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Vanilloid receptor loss in rat sensory ganglia associated with long term desensitization to resiniferatoxin

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A dose-dependent loss of vanilloid receptors (specific [^{3}H]resiniferatoxin binding sites) was found in sensory ganglia of rats 24 h after s.c. administration of resiniferatoxin (RTX), an ultrapotent capsaicin analog. This receptor loss displayed an ED_{50} of 30 μg/kg both in dorsal root and trigeminal ganglia; the ED_{50} was 6-fold higher than the ED_{50} for loss of the neurogenic inflammatory response and 30–60-fold higher than the ED_{50} for desensitization in the standard eye-wiping (chemogenic pain) response. The receptor loss appeared later (24 h) than the loss of the physiological responses (6 h) and showed modest recovery (to 20–30% of control levels) over the following 4 weeks. This vanilloid receptor loss may represent a novel, specific mechanism for vanilloid-induced chronic desensitization.

Although for centuries capsicum has been used as a spice on account of its pungency, only recently have its biological effects been characterized in detail [1, 5, 12]. Capsaicin, the irritant component in capsicum, excites primary afferent neurons involved in neurogenic inflammation, thermoregulation and nociception [1, 5, 6]. This initial excitation is followed by loss of responsiveness to further stimuli [6]. The loss of responsiveness is generally termed 'capsaicin desensitization', although our emerging understanding of capsaicin action suggests that it represents functional impairment due to a combination of receptor desensitization, neuron defunctionalization and neurotoxicity [5].

We demonstrated that resiniferatoxin (RTX), a naturally occurring diterpene esterified with homovanillic acid at the C20 position, acts as an ultrapotent capsaicin analog [13, 17]. We used [^{3}H]RTX to identify specific RTX binding sites [14]. The tissue distribution and pharmacological specificity confirm that this specific [^{3}H]RTX binding represents the vanilloid (capsaicin) receptor [14, 17].

In the present study, we have used the [^{3}H]RTX binding assay to explore whether vanilloid receptor loss might play a role in the chronic desensitization that follows systemic RTX administration in the rat [16].

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Female Sprague–Dawley rats (200–250 g) were employed in our studies; animals were allowed access to food and water ad libitum during the course of the experiments. RTX (Chemicals for Cancer Research, Inc., Chaska, MN, USA) was dissolved in EtOH and administered s.c. into the scruff of the neck under ether anesthesia in a volume of 100 μl. Control animals received an equal volume of solvent. Animal care was provided by Washington Biotech., Inc. (Bethesda, MD, USA); animal experimentation protocols were approved by the institutional animal use committee.

Chemogenic pain perception was assessed in the standard eye-wiping assay [8]: 10^{-3} g/ml capsaicin in 10% EtOH/10% Tween 80/80% physiological saline was instilled into the right eye of rats and the number of protective movements (eye-wipings with the foreleg) was determined.

Neurogenic inflammation was quantitated by measuring extravasated Evans' blue in the skin [13] after the hind paw had been painted with xylene, an effective inducer of neurogenic inflammation [9].

Specific binding of [^{3}H]RTX (37 Ci/mmol; Chemical Synthesis and Analysis Laboratory, NCI-FCRF, Frederick, MD, USA) was examined in membrane preparations from trigeminal and dorsal root ganglia as described [14, 15]. Briefly, animals were sacrificed by decapitation under CO_2 anesthesia: the ganglia removed and collected into ice-cold buffer A containing (in mM)

