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Activation of vanilloid receptor 1 by resiniferatoxin mobilizes calcium from inositol 1,4,5-trisphosphate-sensitive stores

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1 Capsaicin and resiniferatoxin (RTX) stimulate Ca^{2+} influx by activating vanilloid receptor 1 (VR1), a ligand-gated Ca^{2+} channel on sensory neurones. We investigated whether VR1 activation could also trigger Ca^{2+} mobilization from intracellular Ca^{2+} stores.

2 Human VR1-transfected HEK293 cells (hVR1-HEK293) were loaded with Fluo-3 or a mixture of Fluo-4 and Fura Red and imaged on a fluorometric imaging plate reader (FLIPR) and confocal microscope respectively.

3 In Ca^{2+}-free media, RTX caused a transient elevation in intracellular free Ca^{2+} concentration in hVR1-HEK293 cells (pEC_{50} 6.45 ± 0.05) but not in wild type cells. Capsaicin (100 μM) did not cause Ca^{2+} mobilization under these conditions.

4 RTX-mediated Ca^{2+} mobilization was inhibited by the VR1 receptor antagonist capsazepine (pIC_{50} 5.84 ± 0.04), the Ca^{2+} pump inhibitor thapsigargin (pIC_{50} 7.77 ± 0.04), the phospholipase C inhibitor U-73122 (pIC_{50} 5.35 ± 0.05) and by depletion of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores by pretreatment with the acetylcholine-receptor agonist carbachol (20 μM, 2 min). These data suggest that RTX causes Ca^{2+} mobilization from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores in hVR1-HEK293 cells.

5 In the presence of extracellular Ca^{2+}, both capsaicin-mediated and RTX-mediated Ca^{2+} rises were attenuated by U-73122 (10 μM, 30 min) and thapsigargin (1 μM, 30 min). We conclude that VR1 is able to couple to Ca^{2+} mobilization by a Ca^{2+} dependent mechanism, mediated by capsaicin and RTX, and a Ca^{2+} independent mechanism mediated by RTX alone.

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Abbreviations: DRGs, dorsal root ganglion cells; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FLIPR, fluorometric imaging plate reader; FI, fluorescence intensity; hVR1-HEK293, human embryonic kidney cells expressing human VR1; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; MEM, minimum essential medium, PKC, protein kinase C; PLC, phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; RTX, resiniferatoxin; VR1, vanilloid receptor 1

Introduction

Vanilloid receptor 1 (VR1) is a non-selective cation channel present on a subset of sensory neurons that mediates responses to a number of pain-inducing stimuli including heat, protons and chemical irritants (Szallazi *et al*., 1999). The importance of the receptor in the perception of pain has been demonstrated in transgenic mice where VR1 knockout leads to a reduction in thermal hyperalgesia (Caterina *et al*., 2000; Davis *et al*., 2000).

Activation of VR1 by protons, heat, resiniferatoxin and capsaicin all cause opening of the channel leading to rapid influx of Ca^{2+} into the cytosol (Tominaga *et al*., 1998). However, different stimuli are believed to evoke activation of VR1 by different mechanisms. For resiniferatoxin (RTX) and capsaicin, the relationship between binding affinity and EC_{50} for Ca^{2+} entry is markedly different. Whereas RTX binds VR1 with an affinity up to 50 fold higher than its EC_{50} for triggering Ca^{2+} entry, capsaicin inhibits [^{3}H]-RTX binding with approximately 50 fold lower affinity than its EC_{50} for triggering Ca^{2+} entry (Acs *et al*., 1996; Szallasi *et al*., 1999). These data have been interpreted as evidence for distinct binding sites on VR1 for capsaicin and RTX (Szallasi & Blumberg, 1999). Protons appear to mediate their effect on VR1 by yet another mechanism since a mutation in the sixth transmembrane domain of the rat VR1 causes abolition of RTX and capsaicin-mediated responses but still allows protons to activate the channel (Kuzhikandathil *et al*., 2001). Distinct binding sites and/or mechanisms of action for VR1 agonists may provide an opportunity for VR1 to discriminate and integrate nociceptive stimuli.

