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Effect of ricinoleic acid in acute and subchronic experimental models of inflammation

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Observational studies indicate that topical application of ricinoleic acid (RA), the main component of castor oil, exerts remarkable analgesic and anti-inflammatory effects. Pharmacological characterization has shown similarities between the effects of RA and those of capsaicin, suggesting a potential interaction of this drug on sensory neuropeptide-mediated neurogenic inflammation. The aim of this study was to assess RA anti-inflammatory activities in comparison with capsaicin in several models of acute and subchronic inflammation. The acute inflammation was induced by intradermal injection of carrageenan in the mouse or by histamine in the guinea-pig eyelid. In either experiment, the extent of the oedema thickness was measured. Subchronic oedema was induced by complete Freund's adjuvant injection in the ventral right paw of mice. Tissue substance P (SP) was measured in the carrageenan experiments by radioimmunoassay (RIA). It was found that the acute topical application of RA (0.9 mg/mouse) or capsaicin (0.09 mg/mouse) significantly increased the mouse paw oedema induced by carrageenan, while an 8-day repeated topical treatment with the same doses of both compounds resulted in a marked inhibition of carrageenan-induced paw oedema matched by a reduction in SP tissue levels. Similar effects were found against histamine-induced eyelid oedema in guinea-pigs after acute or repeated application of RA or capsaicin. RA and capsaicin given for 1–3 weeks reduced the established oedema induced by Freund's adjuvant, a subchronic model of inflammation, particularly if given by the intradermal route. Either in mouse paw or in guinea-pig eyelid, capsaicin but not RA by itself produced a slight hyperemia and activation of a behavioural response (e.g. scratching of the eyelids). On the basis of the present results, RA may be seen as a new capsaicin-like, non-pungent anti-inflammatory agent suitable for peripheral application.

Key words: Capsaicin, Carrageenan, Castor oil, Inflammation, Ricinoleic acid, Substance P (SP)

Introduction

Ricinoleic acid (RA; [R-(Z)]-12-hydroxy-9-octadecenoic acid) is the main component of castor oil, accounting for about 90% of the total. Preliminary experiments in our laboratories indicated that RA, known for its laxative properties,[1] could have a pro- or anti-inflammatory action following acute or repetitive local application, respectively. These activities closely resemble those previously described for capsaicin or capsaicinoid analogues called vanilloids (olvanil, resiniferatoxin, scutigeral).[2–4] On the other hand, part of the RA chemical structure has been used as a basis for the development of novel capsaicin-like compounds.[5] The cloning of the vanilloid receptor/ion channel, termed VR1 is very recent[6] and it has been shown that VR1 is activated by capsaicin and a number of other capsaicin-like drugs (vanilloid receptor agonists). Vanilloids specifically act through the activation and then the desensitization of a subset of primary afferent nerves involved in the genesis of neurogenic inflammation.[7] Notably their acute pro-inflammatory effect is due to the stimulation of the release of sensory neuropeptides from peripheral axons of these nerves. In a subsequent phase there is a desensitization of these fibres and, if the dose and the duration of exposure to the agonists are appropriate, a depletion of the neuropeptide content of these nerves occurs.[8] Desensitization and/or neuropeptide depletion determines an anti-inflammatory effect blocking the induction of neurogenic inflammation by endogenous or exogenous inflammatory stimuli.[7]

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Noteworthy is the fact that we found that RA is devoid of the pungent properties typical of capsaicin and that it does not exert any hyperalgesic effect towards heat and chemical nociceptive stimuli (Vieira *et al.*, submitted – ref. 9). These similarities and the differences (lack of pungent properties) with capsaicin render RA a promising analgesic and anti-inflammatory agent via its local application. Long-term topical capsaicin application in rats has been shown to produce a reversible impairment of the primary afferent fibres in the skin.[10]

In view of the above, we have assessed the effect of acute or repetitive RA topical treatment in several models of both acute and subchronic inflammation.