KCl 5, NaCl 5.8, $MgCl_2$ 2, $CaCl_2$ 0.75, glucose 12, sucrose 137, HEPES 10, pH 7.4. Ganglia were homogenized with the aid of a Polytron tissue homogenizer. The homogenate was centrifuged at 1,000×*g* for 10 min, the pellet was discarded and the supernatant was centrifuged at 20,000×*g* for 15 min. The pellet was resuspended in buffer A, washed twice and finally resuspended at a concentration of approximately 1 mg protein per ml. 25–30 μg aliquots of the particulate fraction protein in 0.5 ml of buffer A containing 0.25 mg/ml bovine serum albumin (Sigma, Cohn fraction V) were incubated in triplicate for 10 min at 37°C with 60 pM [^{3}H]RTX in the absence or presence of 100 nM non-radioactive RTX to determine non-specific binding. Bound and free ligand were then separated by pelleting the membranes in a Beckman 12 microfuge; the tip of the microfuge tube containing the pelleted membranes was cut off with a razor blade; and the bound radioactivity determined by scintillation counting. In control ganglia specific [^{3}H]RTX binding was 47±7 fmol/mg protein (mean±S.E.M.; 8 determinations); non-specific binding represented 58±3% of the total binding (mean±S.E.M.; 8 determinations).

For neural counts, the left dorsal root ganglia of lumbar segments 1–3 from 3 rats per group were removed, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and embedded in paraffin. In every eighth section (10 μm thick, stained with Cresyl violet) all neurons containing nucleoli were counted, and the total neuron number calculated according to the method of Konigsmark [10].

Xylene-induced Evans' blue extravasation [9] and determination of protective wiping movements upon intraocular capsaicin instillation [8] are standard assays to characterize the efferent (neurogenic inflammation) and afferent (chemogenic pain perception) functions of capsaicin-sensitive sensory neurons: the loss of these responses has been in use for decades to measure capsaicin-induced desensitization [1]. In accord with our previous findings [13, 16], s.c. administered RTX caused a dose-dependent, complete loss of response in both assays (Fig. 1A,B). Desensitization of the ocular chemogenic pain pathway required less RTX (ED_{50}=0.3–1.0 μg/kg; Fig. 1A,B) than did the desensitization of the neurogenic inflammatory response (ED_{50}=5 μg/kg; Fig. 1A,B); and, in

