

Resiniferatoxin, a potent capsaicin-like stimulator of peripheral nociceptors in the neonatal rat tail *in vitro*

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1 A spinal ventral root response was measured following the activation of peripheral fibres by noxious (heat at 48°C, capsaicin, bradykinin) and innocuous (brush) stimuli in a preparation of the neonatal rat spinal cord-tail maintained *in vitro*.

2 Following superfusion of the tail with 0.1–1.0 nM of the potent irritant, resiniferatoxin (RTX), brief, irregular depolarization and a selective loss of capsaicin sensitivity was produced. RTX 10–100 nM evoked a tonic response, initiated transient irregular depolarizations and desensitization to further applications of RTX and capsaicin but not to other stimuli. Following RTX 1 μM a prolonged loss of sensitivity to all noxious stimuli was produced.

3 When a selective desensitization to capsaicin was produced by a long application of capsaicin, RTX was also ineffective.

4 Superfusion of the tail with 4β-phorbol, 12, 13-dibutyrate (PDBu), a protein kinase C activator, stimulated capsaicin-sensitive peripheral fibres. Prolonged administration of PDBu attenuated or abolished further responses to PDBu and bradykinin but responses to RTX and capsaicin were unchanged. The protein kinase C inhibitor staurosporine (50–200 nM), attenuated the effects of PDBu and bradykinin but not those of RTX or capsaicin.

5 The present data suggest that neither RTX nor capsaicin act on peripheral nociceptors via a phorbol ester-like stimulation of protein kinase C. Rather, RTX acts on nociceptors by a similar mechanism to capsaicin. These effects may be the basis for the irritant properties of RTX and may further relate to the antinociceptive actions observed *in vivo*. RTX is therefore a potent new tool with which to investigate the properties of nociceptive neurones and provides a prototype for further development of antinociceptive agents.

Introduction

Resiniferatoxin (RTX), a diterpene derivative obtained from the plant family *Euphorbiaceae*, is a potent irritant whose mechanism of action is not understood (Hergenhahn *et al.*, 1974; Williamson *et al.*, 1980). RTX has structural analogy with other irritants, including tumour promoting phorbols (Evans & Schmidt, 1979) which produce their effects via the activation of cellular protein kinase C (Castagna *et al.*, 1982). RTX appears not to be a tumour promoter, is a poor activator of brain protein kinase C (Ellis *et al.*, 1987) and does not compete with phorbol esters for protein kinase C binding sites (Driedger & Blumberg, 1980). It is known that the 4-OH, 3-MeO phenylacetic acid substituent at C20 of RTX is an important determinant of its activity (Williamson *et al.*, 1980). This moiety also features in the structure of capsaicin, a compound known for its irritant and algescic properties. The actions of capsaicin have been studied more extensively and this vanillylamide appears to produce its effects via the selective activation of a subset of sensory neurones including polymodal nociceptors and warm thermoceptors (Fitzgerald, 1983; Buck & Burks, 1986; Bevan *et al.*, 1987; Szolcsanyi, 1989).

In the present study we have tested whether RTX activates peripheral nociceptors and whether its effects are produced via a capsaicin-like or phorbol ester-like mechanism of action. For these studies we have used an *in vitro* preparation of the neonatal spinal cord-tail (Yanagisawa & Otsuka, 1984; Dray *et al.*, 1988; 1989) where peripheral fibres may be activated by a number of noxious stimuli (Yanagisawa *et al.*, 1984; Fitzgerald, 1985; 1986) to evoke a spinal nociceptive response.

Methods

In these experiments activation of peripheral fibres in an *in vitro* preparation of the spinal cord with attached tail was assessed. The spinal cord and tail were isolated from 1–2 day old rats following decapitation. The most superficial layer of

