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European Journal of Pharmacology 378 (1999) 17–22

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Intravesical resiniferatoxin desensitizes rat bladder sensory fibres without causing intense noxious excitation. A c-*fos* study

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Received 25 February 1999; received in revised form 11 June 1999; accepted 18 June 1999

Abstract

In this study the desensitizing power of increasing concentrations of resiniferatoxin applied topically to the bladder mucosa, and the irritating properties of the most effective desensitizing dose, were determined with the aid of the spinal expression of the proto-oncogene c-*fos*. Desensitization was assessed by the decrease in the number of Fos-immunoreactive spinal neurons induced by the intravesical instillation of 1% acetic acid, when the latter was preceded by resiniferatoxin in concentrations between 1 and 1000 nM. Irritation, as shown by the noxious excitation of vesical sensory innervation, was measured by the c-*fos* response evoked by a single application of resiniferatoxin. As to the desensitizing power, resiniferatoxin produced a dose-dependent effect with a maximum at 100 nM, which decreased Fos-immunoreactive cell numbers to less than 10% of controls. No further decrease of c-*fos* activation occurred at 1000 nM. As to the irritating power, the saturation dose of resiniferatoxin (100 nM) produced a very weak c-*fos* activation in lumbosacral spinal cord segments. These data show that in an effective desensitizing concentration, resiniferatoxin is virtually devoid of nociceptive effects, in agreement with current clinical observations.

Keywords: Bladder; Desensitization; Vanilloid; Resiniferatoxin; Primary afferent

1. Introduction

Resiniferatoxin, which occurs in extracts of some plants of the genus *Euphorbia*, contains in its molecular structure the same homo-vanillyl group that occurs in capsaicin (Szallasi and Blumberg, 1989). This structural similarity is probably the reason why both compounds activate a special recognition site in the cell membrane of type C sensory neurons, generally known as the vanilloid receptor (Szallasi et al., 1994; Caterina et al., 1997). Such an activation causes immediate noxious excitation with local pain, followed by a prolonged state of desensitization or neuronal insensitivity to subsequent noxious stimulation (Szallasi and Blumberg, 1996).

Resiniferatoxin is an extremely potent vanilloid, exerting desensitizing effects in nanomolar concentrations (Szallasi and Blumberg, 1989; Maggi et al., 1990; Winter et al., 1990; Craft et al., 1993). In the particular case of the urinary bladder, desensitization, as shown in the rat by the decrease in aversive behavior associated with noxious bladder stimulation, occurred even following the intravesical administration of resiniferatoxin in concentrations as low as 10 nM (Craft et al., 1993, 1995). The desensitization was dose dependent, being higher and longer lasting in rats instilled with 100 nM resiniferatoxin instead of 10 nM (Craft and Porreca, 1994; Craft et al., 1995). The greater efficacy of higher resiniferatoxin concentrations was also observed in patients with detrusor hyperactivity in whom the instillation of 50–100 nM solutions caused clinical and urodynamic improvement for several months (Cruz et al., 1997b), whereas 10 nM resiniferatoxin provided alleviation for only 2 weeks (Lazzeri et al., 1997). However, neither of these experimental or clinical studies determined the saturation dose of resiniferatoxin for bladder desensitization.

In contrast to its desensitizing power, the irritating effect of resiniferatoxin is still a matter of controversy. Intravesical injection of even small desensitizing doses of resiniferatoxin in awake rats was immediately followed by

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00451-3

abdominal licking (Craft et al., 1993), with a decrease of the bladder capacity at which reflex micturition occurred (Ishisuka et al., 1995). Therefore, the desensitizing doses of resiniferatoxin were considered noxious to the animal. However, the instillation of 10–100 nM resiniferatoxin solutions in non-anaesthetized patients caused scarce or no discomfort (Cruz et al., 1997b; Lazzeri et al., 1997). This discrepancy between experimental and clinical findings led us to undertake the present investigation using a distinct experimental approach consisting of the analysis of the c-*fos* expression occurring in the rat spinal cord upon noxious stimulation. The degree of activation of this immediate early gene, as shown by the number of spinal sensory neurons immunoreactive for the Fos-protein, allows an accurate quantitative evaluation of the nociceptive input reaching the spinal cord along nonmyelinated primary afferent C-fibres (Hunt et al., 1987; Herdegen et al., 1991). As a matter of fact, the decline in sensory input following desensitization with intravesical capsaicin had been previously assessed quantitatively by us, revealing a dose-dependent decrease in Fos-immunoreactive cell numbers on subsequent noxious stimulation (Cruz et al., 1996).

