TRP and ASIC channels mediate the antinociceptive effect of citronellyl acetate

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ABSTRACT

Background: Citronellyl acetate (CAT), a monoterpene product of the secondary metabolism of plants, has been shown in the literature to possess several different biological activities. However, no antinociceptive abilities have yet been discussed. Here, we used acute pain animal models to describe the antinociceptive action of CAT.

Methods: The acetic acid-induced writhing test and the paw-licking test, in which paw licking was induced by glutamate and formalin, were performed to evaluate the antinociceptive action of CAT and to determine the involvement of PKC, PKA, TRPV1, TRPA1, TRPM8 and ASIC in its antinociceptive mechanism. To do so, we induced paw-linking using agonists.

Results: CAT was administered intragastrically (25, 50, 75, 100 and 200 mg/kg), and the two higher doses caused antinociceptive effects in the acetic acid model; the highest dose reduced pain for 4 h after it was administered (200 mg/kg). In the formalin test, two doses of CAT promoted antinociception in both the early and later phases of the test. The glutamate test showed that its receptors are involved in the antinociceptive mechanism of CAT. Pretreatment with CAT did not alter locomotor activity or motor coordination. In an investigation into the participation of TRP channels and ASICs in CAT's antinociceptive mechanism, we used capsaicin (2.2 μg/paw), cinnamaldehyde (10 mmol/paw), menthol (1.2 mmol/paw) and acidified saline (2% acetic acid, pH 1.98). The results showed that TRPV1, TRPM8 and ASIC, but not TRPA1, are involved in the antinociceptive mechanism. Finally, the involvement of PKC and PKA was also studied, and we showed that both play a role in the antinociceptive mechanism of CAT.

Conclusion: The results of this work contribute information regarding the antinociceptive properties of CAT on acute pain and show that, at least in part, TRPV1, TRPM8, ASIC, glutamate receptors, PKC and PKA participate in CAT's antinociceptive mechanism.

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1. Introduction

Citronellyl acetate (3,7-dimethyl-6-octen-1-yl acetate, abbreviated CAT; Fig. 1) is a monoterpene product of the secondary metabolism of plants. It is frequently used in perfume and is known for its pleasant smell. Citronellyl acetate is present mainly in Eucalyptus citriodora; Shen et al. [1] proved that it is a potent antihepatoma agent with pro-apoptotic activity in HepG2 cells (human hepatoma cells). It also has several other biological activities, including fungicidal [2], larvicidal [3], bactericidal [4] and repel-

ling/insecticidal effects [5]. CAT is also present in less significant amounts in the volatile extract of the pericarp of the Zanthoxylum schinifolium fruit. Paik et al. [6] demonstrated that this fruit induces caspase-3-independent apoptosis in HepG2 cells and inhibits tumor growth in huh-7 cells (also human hepatoma cells) in mice. Fang et al. [7] also showed that CAT has marginal antitumor activity. To date, there have been no studies that examine CAT's antinociceptive activity.

Pain can be defined as an unpleasant sensory and emotional experience associated with real or potential tissue damage, and it can occur without injury, though subjects still describe it in subjective terms as if the tissue damage had actually occurred [8]. Nociception, or the pain response, is necessary for the survival of the organism, allowing it to maintain its integrity. Pain can be classified as acute or chronic depending on its duration and it can also

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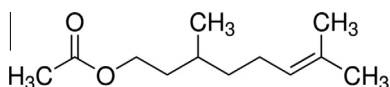E-mail address: emilianovasconcelos@yahoo.com.br (E.R.V. Rios).

Fig. 1. The chemical structure of citronellyl acetate.

be described as neuropathic, which results from damage to the central nervous system [9]. Pain management is an essential aspect of modern medicine and quality of life, yet current therapies are often insufficient due to severe unwanted side effects or limited effectiveness.

