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Involvement of Calmodulin Inhibition in Analgesia Induced With Low Doses of Intrathecal Trifluoperazine

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ABSTRACT—We examined which of the known properties of trifluoperazine, including calmodulin inhibition, are involved in its analgesic effect. Furthermore, we tried to find any possible interaction between opioidergic system and calmodulin inhibition-induced analgesia. Intrathecal trifluoperazine (1, 10, 100 μg) showed a biphasic effect in the formalin test; i.e., analgesia at relatively low doses (1, 10 μg) and hyperalgesia at a high dose (100 μg). No analgesic effects were observed after intrathecal injection of sulpiride (1, 10, 100 μg), atropine (0.1, 1, 10 μg), phentolamine (0.1, 1, 10 μg) and brompheniramine (0.1, 1, 10 μg). Meanwhile, intrathecal calmidazolium (10, 50, 250 μg) induced a dose-dependent analgesia. Histamine (1 μg), physostigmine (1 μg), bromocriptine (1 μg) and norepinephrine (1 μg) did not affect trifluoperazine-induced analgesia. Calcium (20 μg) attenuated the antinociceptive effect of trifluoperazine and inhibited the analgesic effect of calmidazolium. Finally, naloxone (2 mg/kg) decreased trifluoperazine-induced antinociception but did not have any effects on calmidazolium-induced analgesia. We concluded that calmodulin inhibition may be involved in the analgesia produced by trifluoperazine. With increasing doses of trifluoperazine, the algesic effect seems to overcome the analgesic effect. It is also suggested that the opioidergic system does not interact with calmodulin inhibition-induced analgesia even though this system has a possible role in trifluoperazine-induced analgesia.

Keywords: Subarachnoid space, Neuroleptic, Pain relief, Calmodulin antagonist

Several lines of evidence have implicated that antipsychotic drugs, including phenothiazines and butyrophenones, have analgesic effects (1). However, the antinociceptive effects of these drugs are not well established and mostly depend upon clinical observations (2). It seems important, nonetheless, that possible mechanisms of action should be investigated as this may lead to better understanding of drug actions and facilitate the design of new drug entities that help to control pain.

Phenothiazine neuroleptics are known to block a variety of receptors, including dopaminergic, cholinergic, adrenergic and histaminergic receptors (3). In addition, some phenothiazine derivatives inhibit the action of calmodulin (4 – 6). Calmodulin functions as an intracellular mediator for calcium ions. Among phenothiazine neuroleptics, trifluoperazine is one of the most potent calmodulin inhibitors (5, 6). Several recent reports have shown that the intracellular blockade of the calcium functions by calmodulin inhibitors can induce analgesic effects (7, 8).

The main aim of the present work was to clarify whether the blockade of calmodulin is involved in the analgesic effect of trifluoperazine or not. Employing the formalin test, we compared the analgesic effect of trifluoperazine with those of a specific D_2 dopamine receptor antagonist (sulpiride), an anticholinergic agent (atropine), an α-adrenergic receptor antagonist (phentolamine), an antihistamine (brompheniramine) and a specific calmodulin inhibitor (calmidazolium). Also, we examined the effects of histamine, physostigmine, bromocriptine, norepinephrine and calcium on trifluoperazine-induced analgesia. In an attempt to highlight the role of calmodulin inhibition in the trifluoperazine-induced analgesia, the agents tested, except for trifluoperazine and calmidazolium, were selected in such a manner that would not affect the calmodulin function (9, 10).

Since some studies indicate an involvement of an opioid mechanism of action in antipsychotic analgesia (11, 12),

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we also tried to find a possible relationship between the opioidergic system and calmodulin inhibition-induced analgesia of trifluoperazine and calmidazolium.

MATERIALS AND METHODS

Animals

This study was approved by the Animal Care Use Committee of Kumamoto University. Male Wistar rats, weighing 250 – 350 g (from Inoue Co., Ltd., Kumamoto) with free access to laboratory food and water, were used. Rats were randomly assigned to an experimental or a control group and received injection of either drugs or solvents, respectively. Each animal was used only once and sacrified by overexposure to ether on termination of the experiment.

