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Original Article

Peripheral antinociceptive effect of exogenous acetylcholine seems to be mediated by M_1 and nicotinic receptors

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ABSTRACT

The purpose of this study is to identify the cholinergic receptor subtype that mediates the peripheral antinociceptive effect of acetylcholine. To induce hyperalgesia, rat paws were treated with intraplantar prostaglandin E_2 (PGE_2, 2 μg). The nociceptive thresholds to pressure (grams) were measured by paw flexion reaction using an algesimeter apparatus 3 h following injection. Intraplantar administration of acetylcholine (ACh; 50, 100, 200 and 400 μg) caused dose-dependent antinociception in PGE_2-induced hyperalgesia. The subtype-selective muscarinic receptor antagonists for M_1 (telenzepine; 3, 6 and 12 μg), M_2 (dimethindene; 40 and 80 μg), M_3 (4-DAMP, 40 and 80 μg), and M_4 (tropicamide; 40 and 80 μg) as well as the nicotinic antagonist (mecamylamine; 25, 50 and 100 μg) were all co-administered with acetylcholine (200 μg). Only telenzepine and mecamylamine antagonized the antinociceptive effect of ACh. These data suggest the presence of M_1 and nicotinic cholinergic receptors at the peripheral level and that exogenous acetylcholine induces receptor activation with consequent antinociception.

KEYWORDS: acetylcholine, telenzepine, mecamylamine, peripheral antinociception.

INTRODUCTION

The spinal cholinergic system has many different physiological functions, including the inhibition and modulation of nociceptive signaling [1]. Acetylcholine (ACh)-induced analgesia results from ionotropic or metabotropic receptor binding [2]. The muscarinic and nicotinic receptor agonists and the inhibitors of acetylcholinesterase induce antinociception, measured by several algesimeter tests [1, 3, 4], after intrathecal or systemic administration. The antinociceptive effect of cholinergic agonists is mediated primarily by muscarinic receptors [5, 6]; however, some studies have suggested that it is mediated by nicotinic receptors [7, 8]. Although the central antinociceptive effect by activation of muscarinic receptors is well documented [9], the peripheral nociceptive afferents also express these receptors, which can result in antinociception when activated [10-13]. A study in 1998 [14] showed that the acetylcholinesterase inhibitor neostigmine, when injected directly into a rat's knee joints, induced antinociception that was reversed by atropine, demonstrating that there is also a peripheral cholinergic effect.

Antinociception induced by ACh has been linked to low levels of cytosolic Ca^{2+} [15]. Because dibutyryl cyclic GMP mimicked the ACh-induced antinociception, it was suggested that cholinergic agents could cause antinociception by increasing the cyclic GMP in the nociceptor [10]. This result is consistent with the findings that showed that ACh caused accumulation of cyclic GMP in various tissues [16]. This hypothesis was confirmed by a study showing that this type of antinociception was blocked by methylene blue, an inhibitor of guanylate cyclase, and by an inhibitor of nitric oxide (NO) synthase [11].

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Considering the multiple cholinergic receptor subtypes currently known (M_1, M_2, M_3, M_4 and N), the aim of this study was to identify which cholinergic receptor subtype mediates the ACh-induced peripheral antinociception using prostaglandin E_2 (PGE_2)-induced hyperalgesia.

SUBJECTS AND METHODS

Animals

Male Wistar rats weighing 180-220 g obtained from Animal house of Federal University of Minas Gerais (CEBIO-UFMG) were housed in individual cages under controlled light and temperature conditions, with water and rat chow *ad libitum* right up to the experiment. The experiments were conducted according to the National Research Council's guidelines.

Measurement of hyperalgesia

Hyperalgesia was induced by subcutaneous injection of PGE_2 into the plantar surface of the rats' hindpaw and measured according to the paw pressure test [17]. An algesimeter was used (Ugo-Basile, Italy) with a cone-shaped rounded tip paw-presser which applies a linearly increasing force to the hindpaw. The weight in grams (g) required to elicit a nociceptive response such as paw flexion was determined as the nociceptive threshold. A cut-off value of 300 g was used to prevent damage to the paws. The nociceptive threshold was measured in the right paw and determined as the average of the three consecutive trials recorded before and 3 h after hyperalgesic agent injection.