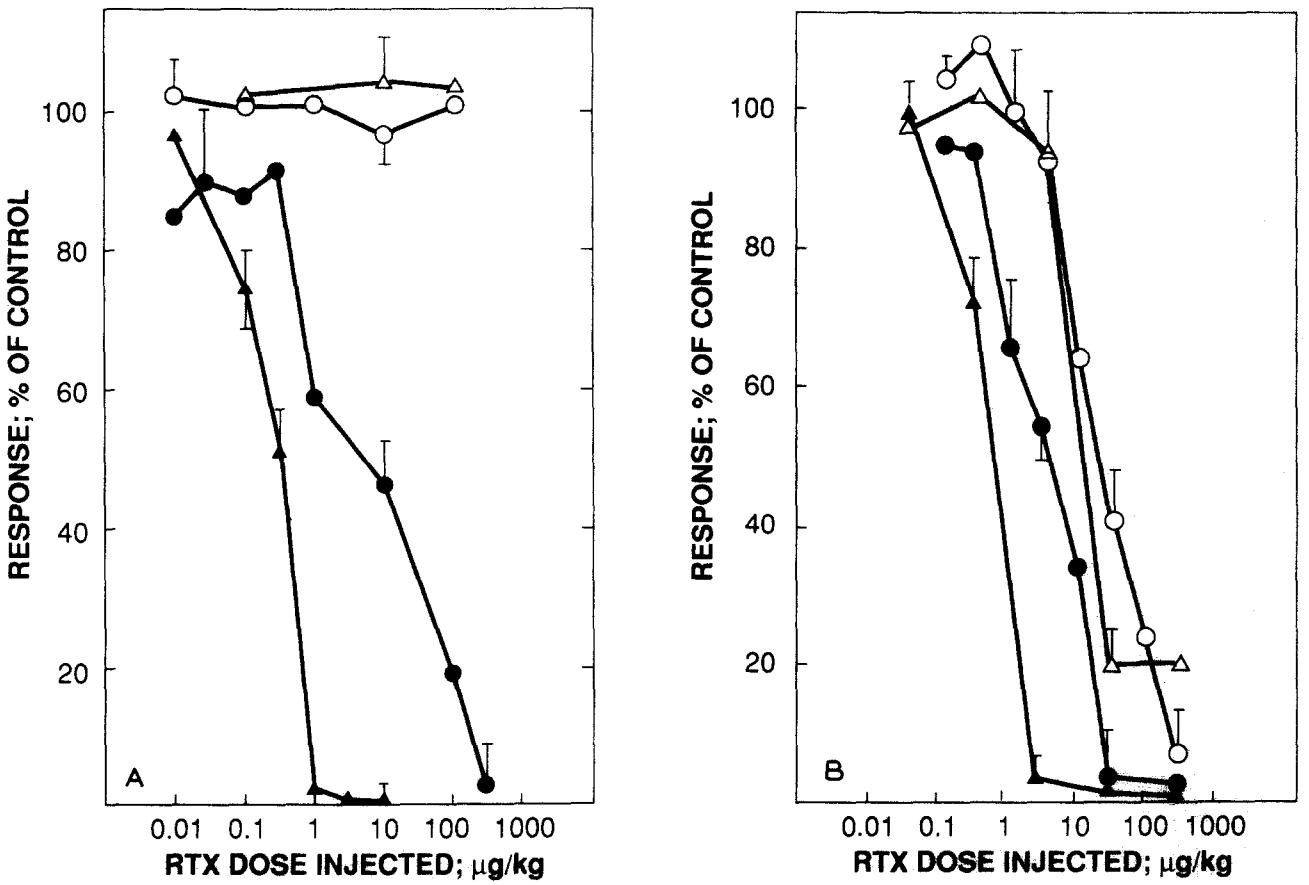

Fig. 1. Dose-dependence of the loss of the neurogenic inflammatory (●) and the chemogenic pain response (▲) as well as the loss of specific [^{3}H]resiniferatoxin binding sites in dorsal root (○) and trigeminal (△) ganglia of rats 6 h (A) or 24 h (B) after s.c. resiniferatoxin (RTX) administration. Each point represents the mean of at least 5 determinations±S.E.M. A second experiment gave similar results. Neurogenic inflammation was quantitated by measuring Evans' blue extravasation 5 min after xylene application. Chemogenic pain response was assessed by counting protective (eye-wiping) movements in response to capsaicin instillation into the eye.

contrast to the 60% recovery of the Evans' blue extravasation response in the paw, the eye remained fully refractory to capsaicin during the 4 weeks course of the experiment (Fig. 2). In sharp contrast to the absence of the eye-wiping response, rats from the same experimental group (28 days after treatment with 300 μg/kg RTX) *did* extravasate Evans' blue into the eye-lids in response to the 1% capsaicin solution indicating that efferent and afferent functions may recover differentially (data not shown).

Whereas the subacute (6 h) desensitization occurred without any detectable loss of vanilloid receptors (Fig. 1A), the chronic (24 h) desensitization was accompanied by an 80–90% loss of specific [^{3}H]RTX binding sites both in dorsal root and trigeminal ganglia (Fig. 1B). The receptor loss displayed an ED_{50} of 30 μg/kg in both ganglia, a dose 6-fold higher than the ED_{50} for the loss of the xylene-induced Evans' blue extravasation response and 30–60-fold higher than the ED_{50} for the loss of chemogenic pain perception (Fig. 1B). [^{3}H]RTX binding showed limited (20–30%) recovery up to the 28th day after treatment. All of the RTX-treated animals (300 μg/kg) remained in apparent good condition (active; normal appetite; no sign of clinical stress) during the whole length of the experiment, and the receptor loss could not be explained by gross neuron loss. We determined the number of dorsal root ganglia (DRG) neurons at 3 segmental levels (L_1–L_3) 28 days after treatment and found the neuron numbers to be similar in rats given RTX and those given vehicle only: 4,400±300 and 4,700±500, respectively (mean neuron number±S.E.M.).