Drugs

Calmidazolium (R24571; 1-[bis-(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]-ethyl]-1*H*-imidazolium chloride) was dissolved in 10% dimethyl sulfoxide (DMSO). The solution was sonicated for some minutes prior to its administration. Sulpiride and bromocriptine were dissolved in a small amount of acetic acid and diluted with distilled water; pH was adjusted to 6.7 by adding NaOH. Atropine, physostigmine, histamine, trifluoperazine, naloxone, phentolamine, brompheniramine and calcium chloride were dissolved in distilled water. All drugs used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Drugs were injected intrathecally (i.t.) and dissolved by such a manner that the final dose was administered at a volume of 10 μl. Intrathecal injections were given by chronic catheterization of subarachnoid space according to a modification of the method described by Yaksh and Rudy (13). In brief, the animals were anesthetized with ketamine (100 mg/kg) and mounted in a conventional stereotaxic instrument. A midline incision was made on the skull extending from a line between the ears to a point approximately 2-cm caudal. A polyethylene (PE-10) catheter was advanced 8.5-cm caudally through an incision in the atlanto-occipital membrane to the level of the lumbar enlargement. After surgery rats were housed in the individual cages and allowed to recover for 7 days.

Formalin test

In the formalin test, the rats were adapted in standard transparent cages approximately 1 h before injection of formalin. Transparent cages were also used as an observation chamber after injection of formalin. A mirror was positioned behind the chamber and provided an unobstructed view of the right hindpaw. Fifty microliters of formalin (2.5% in saline) was subcutaneously injected under the skin of the dorsal surface of the right hindpaw of the rats using a microsyringe with a 26-gauge needle. Each rat was immediately returned to the observation chamber after the injection. The measurment of early response started immediately and lasted for 5 min. The recording of the late response started 15 min after the formalin injection and lasted for 15 min. In both phases, only licking and biting of the injected hindpaw was defined as a nociceptive response and the total time of the response was measured with a hand-held stopwatch during the test period.

Rotarod test

Motor performance was evaluated by using a rotarod apparatus (MK-650; Muromachi Kikai Co., Ltd., Tokyo). Animals were placed on a cylinder (7 × 9 cm) moving at 10 rpm. The time at which the rat was unable to stay on the cylinder, i.e., fell down, was recorded. If an animal could maintain its position on the cylinder for 5 min, the trial was halted and considered as an evidence of full locomotor activity. On the day prior to the experiment, all the rats were habituated to the apparatus and trained, in order to establish a more reproducible baseline. The baseline scores were obtained on the day of the experiment, with 4 measurements made for each rat at 10 – 15 min intervals. The mean of these 4 measurements was considered as the basal score. The effect of drugs on rotarod performance was measured by using a single measure 10 – 15 min after i.t. administration of drugs or solvents. Finally, the percentage of baseline score obtained in each trial was calculated by the following formula:

$$\%\ \text{of rotarod score} = \text{Experimental score} / \text{Basal score} \times 100$$

When a treated rat reached its own basal score or stayed on the rotarod for 5 min, the trial was interrupted and a 100% rotarod score was assigned.

Statistical analyses

Results were expressed as the mean ± standard error of mean (S.E.M.). A Bartlet test was conducted to analyze the uniformity of variance in each group. Because of non-uniformity in some groups, Sheffe's test was used to compare each treatment with the corresponding control group. The accepted level of significance for all tests was $P<0.05$.

RESULTS

Effects of i.t. drug administration on the formalin test

The subcutaneous injection of 50 μl of formalin (2.5%) into the plantar surface of a hindpaw induced a biphasic pain behavior response, such as biting and licking the injected paw. This response reached its first maximum around 5 min after the injection (phase I), it then decreased for a period of 5 – 10 min, and finally rose again after about

15 min (phase II), showing a pattern of intense and persistent pain behavior during the remaining period of testing.

