Activation of vanilloid receptor type I in the endoplasmic reticulum fails to activate store-operated Ca^{2+} entry

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To evaluate interaction of vanilloid receptor type 1 (TRPV1) with endogenous Ca^{2+} signalling mechanisms, TRPV1 was expressed in *Spodoptera frugiperda* (Sf9) insect cells using recombinant baculovirus. Stimulation of TRPV1-expressing cells, but not control Sf9 cells, with resiniferatoxin (RTX), capsaicin or anandamide, produced an increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), with EC_{50} values of 166 pM, 24.5 nM and 3.89 μM respectively. In the absence of extracellular Ca^{2+} , both capsaicin and RTX caused an increase in $[\text{Ca}^{2+}]_i$ with EC_{50} values of approx. 10 μM and 10 nM respectively. This TRPV1-induced release of Ca^{2+} from intracellular stores was not blocked by U73122, suggesting that phospholipase C was not involved. Substantial overlap was found between the thapsigargin- and RTX-sensitive internal Ca^{2+} pools, and confocal imaging showed that intracellular TRPV1 immunofluorescence co-localized with the endoplasmic reticulum targeting motif KDEL. To determine if TRPV1-induced mobilization of intracellular Ca^{2+} activates endogenous store-operated Ca^{2+} entry, the effect

of 2-aminoethoxydiphenyl borate (2-APB) on Ba^{2+} influx was examined. 2-APB blocked thapsigargin-induced Ba^{2+} influx, but not RTX-induced Ba^{2+} entry. In the combined presence of thapsigargin and a store-releasing concentration of RTX, the 2-APB-sensitive component was essentially identical with the thapsigargin-induced component. Similar results were obtained in HEK-293 cells stably expressing TRPV1. These results suggest that TRPV1 forms agonist-sensitive channels in the endoplasmic reticulum, which when activated, release Ca^{2+} from internal stores, but fail to activate endogenous store-operated Ca^{2+} entry. Selective activation of intracellular TRPV1, without concomitant involvement of plasmalemmal Ca^{2+} influx mechanisms, could play an important role in Ca^{2+} signalling within specific subcellular microdomains.

Key words: 2-aminoethoxydiphenyl borate, endoplasmic reticulum, fura 2, insect cell, store-operated channel.

INTRODUCTION

The Ca^{2+} signalling cascade is a ubiquitous cellular mechanism by which external signals control several crucial aspects of cell and tissue functions (for reviews see [1–4]). The cascade is initiated by the binding of hormones, growth factors or neurotransmitters to specific membrane receptors, linked via heterotrimeric G-proteins to phospholipase C (PLC). PLC, via the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generates two second messengers, namely $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. $\text{Ins}(1,4,5)\text{P}_3$ diffuses to the endoplasmic reticulum (ER), and, on binding to a specific receptor, initiates a rapid and regenerative release of Ca^{2+} from internal stores leading to a transient increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The release of Ca^{2+} from internal stores is accompanied by the influx of Ca^{2+} from the extracellular space which, in the continued presence of receptor activation, gives rise to a sustained increase in $[\text{Ca}^{2+}]_i$. Thapsigargin, a specific high-affinity inhibitor of the sarcoplasmic/ER Ca^{2+} -ATPase pump, depletes the internal Ca^{2+} store and concomitantly activates Ca^{2+} entry in the absence of measurable phosphoinositide hydrolysis. Thus store depletion by itself appears to be sufficient to trigger activation of Ca^{2+} influx via surface-membrane channels. The channels responsible for Ca^{2+} entry following store depletion have been called store-

operated channels (SOCs) and the influx of Ca^{2+} has been termed store-operated Ca^{2+} entry (SOCE). The mechanism by which information on the repletion status of the internal store is conveyed to the plasmalemmal channels remains an area of intense investigation.

Although the exact molecular identity of SOCs is unknown, some members of the transient receptor potential (TRP) family of Ca^{2+} -permeable non-selective cation channels have been suggested as possible candidates (for reviews see [5–7]). The TRP channel family, originally identified as light-activated channels in *Drosophila* photoreceptor cells, is composed of three major subfamilies in mammalian cells: TRPC, TRPV and TRPM [8]. The vanilloid receptor VR1 is the founding member of the TRPV subfamily [9–11]. VR1 (currently designated as TRPV1 [8]) is a membrane component of a subset of primary pain-sensing neurons known as nociceptors [9]. TRPV1 is unique among TRP family members in that it has a specific non-endogenous agonist, capsaicin, which is the active ingredient in pungent chilli peppers. The vanilloid receptor also has a well-defined pharmacological profile [10,11]. In addition to capsaicin, TRPV1 is also stimulated by an ultra-potent agonist resiniferatoxin (RTX) and by the endogenous cannabinoid receptor agonist anandamide. Agonist stimulation of TRPV1 is also blocked by the competitive antagonist capsazepine. Recent studies [12,13] suggest that

Abbreviations used: 2-APB, 2-aminoethoxydiphenyl borate; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; fura 2/AM, fura 2 acetoxymethyl ester; HBS, Hepes-buffered saline; PLC, phospholipase C; RTX, resiniferatoxin; MBS, Mes-buffered saline; MEM, minimum essential medium; SOC, store-operated channel; SOCE, store-operated Ca^{2+} entry; TRP, transient receptor potential; TRPV1, vanilloid receptor type 1 (usually designated as VR1).

