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Differential effects of intraplantar capsazepine and ruthenium red on capsaicin-induced desensitization in mice

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Abstract

Intraplantar injection of capsaicin (1.6 μg/paw) into the mouse hindpaw produced an acute paw-licking/biting response. This study was designed (1) to investigate the antinociceptive effects of intraplantar administration of capsazepine, a competitive vanilloid receptor antagonist, and ruthenium red, a noncompetitive antagonist, in the nociceptive licking/biting response induced by intraplantar injection of capsaicin, and (2) to determine whether these compounds were able to prevent capsaicin-induced desensitization in mice. Both capsazepine and ruthenium red produced a dose-dependent reduction in the capsaicin-induced nociceptive response. In licking/biting response to intraplantar capsaicin, ruthenium red was more potent than capsazepine in producing antinociceptive activity as assayed by the capsaicin test. The first injection of capsaicin induced a profound desensitization to the second and third injections of capsaicin at the interval of 15 or 30 min. The capsaicin-induced desensitization was prevented dose-dependently by antinociceptive doses of capsazepine, whereas ruthenium red in doses exhibiting antinociceptive activity was without effect on capsaicin-induced desensitization. The present results suggest that both capsazepine and ruthenium red can produce a local peripheral antinociceptive action, which may be mediated by inhibiting the membrane ion channel activated by capsaicin. In addition, these data suggest that capsazepine may act in the mechanism clearly different from ruthenium red in the capsaicin-induced nociceptive desensitization.
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Keywords: Capsaicin; Intraplantar injection; Capsazepine; Ruthenium red; Antinociception; Desensitization; Mouse

1. Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-noneamide), the pungent main ingredient of chili peppers, specifically acts on unmyelinated C-fibers and thinly myelinated Aδ primary sensory neurons (Fitzgerald, 1983; Szolcsanyi, 1983; Buck and Burks, 1986; Maggi and Meli, 1988; Bevan and Szolcsanyi, 1990; Holzer, 1991; Jancso, 1992). Functionally, such neurons are thought to be the sites of release of proinflammatory mediators in the periphery and to transmit nociceptive information to the spinal cord level (Szallasi and Blumberg, 1993). Recently, a functional vanilloid receptor (VR), termed VR subtype 1 (VR1), has been cloned (Caterina et al., 1997). Studies in "knockout mice" lacking the VR1 receptor showed a marked reduction in nocifensive behavioral responses to capsaicin or noxious heat (Caterina et al., 2000) and in carrageenan-induced thermal hyperalgesia (Davis et al., 2000). Therefore, capsaicin is a useful tool for the study of nociceptive sensory neurons in mammals. The activation of nociceptors by capsaicin is believed to be mediated by the activation of a ligand-gated ion channel (Wood et al., 1988; Oh et al., 1996). Using patch-clamp techniques, it has been proposed that a functional capsaicin receptor termed VR1 is a heat-gated ion channel that is able to mediate responses of small-diameter sensory neurons to moderate thermal stimuli (Leem et al., 1993; Caterina et al., 1997). In the electrophysiological study, the channel for VR1 is permeable to cations, particularly Ca^{2+} and Na^{+} (Bleakman et al., 1990; Caterina et al., 1997). Accordingly, stimulation of VR1 opens a Ca^{2+} and Na^{+} entry into the sensory neurons (Bevan and Szolcsanyi, 1990). In primary afferent neurons, capsaicin activates a cation-selective inward current

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(Marsh et al., 1987) and causes a marked accumulation of intracellular Ca^{2+} (Wood et al., 1988). It is generally held that the influx of extracellular Ca^{2+} is essential for capsaicin-evoked transmitter release (Gamse et al., 1979; Bernath and Vizi, 1987) and for its desensitization (Santicioli et al., 1987). Exposure to capsaicin can elicit a functional desensitization to subsequent noxious stimuli, suggesting alleviation for some painful conditions (Maggi and Meli, 1988; Bevan et al., 1992). These capsaicin-induced responses are thought to be antagonized by capsazepine, a first competitive antagonist for the VR1, and ruthenium red, another functional antagonist for capsaicin with the property of inhibiting transmembrane and mitochondrial Ca^{2+} transport (Dray et al., 1990a,b; Urban and Dray, 1991; Bevan et al., 1992; Perkins and Campbell, 1992; Szallasi, 1995). However, the effect of ruthenium red on capsaicin-induced desensitization is controversial; the sensitizing effect of capsaicin was antagonized by ruthenium red in sensory neurons or fibers of rat urinary bladder (Maggi et al., 1988; Amann, 1990), whereas ruthenium red was unable to prevent capsaicin-induced desensitization in the dorsal root ganglion and spinal cord (Geppetti et al., 1991). The selectivity of capsazepine for the VR makes it an extremely useful tool to investigate in the pharmacological and physiological fields. In contrast to capsazepine, the effect of ruthenium red is characterized by noncompetitive antagonism of capsaicin-induced primary afferent stimulation, as it does not bind to the VR1 (Bevan et al., 1992; Maggi et al., 1993).

