



Neuropharmacology and analgesia

Participation of potassium channels in the antinociceptive effect of docosahexaenoic acid in the rat formalin test



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ABSTRACT

Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid (PUFA) that has shown gastroprotective, cardioprotective, neuroprotective, anti-inflammatory and antinociceptive effects in different models. However, its action mechanism is still not well-defined. Reports indicate that some PUFAs regulate potassium (K⁺) channels in ventricular myocytes of rodents. As a result, the aim of this study was to evaluate the probable participation of K⁺ channels in the antinociceptive effect of DHA. The rat paw 1% formalin test was used to assess nociception and antinociception. Fifty microliters of formalin solution were administered subcutaneously in the paw, and the number of flinches were quantified. Rats were treated with local peripheral administration of DHA (100–1778 µg/paw) or diclofenac (10–300 µg/paw). The antinociception of DHA was evaluated with and without the local pretreatment of K⁺ channel blockers. DHA and diclofenac produced dose-dependent antinociceptive effects during the second phase ($P < 0.05$). Local peripheral administration of tolbutamide and glibenclamide (K_v6.1-2; ATP-sensitive K⁺ channel blockers); iberiotoxin and charybdotoxin (K_{Ca}1.1; big conductance calcium-activated K⁺ channel blockers); apamin and dequalinium (K_{Ca}2.1–3; small conductance Ca²⁺-activated K⁺ channel blockers); but not by 4-aminopyridine and tetraethylammonium (K_v; voltage-gated K⁺ channel blockers) reverted the DHA-induced antinociceptive effect. It is concluded that big- and small-conductance Ca²⁺-activated K⁺ channels and ATP-sensitive K⁺ channels are activated by DHA to produce local antinociception on the rat formalin test.

1. Introduction

Fatty acids are important constituents of lipids. Fatty acids are carboxylic acids with hydrocarbon chains that are divided into saturated and unsaturated. Polyunsaturated FAs (PUFAs) possess two or more double bonds (Fritsche, 2015; Tvrzicka et al., 2011). Linoleic (18:2n-6) and α-linolenic acids (18:3n-3) are considered essential PUFAs because they cannot be synthesized by humans and these must be taken from the diet (Fritsche, 2015; Tvrzicka et al., 2011). Linoleic acid is mainly metabolized to γ-linolenic, dihomo-γ-linolenic, and arachidonic acid. On the other hand, metabolism of α-linolenic acids gives eicosapentaenoic, docosahexaenoic (DHA) and docosapentaenoic acids. α-linolenic acids is found in seeds and leaves of some plants and eicosapentaenoic acid and DHA are found in significant quantities in flesh of both lean and oily marine fish (Fritsche, 2015; Tvrzicka et al.,

2011).

DHA has shown gastroprotective (Pineda-Peña et al., 2012), cardioprotective (Endo and Arita, 2016), neuroprotective (Molz et al., 2015), anti-inflammatory (Calder, 2015) and antinociceptive effects (Arroyo-Lira et al., 2014; Nakamoto et al., 2011). DHA's antinociceptive effect has been studied in several murine thermal and chemical nociception models (tail flick, acetic acid-induced writhing and formalin tests) (Arroyo-Lira et al., 2014; Nakamoto et al., 2010). Systemic release of β-endorphin and successive activation of opioid receptors has been demonstrated in the DHA-induced antinociceptive effects in rodents (Nakamoto et al., 2011). New findings showed that the intracerebroventricular and intrathecal administration of DHA induced antinociceptive effect, and this is due to the activation of G-protein coupled receptor 40 (Nakamoto et al., 2012). Opioid receptors belong to the G_i/G_o-protein-coupled receptors. Opioid receptors are coupled to

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inhibition of voltage-operated calcium conductances and activation of inwardly rectifying potassium conductance (Grudt and Williams, 1995). The role of potassium channels is shared in antinociception for activation of α_2 -adrenoceptors, GABA(B), muscarinic M(2), adenosine A(1), serotonin 5-HT(1A) and cannabinoid receptors (Ocaña et al., 2004). Recently, it has been reported that PUFAs regulate big conductance calcium-activated potassium (K^+) channels from the inner mitochondrial membrane (Olszewska et al., 2014) and delaying (Ik) and inwardly (Ik1) rectifier potassium currents in rat ventricular myocytes related to antiarrhythmic mechanisms (Song et al., 2013).