The search for new molecules that possess antinociceptive activity is constant, since citronellyl acetate is chemically similar to citronellol and citronellal which have several biological activities [25,26], including antinociceptive activity, we decide to investigate if the citronellyl acetate also has this action. So the aim of this work was to demonstrate the antinociceptive activity of citronellyl acetate in both physically and chemically induced acute pain models and to relate this therapeutic effect to a possible mechanism of action.

2. Materials and methods

2.1. Animals

In all of our experiments, male Swiss mice over 4 weeks of age were used (26–32 g). The animals were kept in a temperature-controlled room at 25 ± 2 °C with a 12/12 h light/dark cycle (the light was turned on at 6:00), with food and water provided ad libitum. The animals were acclimatized to the laboratory conditions for at least 1 h before testing and were deprived of food but given free access to water for 8 h before the experiment. These experiments were performed from 09:00 to 13:30 in a quiet room in which the conditions described above were maintained. Each animal was used only once. The number of animals and the intensity of the noxious stimuli were the minimum necessary to demonstrate consistent effects of the drug treatments.

The experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Ceará. The experiments were carried out in accordance with current guidelines for the care of laboratory animals, as well as ethical guidelines for investigations of experimental pain in conscious animals [10].

2.2. Drugs

All of the substances used in this study, including CAT, were purchased from Sigma–Aldrich® (St. Louis, MO, USA), with the exception of morphine, which was purchased from Cristália® (Fortaleza, CE, Brazil). The capsaicin was dissolved in 1% ethanol and 1% Tween 80 in saline (1:1:8). CAT is an oily substance, but it is easily emulsified in a solution of 2% Tween 80 in distilled water that has been sonically agitated for 5 min before administration. The vehicle, administered alone, had no effect on the nociceptive responses in the mice.

2.3. Protocols

The analgesic activity of CAT was evaluated in animal models of the following pain conditions: chemical nociception, including both abdominal writhing induced by acetic acid and hind paw licking induced by formalin, glutamate, capsaicin, cinnamaldehyde, menthol, acidified saline, PMA (Phorbol 12-myristate 13-acetate) and 8-Br-cAMP. Conscious mice were used in all of the nocifensive tests. CAT was administered intragastrically by gavage. The doses selected, 25, 50, 75, 100 and 200 mg/kg, were based on the results

of preliminary experiments. The control groups were treated with a volume of the vehicle similar to that used to dilute the CAT.

2.4. Abdominal writhing induced by acetic acid

The animals were divided into 11 groups ($n = 8$–9), each of which received either vehicle (2% Tween 80 in distilled water), CAT (25, 50, 75, 100 or 200 mg/kg) or indomethacin (10 mg/kg, p.o.) as a standard drug. After 60 min (for all CAT groups) and after 30, 60, 120, 240 and 360 min (for the 200 mg/kg group), the animals received 0.6% acetic acid intraperitoneally (10 μL/g of weight). After 10 min of acetic acid administration, the number of writhings over a period of 20 min was recorded for each animal. A writhing was identified as an extension of the hind legs accompanied by constriction of the abdomen [11]. Dose–response and time course curves were produced because the pharmacokinetic of CAT is poorly understood.

In the investigation into CAT's mechanism of action, others animal groups were treated with L-arginine (150 mg/kg, i.p.) and received L-NAME (10 mg/kg, i.p.) 15 min later; they were then observed for writhings after an additional 15 min. Another group of animals received L-arginine (150 mg/kg, i.p.) and, after 15 min, 200 mg/kg CAT. Thirty minutes after the CAT treatment, the animals were tested using the writhing model described above. The control groups received only L-NAME (10 mg/kg, i.p.) or L-arginine (150 mg/kg, i.p.).

The role of ATP-dependent potassium channels in the antinociceptive effect of CAT was investigated through the administration of glibenclamide (2 mg/kg, i.p.), a K_{ATP}^{+} channel blocker, 15 min before the animals received either CAT (200 mg/kg, p.o.) or vehicle. Thirty minutes after the CAT treatment, the animals were tested using the writhing model described above.

