Research Article

Inhibitory effects of cardamonin on compound action potentials in frog sciatic nerves and the possible involvement of opioidergic pathway

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Abstract

Introduction: Active compounds derived from plants are able to inhibit nerve conduction. Cardamonin, a naturally occurring chalcone, manifests anti-nociceptive, anti-inflammatory and anti-neuropathy properties. Consequently, cardamonin may potentially inhibit nerve action potential, whereby, it affects the nerve conduction. Compound action potential is the sum of the activity which is measured from a nerve trunk. **Objective:** The experiment was carried out to investigate the inhibitory effect of cardamonin on compound action potentials and its possible mechanism of action on frog sciatic nerve. **Methodology:** LabTutor software was used to record compound action potentials in frog sciatic nerve. Sciatic nerve was isolated from the frog and soaked in Ringer's solution. Stimulating electrodes were used to stimulate the nerve and recording electrodes were used to record compound action potentials. Compound action potential of the nerve were recorded before and after treatments [vehicle, cardamonin (0.5, 1 & 2 mg/ml) & morphine (3mg/ml)]. Participation of opioid system was investigated by pre-treating the nerve with naloxone and followed by cardamonin. All the data were recorded and analysed via LabTutor software. The data were analysed by using Two-way ANOVA followed by Bonferonni's post hoc test with significant value at $P < 0.05$. **Results:** The outcomes showed that all the doses of cardamonin significantly reduced the peak amplitude of compound action potential in frog sciatic nerves. Besides, co-treatment of naloxone and cardamonin significantly ($P < 0.001$) reversed the effect of cardamonin on peak amplitude of compound action potential, suggesting the involvement of opioid receptors to inhibit nerve conduction. **Conclusion:** Cardamonin reduces the nerve signal conduction via activation of opioid receptors to modulate pain and contribute to the analgesic effects.

Keywords: Cardamonin, Sciatic nerve, Compound Action Potentials, Opioid receptor

1.0 Introduction

Cardamonin (2', 4'-dihydroxy-6'-methoxychalcone), an active compound found in various zingiberous plant species (Gonçalves, Valente, & Rodrigues, 2014) such as *Alpinia blepharocalyx* (Dong, Chen, Xu, Kadota, & Namba, 1998), *Alpinia rafflesiana* (Chow et al., 2012) and *Boesenbergia pandurata* (Trakoontivakorn et al., 2001). Extensive studies had reported that cardamonin exhibits several biological properties, importantly, anti-nociceptive (Mi Kyung Park et al., 2014; M. K. Park et al., 2014; Wang et al., 2016), anti-neuropathic pain (Sambasevam et al., 2017), anti-inflammatory (Ahmad et al., 2006; Israf, Khaizurin, Syahida, Lajis, & Khozirah, 2007; Lee et al., 2006), anti-HIV (Tewtrakul, Subhadhirasakul, Puripattanaavong, & Panphadung, 2003), antineoplastic (Trakoontivakorn et al., 2001), antioxidant (Bajgai, Prachyawarakorn, Mahidol, Ruchirawat, & Kittakoop, 2011) and hypoglycemic (Yamamoto et al., 2011) activities. Besides, cardamonin had been reported to modulate receptors and ion channels. For instance, cardamonin isolated from *A. katsumadai*, act as a selective TRPA1 antagonist responsible for the anti-nociceptive effect (Wang et al., 2016), in which modulation of TRP channels will inhibit nerve excitability (Ohtsubo, Fujita, Matsushita, & Kumamoto, 2015). Taken together, it is possible that analgesic effects produced by cardamonin are due to inhibition of synaptic transmission and action potential conduction in pain pathways.

Compound action potentials (CAPs) are seen in nerve trunks which contains hundreds of nerve fibres in parallel with various sizes of diameter and thresholds. The recorded potential from the nerve trunk is the sum of the excitability of nerve fibers (Marieb & Hoehn, 2007). Moreover, action potential is a signal which travel along the nerve fibres resulting in electrical membrane potential to rapidly rise or fall (Lodish, 2008). Previous studies had revealed that plant-derived active compounds are able to reduce the peak amplitude of compound action potentials. For instance, peppermint component menthol (Kawasaki, Mizuta, Fujita, & Kumamoto, 2013), wasabi component allyl isothiocyanate and cinnamon component cinnamaldehyde (Matsushita, Ohtsubo, Fujita, & Kumamoto, 2013) as well as aroma-oil compounds (Ohtsubo et al., 2015) had been proven to have depressive action on CAPs. Thus, it is possible that cardamonin also may induce reduction of action potential in peripheral nerve.

