed out and replaced with free-carvone saline. **F** As in **B**, the inhibition of the CAP of a nerve incubated in 10 mM lidocaine for 3.5 min (n = 4). **G** As in **C**, the recovery of the CAP after 10 mM lidocaine was washed out and replaced with lidocaine-free saline (n = 4).

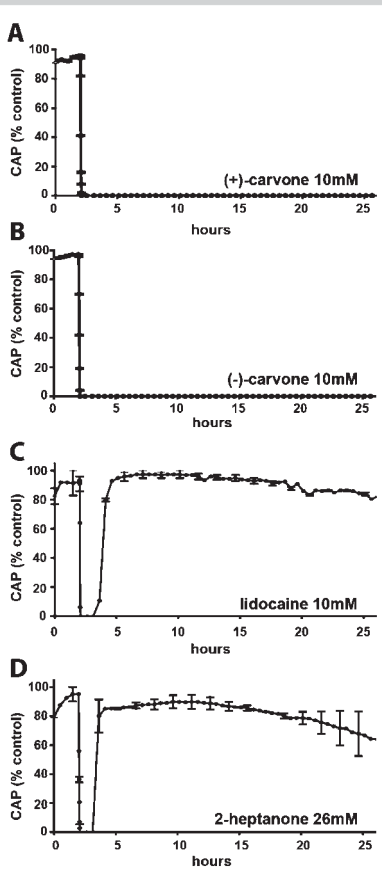

Fig. 2 Time-response curve for the decrease and recovery in the amplitude of the evoked CAP of the sciatic nerve fibres of the frog as a function of incubation time. After incubation of the nerve in normal saline for 2 h, the nerve was exposed for 1 h to 10 mM of (+)-carvone (**A**), (−)-carvone (**B**), lidocaine (**C**), and 26 mM of 2-heptanone (**D**) (n = 4, in all four cases). The exposure period starts with the fast decrease in the amplitude of the CAP. Then, it was washed out thoroughly with normal saline and left in it for the rest of the experiment. Records were obtained every 1 h and the CAP was monitored for 24 h. The higher amplitude value recorded throughout the experiment was considered to be 100%; values are means ± SEM.

of (−)-carvone showed an instant recovery in the amplitude of the eliminated CAP up to 83.0% of the initial value (n = 5) (**Fig. 1E**). It is worth noting that after full recovery from the inhibition caused by the two carvones, the amplitude of CAP remained constant for over 24 h (**Fig. 1C** and **E**). This clearly shows that the incubation in 10.0 mM of (+)- or (−)-carvone for 6 or 7 min had no toxic effects on the sciatic nerve fibres (83–87% recovery). The pattern of recovery of CAP for nerves incubated in 20.0 mM of (+)- or (−)-carvone was identical to those of 10.0 mM (data not shown). Finally, the pattern of recovery of CAP after exposure to 10.0 mM lidocaine and intense washout (**Fig. 1F**) was similar to that of carvone, but the recovery was 98% (n = 4) (**Fig. 1G**).

In order to investigate any possible toxic effect due to prolonged exposure, the nerves were incubated in the two carvones for a longer period, 60 min, instead of 6–7 min. Exposure of the sciatic nerve to 10.0 mM of (+)- or (−)-carvone for 1 h was extremely toxic. CAP never recovered even after 24 h in normal, carvone-free saline (**Fig. 2A** and **B**). This contrasts dramatically with the 83–87% recovery after incubation in carvone-enriched saline for 6–7 min. It also contrasts with the results for lidocaine and 2-heptanone, a natural compound with local anaesthetic action [15]. There was a full recovery of the CAP to 98% after 1 h of incubation in 10.0 mM lidocaine, which remained at this level for 24 h (**Fig. 2C**). Also, for a much higher concentration of 2-heptanone, 26.0 mM, the recovery was 80–85% with CAP remaining again at this level for 20 h (**Fig. 2D**, n = 4). This is a clear indication that lidocaine and 2-heptanone, i.e., compounds with local anaesthetic properties that were examined using the same nerve

preparation and applied on the nerve for 1 h, do not exhibit any neurotoxic effect on the sciatic nerve fibres of the frog, unlike the two carvones.

Both enantiomeric forms of carvone were investigated in this study for their local anaesthetic activity using a commonly applied *ex vivo* electrophysiological method based on the sciatic nerve of the frog. The same nerve preparation was used by other researchers in similar studies on carvones [6]. Compared with lidocaine, the two carvones were found to have an identical pattern of action, but were 3–4 times less effective. It is interesting, the fact that when linalool, another constituent of essential oils, was examined against lidocaine, it was found to be 3.6 times less effective [14], indicating a possible common function on the peripheral nervous system. The local anaesthetic effects of carvones, as expressed by the inhibition of CAP, which is partially extinguished by nerve washing, could be a result of blockages of VGNaC, which is considered to be the main target of local anaesthetics [16,17]. The same mechanism has been proposed for the local anaesthetic action of linalool [18]. Yet another mechanism was proposed for the action of (−)-carvone, which was recently identified as a novel agonist of TRPV-1 channels [9], thus being a promising target for the development of analgesic drugs [10,11]. Although the mechanism of inhibition of the action potential by carvones is not clear, there is already a number of publications suggesting their use as local anaesthetics. The antinociceptive activity of (−)-carvone *in vivo* using different experimental models of pain has already been investigated [4]. However, in clinical practice, local anaesthetics are injected into the region near the nerve and remain there for a certain period of time until they are metabolised, which requires 90–120 min in the case of lidocaine [19]. We simulated the above-prolonged exposure using the isolated nerve preparation and exposed the nerve for 1 h in either (−) or (+)-carvone; both compounds proved to be neurotoxic when compared with lidocaine and 2-heptanone. For reasons unknown at the moment, carvones appear to have a delayed neurotoxicity, although in some other cases, (−)-carvone, at pharmacologically active concentrations, did not reveal significant cytotoxicity in HEK293 cells [10].