2.5. Paw licking induced by formalin, glutamate, capsaicin, menthol, cinnamaldehyde, acidified saline, PMA and 8-Br-cAMP

The animals were divided into groups (eight animals per group) and pre-treated with either vehicle or CAT (100 or 200 mg/kg, p.o.). Morphine (7.5 mg/kg, i.p.), ruthenium red (a nonselective TRP antagonist, 3 mg/kg, i.p.), (+)-MK 801 (a potent and selective NMDA receptor antagonist, 1 mg/kg, i.p.) and camphor (a TRPA1 antagonist, 7.6 mg/kg, s.c.) were used as standard drugs. After either 30 or 60 min of treatment, the animals received a 20 μL intraplantar injection (i.pl.) in the right hind paw of one of the following inducers: 1% formalin, 10 μmol/paw glutamate, 1.6 μg/paw capsaicin, 1.2 μmol/paw menthol, 10 nmol/paw cinnamaldehyde, acidified saline (2% acetic acid in 0.9% saline, pH 2.04), 500 pmol/paw PMA (a PKC activator) or 500 nmol/paw 8-Br-cAMP (a PKA activator). The duration of paw licking was recorded in seconds over periods of 0–5 min (early phase) or 20–25 min (late phase) after administration in the formalin test, 0–5 min in the capsaicin test, 0–10 min in the glutamate test and 0–20 min in the acidified saline test [12–16].

2.6. Evaluation of locomotor activity

2.6.1. Rotarod test

A rotarod apparatus (Ugo Basile, model 7650, Italy) was used for the rotarod test. This test evaluates the possible muscle relaxation or motor incoordination effects produced by drugs in animals [17]. For this test, the mice were divided into five groups (eight animals per group); the animals were administered either citronellyl acetate (25, 50, 100 or 200 mg/kg, p.o.) or vehicle (2% Tween 80 in distilled water). Sixty minutes later, the test animals were placed on all four paws on a 2.5 cm cylinder rotating at 12 rpm. We recorded the time that the animals remained on the cylinder in seconds, as

well as the number of falls, with a maximum of three attempts on the cylinder [18].

2.6.2. Spontaneous locomotor activity test (open field test)

A cube of transparent acrylic with a black floor (30 × 30 × 15 cm) was divided into nine equal quadrants for this test. The mice were divided into five groups (eight animals per group), each of which received either citronellyl acetate (25, 50, 100 or 200 mg/kg, p.o.) or vehicle (2% Tween 80 in distilled water). After 60 min, the animals were placed on the central quadrant to begin the test. The outcome measured was the number of quadrants the mice contacted with all four legs (spontaneous movement) over the course of 5 min.

2.7. Statistical analysis

The results are presented as the mean ± the standard error of the mean (SEM). The statistical differences between the groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparisons test. To analyze the data from the hot plate and mechanical inflammatory hypernociception tests, two-way ANOVA followed by a Bonferroni post hoc test was used. GraphPad software (GraphPad Software, San Diego, CA, USA) was used in these analyses. Values of $P < 0.05$ were considered significant. The ED_{50} was estimated using non-linear regression.

3. Results

3.1. Dose–response and time course curves

According to the dose–response curve, an ED_{50} of 74.42 mg/kg (between 67.65 and 81.88 mg/kg, $P < 0.05$) was required to achieve an antinociceptive response in the acetic acid-induced writhing test, the outcomes of which were 60 min after acid administration. The curves concern only the CAT effect on the writhing test. In the time course curve, we observed that, from 30 to 240 min after administration, CAT had an antinociceptive effect, with the largest effect occurred between 60 and 120 min (Fig. 2).