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Figure 1 Time course of TRPV1 expression in Sf9 insect cells

(A) Membrane preparations were isolated as described in the Materials and methods section at 0 (uninfected Sf9 cells), 12, 18, 28 and 48 h after infection with recombinant baculovirus containing TRPV1 cDNA. Protein expression was determined by Western-blot analysis using rabbit anti-TRPV1 polyclonal antibody. (B) Sf9 cells were infected with TRPV1 baculovirus. At the indicated post-infection times, the cells were harvested, loaded with fura 2 and suspended in MBS. Five traces are shown superimposed. At the time indicated by the arrow, 1 μ M capsaicin (in this and all subsequent Figure legends, final concentrations are indicated) was added to the cuvette and the fluorescence recorded. The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

TRPV1 is tetrameric, but the actual subunit composition or the ability of TRPV1 to form heteromultimers with other members of the TRP channel family remains largely unknown. However, a recently cloned member of the TRPV family, TRPV3, associates with TRPV1 when heterologously expressed, and both TRPV1 and TRPV3 are co-expressed in dorsal-root ganglion cells consistent with heteromultimeric subunit assembly [14]. Although there is little evidence in the literature that TRPV1 channels can be activated by depletion of internal Ca^{2+} store intrinsically, Olah et al. [15] report that activation of heterologously expressed TRPV1 in COS-7 cells and native TRPV1 in dorsal-root ganglion cells gives rise to an increase in $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} . Since the binding site for agonist agents on the channel is assumed to be cytoplasmic [16–19], and TRPV1 agonists are highly lipophilic, this result suggests that both heterologously expressed and native TRPV1 channels may exist in the ER and, when activated, initiate the release of Ca^{2+} from internal stores. Alternatively, agonist stimulation of plasmalemma-associated TRPV1 may activate PLC and initiate the release of stored Ca^{2+} . The ability of TRPV1-induced Ca^{2+} release to activate SOCE, however, remains unknown.

The purpose of the present study was to evaluate the interaction of TRPV1 with endogenous cellular signalling mechanisms. Specifically, the experiments were designed to determine if

TRPV1 (a) is activated by depletion of internal Ca^{2+} stores intrinsically, (b) causes the release of Ca^{2+} from internal stores and (c) activates or inhibits endogenous SOCE. To accomplish these goals, the activity of TRPV1 was determined following heterologous expression in *Spodoptera frugiperda* (Sf9) insect cells using recombinant baculovirus. This eukaryotic expression system has several unique features that are advantageous for the expression of channels and proteins involved in Ca^{2+} signal transduction. First, following infection with recombinant baculovirus, host-cell protein synthesis is decreased within 6 h and is essentially zero after 24 h [20]. Since heterologous protein expression is under the control of the polyhedrin promoter, a late turn-on promoter in the baculovirus life cycle, problems associated with co-expression or up-regulation of host-cell proteins are minimized and the likelihood of recording homomeric channel activity is increased. Secondly, Sf9 cells exhibit G-protein-coupled Ca^{2+} signalling essentially identical with that observed in mammalian cells, including thapsigargin-induced SOCE [21]. Lastly, Sf9 cells lack endogenous Ca^{2+} -activated channels that may contribute to the Ca^{2+} signalling profile observed in other expression systems such as HEK-293 cells or *Xenopus oocytes* [21–24]. The results of the present study demonstrate that TRPV1, functionally expressed in Sf9 insect cells, shares pharmacological characteristics of both the native channels found in nerve and TRPV1 channels heterologously expressed in mammalian cells. However, the results also show that activation of TRPV1 causes an increase in $[\text{Ca}^{2+}]_i$ that reflects both the release of Ca^{2+} from internal stores and the influx of Ca^{2+} from the extracellular space. Furthermore, although the TRPV1- and thapsigargin-sensitive internal Ca^{2+} pools substantially overlap and TRPV1 immunoreactivity co-localizes with a marker for the ER, stimulation of TRPV1 alone failed to activate endogenous SOCE. When TRPV1 and SOCE were simultaneously activated, cation influx was essentially additive suggesting that TRPV1 in the plasmalemma and SOCE are functionally independent. The results are consistent with the hypothesis that only a specific fraction of the ER is linked to SOCE. Furthermore, the selective activation of TRPV1, present in the ER by its endogenous ligand, may play an important role in Ca^{2+} signal transduction within specific subcellular microdomains.

MATERIALS AND METHODS

Materials

Mes-buffered saline (MBS) containing 10 mM CaCl_2 , 60 mM KCl, 17 mM MgCl_2 , 10 mM NaCl, 4 mM D-glucose, 110 mM sucrose, 0.1% BSA and 10 mM Mes (pH adjusted to 6.2 at 22 $^{\circ}\text{C}$ with NaOH) was used with Sf9 cells. The total osmolality of the MBS was approx. 340 mosM. Nominally Ca^{2+} -free MBS was identical with MBS, except that CaCl_2 was isosmotically replaced with MgCl_2 . Hepes-buffered saline (HBS) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 10 mM D-glucose, 1.8 mM CaCl_2 , 15 mM Hepes and 0.1% BSA (pH adjusted to 7.40 at 37 $^{\circ}\text{C}$ with NaOH) was used with mammalian cells. Ca^{2+} -free HBS contained the same salts as HBS without added CaCl_2 . Rabbit and guinea-pig anti-VR1 antibodies were obtained from Chemicon International (Temecula, CA, U.S.A.). Mouse anti-KDEL was from StressGen Biotechnologies (Victoria, BC, Canada). Rhodamine Red-X-conjugated donkey anti-mouse IgG and FITC-conjugated donkey anti-(guinea-pig IgG) were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Fura 2 acetoxymethyl ester (fura 2/AM) was obtained from Molecular Probes (Eugene, OR, U.S.A.). Capsaicin and capsaizipine were obtained from Calbiochem (San Diego,

Figure 2 Concentration–response relationship for TRPV1 agonists

Fura 2-loaded Sf9 cells expressing TRPV1 were suspended in MBS. Agonist-induced change in $[Ca^{2+}]_i$ following receptor stimulation was determined for various concentrations of (A) capsaicin (●, 10 μM; ○, 1 μM; ▼, 100 nM; ▽, 10 nM; ■, 1 nM), (B) RTX (●, 100 nM; ○, 10 nM; ▼, 1 nM; ▽, 100 pM; ■, 10 pM) and (C) anandamide (●, 10 μM; ○, 5 μM; ▼, 2 μM; ▽, 1 μM; ■, 100 nM). The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. values at selected time points. (D) Mean normalized peak response at each concentration was fitted to a binding equation. The best-fit apparent $K_{0.5}$ values were determined for RTX (●, 166 pM), capsaicin (■, 24.5 nM) and anandamide (▲, 3.89 μM).