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On the other hand, descending opioidergic pathway plays role in modulating the pain signal transmitted from the nociceptors in peripheral nerve fibres. Activation of the localised opioid receptors indirectly inhibit the excitatory synaptic transmission triggering anti-nociceptive effects (Gissen, Gugino, Datta, Miller, & Covino, 1987; Jaffe & Rowe, 1996). Activation of opioid receptors (μ , δ , and κ) will decrease the conductance of voltage-gated Ca^{2+} channels and opening of rectifying K^{+} channels, directly inhibiting neuronal hyperexcitability, leads to analgesic effects (Stein, 2016). Based on the previous studies, action potential in nerve fibres was blocked by the treatment of opioid drugs. For instance, sufentanil and fentanyl suppress the peak amplitudes of CAPs measure from peripheral nerve fibres (Gissen et al., 1987) and inhibit action potential (Jaffe & Rowe, 1996).

We previously had reported the involvement of opioid receptors in cardamonin-induced antihyperalgesic and antiallodynic properties (Sambasevam et al., 2017). Thus, we hypothesized that cardamonin may potentially inhibit compound action potential via modulation of opioid receptors localised in peripheral nerve fibres. To our knowledge, there are no data has been reported on the effects of cardamonin on nerve conduction in inhibiting pain circuit. To address this issue, the present study speculates how cardamonin used to relieve pain affect CAPs measured from the frog sciatic nerve.

2.0 Materials & Methods

2.1 Animals

Bullfrogs (*Rana catesbeiana*) weighing 200-300g, housed in the aquarium and fed with living insects and water *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee (IACUC), UPM (AUP No.: U001/2017). All efforts were made to minimize the number of animal used and animal suffering.

2.2 Isolation of frog sciatic nerves

The method used to isolate frog sciatic nerve has been described previously (Kosugi, Mizuta, Fujita, Nakashima, & Kumamoto, 2010; Matsushita et al., 2013; Ohtsubo et al., 2015). Frogs were pithed and the sciatic nerve was dissected from the lumbar plexus to the knee in Ringer solution. The sciatic nerve was soaked in the petri dish which contained Ringer solution and then placed in the nerve chamber for CAPs measurement. The Ringer solution was prepared by using (mM): NaCl, 115.5; KCl, 2.0; CaCl_2 , 1.8; Na_2HPO_4 , 1.3; (pH=7.0).

2.3 Materials

Cardamonin with the purity of $\geq 98\%$ was prepared by dissolving in Tween20 and normal saline at a ratio of 5:95 (v/v). Drugs such as morphine sulphate and naloxone hydrochloride were dissolved in normal saline. All compound and drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.4 Experimental design

Table 1: Experiment I – CAPs inhibition by cardamonin

Experimental groups	Treatment
1	Vehicle (95% normal saline & 5% Tween20)
2	Cardamonin (0.5 mg/ml)
3	Cardamonin (1 mg/ml)
4	Cardamonin (2 mg/ml)
5	Morphine (3 mg/ml)

Table 2: Experiment II – Involvement of opioid pathway

Experimental groups	Treatment
1	Vehicle (95% normal saline & 5% Tween20)
2	Cardamonin (1 mg/ml)
3	Naloxone hydrochloride (0.1 mg/ml)
4	Naloxone hydrochloride + cardamonin

2.5 Recordings of compound action potentials from frog sciatic nerve

LabTutor software (ADInstruments Pty Ltd., Australia) consists of a data capture module known as Powerlab connected to a computer, was used throughout the experiment. As shown in the LabTutor software, the sciatic nerve was placed on the nerve bath chamber where it was connected with two stimulating electrodes and two sets of recording electrodes. Stimulating electrodes were used to stimulate the sciatic nerve, while the recording electrodes were used to record CAPs. The stimulation was started at 10mV, where CAPs curve having 0.5ms duration and varying strength were used. The stimulation, 200mV had been chosen to generate the CAPs. CAPs were recorded before and after the treatments (Table 1 & Figure 1). The procedure was quickly performed to avoid the sciatic nerve from losing moisture. The data were recorded in the LabTutor software. A stimulus artifact was produced followed by a CAP when the nerve was stimulated. The difference between baseline and CAP highest peak value was recorded as the peak amplitude of the CAP (Matsushita et al., 2013; Ohtsubo et al., 2015).