Carvones were also tested as local anaesthetics *in vivo* [7,8] on the mammalian sciatic nerve. A mild effect was reported. At 10 mM, (−)-carvone decreased CAP to 50% and (+)-carvone to 25% in 30 min [5]. Although the pattern of the carvones' local anaesthetic activity is similar to that of lidocaine, their low *in vivo* effect combined with their delayed but significant neurotoxicity described here make the two compounds unsuitable for use as local anaesthetics. Since carvones and plants bearing them are extensively used, and because carvones have been proposed as local anaesthetics, and even cancer chemopreventive agents [3], further information on their mode of action is crucial and can contribute to the development of new compounds with desired properties.

Materials and Methods

To evaluate the local anaesthetic action and the neurotoxic effects of (+)- and (−)-carvone, the sciatic nerve of the frog (*Rana ridibunda*), isolated in a three-chambered recording bath, was used. The same preparation has been used repeatedly for the assessment of local anaesthetic activity of both synthetic and natural compounds [12–14]. The frogs were of either sex, 50–60 g in weight, bought from local suppliers, and kept in tanks at 18 to 22 °C, in 12 h light and 12 h dark. All experimental procedures were conducted in accordance with the protocols outlined by the Aristotle University of Thessaloniki, Greece, regarding the recommended standard practices for biological investigations (prot. No. 16 324/2014; date of approval 13/2/14). The frogs were double pitched, and the sciatic nerve was rapidly dissected from the spinal cord to the knee. The nerve was immersed in a standard oxygenated saline solution (O_2, 100%), pH = 7.2, with the following composition in mM: 111 NaCl, 2 glucose, 2.4 KCl, 2 $CaCl_2$, 1 $NaHCO_3$, and 10 HEPES. The nerve was cleaned, and the epineural sheath was removed under a stereoscope. All experiments were performed at a constant temperature of 26.0 ± 1.0 °C.

The nerve was placed in the three-chambered bath, described in detail elsewhere [12–14]. The carvones were added to 0.1% Tween 80 to make stock solutions of 1.0 M, and then were put in the oxygenated saline of the perfusion chamber to achieve the desired final concentration. At the maximum concentration of the carvones used in this study, the final concentration of Tween 80 in the perfusion chamber was 0.1%, a concentration having no effect on the function and vitality of the nerve, as mentioned above. To assess the local anaesthetic activity of (+)- or (−)- carvone, each compound was diluted in the saline of the perfusion chamber at the desired concentration of 10.0 or 20.0 mM, and single CAPs were recorded and stored every 1 min (example, **Fig. 1 A**). When CAPs were eliminated, the saline with the carvone was washed out thoroughly and then replaced with normal saline (carvone-free saline, second arrow in **Fig. 1 A**). To monitor the recovery of the exposed nerve, samples of CAPs were stored every 1.0 h for over 24 h (second half of **Fig. 1 A**). In these long-term recordings, the saline was stagnant, and the recording bath was shielded airtight to avoid evaporation during the long recording period. The vitality of the nerve was quantified by measuring the amplitude of the evoked CAP, base to peak. The same procedure was followed for the standard local anaesthetic lidocaine, which was diluted directly in the saline of the perfusion chamber.

To further test the possible neurotoxic effects of the two compounds under prolonged exposure, the nerve preparation was left in the saline enriched with each of the tested compounds for 1 h and then the nerve preparation was washed out and bathed in normal saline (free of carvone and lidocaine). In these experiments, for a better comparison of the neurotoxic effect, an additional natural compound with local anaesthetic properties, 2-heptanone [15], was tested.

For data analysis, the amplitude of CAP, measured in Volts, was expressed as a percentage of (i) the last value recorded before the application of the tested compound (for short-term experiments) or (ii) the higher CAP amplitude value recorded throughout the experiment (for long-term experiments), which was considered as 100%. Values deriving from the replicated experiments were averaged and expressed as means ± SEM. Using these values, the mean time-response curves were plotted (for an example, see **Fig. 1 D**). For the experiments of the short-term exposure, and from the mean time-response curves (vitality curves), we estimated the time required for CAP to reach 50% of its initial amplitude at t = 0 for all tested compounds (at 10.0 and 20.0 mM for the carvones and lidocaine) using the program GraphPad Prism 5.0. This period of time here is called IT_{50}. The IT_{50} values of the compounds tested, for the different concentrations in which they were applied, were analyzed by one-way ANOVA and Bonferroni's post hoc tests.

The chemicals used were: (+)-carvone (96%) and (−)-carvone (98%), purchased from Alfa Aesar; $CaCl_2.2H_2O$ (97%), KCl

(99.5%), NaCl (99.5%), $MgCl_2$ (99%), and $NaHCO_3$ (99.7) from Panreac; HEPES (99.5%) from Sigma-Aldrich; glucose (99.5%) from Riedel-de Haen; xanthopren from Heraeus Kulzer; and lidocaine (99.9%) and 2-heptanone (98%) from Sigma-Aldrich.

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Conflict of Interest

There is no conflict of interest among the authors.

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