CA, U.S.A.), and RTX from Alexis Corporation (San Diego, CA, U.S.A.).

Cell culture

Sf9 cells were obtained from A.T.C.C. (Rockville, MD, U.S.A.) and cultured as described previously [22,20] in Grace's insect medium, supplemented with 2 mM L-glutamine, 1% (v/v) penicillin–streptomycin–neomycin solution, 10% (v/v) heat-inactivated foetal bovine serum, 2% (v/v) yeastolate solution and 2% (v/v) lactalbumin hydrosylate solution (Gibco Laboratories, Grand Island, NY, U.S.A.). HEK-293 cells, obtained from A.T.C.C., were maintained with minimum essential medium (MEM), supplemented with 10% foetal bovine serum, 1% penicillin–streptomycin–neomycin solution and 2 mM L-glutamine. For passage, HEK-293 cells were dispersed by trypsin treatment and seeded to a density of approx. 3×10^3 cells/cm². The medium was changed every 2–3 days following seeding.

Generation of a HEK-293 cell line stably expressing TRPV1

The cDNA encoding rat VR1 (TRPV1), a gift from Dr David Julius (University of California, San Francisco, CA, U.S.A.), was subcloned into a bicistronic enhanced green fluorescent protein (EGFP)-containing vector pIRES-EGFP, yielding pIRES-EGFP-TRPV1. Wild-type HEK-293 cells were transfected using

FugeneTM transfection reagent (Roche Diagnostics, Indianapolis, IN, U.S.A.) according to the manufacturer's instructions. Briefly, 35 mm dishes of wild-type HEK-293 cells at approx. 95% confluence were incubated with serum-free MEM. Fugene (2 μl) and pIRES-EGFP-TRPV1 cDNA (approx. 1 μg) were allowed to form a complex in 100 μl of serum-free MEM in a polystyrene tube. After 20 min, this complex was added to the HEK-293 cells and incubated for 24–48 h. Cells were dispersed with trypsin and sparsely seeded on to 100 mm dishes with MEM containing approx. 200 μM G418. After selection, individual colonies were assessed visually using fluorescence microscopy. Green fluorescent colonies were isolated using plastic cloning rings, dispersed with trypsin and re-seeded on to new dishes with fresh media containing G418. Individual colonies were grown to confluence and assayed for functional TRPV1 expression by fura 2 fluorescence assay. To increase the level of TRPV1 expression in the stably expressing HEK-293 cells, confluent dishes were maintained at 26 °C in a 5% CO₂ atmosphere for 96 h, before experiments were performed.

Generation of recombinant baculovirus

The cDNA encoding TRPV1 was subcloned into a baculovirus transfer vector, pVL1393, using standard techniques. Recombinant baculovirus was produced using the BaculoGoldTM Transfection Kit (Pharmingen, San Diego, CA, U.S.A.) as described in the manufacturer's instructions. Recombinant viruses

Figure 3 Agonist-induced release of Ca^{2+} from internal stores in TRPV1-expressing Sf9 cells

TRPV1-expressing Sf9 cells were suspended in nominally Ca^{2+} -free MBS. Cells were stimulated at the indicated time by (A) capsaicin (●, 100 μM ; ○, 10 μM ; ▼, 1 μM ; ▽, 100 nM) or (B) RTX (●, 10 μM ; ○, 100 nM; ▼, 10 nM; ▽, 1 nM). (C) Sf9 cells expressing the M_3 muscarinic receptor were suspended in nominally Ca^{2+} -free MBS. At the time indicated, 100 μM carbachol (CCH) was added in the presence of 10 μM U73122 (○) or 10 μM U73343 (●) added at 50 s. (D) Sf9 cells expressing TRPV1 were suspended in nominally Ca^{2+} -free MBS. At the time indicated, 100 nM RTX was added in the presence of 10 μM U73122 (□) or 10 μM U73343 (■) added at 50 s. The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

were amplified to obtain a high-titre viral stock solution. The virus was stored at 4 °C under sterile conditions and used for infecting Sf9 cells as described previously [21,22].

Infection of Sf9 insect cells with recombinant baculovirus

Sf9 cells in Grace's medium were plated on to 100 mm culture dishes (approx. 10^5 cells/cm²). After incubation for 30 min, an aliquot of viral stock was added (multiplicity of infection approx. 10) and the cells were maintained at 27 °C in a humidified air atmosphere. Unless otherwise indicated, cells were used at 24–28 h post-infection.

Isolation of membrane-associated TRPV1 protein

Sf9 cells were harvested at various post-infection times, subjected to centrifugation at 500 *g* for 5 min, and resuspended at a density of 5×10^6 cells/ml in lysis buffer, containing 20 mM Tris/HCl, 5 mM EDTA, 1 mM EGTA and 1 mM pMSF. The cell suspension was sonicated on ice three times for 10 s with a 10 s rest between pulses using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, U.S.A.) on a power setting of 2.5. The cell lysate was subjected to centrifugation at 6000 *g* for 10 min at 4 °C. The resulting pellet was discarded and the supernatants were centrifuged at 42000 *g* for 30 min. The microsomal pellets were resuspended in lysis buffer at a protein concentration

of 5–10 mg/ml and stored at –80 °C until use. Protein concentration was determined by the method of Lowry using BSA as the standard [24a].