In the present study, we have compared antinociceptive activity of capsazepine, a competitive antagonist of VR1, and ruthenium red, a nonspecific cation-channel blocker, when injected into the plantar surface of the hindpaw in mice. Furthermore, the effect of capsazepine and ruthenium red on capsaicin-induced nociceptive desensitization was also compared.

2. Methods

Experimental procedures were conducted according to protocols approved by the Committee of Animal Experiments of the Tohoku Pharmaceutical University and in accordance with the approval of the guidelines of the ethics of the International Association for the Study of Pain (Zimmermann, 1983).

2.1. Animals

Male ddY strain mice (Kyudo Industries, Kumamoto, Japan) weighing 21–24 g were used. They were maintained in conditions of controlled room temperature (22–24 °C) and relative humidity (50–60%) under reversed daylight conditions (12 h on/12 h off, lights on 0800 h) for at least 24 h before the start of each experiment, with standard laboratory food and water available ad libitum. All experiments took place between 1000 and 1700 h.

2.2. Capsaicin-induced nociceptive response

Experiments were performed essentially according to the method of Sakurada et al. (1992). Approximately 1 h before capsaicin injection, the mice were placed individually in an observation plastic cage (22.0 × 15.0 × 12.5 cm) to allow adaptation to the new environment. The mouse was injected 20 μl of a solution of capsaicin (1.6 μg/paw) under the skin of the plantar surface of the right hindpaw using a microsyringe with a 26-gauge needle. Control animals were similarly injected with 20 μl of vehicle only. Licking/biting behavior induced by intraplantar injection of capsaicin was observed as an indicator of nociceptive response. The accumulated response time (in seconds) spent in licking/biting the capsaicin-injected paw was measured for a period of 3 min immediately after the subcutaneous injection of capsaicin, except in the time course experiment.

2.3. Desensitization studies with capsaicin

Animals were injected with capsaicin into the plantar surface of the hindpaw at intervals of 15, 30, 60, 120, and 240 min. The licking/biting response to the subsequently injected capsaicin was then examined to determine the extent to which repeated treatment with capsaicin elicited a reduction in functional response as a result of the development of desensitization.

2.4. Drugs

The following drugs were used: capsaicin (Sigma, St. Louis, MO), capsazepine (Sigma), and ruthenium red (Sigma). Capsaicin and capsazepine were dissolved in 100% dimethylsulfoxide (DMSO). The concentrated stock solutions were diluted with physiological saline (0.9% wt/vol). Ruthenium red was dissolved in 0.9% saline. Capsazepine or ruthenium red was coinjected with capsaicin in a volume of 20 μl. Intraplantar subcutaneous injections were made with a 28-gauge needle attached to a 50-μl Hamilton syringe.

2.5. Data analysis

The data were given as mean ± S.E.M. of 10 mice in each group. Statistical analyses of the results were evaluated using analysis of variance (ANOVA), followed by the Dunnett's test for multiple comparisons. A difference was considered statistically significant at $P < .05$.

3. Results

3.1. Antinociceptive effects of intraplantar capsazepine and ruthenium red in the capsaicin test

The intraplantar injection of capsaicin (1.6 μg/paw) produced a licking/biting response toward the injected