Peripheral antinociceptive mechanism of several compounds has been described by the activation of potassium channels such as nocistatin (Scoto et al., 2012), ketamine (Romero and Duarte, 2013), diclofenac (Ortiz et al., 2008), ketorolac (Lázaro-Ibáñez et al., 2001), meloxicam (Ortiz et al., 2005), ellagic acid (Ghorbanzadeh et al., 2014) among others. In this sense, the participation of potassium channels in the DHA-induced antinociceptive effect cannot be excluded. Therefore, the aim of this study was to evaluate the probable participation of ATP-sensitive K^+ (K_{ATP}), voltage-gated K^+ (K_V), small and big conductance Ca^{2+} activated K^+ (K_{Ca}) channels in the antinociceptive effect of DHA on the formalin test.

2. Materials and methods

2.1. Animals

Female rats (Wistar strain; aged 7–9 weeks; weight range 180–220 g) were used in our study. Rats were killed in a CO_2 compartment at the end of the experiment. The study was conducted according to the ethical guidelines previously reported by Zimmermann (Zimmermann, 1983), and all experiments were approved by an independent committee at our Institution (CINVESTAV, IPN, Ciudad de México, México).

2.2. Drugs

Docosahexaenoic acid (DHA; D2534), olive oil, glibenclamide, tetraethylammonium chloride, apamin, 4-aminopyridine and diclofenac were purchased from Sigma-Aldrich (Toluca, Mexico). Tolbutamide, dequalinium chloride, iberitoxin and charybdotoxin were purchased from Santa Cruz (Mexico). Formaldehyde and dimethyl sulfoxide (DMSO) were purchased from J.T. Baker. All drugs were dissolved in isotonic saline, except for glibenclamide and tolbutamide, which were dissolved in 20% DMSO. The vehicle used for DHA was olive oil. All drugs were injected in a volume of 50 μ l per paw.

2.3. Measurement of antinociceptive activity

The rat paw 1% formalin test was used to assess nociceptive and antinociceptive effects (Arroyo-Lira et al., 2014; Ortiz, 2011). Fifty microliters of 1% formalin were injected subcutaneously (s.c.) in the right hind paw, and the flinching behavior was quantified. The nociceptive behavior showed a biphasic pattern (Arroyo-Lira et al., 2014; Ortiz, 2011). The number of flinches yielded a biphasic curve, and the area under the curve was calculated for both phases.

2.4. Study design

Responses-doses (Fig. 1) and -time curves (Table 1) were realized in order to determine the antinociceptive dose of DHA and feasible times of its administration before formalin injection. After that, seventy-five min before the formalin injection, local peripheral injections with vehicle or different doses of DHA (100, 175, 300, 562, 1000, 1778 μ g/paw) were assayed. The local peripheral antinociceptive effects induced by diclofenac (10, 30, 100 and 300 μ g/paw) were evaluated as positive control (Ortiz et al., 2002; Ortiz, 2011).