Drug administration

PGE_2 (Sigma, USA) was used to induce hyperalgesia. The cholinergic agonist, acetylcholine hydrochloride (Sigma, USA) was used. The following muscarinic receptor antagonists were used: telenzepine, M_1 antagonist (Tocris, USA); dimethindene, M_2 antagonist (Tocris, USA); 4-difenylacetoxy-N-methylpiperidine methiodide, M_3 antagonist (4-DAMP, Tocris, USA) and tropicamide, M_4 antagonist (Tocris, USA). Mecamylamine (Sigma, USA) was used as a specific nicotinic receptor antagonist. All drugs were dissolved in saline, except PGE_2 (8% ethanol in saline), and injected in a volume of 50 µl/paw.

Experimental protocol

Basal measurement of nociceptive threshold (BM) was done three times and the average value was taken before administration of any drug. The hyperalgesic agent (PGE_2; 2 µg/paw) was then administered at time zero and nociceptive measurements were made after three hours. Doses and times of drug administration were calculated from preliminary experiments (data not shown).

Increasing doses of acetylcholine (ACh; 50, 100, 200 and 400 µg) were administered subcutaneously in the right paw 175 min after local injection of PGE_2. The specific muscarinic receptor antagonists (telenzepine, dimethindene, 4-DAMP and tropicamide) and the nicotinic receptor antagonist (mecamylamine) were administered 35 min before administration of acetylcholine. To exclude a non-local effect of ACh, PGE_2 was injected in both hind paws, while ACh was administered only in the right paw. Local effect was confirmed when only the right paw of the experimental group showed the effect of the tested drug.

Statistical analysis

Data were analyzed for statistical significance by one-way ANOVA analysis of variance followed by Bonferroni's test. The minimum level of significance considered was $P < 0.05$. Statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

The antinociceptive effect of acetylcholine

The administration of ACh (50, 100, 200 or 400 µg/paw) produced an antinociceptive effect against hyperalgesia induced by prior local injection of PGE_2 (2 µg/paw) in a dose-dependent manner. ACh (200 µg paw) alone did not alter the nociceptive threshold (Fig. 1a). PGE_2 was administered in the right (RP) and left paw (LP) and acetylcholine only in the right paw. Acetylcholine (200 µg) increased the nociceptive threshold only in the treated paw, suggesting that at this dose acetylcholine has only a local site of action (Fig. 1b). This dose of acetylcholine is able to induce maximal antinociceptive response without systemic implications; therefore, the dose of 200 µg was