A number of signal transduction components, including protein kinase C (PKC), phospholipase C (PLC) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) are reported to interact with VR1 to regulate channel gating (Chuang *et al*., 2001; Premkumar & Ahern, 2000; Tominaga *et al*., 2001; Vellani *et al*., 2001). In the present study, we

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were interested in whether VR1 activation may itself have a regulatory effect on the phosphoinositide signalling pathway. Our data indicate that VR1 activation stimulates Ca^{2+} mobilization from inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3)-sensitive Ca^{2+} stores *via* activation of phospholipase C. Ca^{2+}-dependent and Ca^{2+}-independent pathways coupling VR1 to mobilization of Ca^{2+} appear to be activated differentially by capsaicin and RTX.

Methods

Materials

Fluo-3 AM and Fluo-4 AM were obtained from Teflabs (Austin, TX, U.S.A.), Fura Red AM was from Molecular Probes (Eugene, OR, U.S.A.), resiniferatoxin was from Alexis Biochemicals (Nottingham, U.K.), capsaicin was from Tocris (Bristol, U.K.) and ruthenium red, U-73122, U-73343 and thapsigargin were from Calbiochem (San Diego, CA, U.S.A.). Membrane Potential Dye was from Molecular Devices (Wokingham, U.K.). All cell culture reagents were obtained from Life Technologies (Paisley, U.K.). All other reagents were from Sigma-Aldrich (Dorset, U.K.).

Cell culture

Human embryonic kidney cells expressing human VR1 (hVR1-HEK293) were grown in minimum essential medium (MEM) supplemented with non essential amino acids, 10% foetal calf serum and 0.2 mM L-glutamine and were maintained under 5% CO_2 at 37°C. Cells were passaged every 3–4 days.

Measurement of $[Ca^{2+}]_i$ and membrane potential using a fluorometric imaging plate reader (FLIPR)

hVR1-HEK293 cells in culture medium (see above) were seeded into black walled clear-based 96-well plates (Costar, U.K.) at a density of 25,000 cells per well and cultured overnight. For $[Ca^{2+}]_i$ measurements, cells were incubated with MEM containing 4 μM Fluo-3AM at 22°C for 2 h (Smart *et al*., 2001), washed three times with Tyrodes medium (mM: NaCl 145, KCl 2.5, HEPES 10, glucose 10, $MgCl_2$ 1.2, $CaCl_2$ 1.5, pH 7.3) in order to remove extracellular Fluo-3AM and incubated for a further 30 min at 25°C. For Ca^{2+} free conditions, $CaCl_2$ was replaced with 1 mM ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA). Fluorescence (λex = 488 nm, λem = 540 nm) from each well was measured in a FLIPR (Molecular Devices, U.K.) before and after the addition of agonists. For membrane potential assays, cells were incubated with Membrane Potential Dye for at least 30 min at 22°C and fluorescence emission (λex = 488 nm, λem = 540 nm) was measured in the FLIPR without washing.

Confocal microscopy

hVR1-HEK293 cells in culture medium (as above) were seeded onto 22 mm diameter polylysine-coated glass coverslips at a density of 50,000 cells per coverslip and cultured overnight. Medium was removed and coverslips were washed three times in Tyrodes medium (mM: NaCl 145, KCl 2.5, HEPES 10, glucose 10, $MgCl_2$ 1.2, $CaCl_2$ 1.5, pH 7.3) supplemented with 1 mg ml^{-1} bovine serum albumen. Cells were incubated with Tyrodes medium containing 2 μM Fluo-4 AM and 10 μM Fura Red AM for 30 min in the dark at 25°C, washed three times in order to remove extracellular dye and incubated for a further 30 min at 25°C. Cells were then imaged on a Perkin Elmer Ultraview confocal microscope running UltraView Version 4.0 software. Fluorescent dyes were excited at 488 nm using an argon ion laser and fluorescence emission was collected using 525/25 nm bandpass and 650 nm longpass filters for Fluo-4 and Fura Red respectively. Unless otherwise indicated, data are expressed as the ratio of fluorescence emissions from Fluo-4 and Fura Red. Ratiometric measurements were used to distinguish changes in intracellular Ca^{2+} concentration from dye leakage and/or photobleaching.