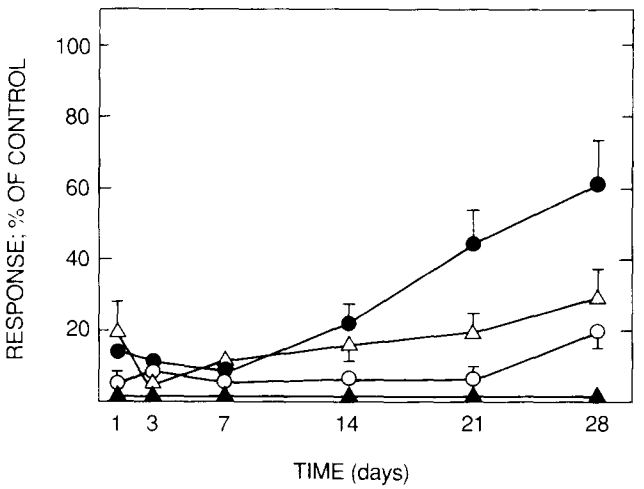

Fig. 2. Time course of desensitization with RTX (300 μg/kg s.c.) of the neurogenic inflammatory (●) and chemogenic pain responses (▲); recovery of [^{3}H]RTX binding in dorsal root ( ) and trigeminal ( ) ganglia. Each point represents 5–10 animals in a single experiment. Error bars indicate ± S.E.M.

The capsaicin-sensitivity of cultured sensory neurons was shown to depend on the presence of nerve growth factor (NGF) in the media [18]. It was speculated that the expression of the vanilloid receptor requires NGF, and that NGF deprivation due to the block by capsaicin of anterograd axonal transport may contribute to the functional impairment of sensory neurons [5, 18]. Nevertheless, our finding that vinblastine (3 mg/kg i.p.), an effective blocker of axonal transport [11], reduced the number of [^{3}H]RTX binding sites by less than 20% in DRG (data not shown) argues against a dominant role of NGF deprivation in the almost complete loss of binding sites following RTX treatment.

Mechanisms for agonist-induced apparent receptor loss include decreased affinity for the agonist, receptor compartmentalization, or a reduction in receptor number [4]. Scatchard analysis of [^{3}H]RTX binding in sensory ganglia of rats pretreated with 30 μg/kg RTX, the ED_{50} for receptor loss, indicated a corresponding reduction in the B_{max} value (25 fmol/mg protein as opposed to 60 fmol/mg protein in control rats; data not shown); the associated decrease in the specific binding prevented the quantification of the affinity of the residual receptors. In control ganglia we measured an affinity of 180±25 pM (mean±range; 2 determinations) in good agreement with our previous determinations (270 pM) [14]. As yet, no indication of receptor translocation from the particulate fraction to the cytosol has been found.

Capsaicin-induced desensitization not only affords a neuropharmacological probe to study sensory neuron functions [1, 5] but also provides a promising therapeutic approach in the relief of neuralgic pain [2, 3, 12]. The mechanisms underlying 'capsaicin desensitization' are poorly understood, but are thought to involve specific desensitization of the vanilloid (capsaicin) receptor as well as a cation influx through a receptor-coupled non-specific cation channel that, in a dose-dependent fashion, may cause neuron defunctionalization and neurotoxicity [5]. Functional impairment is likely to involve block of axon conduction and depletion of neurotransmitters [1, 5]. Whereas a number of indirect arguments supported the concept of short term vanilloid receptor desensitization [5], until recently no technique was available to distinguish between specific (receptor-linked) and non-specific components in chronic vanilloid-induced desensitization. The physiological significance of our finding that an apparent loss of vanilloid receptors accompanies the long term RTX-induced desensitization is unclear. The late onset (24 h) does not match the 4–6 h required for the loss of the physiological responses as well as for the full development of morphological changes [16]. Recep-

tor loss thus appears not to be involved in the initial loss of response, but it is an attractive candidate for a role in the specific chronic desensitization to vanilloid compounds.

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