To determine whether K^+ channels mediated the antinociception of

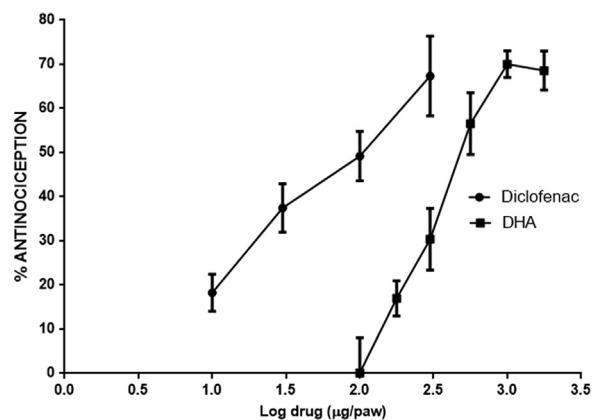


Fig. 1. Comparative dose–effect logarithmic curves for administration of diclofenac and docosahexaenoic acid (DHA) during the second phase of the formalin test. Doses of diclofenac were 10, 30, 100 and 300 μ g/paw and doses of DHA were 100, 175, 300, 562, 1000, 1778 μ g/paw. Each point corresponds to the mean \pm S.E.M. of at least 6–12 rats.

Table 1

Antinociceptive effect of 562 μ g/paw DHA at different times of administration before formalin injection.

Pretreatment time (min)	% of antinociceptive effect
Vehicle	-4.96 \pm 9.02
15	30.27 \pm 7.66
30	17.52 \pm 7.77
45	38.30 \pm 3.25 ^a
60	46.07 \pm 7.29 ^a
75	66.42 \pm 4.07 ^a
90	32.16 \pm 6.74

Values are the mean \pm S.E.M. for 6–12 rats during the phase two. One-way ANOVA followed by Bonferroni's test.

^a P < 0.05 vs. vehicle.

DHA, 10 min prior to DHA (562 μ g/paw) administration rats were also pretreated with K^+ channels blockers. Rats received vehicles or with glibenclamide (50 and 100 μ g/paw) and tolbutamide (150 and 300 μ g/paw) (both K_{ATP} ; ATP-sensitive K^+ channel blockers); 4-aminopyridine (25 and 50 μ g/paw) and tetraethylammonium chloride (100 and 200 μ g/paw) (both K_V ; voltage-gated K^+ channel blockers); apamin (1 and 2 μ g/paw) and dequalinium chloride (25 and 50 μ g/paw) (both K_{Ca} ; small conductance Ca^{2+} -activated K^+ channel blockers); iberitoxin (0.5 and 1 μ g/paw) and charybdotoxin (1 and 2 μ g/paw) (both K_{Ca} ; big conductance calcium-activated K^+ channel blockers) and the antinociceptive effects were assessed. For all the experiments, the doses used of K^+ channels blockers were selected based on previous reports (Alves et al., 2004; De Paz-Campos et al., 2012; Ortiz et al., 2002; Ortiz, 2011). The tester was blinded to the administration of the different treatments.

2.5. Data analysis

Results are presented as mean \pm S.E.M. (six to twelve rats per group). The area under the curve (AUC) of the local peripheral antinociceptive effects produced by each individual and combined (drug + blocker) drug regimen was calculated as previously described (Arroyo-Lira et al., 2014; Ortiz et al., 2002; Ortiz and Castañeda-Hernández, 2008; Ortiz, 2011). The percentage of antinociception for both phases of the assay was determined according to the following equation (Ortiz and Castañeda-Hernández, 2008):

$$\% \text{ antinociception} = [(AUC_{\text{vehicle}} - AUC_{\text{post-drug}}) / AUC_{\text{vehicle}}] \times 100.$$

The dose-response curves were evaluated for significance using the one-way ANOVA and Bonferroni's test. The results were considered

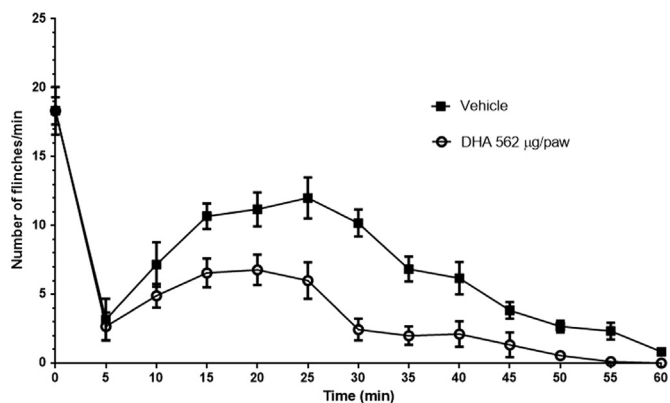


Fig. 2. Time course of the number of flinches per min in rats treated with vehicle or DHA (562 µg/paw) in the 1% formalin test in the rat. Data are presented as mean ± S.E.M. (n=6–12).

statistically significant when $P < 0.05$.