In the present experimental study, we make use of the c-*fos* method to determine the saturation dose of resiniferatoxin for bladder desensitization using increasing concentrations from 1 to 1000 nM. The noxious excitatory (irritating) effect of the most effective desensitizing concentration of resiniferatoxin was evaluated by comparing the c-*fos* activation achieved with that produced by the solution vehicle or urethral catheterization. Preliminary results have been presented in abstract form (Cruz et al., 1997a).

2. Materials and methods

2.1. Animals and preliminary experiments

Adult female Wistar rats from the Gulbenkian Foundation Colony weighing 250 g were used. Resiniferatoxin was purchased from Sigma.

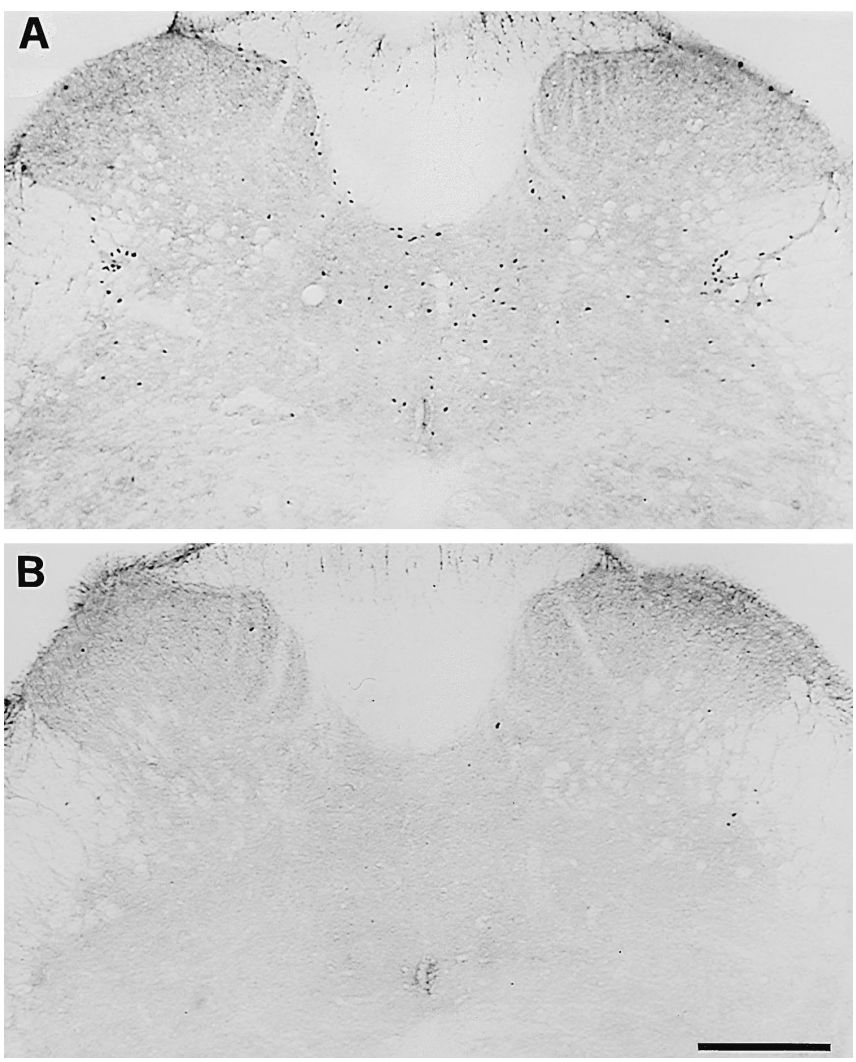

Fig. 1. Photomicrographs of the L6 spinal cord segment, showing the distribution of Fos-immunoreactive cells in the L6 spinal cord segment 2 h after bladder irritation with acetic acid. (A) Pretreatment with vehicle solution; (B) Pretreatment with 100 ml resiniferatoxin. Calibration bar = 250 μm.

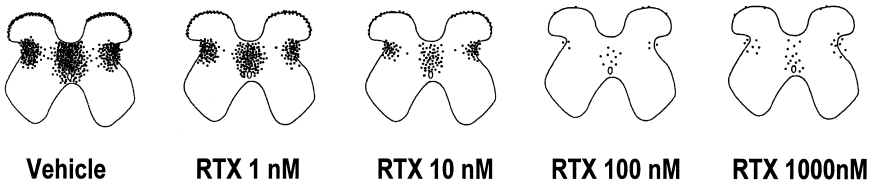

Fig. 2. Camera lucida drawings of the L6 spinal cord segment, showing the distribution of Fos-immunoreactive cells in 10 coronal sections following bladder irritation with acetic acid. Note the progressive decrease in the number of Fos-immunoreactive cells with the increasing concentration of resiniferatoxin (RTX). Each dot represents one Fos-immunoreactive cell.