Materials and methods

Animals

Male albino Dunkin–Hartley guinea-pigs (250–350 g) from Charles River (Calco, Italy) and male Swiss mice (20–25 g) from Harlan Nossan (Correzzana, Milan, Italy) were used. The animals were fed with a standard diet and water *ad libitum*. The experiments were performed in respect of the *Principles of Laboratory Animal Care* (NIH publication no. 85–23, revised 1985) and in accordance with the Italian Health Ministry guidelines for the care and use of experimental animals.

Oedema induction in the paw of mice

Paw oedema was induced by intradermal (i.d.) injection in the right paw of 300 μg/0.04 ml of carrageenan. The thickness of the paw was measured in mm using a micro-calibrator. In acute experiments, the paw thickness was measured before and 1, 2, 3, 4, 5 and 6 h after the carrageenan injection. In chronic experiments, the progression of oedema was assessed on the first day and the eighth day each hour during the 6-h period following the first or second carrageenan injection, respectively. Single or repeated (8 days) topical applications of vehicle (peanut oil), RA (0.9 mg/mouse) or capsaicin (0.09 mg/mouse), at doses chosen in preliminary experiments, were applied on the ventral surface of the right paw. The first and the last application were made 30 min before the injection of carrageenan.

In other experiments, the repeated topical application (8 days) of vehicle (peanut oil), RA (0.9 mg/mouse) or capsaicin (0.09 mg/mouse) or the repeated (4 days) i.d. administration of RA potassium salt (0.03 mg/mouse) or capsaicin (0.003 mg/mouse) on the ventral surface of the right paw was tested in the presence of complete Freund's adjuvant (30 μl), injected in the hind-paw on the first day. The paw volume was monitored once a week for 3 weeks as described above.

Eyelid oedema induction in guinea-pigs

Eyelid oedema was induced by i.d. injection of 10 μg/0.1 ml of histamine in the right superior eyelid of animals under a short anaesthesia by diethyl ether. The thickness of the eyelid was measured in mm using ophthalmic micro-callipers (Dixey, UK). In acute experiments, the eyelid thickness was measured before and 1, 2, 3, 4, 5 and 6 h after the histamine injection or maximal applicable doses of RA (100 mg/guinea-pig) and capsaicin (10 mg/guinea-pig).

In subchronic experiments, the animals were treated topically for 8 or 22 days with vehicle (peanut oil), RA (0.9 mg/guinea-pig) or capsaicin (0.09 mg/guinea-pig), and the progression of the oedema was assessed on the first day during 6 h following the first histamine injection and then the eighth and the 22nd day each hour for a 6-h period after the second injection of histamine.

Radioimmunoassay (RIA)

For the tissue peptide measurements, the paws were rapidly removed and weighed. The samples were extracted with 3 ml of 2 N acetic acid at 95°C for 15 min. They were then homogenized and centrifuged at 10 000 ***g*** for 30 min at 4°C, the supernatants were freeze-dried and stored at –20°C until their content of substance P-like immunoreactivity (SP-LI) was determined by RIA. Lyophilized samples were reconstituted in 10 ml of 50 mM phosphate buffer (pH 7.4), containing 0.3% bovine serum albumin and 10 mM ethylene diamine tetraacetic acid (EDTA). The RIA assay for SP-LI was based on scintillation proximity assay technology, as previously described.[11] The incubation mixture was composed of 100 μl of the reconstituted sample (diluted 1:30), 100 μl of ^{125}I-labelled tracer (about 6000 cpm), 100 μl of diluted antiserum (1:100 000 for SP) and 50 μl of scintillation proximity assay protein A reagent. After an overnight incubation at room temperature under gentle agitation, the samples were counted in a β-scintillation counter (2200CA, Canberra-Packard, USA).