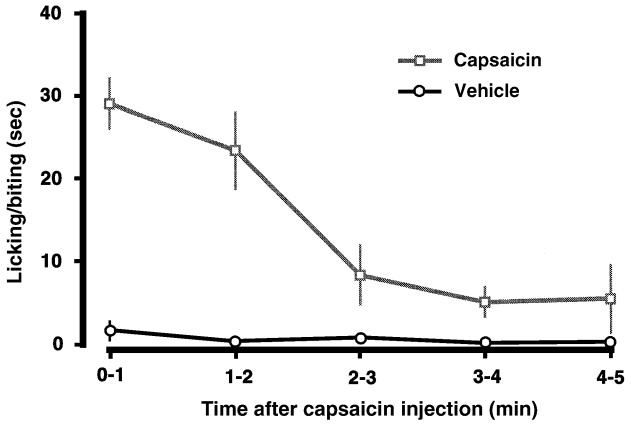

Fig. 1. Time course of paw-licking/biting response induced by injection of capsaicin into the right hindpaw in mice. Nociceptive behavior after intraplantar injection of capsaicin (1.6 μg/paw) was measured as the amount of time spent licking/biting the injected right hindpaw in mice. The data are given as the mean ± S.E.M. for groups of 10 mice.

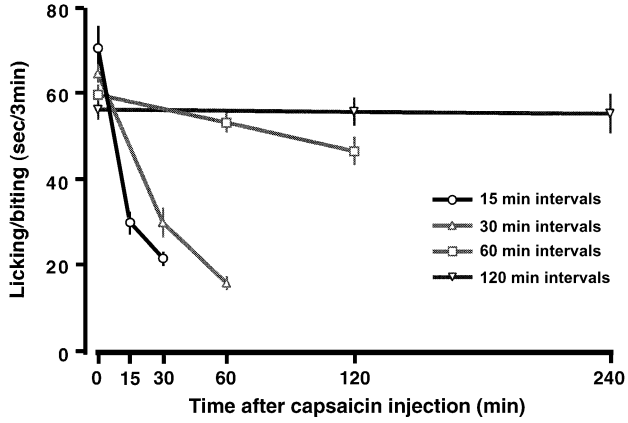

Fig. 3. Desensitization of liking/biting responses to three consecutive injections of capsaicin into the hindpaw in mice. All mice were treated with capsaicin (1.6 μg/paw) into the right hindpaw at intervals of 15, 30, 60, or 120 min. Licking/biting behavior after intraplantar injection of capsaicin (1.6 μg/paw) was measured as the amount of time spent licking/biting the injected right hindpaw at each time. The data are given as the mean ± S.E.M. for groups of 10 mice.

paw. The time course of the licking/biting after intraplantar injection of capsaicin shows that this nociceptive response peaked at 0–1 min and declined markedly at 2–3 min postinjection (Fig. 1). A similar injection of the vehicle control (containing 7.5% DMSO in saline) was without effect. Therefore, the licking/biting response induced by capsaicin was measured for 3 min to see the effects of capsazepine and ruthenium red.

Intraplantar coinjection of capsazepine (20–101 pmol), a competitive VR antagonist, resulted in a dose-dependent inhibition of the capsaicin-induced licking/biting response (Fig. 2). Similarly, ruthenium red in lower doses (8.9–30 pmol), coinjected into the plantar surface, inhibited the capsaicin-induced behavioral response in a dose-dependent manner.

3.2. Effects of intraplantar capsazepine and ruthenium red on capsaicin-induced desensitization

As shown in Fig. 1, 1.6 μg of capsaicin, injected into the plantar region, resulted in licking/biting response with a short duration. Three consecutive injections of capsaicin at 15- or 30-min intervals produced a progressive desensitization to this response (Fig. 3). A slight desensitization was observed in mice injected thrice with 1.6 μg of