3.2. The effects of citronellyl acetate on models of chemically induced nociception

Only the mice pre-treated with the highest doses of CAT (100 or 200 mg/kg) and indomethacin (10 mg/kg, p.o.) exhibited a significant decrease in the number of abdominal writhes that were induced by acetic acid when compared to mice treated only with vehicle. Pre-treatment with two doses of CAT resulted in a decrease in paw-licking time, a nociception-related behavior, during both phases of the formalin test when compared with the group pre-treated with vehicle. Similarly, morphine was effective in reducing this behavior in both the early and late phases in comparison to the vehicle. In the glutamate test, CAT decreased nociceptive behavior after pretreatment with either of the higher doses (100 and 200 mg/kg). Similarly, MK-801 was effective in reducing paw licking. The results of this experiment are summarized in Table 1.
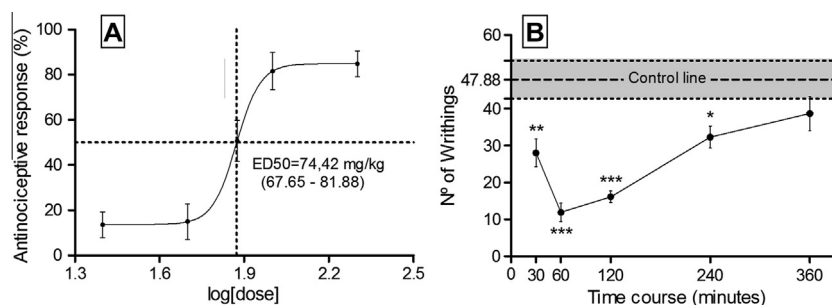

Fig. 2. (A) The dose–response and (B) time-course curves for the antinociceptive effect of citronellyl acetate in the acetic acid-induced abdominal writhing test. The points in the lines represent the group mean ± SEM. Non-linear regression was used to determine the ED_{50} with 95% confidence. Significant differences were indicated by [*]$P < 0.05$, [**]$P < 0.01$ and [***]$P < 0.001$ when compared with the control group (control line). ANOVA, followed by the Newman–Keuls post hoc test, was used.

Table 1
Effect of CAT on the acetic acid-induced writhings, licking induced by intraplantar injection of capsaicin and glutamate in mice.

Treatments groups	Acetic acid-induced writhings (number of writhings)	Formalin test (paw linking time)		Glutamate test (paw linking time)
		1st phase	2nd phase	
Vehicle	33.8 ± 4.315	62.50 ± 3.723	26.88 ± 4.933	39.83 ± 4.43
CAT 25	31.0 ± 4.0	—	—	—
CAT 50	31.29 ± 4.252	—	—	—
CAT 75	23.9 ± 4.157	—	—	—
CAT 100	6.833 ± 2.868[b]	44.63 ± 4.910[a]	11.63 ± 2.976[b]	22.57 ± 3.753[a]
CAT 200	4.818 ± 1.813[b]	44.38 ± 3.836[a]	9.875 ± 2.083[b]	19.43 ± 4.674[b]
Morphine	—	28.57 ± 1.152[b]	1.0 ± 0.7237[b]	—
Indomethacin	5.167 ± 2.522[b]	—	—	—
MK 801	—	—	—	2.714 ± 2.254[b]

Values are mean ± SEM of 8–9 mice.
Morphine (positive control) was administered at the dose of 7.5 mg/kg (i.p.).
Indomethacin (positive control) was administered at the dose of 10 mg/kg (i.p.).
MK 801 (positive control) was administered at the dose of 3 mg/kg (i.p.).
[a] $P > 0.01$ versus vehicle group (one-way ANOVA followed by Newman–Keul's test).
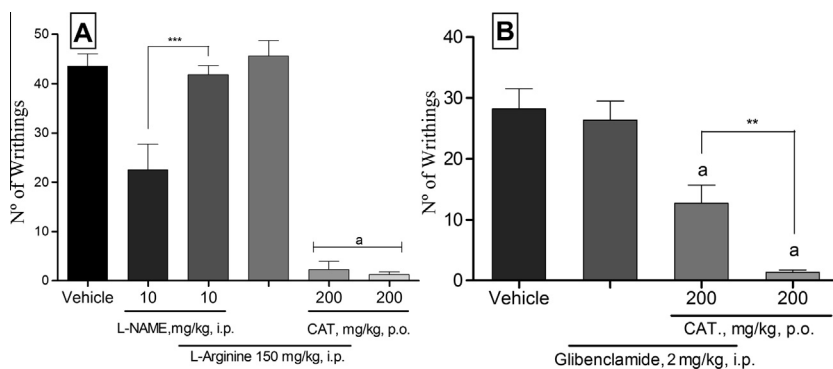[b] $P > 0.001$ versus vehicle group (one-way ANOVA followed by Newman–Keul's test).