Chemicals

RA (99% pure; Sigma) was diluted with peanut oil (Sigma); its soluble potassium salt (MEN 11938 or potassium [R-(Z)]-12-hydroxy-9-octadecenoate from Chemistry Department, Menarini Ricerche spa., Pomezia, Italy) was used for i.d. treatment dissolved in saline. Capsaicin (Serva) was dissolved in 10% ethanol, 10% tween 80 and 80% saline solution and applied locally in peanut oil. Histamine (Sigma) was dissolved in saline, carrageenan (type II, Sigma) in sterile phosphate buffer solution (PBS; Sigma). Complete Freund's adjuvant was purchased from Sigma. [^{125}I]- Bolton Hunter SP and scintillation proximity

assay reagents were obtained from Amersham. The rabbit anti-SP serum (RAS 7451, cross-reactivity < 5% with SP 7–11, < 0.01% with neurokinin A (NKA) was from Peninsula Laboratories.

Statistical analysis

The data are expressed as means ± standard error of the mean (SEM). The statistical significance between groups was assessed using one-way analysis of variance followed by Bonferroni's test. The Mann–Whitney *U* test was used for the "*in vitro*" experiments.

Results

Effects of acute and subchronic topical treatment with RA and capsaicin on carrageenan-induced paw oedema in mice

As shown in Fig. 1, paw oedema induced by carrageenan was markedly increased by the topical application of RA (0.9 mg/mouse) or capsaicin (0.09 mg/mouse). The enhancement by RA or capsaicin of the carrageenan-induced oedema reached its maximum between the second and fourth hours and then decreased over time, but still remained significant at the sixth hour as compared with the vehicle-treated paws (Fig. 1). No enhancement of carrageenan-induced oedema was detected with the vehicle (n = 5) or with a solution at the same pH of 100 mg of RA (n = 5). Topical administration of vehicle, RA up to 10 mg/10 µl or capsaicin up to 1 mg/10 µl did not produce *per se* any oedema in the paws of the mice.

The treatment with RA (0.9 mg/mouse) for 8 days did not result *per se* in any oedematous effect or hyperemic reaction. Conversely, during the first days of treatment, capsaicin (0.09 mg/mouse) produced a slight hyperemic reaction that disappeared within 1–2 h from treatment. On the eighth day, the carrageenan-induced oedema was virtually abolished in RA or capsaicin pre-treated animals (Fig. 2). Thus, the mice were unable to produce any oedema in response to carrageenan.

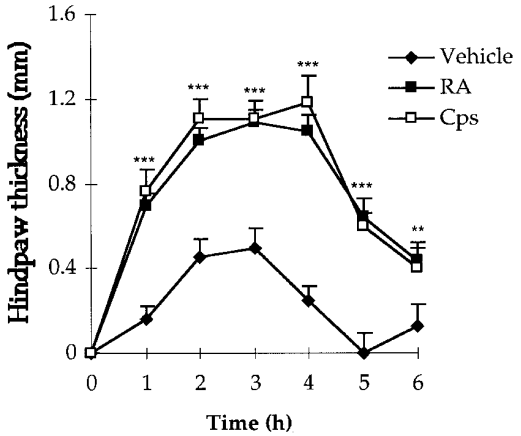

FIG. 1. Effects of vehicle, ricinoleic acid (RA, 0.9 mg/mouse) or capsaicin (Cps, 0.09 mg/mouse) on carrageenan-induced paw oedema expressed in mm. Ricinoleic acid, capsaicin and the vehicle were applied topically 30 min before carrageenan injection. Data are mean ± standard error (SE). n = 5–8 for each group. $^{**}p < 0.01$; $^{***}p < 0.001$ versus vehicle group.

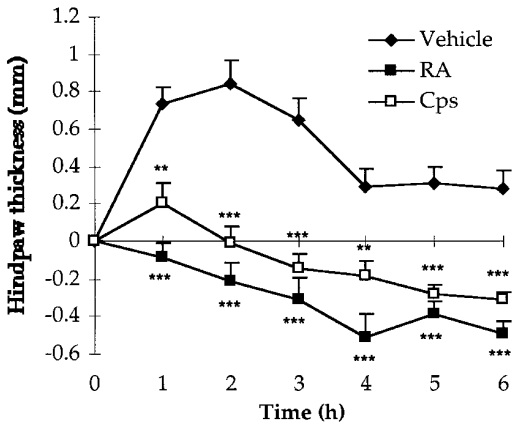

FIG. 2. Carrageenan-induced oedema of mouse paw. Effects of 8-day treatment with ricinoleic acid (RA, 0.9 mg/mouse) or capsaicin (Cps, 0.09 mg/mouse). Data are mean ± standard error (SE). n = 5–8 for each group. $^{**}p < 0.01$; $^{***}p < 0.001$ versus vehicle group.