The i.t. administration of trifluoperazine at various doses (1, 10 and 100 μg/rat) 10 – 15 min before the formalin injection showed biphasic effects, i.e., analgesia in relatively low doses (1 and 10 μg/rat) and hyperalgesia in the higher dose (100 μg/rat, Fig. 1). All doses of sulpiride (1, 10 and 100 μg/rat) induced hyperalgesia. Low and medium doses of atropine (0.1 and 1 μg/rat) did not show any significant effects and the higher dose (10 μg/rat)

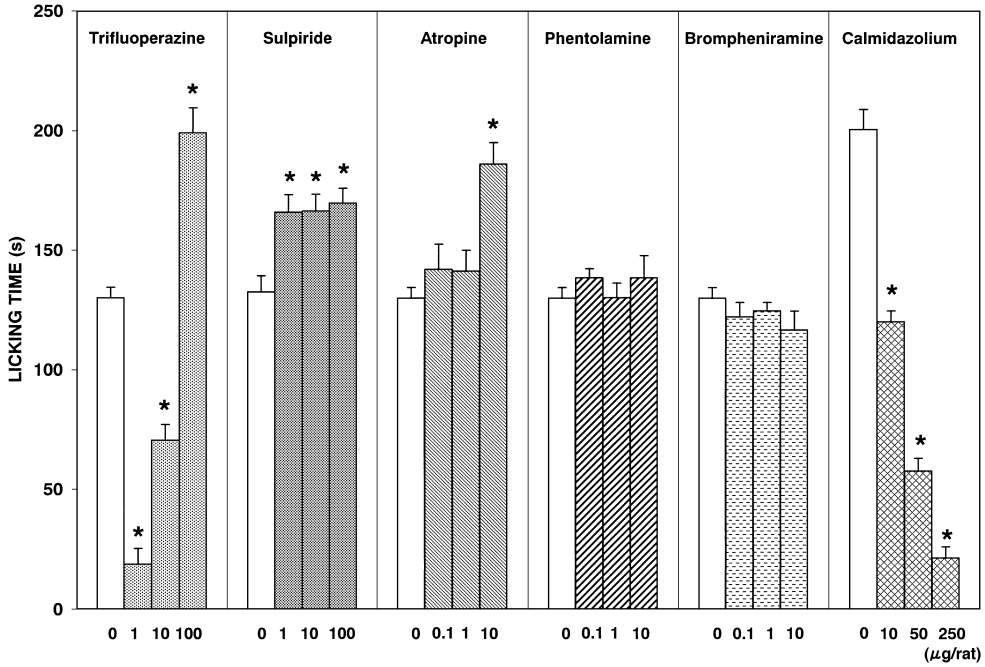

Fig. 1. Effects of i.t. drugs (μg/rat) or vehicle (10 μl), injected 10 – 15 min before testing, on the nociceptive behavior (licking and biting) induced by an intraplantar formalin (2.5%, 50 μl) injection. The mean (± S.E.M.) of licking times of each group (n = 5) during phase II (between 15 to 30 min after formalin injection) is represented. Bartlett and Sheffe's tests were used for statistical analysis, **P*<0.05.