A set of pilot experiments was designed to verify if the proto-oncogene was still expressed 24 h after stimulation. Under halothane anaesthesia (4% for induction and 2% for maintenance) the bladders of three rats were catheterized through the urethra with a polyethylene 22-gauge catheter and emptied. The bladder was filled with 0.5 ml of 1000 nM resiniferatoxin in 10% ethanol in saline for 30 min, after which it was emptied again and rinsed with saline, and the anaesthesia was withdrawn. All rats were anaesthetized intraperitoneally with 1 ml/kg of 35% chloral hydrate and perfused 24 h after the onset of experiments.

2.2. Bladder desensitization

Twenty rats were anaesthetized with halothane and the bladder was catheterized as above. Five groups of four animals each were instilled for 30 min with 0.5 ml of 1, 10, 100 or 1000 nM resiniferatoxin, or its vehicle (10% ethanol in saline). ln the end, bladders were emptied and rinsed with saline. Twenty-four hours later the animals were re-anaesthetized with chloral hydrate. The bladder was filled with 0.5 ml of 1% acetic acid for 15 min. Two hours after the onset of the experiment the rats were perfusion fixed.

2.3. Bladder irritation

Twelve rats were anaesthetized with intraperitoneal chloral hydrate after which the bladder was catheterized through the urethra and emptied. Two groups of four animals were instilled with 0.5 ml of 100 nM resiniferatoxin (previously determined as the most effective resiniferatoxin solution for desensitization) or with 0.5 ml of 10% ethanol in saline, the vehicle solution. Thirty minutes later, the solutions were withdrawn and the bladder was rinsed with saline. A third group of four rats was only catheterized through the urethra and the catheter was left in place for a similar period of time. Two hours after the onset of the experiments the rats were perfusion fixed.

2.4. Fixation, immunocytochemistry, counting and statistics

During all experiments the skin of the lower abdomen and upper thighs was protected with vaseline to minimize contact with the solutions instilled in the bladder. Perfusions were carried out through the ascending aorta with 100 ml of 0.1 M phosphate buffer-saline (PBS) followed by 1000 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Spinal segments L5–S1 were removed and immersed overnight in the same fixative and for 24 h in 30% sucrose in phosphate buffer. Each spinal segment was cut in a freezing microtome into 40-μm coronal sections and one in every three consecutive sections was immersed and stored in a cryoprotective solution at −20°C (Lu and Haber, 1992). When all animal experiments were concluded, the stored sections were washed with PBS and incubated in the same bath with a polyclonal antiserum against Fos protein raised in sheep (Genosys) and diluted at 1:6000. The immune reaction was visualized by the avidin–biotin (ABC) method (Vector). Fos-immunoreactive cells occurring in 10 sections taken at random from each spinal segment were plotted on diagrams of the Rexed's laminae and counted in each animal. Results are presented as the mean number ± standard deviation of cells occurring per 10 sections. Mean differences were compared by one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test for multiple comparisons using Statistica version 4.0 (Statsoft) software. Values of $P < 0.05$ were considered statistically significant.

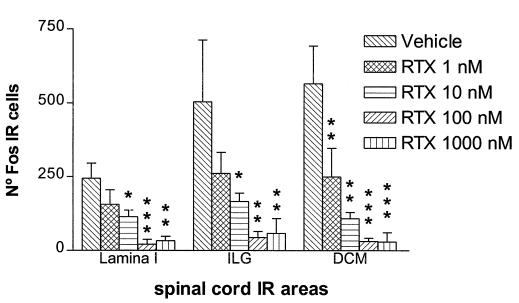

Fig. 3. Histogram showing the average number of Fos-immunoreactive cells counted in L5–S1 segments 2 h after irritation with acetic acid preceded by resiniferatoxin (RTX) treatment. Bars indicate standard deviation. Asterisks indicate statistical significance ($^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$) between mean values in resiniferatoxin- and vehicle-treated animals.