the skin was carefully removed from the distal four fifths of the tail with fine forceps. This exposed cutaneous fibres and their endings to allow activation by bradykinin and to facilitate activation by capsaicin. Damage to deeper skin layers and tissue severely compromised chemical sensitivity. Both the spinal cord and tail were separately superfused with physiological salt solution of the following composition at 24°C (mM: NaCl 138.6, KCl 3.35, CaCl₂ 1.26, MgCl₂ 1.16, NaHCO₃ 21.0, NaH₂PO₄ 0.58, glucose 10) and equilibrated with 95% O₂, 5% CO₂. Activation of fibres in the tail by peripheral stimuli was assessed by recording the depolarizing response produced in a spinal ventral root (L₃–L₅). Recordings were made with a low impedance extracellular glass pipette filled with electrolyte and placed in an electrolyte-filled well containing the selected ventral root. The ventral root potential was recorded d.c. with respect to the spinal cord which was earthed. Signals were amplified by conventional means and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder. Drugs were administered in the tail superfusate, usually for a 10 s period to test for agonistic properties or for a 5 min period to study desensitization. The noxious heat stimulus consisted of a 10 s superfusion with physiological salt solution heated to 48°C and the innocuous stimulus was lightly brushing the surface of the tail with a fine sable-hair paint brush. For noxious chemical stimulation submaximal doses of capsaicin (0.3–0.7 μM) or bradykinin (0.1–0.4 μM) were used. Test stimuli were repeated following an interval of 15 min or 60 min between doses of bradykinin to prevent the occurrence of tachyphylaxis.

In the first series of experiments the effects of RTX and its interactions with capsaicin and other stimulants were assessed. The next series tested whether RTX has similar properties to the selective protein kinase C activator 4β-phorbol 12,13-dibutyrate (PDBu) and whether the effects of RTX were sensitive to the potent protein kinase C inhibitor, staurosporine (Tamaoki *et al.*, 1986). In these experiments the tail superfusate contained no added calcium to enhance the responses produced by PDBu (Dray *et al.*, 1988).

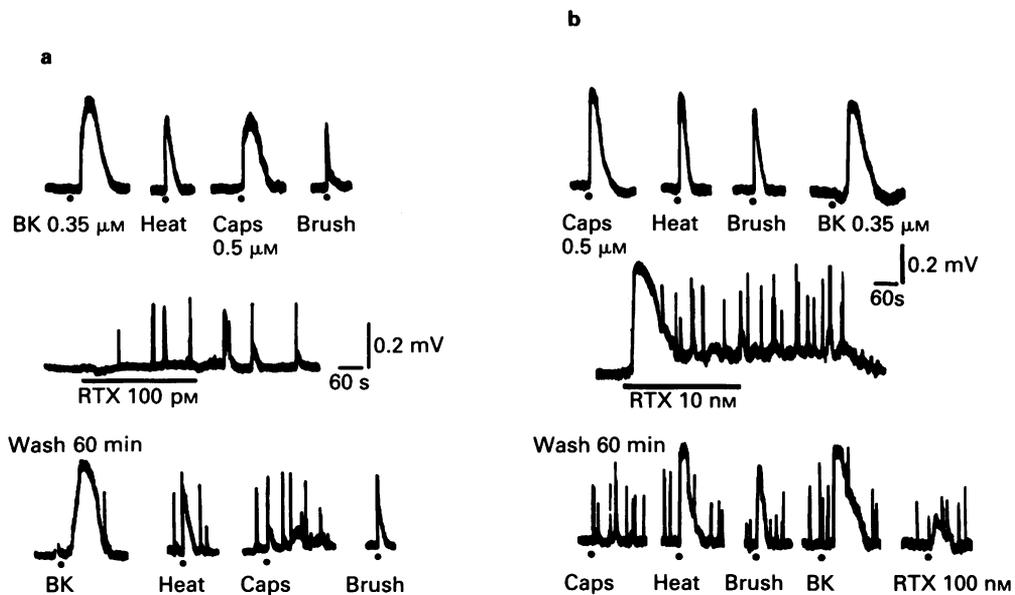


Figure 1 (a) Resiniferatoxin (RTX) did not produce a tonic depolarization but could induce cross-desensitization to capsaicin. The top traces show reproducible nociceptive responses to bradykinin (BK, 0.35 µM), noxious heat (48°C), capsaicin (Caps, 0.5 µM) and an innocuous response to light brush. The prolonged administration of RTX 100 µM (middle trace) did not evoke a tonic response but initiated a series of transient irregular depolarizations. These lasted longer than 4–6 h after the end of the RTX administration. The bottom traces show that following a 60 min wash period, responses to capsaicin were abolished whereas those to other stimuli were unaffected. (b) Depolarization of peripheral fibres by RTX. The top traces show control ventral root responses following application of noxious heat (48°C), a submaximal dose of capsaicin (0.5 µM) or bradykinin (0.5 µM) and innocuous brushing of the tail. The middle trace shows the initial tonic response produced by a prolonged administration of RTX (10 nM). The response was not sustained in the continuous presence of RTX and a series of irregular and transient depolarizations were initiated. These lasted for many hours. Following a 60 min wash period (bottom traces) there was a complete loss of sensitivity to capsaicin and almost complete abolition of RTX sensitivity (tested at 100 nM) whereas responsiveness to other stimuli was unchanged. The administration of brief stimuli are indicated by the dot below the traces while more prolonged administrations are shown as a bar below the trace. The calibration bars are indicated in this and other figures.