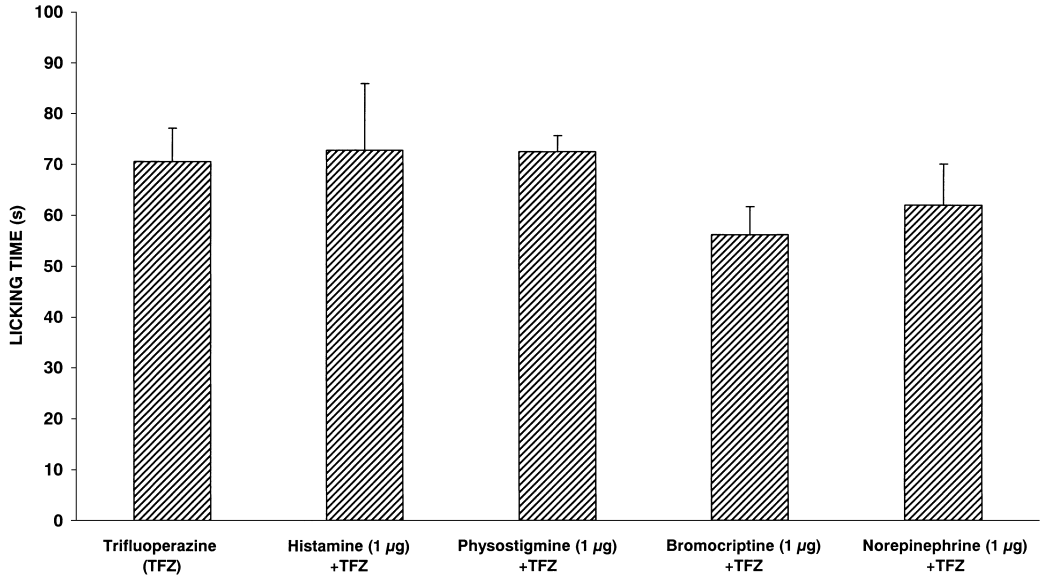

Fig. 2. Effects of i.t. trifluoperazine (TFZ) alone (10 μg/rat) and trifluoperazine (10 μg/rat) plus various agents (μg/rat), injected 10 – 15 min before testing, on the formalin test. The mean (± S.E.M.) of licking times of each group (n = 5) during phase II (between 15 to 30 min after formalin injection) is represented. Bartlett and Sheffe's tests were used for statistical analysis. No significant differences were observed.

caused hyperalgesia. Phentolamine (0.1, 1 and 10 μg/rat) and brompheniramine (0.1, 1 and 10 μg/rat) failed to produce analgesia. Calmidazolium (10, 50 and 250 μg/rat) showed significant analgesia in a dose-dependent manner.

Effects of various agents on trifluoperazine-induced analgesia

Histamine (1 μg/rat), physostigmine (1 μg/rat), bromocriptine (1 μg/rat) and norepinephrine (1 μg/rat) did not affect trifluoperazine-induced analgesia (Fig. 2). Calcium (20 μg/rat, i.t.) attenuated the analgesic effects of trifluoperazine and inhibited calmidazolium-induced antinociception. Naloxone pretreatment [30 min, 2 mg/kg, intraperitoneally (i.p.)] did not show any effect by itself in the formalin test, but partially attenuated the analgesic effect of trifluoperazine, while it did not have any effect on calmidazolium-induced analgesia (Fig. 3).

Since qualitatively very similar patterns of responses were observed during phases I and II of the formalin test, only the results of phase II for the experiments have been depicted.

Effects induced by i.t. injection of various agents on the rotatod test

The effects of all agents on motor coordination are shown in Table 1. None of the drugs within the dosage range used in this study and administered via i.t. injection caused a lack of motor coordination as measured in the rotatod apparatus.

Table 1. Results of rotarod test

Drug	Dose (μg/rat, i.t.)	% of rotarod performance
Trifluoperazine (TFZ)	1	95.75 ± 2.45
TFZ	10	96.35 ± 3.65
TFZ	100	90.75 ± 5.85
Sulpiride (SUL)	1	91.45 ± 8.55
SUL	10	92.35 ± 4.58
SUL	100	95.45 ± 3.55
Atropine (ATR)	0.1	100 ± 0
ATR	1	98.20 ± 1.80
ATR	10	92.25 ± 4.49
Phentolamine (PHN)	0.1	94.40 ± 3.47
PHN	1	90.75 ± 5.37
PHN	10	96.57 ± 3.42
Brompheniramine (BRM)	0.1	95.72 ± 4.27
BRM	1	100 ± 0
BRM	10	96.20 ± 3.8
Calmidazolium (CAL)	10	98.60 ± 1.4
CAL	50	96.10 ± 3.9
CAL	250	96.30 ± 3.7
Histamine	1	93.48 ± 4.58
Physostigmine	1	94.89 ± 7.61
Bromocriptine	1	96.12 ± 6.32
Norepinephrine	1	95.16 ± 3.48
Water	—	95.82 ± 2.50
SUL vehicle	—	96.47 ± 3.52
DMSO	—	96.10 ± 3.9

Comparison was made with the Sheffe's test for each dose and its vehicle.