Effect of topical and i.d. treatment with RA or capsaicin on Freund's adjuvant-induced paw oedema in mice

The intraplantar injection of Freund's adjuvant in one of the hind-paws leads to localized, unilateral inflammation. As shown in Table 1, the paw oedema induced by Freund's adjuvant peaked at the first 2 weeks after the induction and then declined. Topical treatment with RA for 8 days was effective in reducing the oedema formation at 1 week observation time. On the second and third weeks after the oedema induction, RA did not affect the increase in thickness of the paws as compared with the vehicle-treated group. Capsaicin in the applied dose did not induce significant inhibition in the oedema evoked by Freund's adjuvant (Table 1).

Conversely, the i.d. administration of the potassium salt of RA (MEN 11938) was able to exert a marked anti-inflammatory effect that lasted for 3 weeks (Table 1). The values of reduction in oedema formation were 68, 67 and 85% of control at 1, 2 and 3 weeks after Freund's adjuvant injection. Similarly, i.d. capsaicin significantly affected oedema formation, the significance of the effect lasting only for 2 weeks; the oedema was reduced by 50, 57 and 51% at 1, 2 and 3 weeks after its induction (Table 1). The i.d. injection of capsaicin (0.003 mg/mouse), but not RA (0.03 mg/mouse), produced nociceptive reactions during the first 2–3 days of treatment.

Table 1. Paw oedema induced by Freund's adjuvant in mice. Animals were treated by local application of ricinoleic acid (RA; 900 μg/mouse) or capsaicin (90 μg/mouse) for 8 days or intradermally administered for 4 days with MEN 11938 (30 μg/mouse), the potassium salt of RA, or capsaicin (3 μg/mouse). The paw oedema was measured once a week after the injection of Freund's adjuvant

	First week	Second week	Third week
Local application for 8 days			
Vehicle	1.39 ± 0.10	0.79 ± 0.05	0.60 ± 0.10
RA (900 μg/mouse)	0.82 ± 0.10**	0.61 ± 0.13	0.40 ± 0.07
Capsaicin (90 μg/mouse)	0.87 ± 0.13	0.70 ± 0.08	0.48 ± 0.09
Intradermal treatment for 4 days			
Vehicle	1.19 ± 0.09	1.20 ± 0.12	0.63 ± 0.10
MEN 11938 (30 μg/mouse)	0.38 ± 0.10**	0.40 ± 0.13**	0.09 ± 0.13*
Capsaicin (3 μg/mouse)	0.59 ± 0.09**	0.51 ± 0.08**	0.31 ± 0.15

Data are mean ± standard error (SE). n = 8–10 for each group. *p < 0.05; **p < 0.01 versus respective vehicle group.

Effect of topical application of RA or capsaicin on guinea-pig eyelid

The topical administration of capsaicin resulted in a local hyperemic response and activation of behavioural defensive responses including scratching of the eyelid. Neither hyperemia nor aversive behaviour was observed following RA topical treatment. A slight eyelid oedema with the peak at 2 h following application with high doses of RA (100 mg/guinea-pig, 0.23 ± 0.01 mm) or capsaicin (10 mg/guinea-pig, 0.35 ± 0.005 mm) alone was observed.

Effects of acute and subchronic topical treatment with RA and capsaicin on eyelid oedema induced by histamine in guinea-pig

The injection of histamine in the right eyelid rapidly induced an oedema that reached the maximum thickness at the first hour (Fig. 3). During i.d. injection, the animals showed aversive response behaviour, but no scratching response on the eyelid was usually observed. As shown in Fig. 3, the oedema was increased by the topical application of RA (0.9 mg/guinea-pig) and capsaicin (0.09 mg/guinea-pig).