Data analysis

For confocal microscopy studies, calibration of ratiometric measurements to intracellular Ca^{2+} concentrations was unreliable because the relative uptake of dyes was different in individual cells. Results are therefore expressed as the percentage change in Fluo-4 : Fura Red emission intensities in individual cells before and after treatment with agonists.

FLIPR responses were measured as peak fluorescence intensity (FI) minus basal FI (ie before addition of agonist) and were expressed as a percentage of a capsaicin-induced or RTX-induced response where indicated. Data are expressed as mean ± s.e.mean unless otherwise stated. Sigmoidal dose-response curves were fitted using a four parameter logistical fit with variable Hill slopes using GraphPad Prism 3.00 (GraphPad Software Inc., CA, U.S.A.). pk_B values were generated from IC_{50} curves for the antagonist versus a fixed EC_{80} concentration of agonist using the Cheng-Prussof equation, as previously described (Jerman *et al*., 2000).

Results

RTX caused a transient elevation in intracellular free Ca^{2+} concentration in hVR1-HEK293 cells bathed in Ca^{2+} free media (pEC_{50} determined by FLIPR: 6.45 ± 0.05, Figure 1A) suggesting release of an intracellular Ca^{2+} pool. In single cell imaging experiments, intracellular Ca^{2+} rises were observed in 92 ± 5% (n = 6 coverslips; 368 cells examined) of hVR1-HEK293 cells following exposure to 5 μM RTX (Figure 1B). No intracellular Ca^{2+} rises were observed in wild type cells exposed to 5 μM RTX (Figure 1C) or in hVR1-HEK293 cells exposed to 100 μM capsaicin (data not shown).

RTX and capsaicin caused a concentration-dependent depolarization of the membrane potential in hVR1-HEK293 cells in the presence of 1 mM EGTA indicating that both RTX and capsaicin are able to activate VR1 in the absence of extracellular Ca^{2+} (Figure 2).

RTX-mediated Ca^{2+} mobilization was inhibited in a concentration-dependent manner by the VR1 antagonist capsazepine (pIC_{50} 5.84 ± 0.04), the phospholipase C inhibitor U-73122 (pIC_{50} 5.35 ± 0.05) and by depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} stores by thapsigargin (pIC_{50}