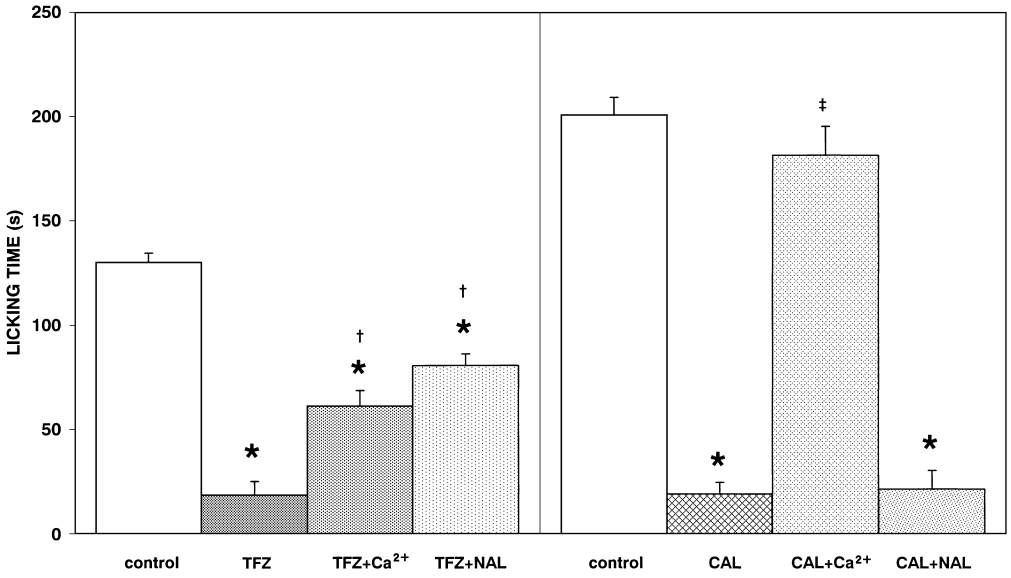

Fig. 3. Effects of calcium (Ca^{2+}; 20 μg/rat, i.t.) and naloxone (NAL; 2 mg/kg, i.p.) on trifluoperazine (TFZ; 1 μg/rat, i.t.)- and calmidazolium (CAL; 250 μg/rat, i.t.)-induced analgesia. The mean (± S.E.M.) of licking times of each group (n = 5) during phase II (between 15 to 30 min after formalin injection) is represented. Bartlett and Sheffe's tests were used for statistical analysis, $P<0.05$: *treated groups vs controls, †treated groups vs trifluoperazine, ‡treated group vs calmidazolium.

DISCUSSION

The antipsychotic trifluoperazine has diverse pharmacological properties, presumably related with its analgesic effect previously observed in clinical practice (1). In the present study, we have clearly demonstrated for the first time that the i.t. administration of trifluoperazine showed biphasic effects; analgesia at the relatively low doses and hyperalgesia at the higher dose and have discussed its relation to the various properties of this drug.

The formalin test is believed to represent a valid model for clinical pain (14). In this test, the first, or acute phase, is thought to result from direct chemical activation of myelinated and non-myelinated nociceptive afferent fibers and the second, or tonic phase, as a consequence of noxious stimulus-evoked changes in the properties of dorsal horn neurons (15).

Since antinociceptive measurement in the formalin test is based on behavioral reactions, a motor deficit could be erroneously interpreted as an antinociceptive effect (8). In order to quantify motor side effects of the drugs, we used a rotarod test, currently considered as a sensitive method to detect motor deficits. Despite several reports on motor side effects of antipsychotic drugs, none of the drugs used in this experiment impaired motor activity perhaps due to negligible brain concentration after spinal subarachnoid administration (13).