Immunoblotting of TRPV1 protein

An equivalent volume of 2 $\times$ sample buffer [60 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 100 mM dithiothreitol and 0.025% Bromophenol Blue] was added to an aliquot of membrane preparation and the reaction was incubated for 1 min at 100 °C. Protein samples (20 μg) were subjected to electrophoresis on 7% (w/v) polyacrylamide gels along with molecular-mass standards (Bio-Rad). Proteins were transferred electrophoretically from the polyacrylamide gels to Millipore PVDF membranes (100 V for 1 h on ice). Blots were probed with an anti-capsaicin receptor (TRPV1) polyclonal antibody at a dilution of 1 : 3000 for 1 h at room temperature. After washing, membranes were probed for 1 h with goat anti-rabbit IgG and detected using ECL[®] (Amersham Life Sciences).

Immunolocalization of TRPV1 protein

Sf9 cells were plated on to polylysine-coated glass coverslips and subsequently infected with recombinant baculovirus containing VR1. Following incubation for 28 h at 27 °C, the coverslips were washed with PBS and fixed with 4% (w/v) paraformaldehyde

in PBS for 15 min. After fixation, coverslips were washed three times with PBS and permeabilized by incubation for 5 min in PBS, containing 0.1 % Triton X-100. Coverslips were blocked with 4 % (w/v) non-fat dry milk/PBS for 1 h and incubated overnight at 4 °C with primary antibodies diluted 1 : 1000 (guinea-pig anti-VR1) or 1 : 200 (mouse anti-KDEL) in 4 % milk/PBS solution. Coverslips were subsequently washed three times with PBS and incubated with FITC-conjugated donkey anti-(guinea-pig IgG) (1 : 200) and/or Rhodamine Red-X-conjugated donkey anti-mouse IgG (1 : 500) for 1 h at room temperature (22 °C). After incubation with secondary antibodies, the coverslips were washed three times and mounted on to glass slides using Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) mounting medium.

Confocal microscopy

Immunofluorescence and DIC images were obtained as single scans using a Leica TCS SP2 confocal microscope and a $\times 100$ oil-immersion objective. Dual imaging of FITC and Rhodamine Red-X were obtained by sequential scans to minimize cross-excitation. Two scans were averaged for each image.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in Sf9 cells using the fluorescent indicator fura 2 as described previously [21,22]. Briefly, Sf9 cells were dispersed, washed and resuspended at a concentration of $1.5\text{--}2 \times 10^6$ cells/ml in MBS containing $2 \mu\text{M}$ fura 2/AM. After 30 min incubation at room temperature, the cell suspension was subjected to centrifugation, resuspended in an equal volume of MBS and incubated for an additional 30 min. For experiments performed on HEK-293 cells, dispersed cells were suspended in HBS containing $20 \mu\text{M}$ fura 2/AM. After a 30 min incubation at 37 °C, the cell suspension was diluted 10-fold with HBS, incubated for an additional 30 min, washed and resuspended in fresh HBS. The cells were washed twice immediately before measurement and fluorescence was monitored using an SLM 8100 spectrophotofluorimeter. Excitation wavelength alternated between 340 and 380 nm, and fluorescence intensity was monitored at an emission wavelength of 510 nm. All measurements on Sf9 cells were performed at 22 °C, and experiments on HEK-293 cells were performed at 37 °C. Calibration of fura 2 associated with the cells was accomplished using Triton lysis in the presence of a saturating concentration of Ca^{2+} followed by the addition of EGTA (pH 8.5). $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al. [25] using a K_d value of 278 and 224 nM for Ca^{2+} binding to fura 2 at 22 °C [26] and 37 °C respectively. For experiments using the Ca^{2+} surrogate Ba^{2+} , fluorescence intensity was measured at an emission wavelength of 510 nm and excitation wavelengths of 350 and 390 nm as described previously [27]. The results shown are mean traces from 'n' number of independent experiments. At selected time points, the means $\pm$ S.E. values are plotted in each Figure.

RESULTS

Functional expression of TRPV1 in Sf9 insect cells

TRPV1 was heterologously expressed in Sf9 insect cells using recombinant baculovirus. Protein expression was driven by the polyhedrin promoter, a late turn-on promoter in the baculovirus life cycle. Membrane-associated TRPV1 protein was monitored by Western-blot analysis using an anti-capsaicin receptor polyclonal antibody. The predicted molecular mass of

Figure 4 Localization of TRPV1 to the ER

TRPV1-expressing Sf9 cells were co-labelled with TRPV1-specific antibodies and a monoclonal antibody directed against the ER retention motif, KDEL, as described in the Materials and methods section. The stained cells were visualized using confocal microscopy. (A) FITC fluorescence (green) associated with TRPV1. (B) Rhodamine Red-X fluorescence (red) associated with KDEL. (C) Overlay of the fluorescence images shown in (A,B). (D) Overlay of the fluorescence images with the DIC image. No fluorescence was observed in the absence of primary antibody, i.e. secondary only. Yellow colour in (C,D) indicates areas of co-localization. Results are representative of two independent experiments.