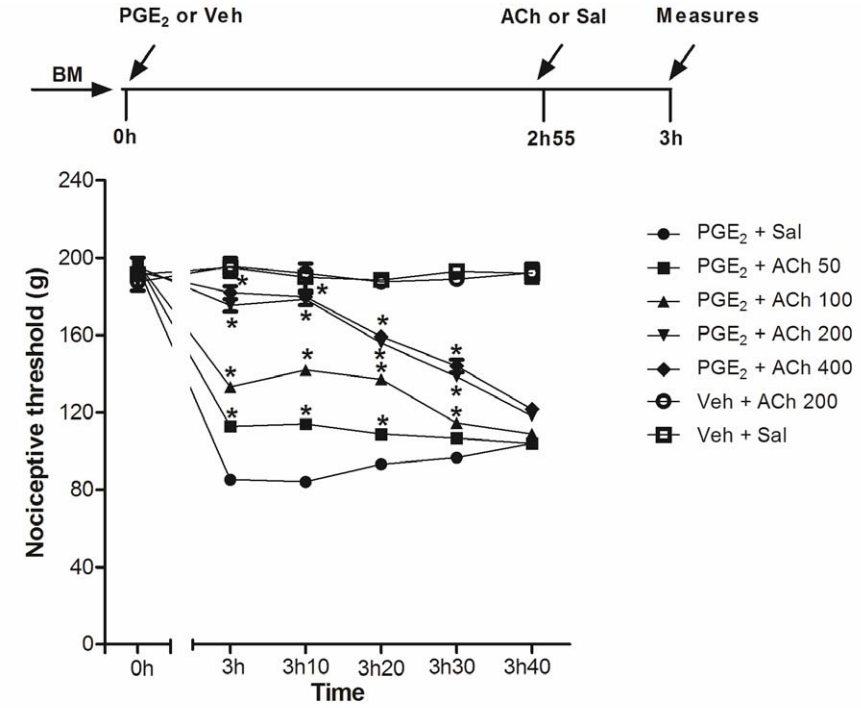

Fig. 1a. Effect of acetylcholine on the hyperalgesia induced by prostaglandin E_2. Acetylcholine (ACh; 50, 100, 200 or 400 μg/paw) reduced hyperalgesia induced by prostaglandin E_2 (PGE_2; 2 μg) in a dose-dependent manner. Each symbol represents the mean ± S.E.M. (n = 5) of nociceptive threshold. *P < 0.05 (PGE_2 + ACh) vs. (PGE_2 + Sal). Veh= vehicle (8% ethanol in saline).

chosen for the subsequent experiments, since the aim of this work was to evaluate the peripheral cholinergic receptors responsible for the acetylcholine-induced antinociception.

Effect of intraplantar administration of selective subtypes of muscarinic antagonists on peripheral antinociception induced by acetylcholine

Evidence that ACh induces peripheral antinociception *via* a specific muscarinic receptor is shown in Fig. 2. In this figure, it is possible to observe that the M_1 antagonist telenzepine (3, 6 and 12 μg/paw) blocked the peripheral antinociceptive effect of ACh (200 μg/paw); moreover, no effect by this antagonist was verified when it alone was injected into normal or hyperalgesic paws (Result not shown). The involvement of other muscarinic receptor subtypes in the peripheral antinociception by ACh was discarded in the present experiments, since the antagonists for subtypes M_2, M_3 and M_4, respectively, dimethindene, 4-DAMP and tropicamide (40 and 80 μg) did not significantly reduce the peripheral ACh effect, as observed in the Figs. 3, 4 and 5. None of the antagonists induced hyperalgesia or antinociception when administered alone (data not shown).

Effect of intraplantar administration of a nicotinic antagonist on peripheral antinociception induced by acetylcholine

Intraplantar administration of mecamylamine at a dose of 25, 50 and 100 μg/paw antagonized the antinociceptive effect of ACh (200 μg) in the hyperalgesia induced by prostaglandin E_2, suggesting the presence of nicotinic receptors in rats' paws (Fig. 6). Mecamylamine, when injected alone, did not induce hyperalgesia or antinociception (data not shown).

DISCUSSION

PGE_2 is the main nociceptor sensitizer to chemical, thermal and mechanical stimulation [18]. *In vitro* studies showed that PGE_2 acts directly on the primary afferent neuron terminals and does not require intermediate cells [19]. PGE_2 rarely activates afferent nociceptive neurons directly [20].