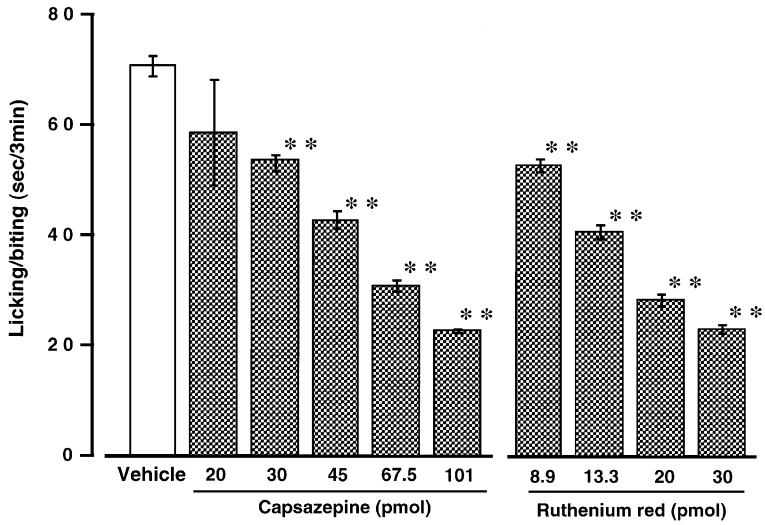

Fig. 2. Effect of capsazepine and ruthenium red on the licking/biting response induced by intraplantar injection of capsaicin in mice. Capsazepine or ruthenium red was coinjected with capsaicin (1.6 μg/paw) in a volume of 20 μl into the right hindpaw. Licking/biting behavior after intraplantar injection of capsaicin, in combination with capsazepine or ruthenium red, was measured for 3 min. The data are given as the mean ± S.E.M. for groups of 10 mice. $^{**}P < .01$, when compared to vehicle-injected control.

capsaicin at 60-min intervals. No desensitization to capsaicin following intraplantar injection at 120-min intervals was noted.

In further experiments, capsaicin was therefore injected into plantar paw at 15-min intervals, in combination with capsazepine or ruthenium red, to test their effects. As shown in Fig. 4A, intraplantar injection of capsaicin was able to desensitize the nociceptive response to a second and a third injection of capsaicin in combination with vehicle. The intraplantar coadministration of capsazepine (30 and 45 pmol) in moderate antinociceptive doses produced a significant protection from capsaicin-induced desensitization. Nociceptive responses induced by the administration of capsaicin, in combination with capsazepine (45 pmol), did not change significantly during three challenges with capsaicin. Ruthenium red, coinjected with capsaicin, had no substantial effects on capsaicin-induced desensitization; there were no significant alterations of licking/biting response to a second and a third challenge with capsaicin in mice coinjected with ruthenium red when compared to vehicle-treated mice (Fig. 4B).

4. Discussion

In the present study, we have confirmed our previous results that acutely administered capsaicin into the plantar surface of the paw elicited a vigorous licking/biting response toward the injected paw, indicating a nociceptive behavioral syndrome (Sakurada et al., 1992). The capsaicin test in mice has been employed to assess the antinociceptive effect of tachykinin neurokinin-1 receptor antagonists, glutamate receptor antagonists, nitric oxide (NO) synthase inhibitor, and morphine (Sakurada et al., 1993a,b, 1994, 1996a,b, 1998). It has been proposed that capsaicin-induced nociceptive response may be mediated by the activation of the capsaicin receptor, also known as the VR1, with a ligand-gated nonselective cation channel in primary sensory neurons (Caterina et al., 1997; Tominaga et al., 1998). In vitro studies on capsaicin-evoked activation of nociceptive neurons have indicated that capsaicin acts on VR1 in sensory neurons (Szallasi and Blumberg, 1993) to induce the influx of cations, particularly Ca^{2+} and Na^{+} ions, through the channel closely coupled to the VR1 (Bleakman et al., 1990; Docherty et al., 1991; Wood et al., 1988). In the