Fig. 3. The effect of the citronellyl acetate-induced antinociception after pretreatment with (A) L-arginine or (B) glibenclamide. Significant differences were indicated by [***]$P < 0.001$, [**]$P < 0.01$ and $a = P < 0.05$ when compared with the vehicle group. ANOVA, followed by the Newman–Keuls *post hoc* test, was used.

Table 2
Effect of CAT on the licking induced by intraplantar injection of capsaicin, acidified saline, cinnamaldehyde, PMA or 8-Br-cAMP in mice.

Treatments groups	Capsaicin	Cinnamal-dehyde	Menthol	Acidified saline	PMA	8-Br-cAMP
Vehicle	52.89 ± 3.74	52.83 ± 3.81	59.75 ± 7.99	105 ± 7.256	133.3 ± 20.11	19.0 ± 3.10
CAT 100	38.67 ± 2.69[c]	50.14 ± 5.65	8.38 ± 2.51[c]	71.44 ± 14.15[a]	—	—
CAT 200	14.86 ± 3.38[b]	49.0 ± 6.44	2.75 ± 0.88[c]	9.75 ± 3.59[c]	38.45 ± 13.49[c]	5.6 ± 2.05[b]
Rut. red	28.71 ± 2.09[c]	—	2.0 ± 0.26[c]	—	—	—
Camphor	—	19.38 ± 2.31[c]	—	—	—	—
CZP	—	—	—	110 ± 35.39	—	—
CZP + CAT	—	—	—	14.75 ± 6.12[c,d]	—	—

Values are mean ± SEM of 7–9 mice.
Ruthenium red (positive control) was administered at the dose of 3 mg/kg (p.o.).
Camphor (positive control) was administered at the dose of 7.6 mg/kg (s.c.).
CZP (Capsaicin) was administered at the dose of 5 mg/kg (i.p.).
CZP + CAT (Capsaicin 5 mg/kg (i.p.)) + CAT 200.
  [a] $P > 0.05$ versus vehicle group (one-way ANOVA followed by Newman–Keul's test).
  [b] $P > 0.01$ versus vehicle group (one-way ANOVA followed by Newman–Keul's test).
  [c] $P > 0.001$ versus vehicle group (one-way ANOVA followed by Newman–Keul's test).
  [d] $P > 0.001$ CZP versus CZP + CAT (one-way ANOVA followed by Newman–Keul's test).

3.3. The involvement of the L-arginine-NO pathway and K_{ATP}^+ channels in the antinociceptive mechanism of CAT

Pretreatment with L-arginine was capable of reversing L-NAME-induced antinociception, but it did not alter the effect of CAT. The outcomes from the control L-arginine-only group were no different than the outcomes from the vehicle group.

In the glibenclamide-plus-CAT group, the antinociceptive effect of CAT was partially reversed by blocking the K_{ATP}^+ channels.

These experiments shown were carried out using the writhing test and the results are summarized in Fig. 3.