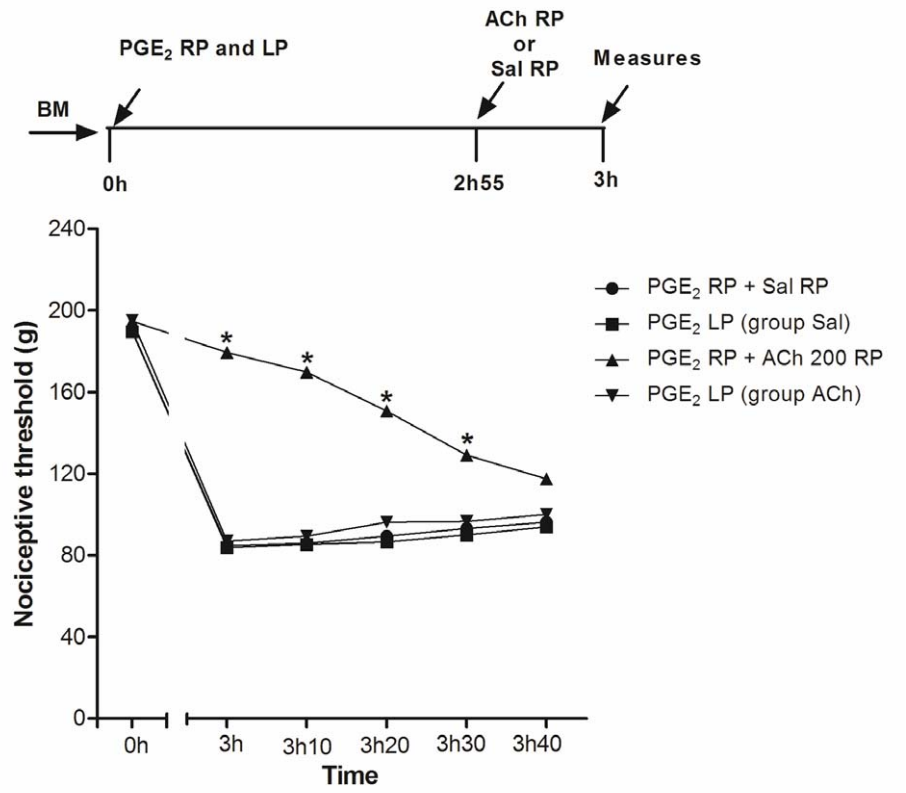

Fig. 1b. Exclusion of non-local effect of acetylcholine (ACh), in the dose of 200 µg. ACh or saline (Sal) was injected only in the right paw (RP) 2h55 after prostaglandin E_2 (PGE_2; 2 µg) that was administered in the right (RP) and left paw (LP). The pressure test was measured in both hind paws. Each symbol represents the mean ± S.E.M. (n = 5) of nociceptive threshold. *P < 0.05 compared with control group (PGE_2 + Sal).

In addition, at concentrations that are found during inflammation, PGE_2 does not evoke pain when injected intradermally into human skin [21], but does decrease the nociceptive threshold in animal behavioral tests [10].

Several studies have shown that the cholinergic drugs are capable of producing central antinociception. The inhibition of pain was observed by intracerebroventricular [22], intrathecal [6, 9] and systemic [8] administration.

Initially, we evaluated the effect of an exogenous administration of acetylcholine on prostaglandin E_2-induced hyperalgesia. We found that intraplantar injection of acetylcholine causes antinociception. These findings are in agreement with various studies [11, 13, 23]. Our study focused on the peripheral antinociceptive effect. To exclude a possible non-local effect, the hyperalgesic agent was injected into both paws, and the evaluated drug was only injected into the right paw. Measurements were taken in both paws. With this strategy, it was possible to determine that acetylcholine induced peripheral antinociception with a dose of 200 µg.

It is well established that the cholinergic system in the spinal cord is involved in sensory modulation and transmission [24]. In another study, the same authors demonstrated that ACh at the spinal level exerted an inhibitory effect on the nociceptive impulses acting on muscarinic receptors [5]. In contrast, it was reported that nicotinic receptors may be involved in antinociception at the spinal level [25]. Furthermore, nicotinic receptors are found in the whole pain pathway [26].

Muscarinic receptors belong to the super-family of G proteins, and five subtypes ($M_{1\text{-}5}$) have been cloned [2]. M_1 receptors are involved in neuronal activity and the release of nitric oxide; M_2 receptors are involved in the inhibition of myocardial contraction; and M_3 receptors are