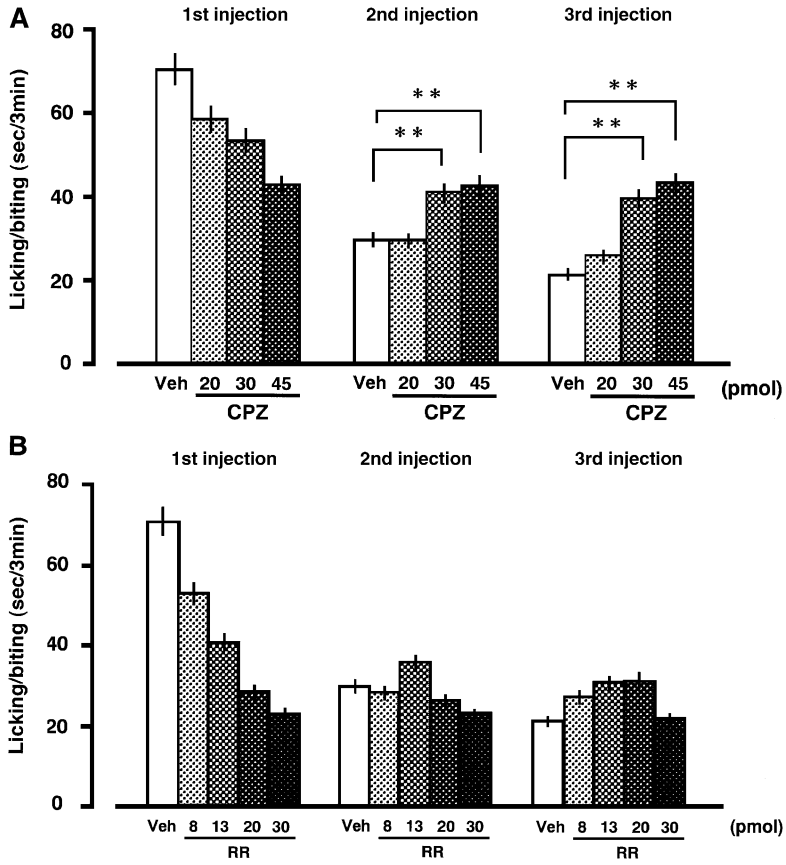

Fig. 4. Effect of capsazepine (A) and ruthenium red (B) on the licking/biting response induced by consecutive injections of capsaicin. Capsazepine or ruthenium red was coinjected with capsaicin (1.6 μg/paw) in a volume of 20 μl into the right hindpaw in mice. Licking/biting behavior after intraplantar coinjection of capsazepine or ruthenium red with capsaicin was measured for 3 min. The data are given as the mean ± S.E.M. for groups of 10 mice. $**P<.01$, when compared to vehicle-injected control. CPZ = capsazepine; RR = ruthenium red; Veh = vehicle.

current study, the characteristic behavioral response evoked by intraplantar capsaicin was antagonized by coadministration of capsazepine, a competitive VR antagonist, indicating an interaction with the VR in the peripheral nerve terminals. This indication corresponds with a recent study that the distribution of expression of VR1 protein is found in the terminals of afferent fibers projecting to the superficial layers (laminae I and II) of the dorsal horn (Tominaga et al., 1998). There are also immunohistochemical studies that VRs are distributed from the peripheral terminals to the central endings (Szallasi, 1995), and VR1-like immunoreactivity is also detected in the peripheral processes of primary afferent neurons as well as the central sensory neurons (Guo et al., 1999). The effect of capsazepine on capsaicin-induced activation of nociceptors has been examined in vitro and in vivo, and has been found to antagonize the effects of capsaicin in a selective and competitive manner (Dickenson and Dray, 1991; Urban and Dray, 1991; Perkins and Campbell, 1992; Bevan et al., 1992). There is an electrophysiological study that intradermal capsazepine into the peripheral receptive field antagonizes the initial increase in C-fiber-evoked activity and the subsequent inactivation of C-fibers evoked by injection of capsaicin (Dickenson and Dray, 1991). Therefore, it seems evident that capsaicin-induced nociceptive behavior can be blocked by capsazepine via the VRs at the peripheral level.

Although capsazepine is able to prevent capsaicin-induced antinociception in acute (paw pressure, tail flick, and hot plate) and chronic (knee joint hyperalgesia) nocifensor models, systemically administered capsazepine had no antinociceptive or anti-inflammatory activity (Perkins and Campbell, 1992). However, Santos and Calixto (1997) have shown that intradermal injection of capsazepine inhibits the early-phase paw licking of formalin-induced response as well as capsaicin-induced nociceptive response, whereas capsazepine does not affect formalin-induced late-phase response. They also found that intracerebroventricular injection of capsazepine is ineffective in these chemical nociceptive models. These data are in line with the present result that intraplanar coadministration of capsazepine produced a dose-dependent antinociceptive activity in the capsaicin test. Taken together, the present results indicate that capsazepine could be effective in reducing the nociceptive response in the chemically induced acute nociceptive response, but not in the pressure and thermal stimuli.

There are a considerable number of studies which found out that ruthenium red inhibits capsaicin-induced stimulation of primary afferents (Szallasi and Blumberg, 1999). The data obtained in the present study show that ruthenium red reduced the licking/biting response to intraplantar capsaicin in a dose-dependent manner. These results are in agreement with a previous report that ruthenium red was three- to fourfold more potent than capsazepine in producing antinociception, as assayed by the capsaicin test (Santos and Calixto, 1997). Ruthenium red also appears to have a relatively selective blocking action on the induction of Ca^{2+} and Na^{+} influxes following activation of VR1 receptors by capsaicin (Bevan and Geppetti, 1994; Wood et al., 1988), whereas conventional blockers for Ca^{2+} and Na^{+} ion channels are ineffective (Amann et al., 1990; Dray et al., 1990a,b; Maggi et al., 1989). It has been shown that ruthenium red, a noncompetitive antagonist with the property of blocking transmembrane Ca^{2+} and mitochondrial Ca^{2+} sequestration, inhibits the capsaicin-mediated excitatory effects on sensory neurons and peripheral nociceptors (Dray et al., 1990a,b). This may be the basis for antagonizing effects of ruthenium red on capsaicin-induced nociceptive response at the peripheral level.