3.4. The effects of CAT on nociception induced by capsaicin, cinnamaldehyde, acid saline, PMA and 8-Br-cAMP

The intragastric administration of CAT (100 or 200 mg/kg) produced a marked and dose-dependent attenuation of pain in the capsaicin-induced nociception model. Ruthenium red was used to reduce paw licking. A similar result was observed in the acidified saline-induced nociception model, in which citronellyl acetate (100 or 200 mg/kg) was capable of decreasing nociceptive behavior. In the cinnamaldehyde-induced nociception model, the results of CAT administration (100 or 200 mg/kg) did not differ from those of vehicle administration. Camphor was also able to reduce paw licking. In the menthol-PMA-induced and 8-Br-cAMP-induced tests, CAT was capable of decreasing pain-related behavior. The results are summarized in Table 2.

3.5. Evaluation of locomotor activity

The intragastric administration of CAT (100 and 200 mg/kg) 60 min before the experiment showed no effect on locomotor activity. In the open-field test, the average numbers of quadrants entered by the mice were as follows: for 100 mg/kg, 42 ± 4.679; for 200 mg/kg, 44.13 ± 2.799; and for the vehicle, 43.25 ± 4.358. In the rotarod test, no differences were observed in the time spent on the cylinder for any dose (100 mg/kg: 55.88 ± 1.407; 200 mg/kg: 56.13 ± 1.663) when the results were compared to those from the control group (56.5 ± 2.035).

4. Discussion

Despite recent advances in the understanding of the molecular mechanisms of pain development and maintenance, there are few classes of effective analgesic drugs. Moreover, because currently used therapeutic drugs have many side effects and limited effectiveness, it is necessary that the search for new active molecules with fewer adverse effects continue.

The open field test and the rotarod test were used to exclude the possibility that the antinociceptive action of CAT is related to non-specific locomotor activity disorders in animals [16]. The results showed that antinociceptive doses of CAT did not alter motor performance in mice.

Initially, CAT administration resulted in antinociceptive activity in an animal model of visceral pain induced by i.p. administration

of acetic acid. The pain response involves the release of several mediators, including prostaglandins E2 and F2 [19], bradykinin, [20] and TNF-α, interleukins 1β and 8 [21]. A large range of substances, such as NSAIDs, narcotics, and anti-histamines, are able to inhibit abdominal writhing via different mechanisms [22]. A dose–response curve was created to determine the effective dose range of CAT in our mice. The results indicate that the highest doses (100 and 200 mg/kg) had some antinociceptive effects, which were not observed for lower doses (25, 50 and 75 mg/kg). It was determined that an ED_{50} of 74.42 mg/kg was required to induce antinociceptive response. This dose range agrees with the results reported by Brito et al. [23] and Quintans-Júnior et al. [24], who evaluated the antinociceptive activities of citronellal and citronellol, respectively, at doses of 25–200 mg/kg administered via i.p. injection.

The formalin-induced paw licking test is a model of persistent pain that can be divided into two phases. In the first phase (0–5 min), the mice engage in nociceptive behavior defined as licking of the injected paw. Nociception in this phase is the result of direct chemical stimulation of the nociceptive afferent myelinated and non-myelinated fibers, mainly the C fibers. This early phase is called the "neurogenic phase", and it can be suppressed by opioid drugs such as morphine [25]. The second phase begins 15 min after the injection of formalin and has distinct characteristics. Hunskaar et al. [25] observed that drugs such as morphine, codeine and orphenadrine, which are centrally acting drugs, shorten the paw licking time in mice following formalin administration in both the first and second phases of the test. However, indomethacin and naproxen, two NSAIDs, only decrease nociception in the second phase of the test. The second phase is called the inflammatory phase; this phase involves the release of local mediators and is sensitive to peripherally acting drugs such as NSAIDs [25,26].

Two tested doses of CAT decreased paw-licking in both phases of the test, which leads us to believe that CAT acts on peripheral nociceptors. Specifically, CAT could modulate the release of inflammatory mediators (such as histamine, serotonin, prostaglandins, and bradykinin) or nociceptors (such as TRP, NMDA, TRPA1 and opioid receptors); it is possible that CAT affects the neurotransmission pathways at the SNC level (such as substance P or CGRP). Other plant-derived oily substances, such as bisabolol [22] and carvacrol [27], also had similar antinociceptive effects.