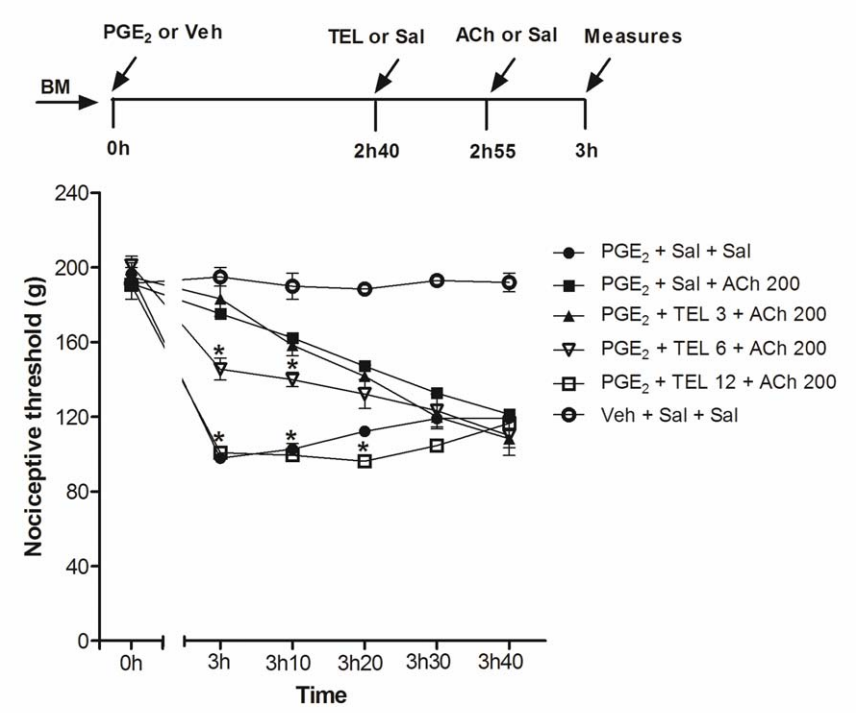

Fig. 2. Antagonism of acetylcholine (Ach)-induced antinociception by intraplantar administration of telenzepine (TEL, 12 μg) *P < 0.05 vs. control group (PGE_2 + Sal + ACh). Veh = vehicle (8% ethanol in saline).

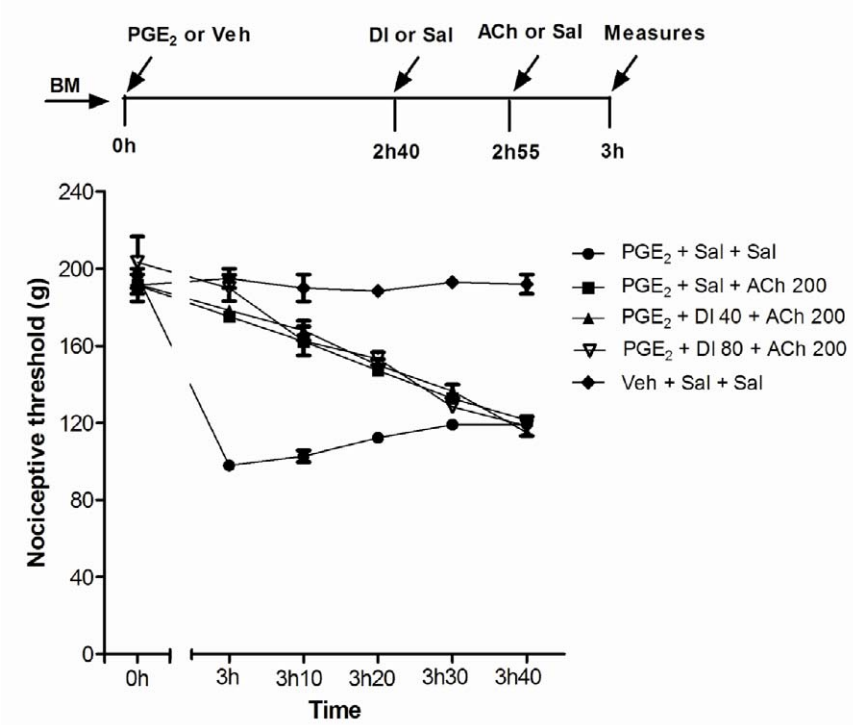

Fig. 3. Effect of intraplantar administration of dimethindene (DI, 40 and 80 μg) on the peripheral antinociception of acetylcholine (ACh, 200 μg) against prostaglandin E_2 induced hyperalgesia (PGE_2, 2 μg/paw). Veh = vehicle (8% ethanol in saline).