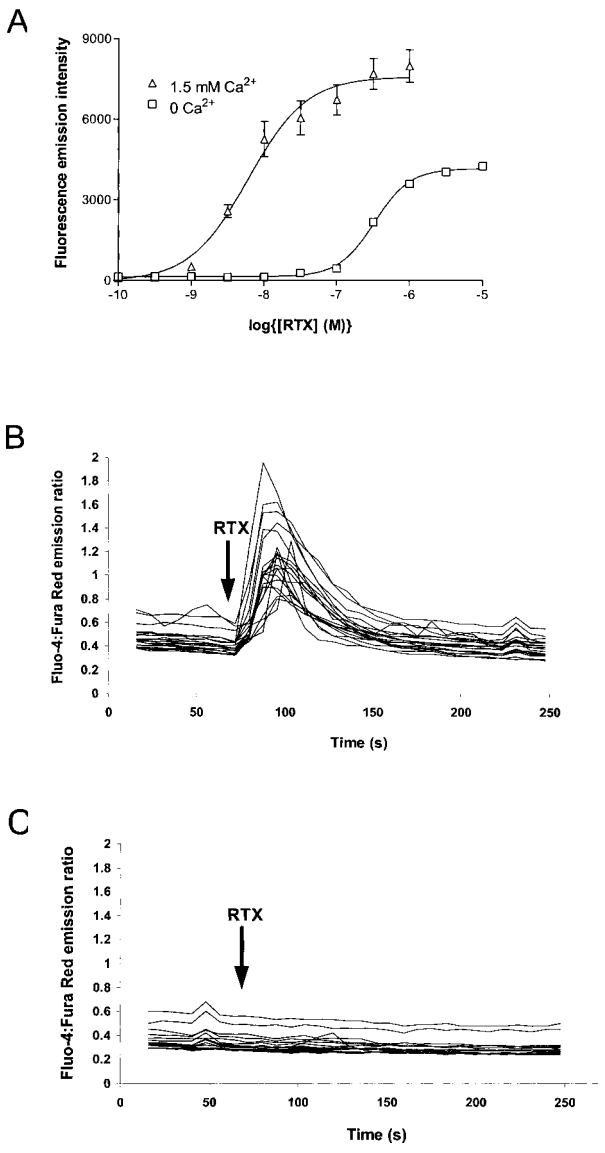

Figure 1 RTX stimulates Ca^{2+} mobilization in hVR1-HEK293 cells. (A) hVR1-HEK293 cells loaded with Fluo-3 were exposed to RTX in the presence or absence of extracellular Ca^{2+}. Changes in fluorescence intensity were recorded using FLIPR as described in Methods. Data are expressed as mean$\pm$s.e.mean. ($n=6$). (B) hVR1-HEK293 and (C) wild type HEK293 cells were loaded with Fluo-4 and Fura Red and exposed to 5 μM RTX in the absence of extracellular Ca^{2+}. Traces show changes in the ratio of Fluo-4 and Fura Red emission in individual cells from a single coverslip as measured by confocal microscopy. Similar data were obtained in three separate experiments.

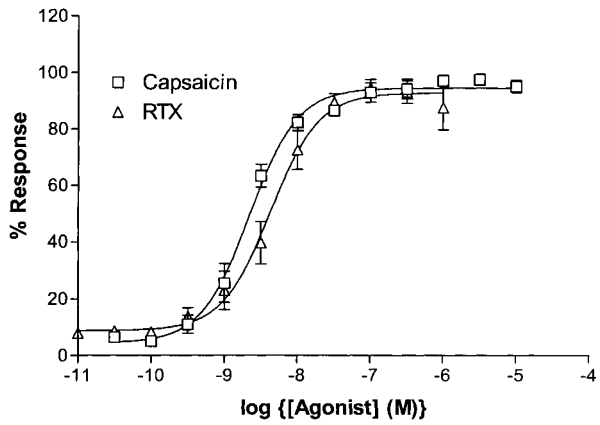

Figure 2 Capsaicin and RTX cause membrane depolarization in hVR1-HEK293 cells in the absence of extracellular Ca^{2+}. hVR1-HEK293 cells loaded with a membrane potential dye were monitored by FLIPR before and after addition of capsaicin or RTX in Ca^{2+}-free medium. Responses were measured as peak increase in fluorescence minus basal, expressed relative to the maximum capsaicin response. Data are mean$\pm$s.e.mean ($n=6$).