Blockade of supraspinal D_2 dopamine receptors can be one of the most prominent features of trifluoperazine as an antipsychotic drug (3). There are some reports that show that spinal or supraspinal blockade of these receptors can affect pain transmission. Most researchers believe that spinal and supraspinal antinociceptive effects of dopamine agonists are mediated mainly via D_2 receptors (16 – 18). In our experiment, i.t. sulpiride also showed hyperalgesia. On the other hand, bromocriptine (D_2-dopamine agonist) could not reduce the antinociceptive effect of trifluoperazine. These suggest that spinal blockade of D_2 dopamine receptors can lead to algesic effects, and it appears that the analgesic effect of i.t. trifluoperazine does not depend on a D_2 blocking property of this drug.

A variety of studies have implicated that the spinal cholinergic system is involved in the modulation of painful stimuli. I.t. muscarinic agonists and cholinesterase inhibitors can produce a sustained antinociception, both of which appear to possess a pharmacology that is suggestive of an M_1 or M_3 receptor subtype (19, 20). Atropine has been reported to attenuate the analgesic effects of physostigmine and even morphine (19, 21), which confirms the role of the cholinergic system in pain transmission. However, there is an ambiguity about the role of nicotinic receptors. Christensen and Smith (22) reported the antinociceptive action of i.t. (–)-nicotine and (+)-nicotine. In contrast, Kahn et al. (19) examined the possible analgesic activity of three nicotinic agonists, epibatidine, cytisine and nicotine, after i.t. injection. All agents elicited dose-dependent algogenic activity, characterized at lower dose by touch-evoked hyperactivity and at higher doses by intermittent vocalization and marked behavioral activity. In addition, i.t. epibatidine elicited a short-lasting, dose-dependent antinociception. Finally they suggested that these analgesic and algogenic responses may be mediated by distinct subtypes of spinal nicotinic receptors. Besides all of these controversies, some reports have suggested that blockade of the cholinergic system also results in an antinociceptive effect in animal studies (23). In this experiment, i.t. injections of atropine did not produce any analgesic effect, but rather, induced hyperalgesia at the higher dose. Meanwhile, physostigmine did not show any effect on trifluoperazine-induced analgesia. So, it seems unlikely that the analgesic effects of trifluoperazine are mediated by its anticholinergic property.

There is a fairly uniform agreement about the role of central norepinephrine in modifying pain transmission. In unanesthetized animals, the i.t. administration of α-adrenergic agonists elicited an increase in the nociceptive threshold in a variety of species (23, 24). These effects seem to be mediated through the α_1 or α_2 adrenergic receptors (23, 25). In our experiment i.t. phentolamine did not show any effect in the formalin test. Also, norepinephrine did not affect trifluoperazine-induced analgesia. Therefore, we concluded that analgesic effect of trifluoperazine could not be due to its α-blocking property.

There are many controversies as to the role of spinal and supraspinal histamine receptors in pain modulation. Although in nearly all reports, intracerebroventricular (i.c.v.) injection of histamine has produced analgesia, the role of known histamine receptors remains to be elucidated (26 – 28). For example, i.c.v. injection of 2-methyl histamine (H_1-agonist) and mepyramine (H_1-antagonist) both showed analgesia in various tests (29, 30). Also, there is at least one report about the analgesic effect of betahistine (H_1-agonist) caused by i.t. injections (31). On the other hand Malmberg-Aiello et al. (32) reported evidence for hypernociception following histamine H_1-receptor activation using a selective histamine H_1-receptor agonist. In their report, 2-(3-trifluoromethylphenyl) histamine dihydrogenmaleate (FMPH) was used to characterize the hypernociception caused by H_1-receptor activation. The effects of FMPH on the pain threshold in the hot plate test were biphasic. In low concentrations, FMPH lowered the reaction latency in the hot plate test while it increased the reaction latency in higher doses. The effects at lower doses are probably due to the activation of histamine H_1 receptors. Their data are in accordance with another experiment, which has been performed on mutant mice lacking H_1

receptors (33). These animals showed significantly fewer nociceptive responses in various pain producing models, which suggests that activation of histamine H_1 receptors could increase sensitivity to noxious stimuli. In our experiment, brompheniramine did not induce analgesic effect and a low dose of histamine did not have any effect on trifluoperazine-induced analgesia. Therefore, it is unlikely that the analgesic effect of trifluoperazine has been conducted through this mechanism.