The peak amplitude of the CAP was dependent on the strength of stimulus given to the sciatic nerve. As the stimulus strength was increased, the CAP peak amplitude was enhanced and attained a maximal value. As done previously (Matsushita et al., 2013; Ohtsubo et al., 2015), the peak amplitude of the maximal CAP was analyzed. All the experiments were conducted at room temperature (22-27°C).

2.6 Involvement of Opioid Receptor

The involvement of opioid receptor was investigated based on (Mizuta, Fujita, Nakatsuka, & Kumamoto, 2008) with minor modification. Each experimental group (Table 2) consists of six frog sciatic nerves. The CAPs of the sciatic nerves were recorded for each treatment procedure (Figure 2). Then, the nerve was soaked in the respective treatment according to the experimental groups. Throughout the experiment, the CAPs were measured and recorded using LabTutor software.

Figure 1: Protocol for the CAP inhibitory effects of cardamonin

Figure 2: Protocol for the involvement of opioid receptors

2.7 Data analysis

Data were indicated as mean $\pm$ SEM and statistically analysed by using GraphPad Prism v5.0 software (GraphPad San Diego, CA). Statistically differences between groups were determined by One-Way Analysis of Variance (ANOVA) followed by Bonferonni's post hoc test. The results were considered significant at $P < 0.05$. In all cases, n refers to the number of sciatic nerves studied.

3.0 Results

3.1 Effects of cardamonin on frog sciatic nerve CAPs

Effects of cardamonin on CAPs were examined in total of 54 sciatic nerves, and when measured the nerves' CAP, an average value of 23.31 mV was obtained, a value similar with those reported previously (Kosugi et al., 2010; Mizuta et al., 2008).

First, the effect of cardamonin on CAPs obtained from the frog sciatic nerve was investigated. Sciatic nerve soaked into 1mg/ml of cardamonin for 30 mins suppress the peak amplitude of the CAP as shown in Figure 3A (upper panel). Figure 3A (lower panel) shows the average time course of the change in peak amplitude after soaking in the cardamonin (1 mg/ml), relative to control obtained from six sciatic nerves. Cardamonin-induced reduction in CAP amplitude at 30 mins following exposure, the peak amplitude of the CAP was reduced to 53.02

$\pm 6.1\%$ ($P < 0.001$) of control. The treated nerve was then returned to drug-free Ringer's solution (washout) for 30 mins, and the CAP peak amplitude recovered to $90.44 \pm 7.8\%$ ($P < 0.05$) of the control.

Figure 3B shows the time course of changes in CAP peak amplitude with an increase in time after soaking the sciatic nerve into vehicle, cardamonin at different concentrations (0.5, 1 and 2mg/ml) and morphine (3mg/ml). Based on the graph, at 30 mins, 0.5mg/ml ($65.95 \pm 2.1\%$, $P < 0.01$), 1mg/ml ($53.0 \pm 6.1\%$, $P < 0.001$) and 2mg/ml ($59.9 \pm 1.9\%$, $P < 0.001$) of cardamonin showed significant reduction of CAP peak amplitude when compared to control group ($84.87 \pm 4.5\%$). However, 1mg/ml of cardamonin produced a pronounced inhibition of CAP at 20 mins of soaking and sustained for 10 mins. Positive control, morphine (3mg/ml) shows significant reduction of CAP peak amplitude ($55.43 \pm 4\%$) when compared to control group.

Figure 3A: Upper Panel - Recording of CAPs before treatment at 0 min, 30 min after exposure to cardamonin (1mg/mL) at 30 min after washout. Lower Panel - Average time course of changes in CAP peak amplitudes following exposure to cardamonin (1mg/mL) for 30 min, relative to baseline. Results are shown as mean $\pm$ S.E.M., n=6 nerves per group.

Figure 3B: Comparison in average time course among CAP peak amplitude changes produced by vehicle, cardamonin (0.5, 1 and 2 mg/mL) and morphine (3mg/mL).

3.2 Effect of opioid receptor antagonist on frog sciatic nerve CAPs

In order to investigate the involvement of opioid pathway in cardamonin-induced reduction of CAP peak amplitude, the sciatic nerves were soaked in a non-specific opioid-receptor blocker, naloxone hydrochloride (0.1 mg/ml) for 20 mins prior to cardamonin (1mg/ml). Figure 4 illustrates the significant reversal ($93.22 \pm 6.2\%$, $P < 0.001$) of reduced CAP peak amplitude produced by cardamonin.