TRPV1 is 95 kDa. TRPV1 protein was non-detectable at 0 and 12 h, but increased in a time-dependent manner from 18 to 48 h post-infection time (Figure 1A), a profile consistent with expression of a protein under control of the polyhedrin promoter. To determine if the TRPV1 protein formed functional channels, TRPV1-expressing Sf9 cells (TRPV1 cells) were loaded with the fluorescent Ca^{2+} indicator, fura 2, and subsequently challenged with capsaicin. Capsaicin ($1 \mu\text{M}$) had no effect on $[Ca^{2+}]_i$ at 0 and 12 h post-infection time. However, stimulation with $1 \mu\text{M}$ capsaicin produced a time-dependent increase in $[Ca^{2+}]_i$ from a resting level of approx. 150 nM to peak values of 0.5, 1.5 and $2.5 \mu\text{M}$ at 18, 28 and 48 h post-infection time respectively ($n = 3$; Figure 1B). Capsaicin had no effect on $[Ca^{2+}]_i$ in uninfected Sf9 cells or in Sf9 cells infected with an unrelated baculovirus, i.e. cells infected with recombinant baculovirus containing the M_5 muscarinic receptor cDNA (see below). These results demonstrate that TRPV1 activity directly correlates with protein expression. All subsequent experiments were performed at 24–28 h post-infection time.

Characterization of TRPV1 expressed in Sf9 insect cells

TRPV1 expressed in mammalian cells can be stimulated by (1) capsaicin, (2) RTX, a high-affinity toxin isolated from *Euphorbia* plants [9], and (3) anandamide, the putative endogenous cannabinoid receptor agonist [28]. All of them, RTX, capsaicin and anandamide, produced a concentration-dependent increase in $[Ca^{2+}]_i$ in TRPV1-expressing Sf9 cells, with EC_{50} values of 166 pM, 24.5 nM and $3.89 \mu\text{M}$ respectively (Figure 2). For each concentration of capsaicin and anandamide tested, $[Ca^{2+}]_i$

Figure 5 Effect of thapsigargin (Tg) and RTX on Ba^{2+} influx in control and TRPV1-expressing Sf9 cells

Two mean traces are shown superimposed in each panel (A, B). Fura 2-loaded control Sf9 cells (i.e. Sf9 cells infected with recombinant baculovirus containing the M_5 muscarinic receptor cDNA) were suspended in nominally Ca^{2+} -free MBS in the absence (●) or presence (■) of $10 \mu\text{M}$ La^{3+} . In each experiment, Ba^{2+} influx through plasmalemmal channels was estimated from the fluorescence change after the addition of 10 mM Ba^{2+} at the indicated time. Cells were treated with thapsigargin (A: 200 nM at 100 s) or RTX (B: 100 nM at 100 s). (C,D) TRPV1-expressing Sf9 cells, loaded with fura 2, were treated with 200 nM thapsigargin or 1 nM RTX. The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

increased to a peak value within 1 min and remained at a sustained level for the duration of the recording. In contrast, the rate of change of $[\text{Ca}^{2+}]_i$, following the addition of RTX, increased as a function of RTX concentration, i.e. both the peak level and the time-to-peak $[\text{Ca}^{2+}]_i$ changed as a function of RTX concentration. Thus, although RTX is the more potent agonist, the kinetics of TRPV1 activation by RTX differs from those of capsaicin and anandamide. To determine if TRPV1 expressed in Sf9 cells could be blocked by capsazepine, a known competitive antagonist of TRPV1 in mammalian cells, the response to either capsaicin or anandamide was measured in the absence and presence of increasing concentrations of capsazepine. Capsazepine produced a concentration-dependent inhibition of the TRPV1-induced response (results not shown).

Effect of TRPV1 stimulation on internal Ca^{2+} stores

To determine if stimulation of TRPV1 causes release of Ca^{2+} from internal stores, capsaicin and RTX were added to TRPV1 cells suspended in Ca^{2+} -free extracellular buffer. Concentrations of capsaicin ($< 100 \text{ nM}$) and RTX ($< 1 \text{ nM}$), which produced dramatic increases in $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} , had little or no effect in the absence of Ca^{2+} . However, higher concentrations of both capsaicin and RTX produced a graded release of Ca^{2+} from internal stores (Figures 3A and 3B). The kinetic differences between capsaicin and RTX observed in the presence of extracellular Ca^{2+} (see Figure 2) were also evident in the absence of extracellular Ca^{2+} (Figure 3), i.e. capsaicin produced an immediate increase in $[\text{Ca}^{2+}]_i$, whereas the response

to RTX was more slowly developing, suggesting a different mechanism of receptor activation for these two agonists.

Upon binding to specific plasmalemmal receptors, many agonist agents cause the release of Ca^{2+} from internal stores through a G-protein-coupled activation of PLC and subsequent generation of $\text{Ins}(1,4,5)\text{P}_3$. The ability of capsaicin and RTX to release Ca^{2+} from internal stores may reflect either the presence of TRPV1 in the ER or the activation of PLC. To determine if TRPV1-mediated release of Ca^{2+} from internal stores is secondary to the activation of PLC, the effect of U73122, an inhibitor of PLC, and U73343, the inactive analogue [29], on TRPV1-dependent change in $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} was examined. As seen in Figure 3(C), U73122 blocked the carbachol-induced release of Ca^{2+} from internal stores in control Sf9 cells expressing the M_5 muscarinic receptor, but had no effect on the RTX-induced release of $[\text{Ca}^{2+}]_i$ from internal stores in TRPV1 cells (Figure 3D). U73343 had no effect on the agonist-induced response in either cell type. Thus the ability of TRPV1 agonists to release Ca^{2+} from internal stores is unrelated to PLC activity and probably reflects the presence of functional TRPV1 channels in the ER. To determine if TRPV1 is present in the ER, confocal images of TRPV1-expressing Sf9 cells, co-labelled with antibodies directed against TRPV1 and antibodies against the ER-targeting motif KDEL [30], were examined. The nucleus occupies a large proportion of the cytoplasm in Sf9 cells and the ER is seen in a narrow region surrounding the nucleus (Figure 4B). Although the level of TRPV1 immunofluorescence was variable from cell to cell, there was substantial co-localization of TRPV1 to the ER (Figure 4C).