The topical application of RA (0.9 mg/guinea-pig) for 8 days did not result *per se* in any oedematous effect or hyperemic reaction. Instead, capsaicin (0.09 mg/guinea-pig) produced hyperemic and painful reactions that disappeared 1–2 h after the treatment. The intensity of these responses gradually decreased starting from 2–3 days and was almost abolished at the end of the treatment. As shown in Fig. 4, the eyelid oedema induced by histamine was markedly reduced after 8 days of topical treatment with RA (0.9 mg/guinea-pig) or capsaicin (0.09 mg/guinea-pig). A similar profile of oedema reduction was also found after 3 weeks of topical treatment with RA (0.9 mg/guinea-pig) or capsaicin (0.09 mg/guinea-pig), the values of eyelid oedema at the peak (first hour) being: 1.37 ± 0.06, 0.70 ± 0.05 and 0.85 ±

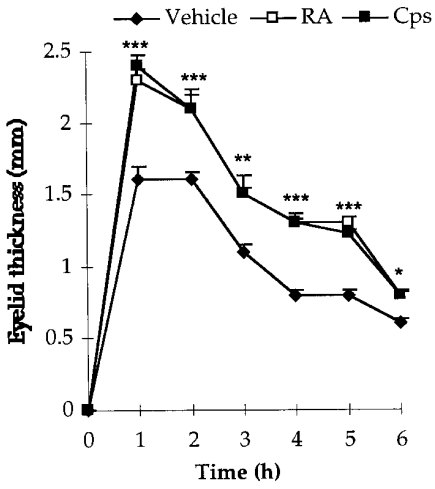

FIG. 3. Histamine-induced oedema of guinea-pig eyelid. Effect of vehicle, ricinoleic acid (RA, 0.9 mg/guinea-pig) and capsaicin (Cps, 0.09 mg/guinea-pig). Ricinoleic acid, capsaicin and the vehicle were administered topically 30 min before histamine injection. Data are mean ± standard error (SE). n = 5–8 for each group. *p < 0.05; **p < 0.01; ***p < 0.001 versus vehicle group.

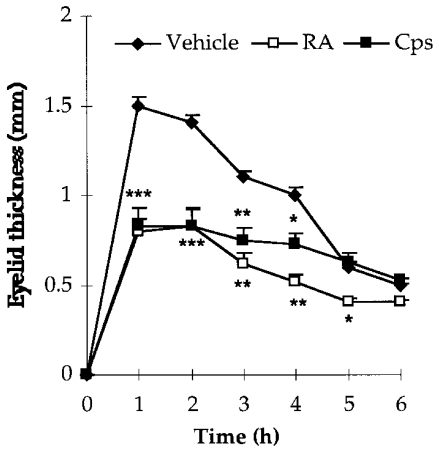

FIG. 4. Histamine-induced oedema of guinea-pig eyelid. Effect of 8-day treatment with ricinoleic acid (RA, 0.9 mg/guinea-pig) or capsaicin (Cps, 0.09 mg/guinea-pig). Data are mean ± standard error (SE). n = 5–8 for each group. *p < 0.05; **p < 0.01; ***p < 0.001 versus vehicle group.

0.05 mm for vehicle-, RA- and capsaicin-treated groups, respectively.

Effect of repeated treatment of RA and capsaicin on mouse paw SP levels

The levels of SP were not different in carrageenan-inflamed and non-inflamed paws (1.30 ± 0.28 and 1.09 ± 0.34 pmol/g tissue). The repeated (8 days) local administration of RA, at doses able to produce marked anti-inflammatory effects (see above), reduced the SP content by 58% in the inflamed paw of mice (0.54 ± 0.12 pmol/g tissue; $p < 0.05$ as compared with the inflamed paw treated with vehicle). Repeated topical capsaicin administration did not significantly reduce SP paw levels (0.73 ± 0.14 pmol/g tissue).

Discussion

The main result of this study is that RA topically administered for 8 days exerts a marked anti-inflammatory effect in several models of inflammation. The anti-inflammatory effect was comparable with that obtained with a 10-fold lower dose of capsaicin.