3. Results

3.1. Peripheral antinociceptive effects of DHA and diclofenac

The local peripheral administration of formalin produced a flinching behavior indicative of nociception. Fig. 1 shows the dose–effect curves for administration of diclofenac and DHA during the second phase of the formalin test. Ipsilateral local peripheral administration of DHA produced dose-dependent antinociceptive effects during the second phase (Fig. 2) ($P < 0.05$) but not during phase one.

3.2. Effect of K^+ channel blockers on the antinociceptive effects of DHA

Local peripheral pretreatment with $K_{ir}6.1-2$; ATP-sensitive K^+ channel blockers.

Glibenclamide or tolbutamide, but not vehicles, significantly dose-dependently reversed the antinociceptive effect of DHA (Fig. 3). In contrast, K_v ; voltage-gated K^+ channel blockers (4-aminopyridine and tetraethylammonium) were not able to prevent DHA-induced antinociceptive effect (Fig. 4). Local peripheral administration of the $K_{Ca}2.1-3$; small conductance channel (apamin and dequalinium) and $K_{Ca}1.1$; big conductance channel (charybdotoxin and iberiotoxin) inhibitors, but not vehicles, reverted the DHA-induced local peripheral antinociception (Figs. 5 and 6). Potassium channel blockers alone did not alter significantly the nociception induced by formalin. Local peripheral treatment with DHA, diclofenac, K^+ channel blockers or the mixture of these drugs did not alter the ambulation or motor capacity in the experimental animals.

4. Discussion

DHA is an essential fatty acid and the most abundant compound present in neural tissue (Fritsche, 2015; Tvrzicka et al., 2011). DHA has shown oral (Arroyo-Lira et al., 2014; Nakamoto et al., 2010), intrathecal (Xu et al., 2010) and intraarticular (Torres-Guzman et al., 2014) antinociceptive effects in several pain models. In the present work, we have demonstrated for the first time that peripheral subcutaneous administration of DHA exhibited antinociceptive effect in a dose-response manner in the rat formalin test. This effect is likely due to a purely local (peripheral) action, as DHA administration in the contralateral paw, with respect to formalin, fails to produce antinociception (data not shown). This allows discarding a systemic drug effect.

Studies have demonstrated that fish oil, rich in PUFAs such as eicosapentaenoic acid and DHA, could relief symptoms found in