Figure 4: Effect of pre-treatment of naloxone (0.1 mg/mL) with cardamonin on the CAPs amplitude of frog sciatic nerve.

4.0 Discussion

The present study demonstrated that cardamonin depresses the CAP peak amplitude measured from frog sciatic nerve. Based on the graphs, cardamonin reduced the peak amplitude of CAP in a concentration-independent manner. Cardamonin reversibly inhibited CAPs peak amplitude, where the peak amplitude was observed to be recovered after washout as shown in Figure 3A. All the doses of cardamonin in the present study demonstrated inhibition of compound action potential. However, 1mg/ml of cardamonin seemed to be the most effective in inhibiting the CAPs when compared to the control group. Morphine, positive control used in this study significantly reduced the CAP when compared with control group. This outcome is further supported by one of the studies which stated that morphine inhibits the CAP peak amplitude via involvement of opioidergic pathway (Jurna & Grossmann, 1977).

Compound action potential, the basic element of nerve activity, is important to regulate physiological and pathological processes (Li et al., 2010). Among the peripheral nerve fibres, sciatic nerve is found to be the largest nerve and frequently used to investigate processes associated with peripheral nervous system (Pandey & Deshpande, 2012). The changes in neuronal excitability observed in the present study suggested that cardamonin possibly act as a local anesthetic agent. This is in agreement with previous study showing that cardamonin possessed anti-nociceptive (Mi Kyung Park et al., 2014) and anti-neuropathic pain activities (Sambasevam et al., 2017). Cardamonin also been reported

that it had antagonistic effect on the TRPA1 receptors and thus block the nerve signal transmission (Caterina et al., 1997; Wang et al., 2016). Consistent with this idea, the nerve conduction possibly inhibited by modulation of TRP receptors as seen in previous studies carried out by using cinnamaldehyde and allyl isothiocyanate on frog sciatic nerve (Matsushita et al., 2013).

Cardamonin, for the first time, revealed its ability in inhibiting the CAPs. Thus, it is possible to investigate the underlying mechanisms. We examined the involvement of opioid pathway by using a non-specific opioid receptor antagonist, naloxone hydrochloride. Based on the findings, cardamonin-induced CAP peak reduction was significantly reversed, indicating the input of opioid receptors in modulating the nerve action potential. We previously reported that cardamonin alleviates pain response observed in chronic constriction injury-induced neuropathic pain in *in vivo* via involvement of opioid receptors localised in nervous system (Sambasevam et al., 2017). Combining with our previous findings, our current results further insights into the depressive action of cardamonin in action potential as an important mechanism in producing analgesic effects in pathological condition.

Opioids are carried from brain to periphery by P-glycoprotein, thus it is possible that centrally-administered opioids act on both central and peripheral nervous system (King, Su, Chang, Zuckerman, & Pasternak, 2001). On the other hand, drugs that does not able to penetrate blood brain barrier, such as N-methyl-morphine demonstrated antinociception in an acetic acid writhing model in mice, suggesting the crucial role of opioids in the peripheral nervous system (Shannon & Lutz, 2002). Taken together, the role of opioids in the periphery seems to be mediated by opioid receptors in the peripheral terminals of afferent fibres (Labuz, Mousa, Schäfer, Stein, & Machelska, 2007). The present study suggests the possible role of cardamonin act as an agonist on the receptors expressed on the peripheral nerve fibres, depresses the action potential, thereby exhibiting analgesic effects (Sambasevam et al., 2017). Besides, cardamonin is classified under flavonoids. Flavonoids exhibits analgesic effects by activating the opioidergic system (Higgs, Wasowski, Loscalzo, & Marder, 2013). This further supports the findings that cardamonin inhibited nerve conduction in the peripheral nerve such as sciatic nerve by activating the localised opioid receptors.

5.0 Conclusion

In conclusion, cardamonin possibly inhibits nerve conduction in a manner independent on their concentrations. The cardamonin-induced inhibition is antagonized by naloxone, suggesting that cardamonin-induced CAP amplitude reduction was due to the activation of opioid receptor.

6.0 Declaration

The authors declare no conflicts of interest in this work.

7.0 Acknowledgements

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8.0 Reference

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