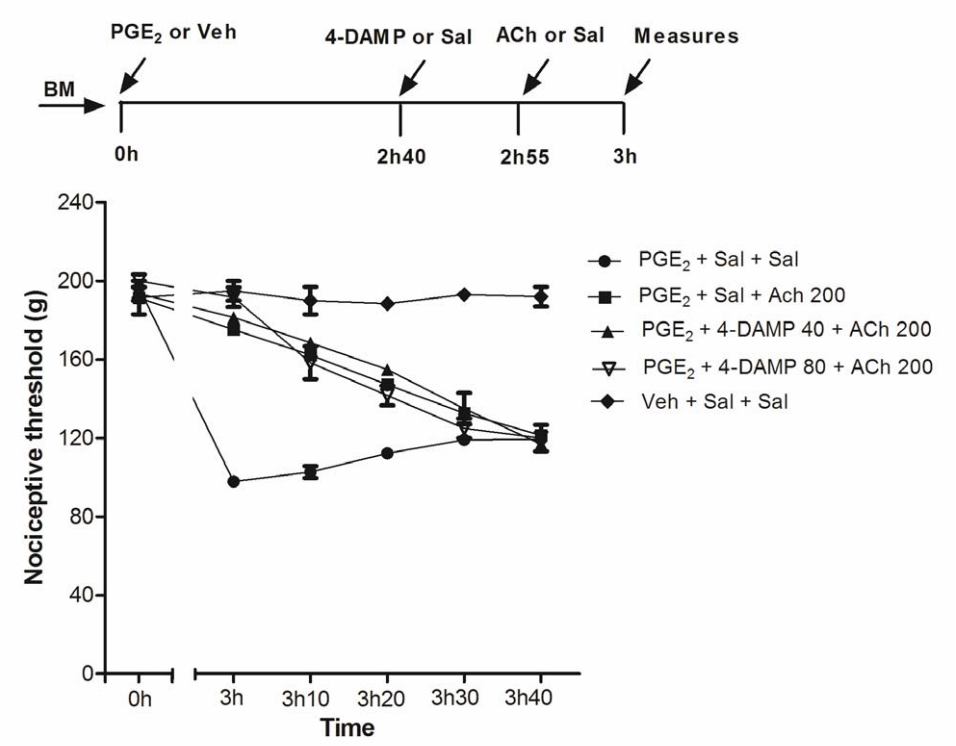

Fig. 4. Effect of intraplantar administration of 4-DAMP (40 and 80 μg) on the peripheral antinociception of acetylcholine (ACh, 200 μg) against prostaglandin E_2 induced hyperalgesia (PGE_2, 2 μg/paw). Veh = vehicle (8% ethanol in saline).

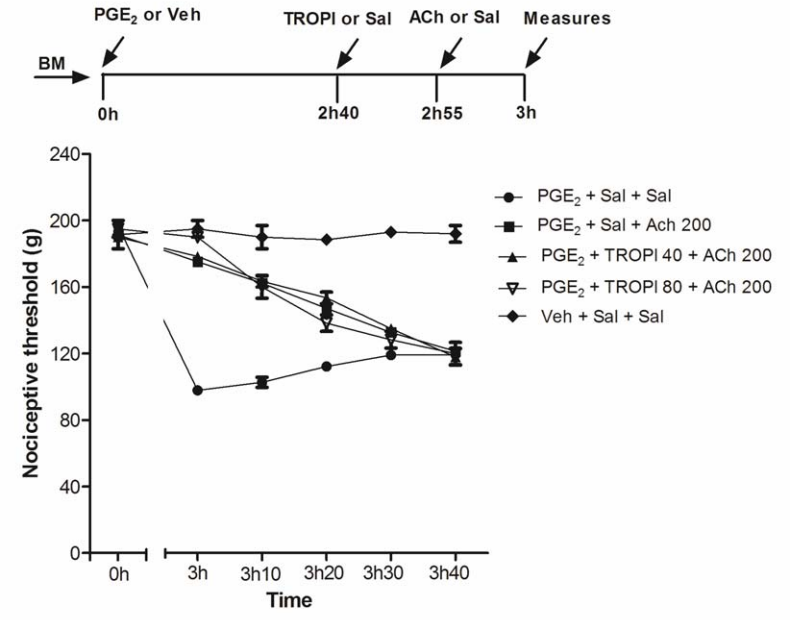

Fig. 5. Effect of intraplantar administration of tropicamide (TRO, 40 and 80 μg) on the peripheral antinociception of acetylcholine (ACh, 200 μg) against prostaglandin E_2 induced hyperalgesia (PGE_2, 2 μg/paw). Veh = vehicle (8% ethanol in saline).