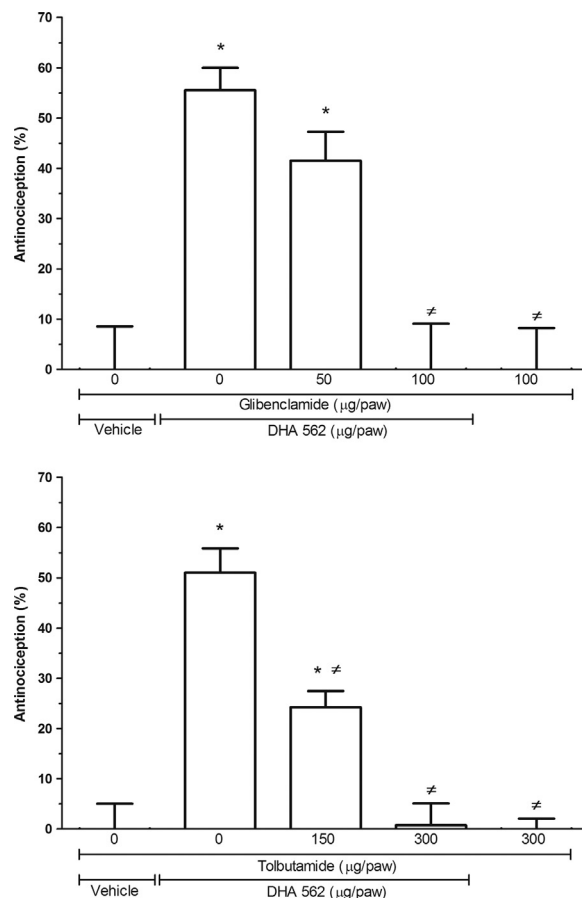


Fig. 3. Effect of $K_{v}6.1-2$; ATP-sensitive K^+ channel blockers glibenclamide and tolbutamide on the antinociceptive effect of DHA (562 µg/paw). DHA was administered 75 min before formalin injection and $K_{v}6.1-2$; ATP-sensitive K^+ channel blockers glibenclamide (50 and 100 µg/paw) or tolbutamide (150 and 300 µg/paw). Data are expressed as the percentage of antinociception. Bars are the means ± S.E.M. for 6–12 rats. One-way ANOVA followed by Bonferroni's test, * $P < 0.05$ vs vehicle, # $P < 0.05$ vs DHA.

arthritis (Proudman et al., 2015). Because of the wide pharmacokinetic distribution of PUFAs through the body, high doses of these are required to be transferred to the affected peripheral tissue to obtain any discernible beneficial effects (Fritsche, 2015; Proudman et al., 2015; Tvrzicka et al., 2011). In peripheral or local painful or inflammatory conditions, another therapeutic option would be the local or peripheral administration of PUFAs. It has previously been demonstrated that eicosapentaenoic acid and DHA are able to permeate through skin (Heard et al., 2003; Puglia et al., 2001; Richards et al., 2006). Previous studies found a greater skin permeation of non-steroidal anti-inflammatory drugs when they are combined with eicosapentaenoic acid or DHA (Heard et al., 2003; Richards et al., 2006). In this sense, the peripheral local antinociception observed in our study strongly support the probable use of DHA in monotherapy or in combination with non-steroidal anti-inflammatory drugs to relieve local peripheral painful or inflammatory conditions. However, the advantage of DHA and its combination with non-steroidal anti-inflammatory drugs at local peripheral level in the clinic situations requires further investigation.

It is well known that activation of potassium channels antagonizes nociceptive responses evoked by noxious stimuli, dampening the hyperexcitability of the nociceptors and counteracting action potential initiation at peripheral nerve terminals, reducing conduction fidelity across the axon or limiting neurotransmitter release at central terminals (Du et al., 2011; Tsantoulas and McMahon, 2014). Potassium channels are the most widely ion channels distributed in neurons