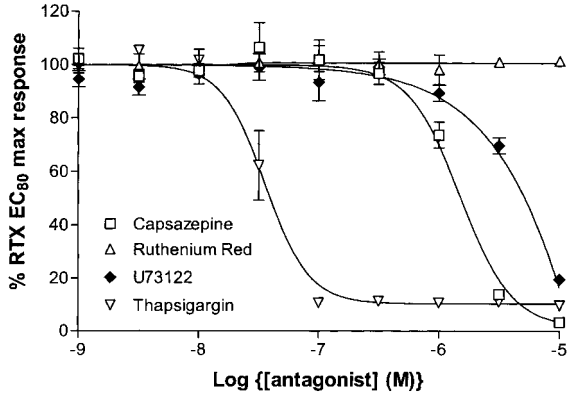

Figure 3 Capsazepine, thapsigargin and U-73122 inhibit RTX-mediated Ca^{2+} mobilization in hVR1-HEK293 cells. hVR1-HEK293 cells loaded with fluo-3 were preincubated with capsazepine, ruthenium red, thapsigargin or U-73122 for 30 min at 22°C in Ca^{2+}-free medium. $[Ca^{2+}]_i$ was then monitored by FLIPR before and after addition of RTX (1 μM). Responses were measured as peak increase in fluorescence minus basal, expressed relative to the control RTX (1 μM) response. Data are shown as mean$\pm$s.e.mean ($n=6$).

7.77$\pm$0.04) (Figure 3). We examined the effects of the VR1 channel blocker ruthenium red on RTX-mediated Ca^{2+} mobilization. At concentrations known to block VR1-mediated Ca^{2+} entry, ruthenium red did not affect RTX-mediated Ca^{2+} mobilization (Figure 3).

In order to verify which intracellular Ca^{2+} pools were released by RTX, hVR1-HEK293 cells in Ca^{2+}-free medium were exposed to RTX (10 μM) before or after depletion of Ins(1,4,5)P_3-sensitive Ca^{2+} pools using the acetylcholine-receptor agonist carbachol. Carbachol-pretreatment of cells (20 μM) reduced the subsequent response to RTX by 76$\pm$16% ($n=3$) whilst pretreatment with 10 μM RTX reduced the carbachol-stimulated response by 92$\pm$10% ($n=3$) (Figure 4). These data suggest that RTX stimulates release predominantly from Ins(1,4,5)P_3-sensitive Ca^{2+} stores in hVR1-HEK293 cells.

We examined whether a component of agonist-mediated responses in Ca^{2+}-containing medium could be due to Ca^{2+} mobilization from internal stores in addition to Ca^{2+} entry. Both thapsigargin (1 μM) and U-73122 (10 μM) reduced RTX- and capsaicin-mediated Ca^{2+} responses in Ca^{2+}-containing medium (Figure 5). These effects were not seen with an inactive analogue of U-73122, U-73343 (Figure 5). Our data suggest that both RTX and capsaicin are able to mobilize Ca^{2+} from intracellular stores in hVR1-HEK293 cells but only RTX is able to mediate this effect in the absence of extracellular Ca^{2+}.