Effect of TRPV1-induced Ca^{2+} release on SOCE

In essentially all cells examined to date, depletion of the internal Ca^{2+} store by thapsigargin, an inhibitor of sarcoplasmic/ER Ca^{2+} -ATPase pumps, is linked to activation of Ca^{2+} -permeable cation channels in the plasma membrane [1,2,4]. The resultant SOCE gives rise to a long-lasting increase in $[\text{Ca}^{2+}]_i$. In most cell types, SOCs are permeable to Sr^{2+} and Ba^{2+} and inhibited by low micromolar concentrations of La^{3+} and Gd^{3+} . Lanthanides are also potent inhibitors of endogenous SOCE in Sf9 insect cells [21–23]. To determine if depletion of internal Ca^{2+} stores by itself activates TRPV1, thapsigargin-induced Ba^{2+} influx in control M5-expressing Sf9 cells was compared with that observed in TRPV1 cells. As seen in Figure 5(A), addition of thapsigargin to control Sf9 cells in the absence of extracellular Ca^{2+} caused a release of Ca^{2+} from internal stores. Subsequent re-addition of Ba^{2+} produced an increase in fluorescence ratio that was blocked by the presence of $10 \mu\text{M}$ La^{3+} , indicative of Ba^{2+} influx via the SOCE mechanism. Addition of RTX (100 nM) had no effect on basal $[\text{Ca}^{2+}]_i$ or on basal Ba^{2+} influx in control M5-expressing cells (Figure 5B). Thus, as expected, RTX has no significant effect on thapsigargin-induced Ca^{2+} signalling in the absence of TRPV1 expression. We next examined Ba^{2+} influx in TRPV1 cells in response to thapsigargin and RTX. As seen in Figure 5(C), expression of TRPV1 had no significant effect on the ability of thapsigargin to release Ca^{2+} from internal stores or on the subsequent La^{3+} -inhibitable Ba^{2+} influx component. In contrast, addition of 1 nM RTX to TRPV1 cells produced a dramatic increase in Ba^{2+} influx, which was relatively insensitive to the presence of La^{3+} (Figure 5D). This concentration of RTX did not release Ca^{2+} from internal stores. These results demonstrate that TRPV1 expression has no effect on endogenous SOCE, and that TRPV1 is not itself an SOC.

Effect of 2-aminoethoxydiphenyl borate (2-APB) on Ba^{2+} influx via SOCE and TRPV1

To determine if TRPV1-mediated Ca^{2+} store release activates endogenous SOCE, we examined the effect of 2-APB, a known inhibitor of SOCE in mammalian cells [31–34]. These experiments were performed in a paired fashion on the same batch of TRPV1-infected Sf9 cells to eliminate problems associated with variation in TRPV1 channel expression levels. As seen in Figure 6(A), 2-APB ($10 \mu\text{M}$) greatly attenuated thapsigargin-induced Ba^{2+} influx in TRPV1-expressing Sf9 cells. The effect was concentration-dependent and no further inhibition was observed at concentrations above $10 \mu\text{M}$ (results not shown). We next compared the effect of 2-APB on Ba^{2+} influx induced by either a non-store-releasing (1 nM) or a store-releasing (100 nM) concentration of RTX (Figures 6B and 6C). In the absence of 2-APB, Ba^{2+} influx was identical in response to both concentrations of RTX. Thus the release of stored Ca^{2+} observed at the higher concentration of RTX does not appear to activate an additional component of Ba^{2+} influx related to SOCE. Consistent with this hypothesis, 2-APB had no effect on Ba^{2+} influx at both concentrations of RTX. To determine if RTX releases Ca^{2+} from a thapsigargin-sensitive compartment, the two agonists were sequentially added to TRPV1-expressing cells in the absence of extracellular Ca^{2+} . As seen in Figure 7, the RTX-releasable pool was greatly reduced by prior treatment with thapsigargin. Likewise, the thapsigargin-releasable component was reduced by prior stimulation with RTX. Overall, the results confirm that activation of TRPV1 channels in the ER releases Ca^{2+} from a thapsigargin-sensitive Ca^{2+} pool, but fail to activate endogenous SOCE.

Figure 6 Effect of 2-APB on thapsigargin- and RTX-induced Ba^{2+} influx in TRPV1-expressing Sf9 cells

TRPV1-expressing Sf9 cells were suspended in nominally Ca^{2+} -free MBS. (A) Two mean traces are shown superimposed. In one, $10 \mu\text{M}$ 2-APB was added at the indicated time, followed by the addition of 200 nM thapsigargin and 10 mM Ba^{2+} . (B) Four mean traces are shown superimposed. RTX, either 1 nM (squares) or 100 nM (circles), was added in the absence (open symbols) or presence (closed symbols) of $10 \mu\text{M}$ 2-APB. (C) The mean $[\text{Ba}^{2+}]_i$ was calculated from the results in (B) after the addition of Ba^{2+} to the cuvette. Experiments are paired, i.e. performed consecutively on the same batch of cells on the same day. The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

Effect of TRPV1 activation in the ER on SOCE

It is possible that activation of TRPV1 in the thapsigargin-sensitive pool prevents or inhibits activation of endogenous SOCE. To test this hypothesis, the effect of 2-APB on Ba^{2+} influx was examined following the release of Ca^{2+} from internal stores by the sequential addition of RTX and thapsigargin. As seen in Figure 8(A), addition of a non-store-releasing concentration of RTX (1 nM) did not prevent thapsigargin-induced Ca^{2+} release, and a substantial component of Ba^{2+} influx seen under this

Figure 7 Overlap of RTX- and thapsigargin-sensitive Ca^{2+} stores

TRPV1-expressing Sf9 cells were suspended in nominally Ca^{2+} -free MBS. Thapsigargin (200 nM) and RTX (100 nM) were added to the cuvette at the times indicated by the arrows. The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

condition was inhibited by the presence of 2-APB (Figure 8B). The 2-APB-sensitive component was essentially the same in cells stimulated with a store-releasing concentration of RTX (100 nM; Figures 8C and 8D). These results demonstrate that activation of TRPV1 does not prevent or inhibit activation of endogenous SOCE.