3. Results

3.1. Fos immunoreactivity 24 h after 1000 nM resiniferatoxin

At 24 h, no Fos immunoreaction could be detected. Therefore, a 24-h interval between resiniferatoxin instillation and the subsequent standard irritation with 1% acetic acid was enough to avoid superimposition between c-*fos* expression caused by the initial resiniferatoxin irritation and that due to 1% acetic acid.

3.2. Bladder desensitization (Fos immunoreactivity induced by 1% acetic acid 24 h after resiniferatoxin instillation)

Acetic acid instilled 24 h after treatment with the vehicle solution (10% ethanol in saline) induced intense bilateral Fos immunoreactivity at L5–S1, with a peak at L6. Positive cells were particularly numerous in the intermediolateral grey matter, in the dorsal commissure and along the lateromedial extension of lamina I (Fig. 1A and Figs. 2 and 3).

ln animals pre-treated with intravesical resiniferatoxin, acetic acid induced fewer Fos-immunoreactive cells in the three locations than in vehicle-treated animals (Figs. 2 and 3). This decrease was dose dependent (Figs. 2 and 3). The maximal effect occurred with 100 nM resiniferatoxin, amounting to an overall 93% decrease (Fig. 1B and Figs. 2 and 3).

3.3. Bladder irritation (Fos immunoreactivity induced 2 h after urethral catheterization, 10% ethanol or 100 nM resiniferatoxin)

Urethral catheterization induced a weak Fos immunoreaction in lamina I, the intermediolateral grey matter and the dorsal commissure (Fig. 4). Bladder instillation of 10% ethanol (resiniferatoxin vehicle) induced a similar Fos immunoreaction (Fig. 4). lnstillation of 100 nM resiniferatoxin caused a moderate increase in c-*fos* expression compared with catheterization or vehicle values in all spinal cord areas, the difference being statistically significant only in lamina I and the intermediolateral grey matter (Fig. 4).

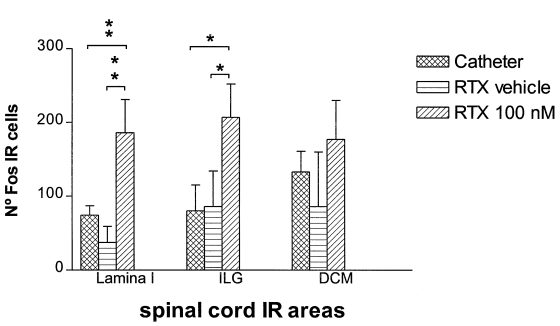

Fig. 4. Histogram showing the average number of Fos-immunoreactive cells counted in L5–S1 segments 2 h after catheterization, application of vehicle, or 100 nM resiniferatoxin (RTX). Bars indicate standard deviation. Asterisks indicate statistical significance ($^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$) between mean values.

4. Discussion

At the experimental level, irritation and desensitization of bladder afferents by a vanilloid have been estimated through the noxious input generated upon the initial administration and by its reduction during subsequent instillation of the same vanilloid or any other irritant, respectively. Methods included nociceptive behaviour (time spent licking the lower abdomen) in freely moving rats (Craft et al., 1993, 1995; Ishisuka et al., 1995), and the spinal c-*fos* response (Cruz et al., 1996; Avelino et al., 1998). In addition, bladder irritation and desensitization by vanilloids were evaluated by the contractile response of the isolated rat urinary bladder (Maggi et al., 1990) or by the change in bladder volume at which reflex micturition occurs in anaesthetized (Maggi et al., 1989, 1990) and non-anaesthetized rats (Ishisuka et al., 1994, 1995).

The c-*fos* technique used in this present study has been often used to investigate bladder nociception in anaesthetized animals. Previous studies have shown that following noxious bladder stimulation c-*fos* expression occurs in the lumbosacral spinal cord with a distribution in lamina I, the intermediolateral grey matter and the dorsal commissure (Birder and deGroat, 1992; Cruz et al., 1994), overlapping the area of termination of bladder sensory fibres (Morgan et al., 1981; Nadelhaft and Booth, 1984), most of which have been shown to be capsaicin sensitive (Jancsó and Maggi, 1987). However, what makes the c-*fos* technique highly adequate is its strict dependence upon noxious sensory input generated in the bladder and conveyed to the spinal cord in the capsaicin-sensitive subset of bladder afferents (Cruz et al., 1994; Cruz et al., 1996). In addition, c-*fos* levels and nociceptive behaviour (number of abdominal stretches) induced by a noxious visceral stimulus have been shown to exhibit a strong correlation in rats subjected to intraperitoneal acetic acid injection (Hammond et al., 1992). The c-*fos* method seemed particularly sensitive since it was capable of detecting low levels of noxious input before behavioural signs became manifest (Hammond et al., 1992).