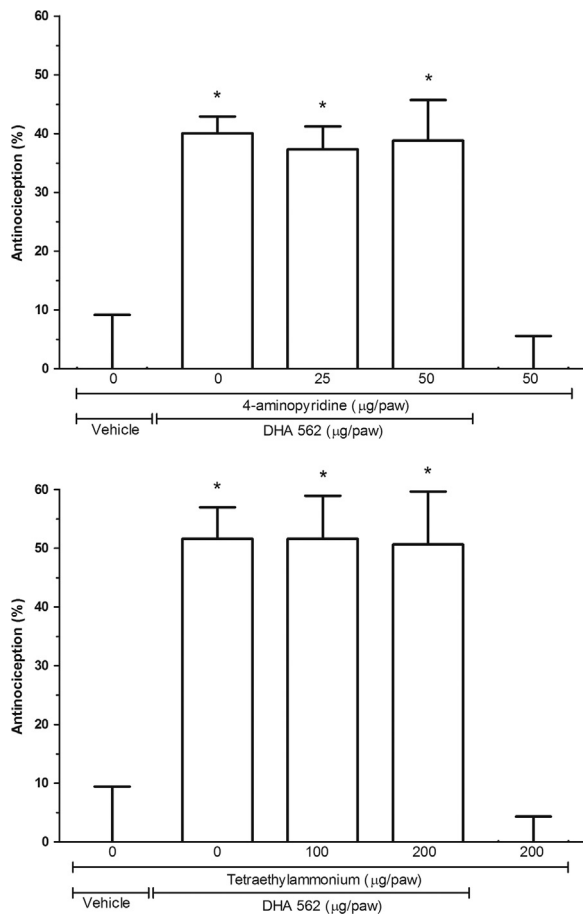


Fig. 4. Participation of the K_v channel in the antinociceptive effect of DHA, administration of DHA (562 $\mu\text{g/paw}$) 75 min previous to formalin injection and K_v channel blockers 4-aminopyridine (25 and 50 $\mu\text{g/paw}$) or tetraethylammonium (100 and 200 $\mu\text{g/paw}$). Data are expressed as the percentage of antinociception. Bars are the means \pm S.E.M. for 6–12 rats. One-way ANOVA followed by Bonferroni's test, * $P < 0.05$ vs vehicle.

(Tsantoulas and McMahon, 2014). Our results reported here suggest the participation of $K_{ir}6.1-2$; ATP-sensitive K^+ channels, $K_{Ca}1.1$; big conductance calcium-activated K^+ channels, $K_{Ca}2.1-3$; small conductance calcium-activated K^+ channels, but not K_v voltage-gated K^+ channels in the peripheral antinociceptive effect of DHA.

$K_{ir}6.1-2$ opening in peripheral and central neuronal systems is related to the antinociceptive effects produced by opioids such as morphine (Ortiz et al., 2002; Rodrigues and Duarte, 2000), and non-steroidal anti-inflammatory drugs such as diclofenac (Ortiz et al., 2002) and lumiracoxib (Lozano-Cuenca et al., 2005). Our results agree with the activation of $K_{ir}6.1-2$ channels on the vasorelaxant actions in aorta induced by DHA (Engler and Engler, 2000). On the other hand, it has been demonstrated that DHA induces antinociception by producing the augment of β -endorphin (Nakamoto et al., 2011). It is probable that this mechanism is related with the activation of $K_{ir}6.1-2$ channels. Nevertheless, previously it was demonstrated that the β -endorphin-induced supraspinal (intracerebroventricular administration) antinociception was not antagonized by the blocker glibenclamide (Raffa and Martinez, 1995). Therefore, the real contribution of $K_{ir}6.1-2$ channels on the DHA- and β -endorphin-induced antinociception await future validation.

Several studies have suggested that $K_{Ca}1.1$; big conductance calcium-activated K^+ channels are the major targets of modulation in vascular tissues by PUFAs and some derivatives (Eichhorn and Dobrev, 2007; Sun et al., 2016; Yan et al., 2014). Our results also provide pharmacological evidence for the involvement of $K_{Ca}1.1$ in the periph-