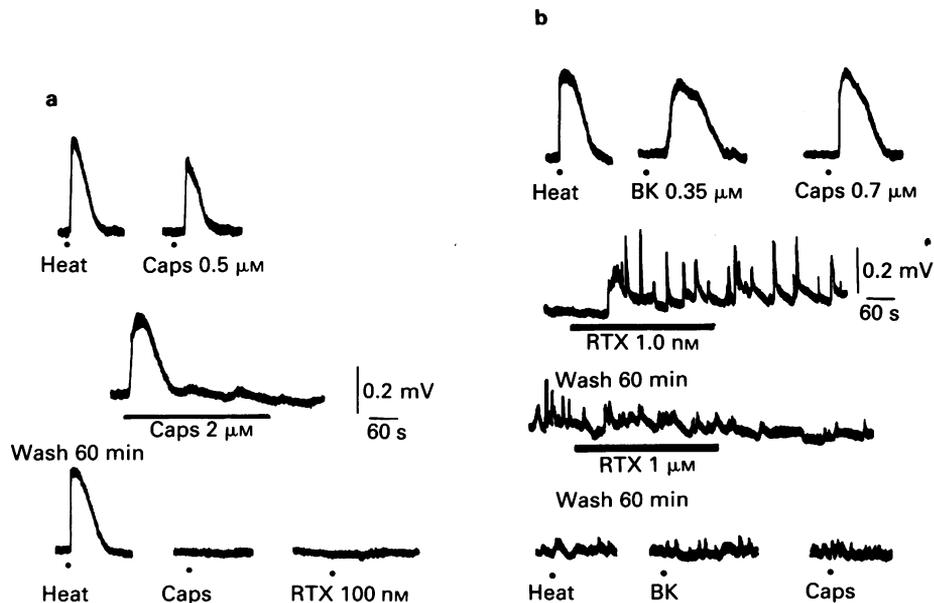


Figure 2 (a) Loss of resiniferatoxin (RTX) sensitivity following selective desensitization with capsaicin. The top traces show depolarizing responses to noxious heat and a brief administration of capsaicin (Caps, 0.5 µM). In the middle trace, the response evoked by capsaicin was not sustained during the continuous presence of capsaicin (2 µM). When retested 60 min later (bottom trace), the response to noxious heat was unchanged but capsaicin sensitivity was completely lost. Moreover neither a tonic depolarization nor transient irregular depolarizations were initiated following the administration of RTX (100 nM). (b) Non-selective impairment of nociceptive responses following RTX. The top traces show control responses to noxious heat (48°C), bradykinin (BK, 0.35 µM) and capsaicin (0.7 µM). Superfusion with RTX, (1 nM) produced a response of small amplitude and initiated a series of irregular, transient depolarizations. A higher dose of RTX (1 µM) did not evoke a subsequent depolarization but following this application the responsiveness to all noxious stimuli was lost (bottom traces) for more than 4–6 h.