The present results also show that consecutive injections of capsaicin in a 15-min interval to the plantar surface of a hindpaw induced a progressively reduced sensitivity to further capsaicin. However, the second and third injections of capsaicin (1.6 μg/paw) at intervals of 60 or 120 min did not induce desensitization, indicating that the dose of capsaicin employed in the present experiment is not in the range of capsaicin-induced neurotoxic action. These results seem to be compatible with a previously reported data that desensitization to intraplantar capsaicin may be involved in the reversible reduction of neurogenic inflammation produced by repeated topical administration of capsaicin to the skin in rats (McMahon et al., 1991). This should be distinguished from a selective sensory neurotoxicity brought about by exposure to high concentrations of capsaicin (Holzer, 1991; Wood et al., 1988). Considerable evidence indicates that repeated and prolonged applications of capsaicin induce a reduced activity of sensory neurons (Buck and Burks, 1986). This phenomenon, referred to as desensitization, appears to arise from desensitization of the VR1 receptor, a nonselective cation channel. In particular, Ca^{2+}-dependent processes contribute to capsaicin-induced desensitization and neuronal degeneration (Cholewinski et al., 1993; Chard et al., 1995; Liu and Simon, 1996). Indeed, capsaicin-induced desensitization is blocked in the absence of extracellular Ca^{2+} in rat dorsal root ganglion (Cholewinski et al., 1993; Koplas et al., 1997) or sensory fibers (Santicioli et al., 1987), and VR1-transfected cells (Caterina et al., 1997). In addition to the presence of extracellular Ca^{2+}, it seems probable that activation of calcineurin, a key intracellular protein, may be involved in the elicitation of capsaicin-induced desensitization (Koplas et al., 1997; Docherty et al., 1996).

In the present study, capsaicin-induced desensitization was prevented dose-dependently by coadministration of capsazepine at doses that were less than those used for inhibiting capsaicin-induced nociception. Based on these previously reported data, it is reasonable to consider that the desensitization in response to intraplantar capsaicin is blocked via VR1 receptors. In contrast, ruthenium red could not prevent desensitization of nociceptive response to intraplantar capsaicin. The differential effect of ruth-

enium red led us to assume that nociception induced by a single injection of capsaicin into the paw and desensitization by consecutive injections of capsaicin may have the participation of distinct mechanisms in the primary afferents. There are contradictory pieces of evidence stating that ruthenium red prevents desensitization of capsaicin-induced stimulatory functions in vitro (Maggi et al., 1988; Chahl, 1989; Dray et al., 1990a,b), and also reduces the desensitizing effect of capsaicin on the release of neuropeptides, substance P, and calcitonin gene-related peptide (CGRP) at peripheral endings of primary afferents in vivo (Amann et al., 1990). Recent reports dealing with ruthenium red have demonstrated that this compound is selective for the site of resiniferatoxin (RTX), an ultrapotent capsaicin analogue, on VRs since capsaicin-induced desensitization is inhibited by ruthenium red only at doses capable of blocking the uptake of Ca^{2+} into rat dorsal root ganglion neurons (Acs et al., 1997). It is noteworthy to compare previous studies stating that desensitization, in response to RTX, is independent of external Ca^{2+}, which is in contrast to capsaicin-induced desensitization, and that the capsaicin-antagonistic action of ruthenium red showed no dependence on external Ca^{2+}. Taken together, it is assumed that ruthenium red may readily antagonize desensitization in response to RTX rather than capsaicin. This assumption is supported by a possible existence of two pharmacologically defined classes of VRs, RTX-type and capsaicin-type (Acs et al., 1997). However, the action mechanism of ruthenium red still remains to be elucidated.

In conclusion, the present study provides evidence that licking/biting response induced by injection of capsaicin into the paw was inhibited by the competitive capsaicin receptor antagonist, capsazepine, and the rather nonspecific cation-channel blocker, ruthenium red. The capsaicin-induced desensitization was prevented by cointraplantar injection of capsazepine, whereas ruthenium red was without effect. These results suggest that desensitization to capsaicin-induced nociceptive response may be mediated via a capsazepine-sensitive VR and a ruthenium red-insensitive pathway.

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