RA, when administered acutely, produced an additive inflammatory effect on carrageenan- or histamine-induced oedema in mice or guinea-pigs, respectively, but at a variance with capsaicin, acute topical RA administration did not produce any nociceptive or hyperemic reaction. Capsaicin is known to induce neurogenic inflammation (e.g. vasodilatation, protein extravasation and oedema) through an acute release of vasoactive peptides, such as SP, neurokinin A and calcitonin gene-related peptide (CGRP) from sensory nerve terminals.[12–14] It is noteworthy that capsaicin-induced oedema or plasma protein extravasation is almost completely absent in genetically tachykinin-deficient mice.[15] A release of tachykinins from sensory terminal nerves leading to an activation of their receptors present on the skin surface may explain the acute additive inflammatory effect of RA in our experimentally induced oedemas.

Although carrageenan injection leads to the generation of a range of inflammatory mediators, the noticeable reduction of paw oedema thickness induced by the NK_1 receptor antagonist CP 96.345 in rats[16] and the almost complete disappearance of paw oedema in mice induced by the other NK_1 receptor antagonist CP 122,721 (Vieira *et al.*, unpublished observations) support the involvement of SP in this inflammatory oedema. Furthermore, in NK_1 receptor knockout mice the carrageenan oedema was significantly reduced.[17]

As concerns the anti-inflammatory effect of RA resulting from an 8-day treatment, a desensitization action on peripheral nerve endings, already described for capsaicin, may explain, at least in part, such a phenomenon. In keeping with the above, the nociceptive reactions observed during the first days only after capsaicin application or i.d. injection disappeared on the second or third day of treatment. It is known that repeated exposure to capsaicin results in a desensitization of chemosensitive sensory afferents,[18] depletion of sensory peptides such as SP[19,20] and a loss of local tissue reactions upon chemical irritation.[20] Desensitization of a flare reaction induced by a prolonged local exposure to capsaicin, i.d. bradykinin and histamine has been described in human skin.[21] The anti-inflammatory effect of RA and capsaicin found in our experimental conditions was more marked towards carrageenan than histamine; different mechanisms of action of these inflammatory agents can be responsible for these differences in desensitization. Histamine has been described to excite small diameter afferent neurones and evoke the release of neuropeptides from vasoactive local nerve endings.[22] It is interesting to note that paw oedema induced by histamine was abolished in capsaicin-denervated rats.[23] Our findings in the guinea-pig eyelid show that the histamine-induced oedema is also dependent on other mediators.

On the other hand, carrageenan paw oedema was blunted by the local application of RA or capsaicin and it was matched by a significant reduction of SP tissue levels at least after the local application of RA.

In contrast to what happens in acute inflammation, in subchronic inflammation induced by Freund's adjuvant, RA was barely more effective than capsaicin in reducing oedema formation. Freund's adjuvant is able to produce a relatively stable inflammation that was decreased partially by local application of RA and was markedly affected by i.d. injection of both RA and capsaicin. The more effective counteraction of the oedema induced by i.d. versus topical administration of both compounds is probably due to a stronger desensitization induced by this route. On the other hand, Freund's adjuvant-induced chronically inflamed tissue in the rat has been reported to be associated with changes in SP content in nerves supplying the inflamed paws[24] and systemic capsaicin administration reduced the oedema formation concomitantly with the reduction of SP in nerve tissues.[25]

The relevance of the data presented should be associated with the observation that RA does not possess the pungent and painful effect of capsaicin but maintains its anti-inflammatory activities. Topical capsaicin has shown therapeutic potential in the treatment of cutaneous disorders such as post-herpetic neuralgia, painful diabetic neuropathy, pruritus, psoriasis, post-mastectomy pain syndrome, vulvar vestibulitis,[26–29] but its utility appears to be limited primarily by its irritant properties. RA has the potential to be a new capsaicin-like substance endowed with anti-inflammatory effects on several models of inflammation without the pungent characteristics of capsaicin.

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