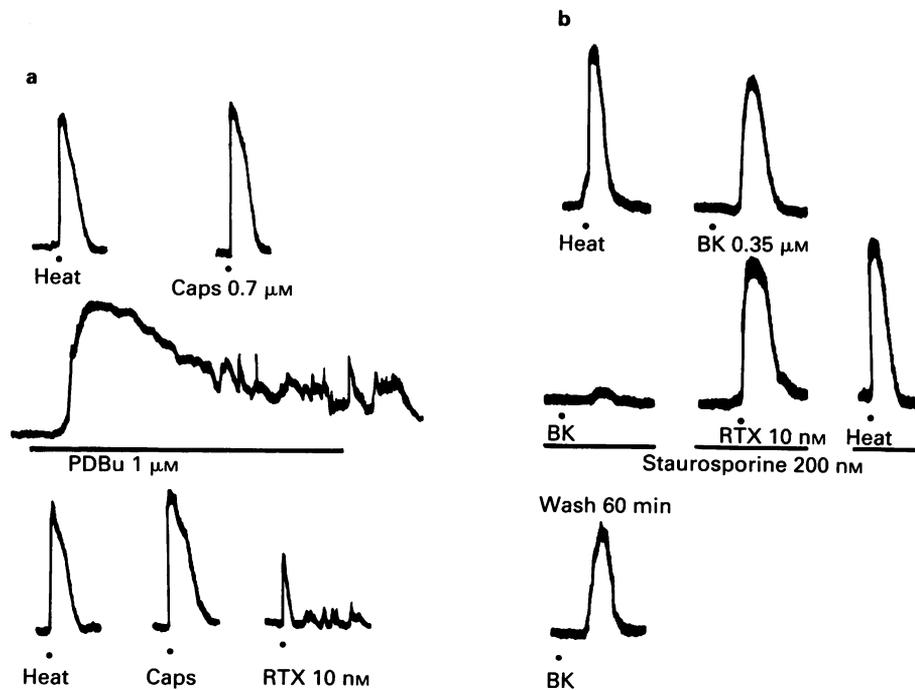


Figure 3 (a) Resiniferatoxin (RTX) evokes a response following desensitization by a phorbol ester. The top traces show control responses to noxious heat and capsaicin. During the prolonged superfusion of the tail with 4β -phorbol 12,13-dibutyrate (PDBu, $1\ \mu\text{M}$) there was a large depolarization which was not sustained throughout the presence of PDBu. The subsequent administration of PDBu was ineffective (not shown). Following a 60 min wash period, further tests showed that the responses to noxious heat and capsaicin were unchanged and RTX was also able to evoke a response. (b) Staurosporine did not affect the response to RTX. As before, the top traces show control responses to noxious heat and bradykinin (BK). Staurosporine ($200\ \text{nM}$) was superfused for 15 min to equilibrate with the tissue before each stimulus was retested. The middle traces show almost complete abolition of the response to bradykinin but responses to RTX and noxious heat were still produced. The response to bradykinin partially recovered (bottom trace) after a 60 min wash period.

Drugs were obtained from the following sources: capsaicin (Sigma, $10\ \text{mM}$ stock solution made up in DMSO and diluted with physiological solution immediately before use); resiniferatoxin (Fluka); bradykinin (synthesized at Sandoz); 4β -phorbol 12,13-dibutyrate (Avanti Polar Lipids); staurosporine (Fluka).

Results

In confirmation of previous observations (Dray *et al.*, 1989), brief (10 s) applications of noxious heat or administrations of capsaicin at a submaximal dose (EC_{50} approx $500\ \text{nM}$) evoked brief (50–103 s) responses which were reproducible for several hours. A prolonged (5 min) administration of capsaicin $1\text{--}2\ \mu\text{M}$ also produced a response but this response was not sustained in the continued presence of capsaicin. Following this, capsaicin was ineffective for many hours but the responsiveness to other noxious stimuli (heat, bradykinin), as well as responses to innocuous light brushing of the tail were unchanged.

At the lowest doses of RTX that were tested (1 and $10\ \text{pM}$, 10 s to 5 min of 5 experiments), no detectable responses were observed and neither were the responses to other stimuli subsequently affected. However, following a prolonged administration at $0.1\text{--}1.0\ \text{nM}$ (5 min, $n = 8$) transient and irregular depolarizations were initiated and responses to further test doses of capsaicin or RTX were abolished without notable changes in the responses produced by other stimuli (Figure 1a). No recovery of RTX or capsaicin sensitivity was observed up to 6 h afterwards.

At doses of 10 to $100\ \text{nM}$ applied for 10 s ($n = 6$ preparations) (Figure 1b), a ventral root depolarization was observed following the first administration of RTX. A dose-response profile for RTX was not obtained as the amplitude of the response was attenuated upon repeated administrations,

probably due to the rapid occurrence of tachyphylaxis (Figure 1b). A potency estimate for RTX and capsaicin was obtained by comparing the response to a submaximal dose of capsaicin with that produced by a subsequent single administration of RTX. These data indicated that RTX was approximately 50–100 times more potent than capsaicin.

Following the tonic phase of the RTX-evoked response, brief and irregular depolarizations occurred repeatedly for more than 4–6 h, despite continuous washing of the tissue (Figure 1b). During this period the response to capsaicin, administered to the tail, was abolished but the response to bradykinin, noxious heat or innocuous brush was unchanged (Figure 1a, b). At the highest doses of RTX used in this study ($1\text{--}10\ \mu\text{M}$) responsiveness to all noxious peripheral stimuli was subsequently abolished and no recovery was observed within 4–6 h (Figure 2a).