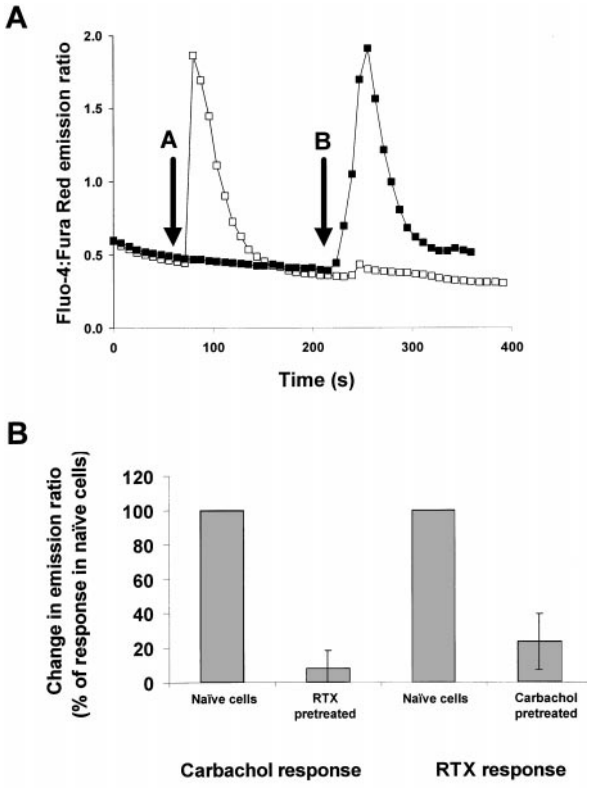

Figure 4 Carbachol and RTX mobilize the same intracellular Ca^{2+} pool in hVR1-HEK293 cells. hVR1-HEK293 cells plated onto glass coverslips were co-loaded with Fluo-4 and Fura Red and imaged by confocal microscopy. Cells were perfused with Ca^{2+}-free Tyrodes medium and treated sequentially with carbachol and RTX (as indicated). (A) Representative traces showing effect of pretreatment with 20 μM carbachol (arrow A, open squares) or buffer (arrow A, closed squares) on subsequent responses to 10 μM RTX (arrow B). Data show changes in mean emission intensity ratios in 91 (closed squares) and 44 (open squares) cells. (B) Pooled data from three independent experiments showing effect of carbachol (20 μM) or RTX (10 μM) pretreatment on subsequent responses to RTX and carbachol.

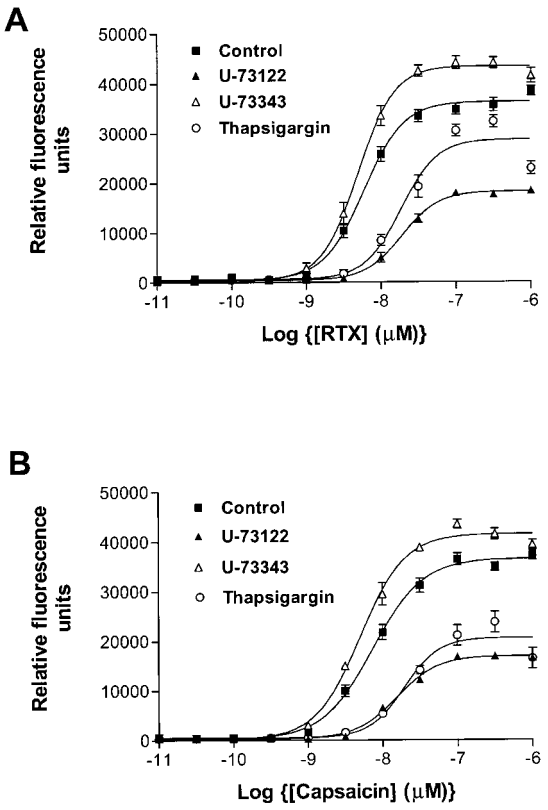

Figure 5 Thapsigargin and U-73122 inhibit RTX- and capsaicin-mediated increases in intracellular Ca^{2+} concentration in Ca^{2+}-containing medium. hVR1-HEK293 cells loaded with fluo-3 were preincubated with thapsigargin (1 μM), U-73122 (10 μM) or U-73343 (10 μM) for 30 min at 22°C. $[Ca^{2+}]_i$ was then monitored by FLIPR before and after the addition of (A) RTX or (B) capsaicin. Responses were measured as peak increase in fluorescence minus basal. Data are mean $\pm$ s.e.mean ($n=6$).

Discussion

RTX is widely accepted to trigger Ca^{2+} entry in cells expressing VR1 by opening an intrinsic non-selective cation channel in the receptor. Here, we have demonstrated that RTX also stimulates Ca^{2+} mobilization from intracellular stores. This response was absent in wild type HEK293 cells and was inhibited by the VR1-specific antagonist capsazepine, providing strong evidence of a direct involvement of the VR1 receptor. Several lines of evidence suggest that RTX-mediated Ca^{2+} release is not simply a consequence of VR1 channel opening. Firstly, Ca^{2+} mobilization did not depend on Ca^{2+} entry through the channel since it occurred in Ca^{2+} free media. Secondly, Ca^{2+} mobilization was not blocked by ruthenium red, a VR1 channel blocker. Thirdly, although both capsaicin and RTX allowed entry of Na^+ through the channel and caused membrane depolarization in Ca^{2+} free media, only RTX was able to trigger Ca^{2+} release from internal stores under these conditions.