CAT had an interesting antinociceptive effect in the chemically induced models. It is known that adding acetic acid into the peritoneal cavity promotes local irritation due to the liberation of various endogenous mediators; this irritation can also be mediated by the dissociation of protons stimulating the TRPV1 and ASIC channels located in primary afferent neurons [28–30].

Thus, the next step in this work was to evaluate the possible mechanisms involved in this action. Because the TRP and ASIC channels have an important role in the detection of noxious stimuli, we evaluated the involvement of these channels in the antinociceptive mechanism of CAT [31,32].

In this study, we found that CAT inhibits the nociceptive response induced by intraplantar injections of capsaicin or menthol, which are selective agonists of TRPV1 and TRPM8, respectively. However, CAT did not inhibit the response caused by cinnamaldehyde, a selective agonist of TRPA1.

TRPV1 functions as a polymodal receptor at peripheral nerve terminals; it modulates synaptic transmissions at the first sensory synapses between the dorsal root ganglion/trigeminal ganglion/nodose ganglion neurons and the dorsal horn/caudal spinal trigeminal nucleus/nucleus tractus solitarius neurons [33]. Capsaicin acts by lowering the "physiological" thermal activation threshold of TRPV1. Our results showed that CAT reverses this action, most likely by reversing the capsaicin-induced sensitization of TRPV1.

Intradermal capsaicin-induced nocifensive behavior was alleviated by intradermal administration of menthol, suggesting that menthol is effective, even for acute pain [34]. However, during TRPV1-induced hypersensitivity, TRPM8 is downregulated. Therefore, the activation of TRPM8 seems to induce a soothing sensation that alleviates hyperalgesia [34]. TRPV1 and TRPM8 are modulated in opposite manners; therefore, CAT seems to directly block the effects of capsaicin and indirectly block the effects of menthol.

The involvement of TRPA1 in cold allodynia and mechanical hyperalgesia has been demonstrated using behavioral models [35]. However, its role in noxious cold and mechanical sensations is still controversial [33]. Recent studies have shown that formalin activates the primary afferent sensory neurons through specific and direct action on TRPA1, which is highly expressed by a subset of C-fiber TRPV1 positive nociceptors [36]. In our evaluation of the role TRPA1 plays in CAT's antinociceptive mechanism, we found that CAT treatment did not change the nociceptive response caused by cinnamaldehyde. This result indicates that although CAT had an antinociceptive effect in the formalin test, that effect is related not to TRPA1 but to some other molecule in a formalin-triggered pathway, such as PGE2, NO, glutamate or kinins.

The activation of PKC both potentiates and prolongs the TRPV1-mediated responses when compared to the activation of PKA, which only potentiates the responses transiently. However, the stimulation of PKC results in the downregulation of TRPM8 [34]. TRPV1 and TRPM8 are modulated by PIP2, but in opposite manners. The depletion of PIP2 caused by the activation of PLC decreased TRPM8 channel activity [37], whereas it increased the blocking of TRPV1 by PIP2, enhancing that channel's activity [38]. PKC plays a pivotal role in pathological somatic pain; its phosphorylation not only sensitizes TRPV1 but also promotes its translocation from the cytosol to the plasma [39,40]. The activation of PKC by phorbol ester was found to depolarize sensory neurons [41,42]. PKC activation profoundly sensitized heat-mediated responses in sensory neurons, which could be attenuated by PKC inhibitors [34]. ASIC are activated by extracellular protons and are modulated by PKC [43].