Our present findings showing that bladder desensitization induced by intravesical resiniferatoxin is dose dependent confirms data from other investigators using the behavioural approach (Craft et al., 1995). We have, however, ascertained that desensitization was maximal and practically total at 100 nM, as this saturating concentration could not be enhanced by using 1000 nM. In addition, our data confirm previous conclusions on the high potency of resiniferatoxin (Maggi et al., 1990; Craft et al., 1995) since

at 100 nM it exerted the same desensitizing effect as a 10,000 times more concentrated (1 mM) capsaicin solution (Cruz et al., 1996).

As to the irritating power of resiniferatoxin, the best desensitizing dose, 100 nM, only produced about 2 times more Fos-immunoreactive cells than simple urethral catheterization in lamina I and the intermediolateral grey matter, and it had no effect on c-*fos* expression in the dorsal commissure. In contrast, 1 mM capsaicin increased the number of Fos-immunoreactive cells 10 times in lamina I, 5 times in the intermediolateral grey matter and 4 times in the dorsal commissure (Avelino et al., 1998). It is difficult to reconcile this relatively mild irritating effect of 100 nM resiniferatoxin, in particular in lamina I where most nociceptive specific neurons are located (Laird and Cervero, 1989), with the observation that the instillation of a similar solution reduced the bladder capacity of awake rats by about 50% (Ishisuka et al., 1995). However, in spite of this reduction, the rats in that study seemed to exhibit nociceptive behaviour only occasionally, as shown by the short time they spent licking the lower abdomen (Ishisuka et al., 1995).

The apparent contradiction between those data and our present findings may result from a different sensitivity of the two types of bladder C-afferent fibres to resiniferatoxin. About 70% of bladder fibres have a low threshold for response and encode intravesical pressure in the physiological range, which indicates a preferential role in micturition control (Sengupta and Gebhart, 1995). The remaining 30% are high-threshold fibres which only respond to high intravesical pressures in the noxious range, a characteristic that suggests a specific involvement in bladder nociception (Sengupta and Gebhart, 1995). Although both types were shown to be chemosensitive (Sengupta and Gebhart, 1995), resiniferatoxin administration may be sufficient to irritate the low-threshold fibres and trigger reflex micturition but insufficient to irritate high-threshold C-fibres and generate intense noxious input. Capsaicin (100 μM), could irritate both types, as shown by the simultaneous reduction of the bladder capacity by more than 75% with frequent licking of the lower abdomen (Ishisuka et al., 1995). In any case, the most reliable sign of noxiousness should come from human patients who can describe pain directly to the investigator. In point of fact, patients receiving 10 to 100 nM resiniferatoxin solutions intravesically without any form of bladder anaesthesia reported no unpleasant sensations or only slight discomfort in spite of reflex bladder contractions occurring at the beginning of treatment (Cruz et al., 1997b; Lazzeri et al., 1997). The same patients treated with 1 mM capsaicin reported a severe burning sensation in the lower abdomen accompanying reflex detrusor contractions (Cruz et al., 1997c).

Rat models of bladder desensitization may give clinically relevant information, as recently suggested by Craft et al. (1995) who commented on several similarities between the desensitizating power of similar capsaicin concentrations in rats and humans. The same parallelism seems to hold true for resiniferatoxin. A 10 nM resiniferatoxin solution, which attenuates nociceptive behaviour during 1 week in the rat (Craft et al., 1995), and reduces c-*fos* expression to 50% of controls as shown here, in addition produces short-lasting clinical improvement that does not last longer than 2 weeks (Lazzeri et al., 1997). Similarly, the desensitization provided by 100 nM resiniferatoxin was complete in the rat, as shown by behavioural or c-*fos* methods, and assured a long-lasting improvement, frequently exceeding 6 months, in hyperreflexic patients (Cruz et al., 1997b).

In conclusion, by making use of spinal c-*fos* expression it is shown that intravesical resiniferatoxin exerts a dose-dependent desensitizing effect on bladder sensory innervation. The maximal saturating concentration of 100 nM was almost devoid of acute irritative properties. lt is suggested that the clinical efficacy of an equivalent resiniferatoxin concentration in patients with detrusor hyperactivity (Cruz et al., 1997b) is due to the reduction of sensory transmission caused by this therapeutic agent.

Acknowledgements

This study was supported by funds from JNICT project PECS/C/SAU/185/95.

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