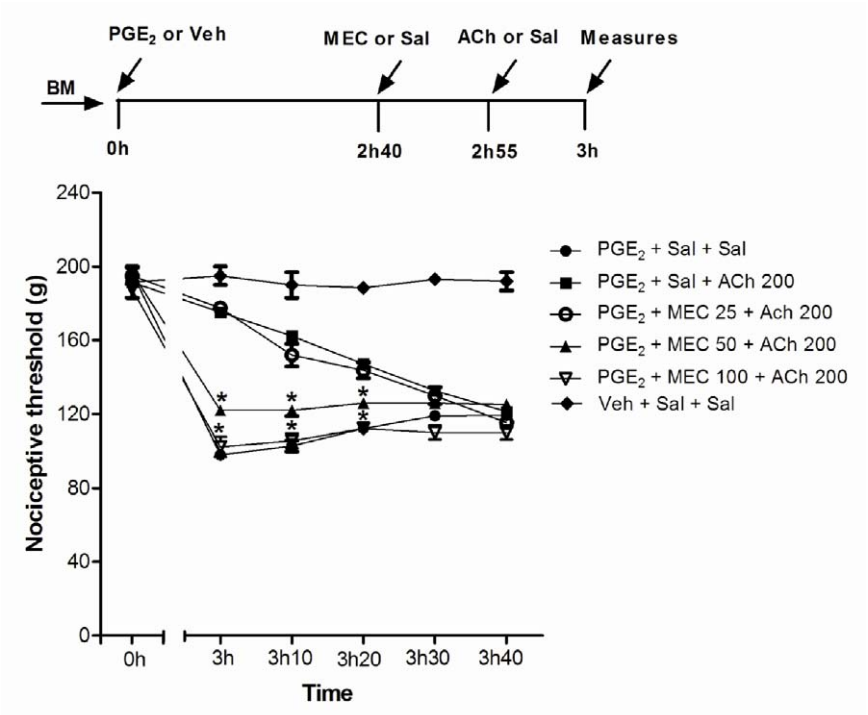

Fig. 6. Antagonism of acetylcholine (Ach)-induced antinociception by intraplantar administration of mecamylamine (MEC; 25, 50 and 100 µg) against prostaglandin E_2 induced hyperalgesia (PGE_2, 2 µg/paw). *P<0.05 compared with control group (PGE_2 + Sal + ACh). Veh = vehicle (8% ethanol in saline).

involved in smooth muscle contraction and salivation. However, the functions of M_4 and M_5 are not well defined [27]. M_1, M_3, M_5 receptors are coupled to the G_q protein that generates inositol 1,4,5-triphosphate (IP_3) leading to the mobilization of intracellular calcium and the stimulation of protein kinase C. M_2 and M_4 receptors are coupled to the G_i protein, which promotes the inhibition of adenylyl cyclase [2]. Clearly, an important question addresses which specific muscarinic receptor subtypes mediate the antinociceptive effect of acetylcholine. The involvement of different muscarinic receptor subtypes in antinociception at the central level is a complex subject. Strikingly, cholinergic-induced analgesia was markedly reduced in M_2 receptor KO mice [28].

At the peripheral level, it was suggested that the activation of muscarinic receptors present in skin nociceptors can suppress the transmission of pain impulses [29]. Aiming to assess these receptor subtypes, we used specific muscarinic antagonists. Only telenzepine, a M_1 antagonist, was able to antagonize the antinociceptive effect exerted by exogenous acetylcholine on prostaglandin E_2-induced hyperalgesia in the rat paw. M_2, M_3 and M_4 receptors were not activated by acetylcholine. These findings suggest that at the peripheral level, exogenous acetylcholine exerts its antinociceptive effect only through M_1 muscarinic receptors.

Nicotinic cholinergic receptors are pentameric proteins that function as Na^+ channels [30]. Although it has been demonstrated that analgesia induced by systemic or central administration of nicotinic agonists is blocked by mecamylamine, a nonselective nicotinic antagonist [31], the lack of a specific antagonist, represents a problem in the elucidation of the subtypes of nicotinic receptors involved in antinociception [32]. However, in our study, mecamylamine also antagonized the peripheral antinociceptive effect of acetylcholine on PGE_2-induced hyperalgesia.

CONCLUSION

The findings in the present study suggest that at the peripheral level, acetylcholine exerts its antinociceptive effect by acting on muscarinic M_1 and nicotinic cholinergic receptors.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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