During inflammation, prostaglandins are released into the bloodstream and increase the levels of intracellular cyclic adenosine monophosphate (cAMP) in sensory neurons [44]. This effect can be mimicked by the addition of membrane-permeable cAMP analogs [45]. NSAIDs and opiates decrease cAMP levels. There is substantial evidence that the presence of cAMP and PKA sensitizes TRP channels, suggesting that analgesics work by reducing TRP channel sensitization. NSAIDs relieve pain by blocking cyclooxygenases and reducing the production of PGs. PGE2 and forskolin enhance the flow caused by capsaicin [46,47].

CAT decreased the pain behaviors caused by intraplantar administration of both PMA (a PKC activator) and 8-Br-cAMP (a PKA activator). Given the extensive evidence implicating PKC in signaling mechanisms that lead to nociception and hyperalgesia [16], we hypothesized that this could be a relevant target for the antinociceptive action of CAT.

Some studies have demonstrated that the expression of ASICs is enhanced by pro-inflammatory mediators [48,49]. At the peripheral level, ASIC3 is important for inflammatory pain. Its expression and activity are potentiated by several pain mediators present in the "inflammatory soup" that sensitizes nociceptors [50]. Some evidence suggests that ASIC and TRPV1 have complementary roles in the proton sensitivity of sensory neurons [16]. In this study, it was found that CAT effectively inhibits the nociceptive responses induced by the i.pl. injection of acidic saline; CAT also inhibits the blockage of TRPV1 by capsazepine. This result confirms those from the capsaicin-induced nociception model and suggests that ASICs are involved in the antinociceptive mechanism of CAT.

Subcutaneous injections of NO in humans induce painful sensations [51], and animal experiments have suggested that the generation of NO and subsequently increased levels of cGMP are involved in the mechanisms of peripheral antinociception, particularly in inflamed tissues [52]. Thus, to investigate CAT's antinociceptive mechanism, we used L-arginine to evaluate its involvement in the L-arginine-NO pathway. Our results did not suggest that CAT is involved in this pathway because pretreatment with L-arginine did not reverse its antinociceptive effect. Finally, following that investigation, we attempted pre-treatment with glibenclamide, a K_{ATP}^+ channel blocker. This pre-treatment partially reversed the antinociceptive effect of CAT in the acetic acid-induced writhing test, suggesting the possible involvement of this channel in CAT's mechanism of action. Several studies have associated the K_{ATP}^+ channel with pain; its opening leads to a hyperpolarization of the cell membrane, which results in a decrease in the cell's excitability [53,54]. In the present study, we analyzed only the participation of the K_{ATP}^+ channel, but we cannot disregard the possible participation of other K^+ channels in the antinociceptive effect. However, the results of this test suggest that the modulation of the K_{ATP}^+ channel plays an important role in the antinociceptive mechanism of CAT.

Finally, we investigated the glutamatergic system's participation in the antinociception caused by CAT. It is well established that glutamate is a major excitatory neurotransmitter involved in the transmission of nociceptive signals. Furthermore, the nociceptive neurons activated by glutamate may release several inflammatory mediators and neuropeptides that could be involved in nociceptive transmission, in both the central and peripheral nervous systems [16]. In the present study, we observed that CAT effectively inhibited the nociceptive response induced by the intraplantar injection of glutamate. This nociceptive response is present in the peripheral, spinal, and supraspinal neurons. We hypothesized that CAT-induced antinociception partially results from the inhibition of ionotropic or metabotropic glutamate receptors.

In conclusion, the results of this study demonstrate that the oral administration of citronellyl acetate results in pronounced systemic antinociceptive effects in mouse models of acute nociception induced by acetic acid, formalin, capsaicin, menthol, acidified saline, PMA, 8-Br-cAMP and glutamate. We also demonstrated that the K_{ATP}^+ channel is involved in this mechanism. Therefore, we suggest that CAT acts, at least in part, by modulating TRPV1, K_{ATP}^+, and ASIC, as well as glutamate receptors via PKC and PKA. To better clarify this mechanism, further experimentation is required. However, this study was the only first step towards the identification of new, potentially therapeutic effects of this molecule.

Acknowledgements

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