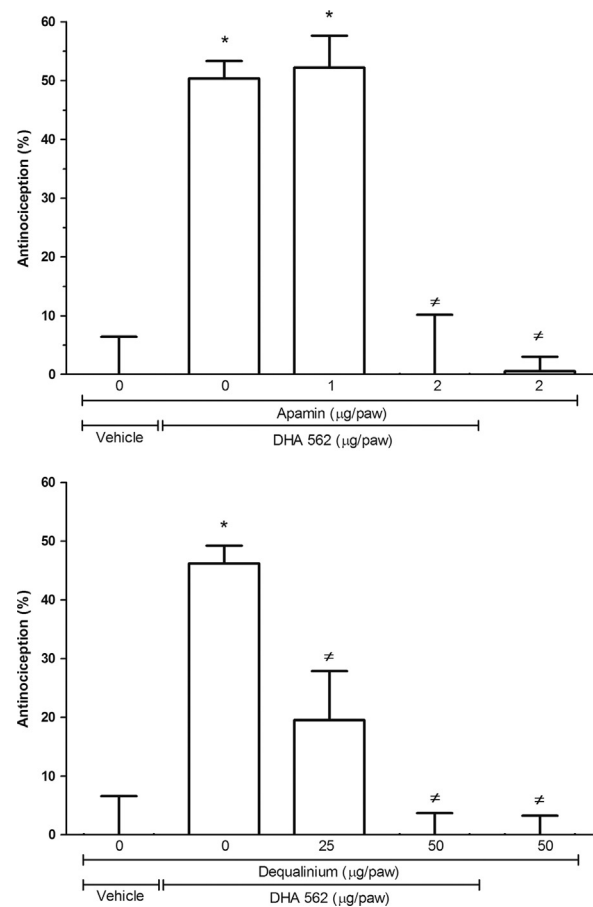


Fig. 5. Participation of the $K_{Ca}2.1-3$; small conductance calcium-activated K^+ channels in the antinociceptive effect of DHA, administration of DHA (562 $\mu\text{g/paw}$) 75 min previous to formalin injection and $K_{Ca}2.1-3$ channels inhibitor apamin (1 and 2 $\mu\text{g/paw}$) or dequalinium (25 and 50 $\mu\text{g/paw}$). Bars are the means \pm S.E.M. for 6–12 rats. One-way ANOVA followed by Bonferroni's test, * $P < 0.05$ vs vehicle, $\neq P < 0.05$ vs DHA.

eral mechanism of action of DHA. The local administration of the $K_{Ca}1.1$; big conductance calcium-activated K^+ channel blockers charybdotoxin and iberiotoxin inhibited the antinociception of DHA. Our data agree with earlier studies where it has been demonstrated that PUFAs such as DHA or eicosapentaenoic acid regulate the $K_{Ca}1.1$ from the inner mitochondrial membrane (Olszewska et al., 2014) and are potent vasodilators that directly activate $K_{Ca}1.1$ channels in the vascular smooth muscle cells (Lai et al., 2009; Sun et al., 2016; Wang et al., 2011; Yan et al., 2014). $K_{Ca}3.1$; intermediate conductance calcium-activated channels- and $K_{Ca}2.1-3$; small conductance calcium-activated K^+ channels are classified separately from the $K_{Ca}1.1$; big conductance calcium-activated K^+ channels, because of structural and functional differences (Kuzmenkov et al., 2015; Wei et al., 2005). In our study, the $K_{Ca}2.1-3$ blockers apamin and dequalinium were able to block the antinociceptive action of DHA, suggesting that DHA does activate these channels. Our data are in agreement with the inhibition of the K^+ currents in rat coronary arterial smooth muscle cells by apamin (Wang et al., 2011). However, our data did not agree with the lack of effect of apamin in the relaxation induced by DHA on the rat aortic rings (Sato et al., 2014). Since charybdotoxin is also a $K_{Ca}3.1$; intermediate conductance calcium-activated channel inhibitor (Kuzmenkov et al., 2015; Wei et al., 2005), the blockade of the DHA-induced antinociception by this blocker suggests the probable participation of that channel. In this regard, it is necessary to realize more experiments with other $K_{Ca}3.1$ inhibitors such as maurotoxin or 4-phenyl-4H-pyran 11, to ensure the participation of $K_{Ca}3.1$ on the effects of DHA (Wei et al., 2005).

