Peripheral antinociceptive effect of exogenous acetylcholine seems to be mediated by M₁ 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 E₂ (PGE₂, 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 PGE₂-induced hyperalgesia. The subtype-selective muscarinic receptor antagonists for M₁ (telenzepine; 3, 6 and 12 μg), M₂ (dimethindene; 40 and 80 μg), M₃ (4-DAMP, 40 and 80 μg), and M₄ (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 M₁ 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 Ca²⁺ [15]. Because dibutryl 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].
Considering the multiple cholinergic receptor subtypes currently known (M₁, M₂, M₃, M₄ and N), the aim of this study was to identify which cholinergic receptor subtype mediates the ACh-induced peripheral antinociception using prostaglandin E₂ (PGE₂)-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 PGE₂ 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

PGE₂ (Sigma, USA) was used to induce hyperalgesia. The cholinergic agonist, acetylcholine hydrochloride (Sigma, USA) was used. The following muscarinic receptor antagonists were used: telenzepine, M₁ antagonist (Tocris, USA); dimethindene, M₂ antagonist (Tocris, USA); 4-difenylacetoxy-N-methylpiperidine methiodide, M₃ antagonist (4-DAMP, Tocris, USA) and tropicamide, M₄ antagonist (Tocris, USA). Mecamylamine (Sigma, USA) was used as a specific nicotinic receptor antagonist. All drugs were dissolved in saline, except PGE₂ (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 (PGE₂, 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 PGE₂. 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, PGE₂ 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 PGE₂ (2 µg/paw) in a dose-dependent manner. ACh (200 µg paw) alone did not alter the nociceptive threshold (Fig. 1a). PGE₂ 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...
Ach induces antinociception *via* M1 and nicotinic receptors

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 M1 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 M2, M3 and M4, 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 E2, 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**

PGE2 is the main nociceptor sensitizer to chemical, thermal and mechanical stimulation [18]. *In vitro* studies showed that PGE2 acts directly on the primary afferent neuron terminals and does not require intermediate cells [19]. PGE2 rarely activates afferent nociceptive neurons directly [20].
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 $\mu$g.

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$_1$-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

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 $\mu$g.

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Ach induces antinociception via M<sub>1</sub> and nicotinic receptors

Fig. 2. Antagonism of acetylcholine (Ach)-induced antinociception by intraplantar administration of telenzepine (TEL, 12 μg) *P < 0.05 vs. control group (PGE<sub>2</sub> + 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<sub>2</sub> induced hyperalgesia (PGE<sub>2</sub>, 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 E2 induced hyperalgesia (PGE2, 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 E2 induced hyperalgesia (PGE2, 2 μg/paw). Veh = vehicle (8% ethanol in saline).
Ach induces antinociception via M₁ and nicotinic receptors.

Exogenous acetylcholine on prostaglandin E₂-induced hyperalgesia in the rat paw. M₂, M₃, and M₄ receptors were not activated by acetylcholine. These findings suggest that at the peripheral level, exogenous acetylcholine exerts its antinociceptive effect only through M₁ 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 PGE₂-induced hyperalgesia.

**CONCLUSION**

The findings in the present study suggest that at the peripheral level, acetylcholine exerts its antinociceptive effect by acting on muscarinic M₁ 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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