The involvement of calcium in the transmission of nociceptive signals has been demonstrated at the spinal level. Specifically, spinal sensitization induced by persistent nociceptive stimulation seems to be related to an increase in cytosolic calcium and the subsequent activation of several enzymes, some of which are calcium-calmodulin dependent (7). In our experiment, the i.t. administration of calmidazolium induced a dose-dependent decrease of the nociceptive responses obtained in both phases of the formalin test. In addition, this effect of calmidazolium was inhibited by simultaneous administration of calcium (20 μg /rat). Trifluoperazine-induced analgesia was also attenuated by calcium. These suggest that calmodulin activation in the spinal cord is involved in the development of pain in both phases of the test, and it supports the possibility that analgesia produced through calmodulin inhibition may partially contribute to the effect induced by i.t. administration of trifluoperazine. The effect of these agents (calmidazolium and trifluoperazine) on the second phase relates well with previous studies describing the fact that NMDA-antagonists (34) and Ca^{2+} chelators (35) also inhibit it. In other words, both the blockade of Ca^{2+} entry prior to calmodulin activation and the inhibition of further steps in the formation of calcium-calmodulin complexes reduce the nociceptive behavior of the second phase of the test. Accordingly, the inhibition of an intermediate process, i.e., calcium-calmodulin activation, should produce the same result. In contrast, the first phase of the formalin test is often considered to be unrelated to spinal Ca^{2+} availability. Supporting this view, the i.t. injection of NMDA-antagonists (34) and calcium chelators (35) do not modify nociceptive behavior in the first phase of the test. Indeed, extracellular Ca^{2+} is not the only source by which cytosolic Ca^{2+} can rise since an increase of intracellular Ca^{2+} can be induced by a receptor-mediated release of intracellular Ca^{2+} stores. Glutamate and substance P, which are released in the spinal cord during the first phase of the formalin test (36, 37), can induce (through metabotropic receptors) the release of intracellular Ca^{2+}. Calmodulin can be activated in response to these receptor-mediated increases of cytosolic Ca^{2+}. In this way, the analgesic effect of calmidazolium and trifluoperazine in the first phases of the formalin test, which is a model of phasic pain, can be explained. In addition, naloxone could partially attenuate the analgesic effect of trifluoperazine but without any effect on calmidazolium-induced analgesia, which suggests a partial role for the opioidergic system in trifluoperazine-induced antinociception. Furthermore, it showed that calmodulin inhibition-induced analgesia was not mediated through the opioidergic system. In summary, we have concluded that trifluoperazine can exert an analgesic effect in low doses and inhibition of calmodulin has at least a partial role in the spinal component of this effect. Finally, as the dosage increases, the antinociceptive effect seems to be overcome by the algesic effect of the drug through the blocking of cholinergic or dopaminergic receptors. On the other hand, we cannot rule out the effects of trifluoperazine on other receptors to explain this dual effect. For example trifluoperazine has an effect on the agonist binding properties and the activation of the human and rat vanilloid receptors. Binding of [^{3}H]resiniferatoxin (a potent capsaicin analog) to membrane preparations of human dorsal horn and rat whole spinal cord is affected by trifluoperazine in a biphasic fashion, with an initial enhancement preceding inhibition.

This study might provide at least another mechanism for the analgesic effects of phenothiazine neuroleptics. Further characterization of the effects of these drugs and the rational design of more potent and selective compounds may provide a new direction for pain therapy.

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