Stimulation of HEK-293 cells stably expressing TRPV1

The baculovirus/Sf9 cell expression system is commonly used to obtain high levels of protein expression. Since TRPV1 agonists are lipophilic, and the ligand-binding site on TRPV1 is assumed to be cytoplasmic [17], we considered the possibility that functional TRPV1 retained in internal membrane as a result of high expression in Sf9 cells may give rise to agonist-induced release of Ca^{2+} from internal stores. A recent study found evidence for the presence of functional TRPV1 channels in the ER of COS-7 cells transiently expressing TRPV1 and in cultured primary DRG neurons [15]. We, therefore, re-examined the effect of capsaicin and RTX on fura 2-loaded HEK-293 cells stably expressing TRPV1. As seen in Figures 9(A) and 9(B), RTX and capsaicin caused a time- and concentration-dependent increase in $[\text{Ca}^{2+}]_i$, with an EC_{50} value of 52 pM and 221 nM respectively. In comparison with Sf9 cells, these values are 3-fold lower for RTX, but 10-fold higher for capsaicin (see above). However, kinetically, the responses to capsaicin and RTX were virtually identical in HEK-293 cells compared with Sf9 cells. Capsaicin produced an immediate response at each concentration tested, whereas the

Figure 8 Effect of 2-APB on thapsigargin-induced Ba^{2+} influx in the absence or presence of RTX-induced store release in TRPV1-expressing Sf9 cells

The experimental method is similar as described in the legend to Figure 7, with the exception that thapsigargin was added after either 1 nM RTX (A) or 100 nM RTX (C) in the absence ($\circ$) or presence ($\bullet$) of 10 μM 2-APB. (B, D) Mean $[\text{Ba}^{2+}]_i$ was calculated from the data in (A, B) respectively after the addition of Ba^{2+} to the cuvette. Also shown is the 2-APB-sensitive component ($\blacktriangle$), which was obtained by subtracting the Ba^{2+} influx in the presence of 2-APB from that obtained in the absence of 2-APB. Experiments are paired, i.e. performed consecutively on the same batch of cells on the same day. The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

Figure 9 Concentration–response relationship for TRPV1 agonists in HEK-293 cells stably expressing TRPV1

Fura 2-loaded HEK-293 cells stably expressing TRPV1 suspended in HBS were treated with (A) capsaicin (●, 10 μ M; ○, 1 μ M; ■, 100 nM; □, 10 nM; ▲, 1 nM) and (B) RTX (●, 100 nM; ○, 10 nM; ■, 1 nM; □, 100 pM; ▲, 10 pM; △, 1 pM; ▼, 100 fM). (C, D) HEK-293 cells were suspended in nominally Ca^{2+} -free HBS and treated with (C) capsaicin (●, 100 μ M; ○, 10 μ M; ■, 1 μ M; □, 100 nM) or (D) RTX (●, 1 μ M; ○, 100 nM; ■, 10 nM; □, 1 nM; ▲, 100 pM; △, 10 pM). The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

time-to-peak was inversely related to RTX concentration. Notably, both capsaicin and RTX produced an increase in $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} (Figures 9C and 9D), consistent with the hypothesis that these agents release Ca^{2+} from internal stores. No effect of either RTX or capsaicin on $[\text{Ca}^{2+}]_i$ was observed in non-transfected HEK-293 cells (results not shown). Thus the response observed is dependent on TRPV1 expression. Despite the difference in apparent affinities of TRPV1 for RTX and capsaicin, the overall profile is remarkably similar between HEK-293 and Sf9 cell expression systems. Furthermore, these results are consistent with TRPV1 localization to both internal and surface membranes.

We next examined the effect of 2-APB on Ca^{2+} influx induced by depletion of the stores by either thapsigargin or RTX. 2-APB attenuated thapsigargin-induced Ca^{2+} entry (Figure 10A), but had no significant effect on RTX-induced Ca^{2+} influx (Figure 10B), although there was a tendency for $[\text{Ca}^{2+}]_i$ to decrease to a lower level at longer times after re-addition of Ca^{2+} to the cuvette in the presence of 2-APB. These results suggest that endogenous SOCE in HEK-293 cells is not activated by RTX-induced release of Ca^{2+} from internal stores. To determine if TRPV1 activation inhibits endogenous SOCE, the effect of 2-APB was examined following the sequential release of Ca^{2+} stores by RTX and thapsigargin (Figure 10C). As was seen in the Sf9 cell, a substantial component of Ca^{2+} influx was blocked by 2-APB under this condition. The magnitude of the 2-APB-sensitive component seen in Figure 10(C) was

only slightly reduced from that seen in Figure 10(A). Thus Ca^{2+} influx via surface-membrane TRPV1 and endogenous SOCE are essentially additive, suggesting that the two pathways are completely independent. Importantly, the results show that RTX-induced Ca^{2+} release in HEK-293 cells stably expressing TRPV1 fails to activate endogenous SOCE (Figure 10B).