When capsaicin was administered for a prolonged period ($2\ \mu\text{M}$, 5 min) to produce a selective desensitization (Dray *et al.*, 1989), the subsequent administration of RTX ($100\ \text{nM}$, $n = 5$) did not evoke a tonic depolarization or any transient, irregular depolarizations (Figure 2b).

Superfusion of the tail with PDBu ($1\ \mu\text{M}$, $n = 5$) produced an activation of capsaicin-sensitive fibres and a ventral root response as described previously (Dray *et al.*, 1988). During a prolonged superfusion with PDBu ($1\ \mu\text{M}$, 5 min) the response was not sustained throughout the application (Figure 3a) and an acute desensitization to subsequent administrations of PDBu was produced, possibly due to inactivation of intracellular protein kinase C. However, subsequent administration of RTX or capsaicin was still able to evoke a response (4 of 4 experiments, Figure 3a). Alternatively prolonged superfusion with the potent protein kinase C inhibitor, staurosporine ($50\text{--}200\ \text{nM}$, 15–20 min, $n = 4$) (Tamaoki *et al.*, 1986) abolished the effect of PDBu, reversibly attenuated the responses to bradykinin (Figure 3b) but did not affect the responses to noxious heat, RTX or capsaicin (Figure 3b).

Discussion

Several features of the effects induced by RTX suggest that it produces potent interactions with capsaicin-sensitive peripheral fibres by mechanisms closely resembling those of capsaicin rather than a phorbol ester. Thus RTX produced a tonic depolarization of peripheral fibres followed by a period of transient, irregular depolarizations. On repeated administration apparent desensitization to RTX and cross-desensitization to capsaicin but not to other stimuli occurred. Indeed desensitization to RTX and cross-desensitization to capsaicin could be seen after doses of RTX too low to produce a substantial tonic response but sufficient to induce transient, irregular depolarizations. Conversely, desensitization following a prolonged administration of capsaicin (2 μM) was accompanied by a selective inability of RTX to induce either tonic or transient depolarizations. The rapid occurrence of desensitization to RTX precluded an accurate determination of its potency as an activator of peripheral fibres. Nevertheless, the comparison of a submaximal response to capsaicin with a subsequent response to RTX suggested that its potency is some 50–100 times greater.

Higher doses of capsaicin (20–50 μM) have also been shown to produce a tonic response followed by irregular depolarizations, but these subside within 10–20 min after the termination of the administration of capsaicin (Dray *et al.*, 1989). Such concentrations of capsaicin also induced a prolonged non-selective impairment of nociceptive responses (Dray *et al.*, 1989). Indeed exposure to higher doses of RTX (1 μM) also produces a prolonged and non-selective impairment of responsiveness to noxious stimuli.

The present data indicate a number of similarities between

the effects of RTX and those of capsaicin. The basis for these effects and why RTX is more potent and longer lasting than capsaicin is not presently understood. However, the similarities of action are consistent with other studies showing that RTX and capsaicin act on the same population of sensory neurones maintained in culture and increase membrane permeability to cations, elevate intracellular cyclic AMP concentrations and produce selective sensory neurotoxicity (Winter *et al.*, 1989). *In vivo* studies further show that RTX, like capsaicin, produced pain, neurogenic inflammation and hypothermia (de Vries & Blumberg, 1989; Szallasi & Blumberg, 1989), while acute systemic administration of these substances produces antinociceptive and anti-inflammatory activity (Campbell *et al.*, 1989; Szallasi & Blumberg, 1989).

It was clear from the present experiments that the pattern of activity observed with RTX was unlike that seen following activation of peripheral fibres with a phorbol ester (Dray *et al.*, 1988). In these experiments tonic depolarization and tachyphylaxis induced by PDBu attenuated the response to bradykinin rather than that to RTX or capsaicin. Moreover, the protein kinase C inhibitor staurosporine consistently attenuated the effects of bradykinin and PDBu but not those of capsaicin or RTX. The present experiments therefore support the likelihood that the irritant properties of RTX are due to the activation of capsaicin-sensitive nociceptive fibres through an interaction with a capsaicin-recognition site and/or through activation of a similar transduction mechanism. Capsaicin-like actions of RTX may also relate to the antinociceptive properties of RTX observed *in vivo*. Finally, the remarkable potency and selectivity of RTX observed in this and other studies indicate that it will be a valuable tool for studying sensory neurone function.

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