The voltage-gated K^+ channels (K_v) are the most abundant group of

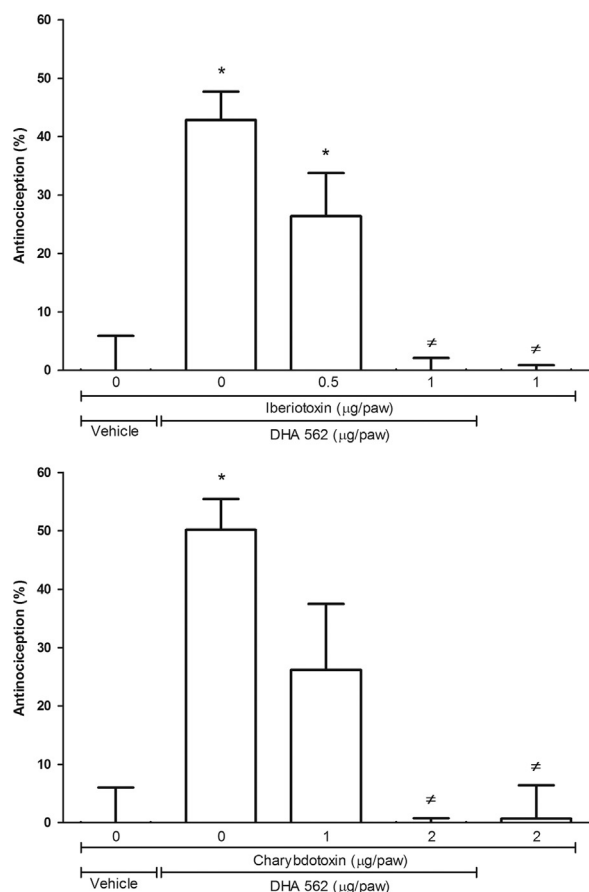


Fig. 6. Participation of the $K_{Ca1.1}$; big conductance calcium-activated K^+ channel in the antinociceptive effect of DHA, administration of DHA (562 $\mu\text{g/paw}$) 75 min previous to formalin injection and $K_{Ca1.1}$ channels inhibitor iberiotoxin (0.5 and 1 $\mu\text{g/paw}$) or charybdotoxin (1 and 2 $\mu\text{g/paw}$). Data are expressed as the percentage of antinociception. Bars represent the means \pm S.E.M. for 6–12 rats. One-way ANOVA followed by Bonferroni's test, * $P < 0.05$ vs vehicle, $^{\#}P < 0.05$ vs DHA.

human potassium channels (Gutman et al., 2005). According to the International Union of Pharmacology (IUPHAR), twelve different families of K_v channels have been identified. In general, the K_v1 – K_v4 families are inhibited by tetraethylammonium, 4-aminopyridine and other drugs (Gutman et al., 2005; Kuzmenkov et al., 2015). In our study, the K_v channels does not appear to be a mechanism involved in the DHA-induced peripheral antinociception since tetraethylammonium and 4-aminopyridine were not able to inhibit this effect. The participation of K_v channels in biological effects induced by DHA is controversial. In endothelium-denuded rat thoracic aorta, tetraethylammonium, but not 4-aminopyridine, reverted the DHA-induced relaxation (Sato et al., 2014). In this case, the K_v channel role in the DHA-induced effect was ruled out. An additional publication found no significant effect of tetraethylammonium on the DHA-induced relaxation in isolated aorta from male spontaneously hypertensive rats (Engler and Engler, 2000). In another different study, the application of tetraethylammonium and 4-aminopyridine inhibited the DHA-induced K^+ currents in rat coronary arterial smooth muscle cells, suggesting the participation of K_v channel in this effect (Wang et al., 2011). In contrast, it was demonstrated that DHA at concentration $> 20 \mu\text{M}$ was able to block the K_v currents in coronary arterial smooth muscle cells (Lai et al., 2009). It is important to point out that these evidences were obtained in vascular tissues. Therefore, it is necessary to evaluate the lack of involvement of K_v channels in the DHA-induced antinociception in other kind of tissues.

5. Conclusion

It is concluded that big- and small-conductance Ca^{2+} -activated K^+ channels ($K_{Ca1.1}$, $K_{Ca2.1-3}$) and ATP-sensitive K^+ channels ($K_{ir6.1-2}$) are activated by DHA in order to produce local antinociception on the 1% rat formalin test. Because the lack of activity of tetraethylammonium and 4-aminopyridine in reverting the antinociceptive effect of DHA, it is unlikely that K_v channels are involved in the DHA-induced peripheral antinociceptive effect.

Conflict of interest statement

The authors report no conflict of interest.

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