If VR1 channel gating is not sufficient to couple RTX binding to Ca^{2+} mobilization, how then is the signal propagated? RTX is known to have weak phorbol ester-like activity and causes translocation of protein kinase C to the membrane in sensory neurones (Harvey *et al*., 1995), raising the possibility that direct PKC activation by RTX may be a contributory factor. However, this does not explain the dependence on VR1 as evidenced by a lack of response to RTX in non-transfected HEK293 cells and inhibition of RTX-mediated Ca^{2+} mobilization by capsazepine. We propose that RTX modulates a direct interaction between VR1 and the machinery responsible for Ca^{2+} release. Ca^{2+} is released from the sarcoplasmic and endoplasmic reticula either through Ca^{2+}-activated ryanodine receptors or through Ins(1,4,5)P_3 receptors. Recently, Eun *et al*. (2001) reported that capsaicin (1 μM) was able to mobilize ryanodine-sensitive but not Ins(1,4,5)P_3-sensitive Ca^{2+} pools in rat dorsal root ganglion cells (DRGs). Our data on human VR1 in HEK293 cells indicate that RTX causes Ca^{2+} mobilization through Ins(1,4,5)P_3 receptors following PLC-mediated hydrolysis of PtdIns(4,5)P_2 to Ins(1,4,5)P_3. The response to RTX was blocked by inhibition of PLC using the selective PLC inhibitor U-73122 and by agents that selectively depleted the Ins(1,4,5)P_3-sensitive Ca^{2+} pool (thapsigargin and carbachol). Although some species differences are known to exist between human and rat VR1 (McIntyre *et al*., 2001), these are unlikely to explain the contrasting data between our study and the findings of Eun *et al*. (2001). Human VR1 and

rat VR1, for example, show similar sensitivities to capsaicin when expressed in HEK293 cells (McIntyre *et al*., 2001; Smart *et al*., 2001) but whereas 100 μM capsaicin did not cause Ca^{2+} mobilization in our experiments in Ca^{2+} free media, it was effective in primary rat DRGs at 1 μM. The discrepancies may be explained by differential mechanisms of activation of VR1 by RTX and capsaicin or by an ability of VR1 to associate with different signalling pathways in different cell types.

A surprising observation in our study was the attenuation of both RTX- and capsaicin-mediated Ca^{2+} rises by U-73122 and thapsigargin in the presence of extracellular Ca^{2+}. We conclude that two mechanisms of VR1-mediated Ca^{2+} mobilization exist in hVR1-HEK293 cells: a Ca^{2+}-dependent pathway, mediated by capsaicin and possibly by RTX; and a Ca^{2+}-independent pathway, showing specificity for RTX. The contrasting effects of capsaicin and RTX on Ca^{2+} release observed here support previous reports that capsaicin and RTX activate VR1 by different mechanisms, and possibly through different binding sites (Acs *et al*., 1997; Biro *et al*., 1998; Szallasi *et al*., 1999). RTX- and capsaicin-mediated responses seen in our system appear to converge on the activation of phospholipase C and subsequent mobilization of Ins(1,4,5)P_3-sensitive Ca^{2+} stores. In line with these observations, several groups have observed interactions between VR1 and elements of the phosphoinositide signalling pathway including PLC-γ, PtdIns(4,5)P_2 and PKC (Chuang *et al*., 2001; Premkumar & Ahern, 2000; Tominaga *et al*., 2001; Vellani *et al*., 2001).

In conclusion, we have provided evidence that VR1 receptor agonists are able to stimulate Ca^{2+} mobilization from Ins(1,4,5)P_3-sensitive stores in addition to their established effects on Ca^{2+} entry. Since capsaicin and RTX appear to stimulate Ca^{2+} mobilization by different mechanisms, we can begin to envisage a tightly coupled relationship between Ca^{2+} entry and Ca^{2+} mobilization mediated by VR1 that may be differentially controlled by the many pain-producing stimuli that activate the receptor.

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