DISCUSSION

In the present study, TRPV1 was functionally expressed in Sf9 insect cells and HEK-293 cells. TRPV1 expressed in both cell types exhibited pharmacological characteristics similar to those reported for native channels and for TRPV1 heterologously expressed in other cell types. Specifically, TRPV1 was activated by RTX, capsaicin and anandamide with the appropriate potency order and the response was antagonized by capsazepine. However, slight differences in agonist potencies were noted. Based on $^{45}\text{Ca}^{2+}$ uptake in neurons, mast cells and glioma cells, the EC_{50} for capsaicin and RTX ranged from 300 to 700 nM and from 0.5 to 2.1 nM respectively [35]. A survey of the literature shows that the EC_{50} values for capsaicin and RTX in heterologous expression systems ranged from 33 to 1000 nM and from 0.1 to 39 nM respectively [9,15,36–42]. In the present study, TRPV1 expressed in HEK-293 cells shows a similar agonist sensitivity (EC_{50} values of 221 and 0.05 nM for capsaicin and RTX respectively). However, in Sf9 cells, the apparent

Figure 10 Effect of 2-APB on thapsigargin- and RTX-induced Ca^{2+} influx in TRPV1-expressing HEK-293 cells

TRPV1-expressing HEK-293 cells were suspended in nominally Ca^{2+} -free MBS. (A) Two mean traces are shown superimposed. In one, $50 \mu\text{M}$ 2-APB was added at the indicated time followed by the addition of 200 nM thapsigargin and 10 mM Ca^{2+} . (B) RTX (100 nM) was added in the absence ($\circ$) or presence ($\bullet$) of $50 \mu\text{M}$ 2-APB. (C) RTX (200 nM) and thapsigargin (200 nM) were added at the indicated times in the absence ($\circ$) or presence ($\bullet$) of $50 \mu\text{M}$ 2-APB. Experiments are paired, i.e. performed consecutively on the same batch of cells on the same day. The lines shown are mean values from three independent experiments. Symbols represent means $\pm$ S.E. at selected time points.

affinity for capsaicin was increased by 10-fold to 24 nM , whereas the affinity for RTX was reduced 3-fold to 0.16 nM . There are three major differences between the Sf9 and HEK-293 cell experiments. First, the activity of TRPV1 in the Sf9 cells quite probably reflects homomeric channels, whereas in HEK-293 cells it is possible that other members of the TRP channel family, endogenously expressed in this cell line, may contribute subunits to the functional channels observed. Secondly, the Sf9 cell experiments were performed at 22°C , whereas the HEK-293 experiments were performed at 37°C . Although TRPV1 channel activity appears to be relatively insensitive to changes in temperature over this range [9], the EC_{50} for capsaicin was reported to change from 125 to 18 nM in stably expressing

HEK-293 cells when temperature was decreased from 37 to 22°C [42]. Thirdly, the HEK-293 experiments were performed at $\text{pH } 7.4$, whereas buffers in the Sf9 cells have a $\text{pH } 6.2$. It is well established that decreasing the pH values increases the sensitivity of TRPV1 to agonist agents [37]. Thus the differences in temperature and pH could explain the increase in the apparent capsaicin affinity in Sf9 cells relative to HEK-293 cells seen in the present study. The reason for the decrease in RTX potency in Sf9 versus HEK-293 cells is not known. However, the kinetics of channel activation by capsaicin and RTX is the same in both cell types. Capsaicin produced a rapid increase in $[\text{Ca}^{2+}]_i$ and the time-to-peak $[\text{Ca}^{2+}]_i$ was independent of concentration. In contrast, RTX produced a slower increase in $[\text{Ca}^{2+}]_i$ and the time-to-peak was inversely related to RTX concentration. Similar profiles have been previously noted [43,44], but the underlying mechanisms for the kinetic difference remain unknown. Overall, however, the general pharmacological profile for TRPV1, the relative potencies for agonists and the kinetics of channel activation for TRPV1 expressed in Sf9 cells are similar to native channels and to channels heterologously expressed in other cell types. Since large amounts of protein can be generated using this expression system, the Sf9 cell may be useful for the ultimate biochemical purification of the channel in sufficient quantities for structural studies.

When TRPV1-expressing cells were challenged with either RTX or capsaicin in the absence of extracellular Ca^{2+} , we observed a concentration-dependent increase in $[\text{Ca}^{2+}]_i$. This increase was unrelated to activation of PLC, since U73122, an inhibitor of PLC, had no effect on RTX- or capsaicin-induced mobilization of intracellular Ca^{2+} . The immunohistochemical results of the present study confirm localization of TRPV1 channels to the ER. Furthermore, the RTX-sensitive internal stores partially, but substantially, overlapped the thapsigargin-sensitive pool. Together, the results suggest that TRPV1, present in the ER, is functional and can be activated by exogenously applied lipophilic agonists. However, in stark contrast with thapsigargin, RTX-induced release of Ca^{2+} from internal stores failed to activate 2-APB-sensitive cation influx. This result was obtained in both Sf9 and HEK-293 cells stably expressing TRPV1. Furthermore, experiments in which stores were depleted by sequential addition of both thapsigargin and RTX demonstrated that SOCE was not inhibited by activation of TRPV1 in the ER. Since the TRPV1 and thapsigargin-sensitive pools substantially overlap, yet only thapsigargin activates SOCE, it seems reasonable to conclude that there must be an internal store that is not 'linked' to SOCE. A similar conclusion was recently derived from studies of the Ca^{2+} release activated current, I_{CRAC} , in TRPV1-transfected RBL cells [51].

TRPV1-induced release of Ca^{2+} from internal stores has been previously reported in dorsal root ganglion cells *in vivo* [15,49] and in TRPV1-transfected cells *in vitro* [15,50,51]. The mobilization of Ca^{2+} via ER-localized TRPV1 could explain effects of capsaicin in neurons that appeared to be independent of extracellular Ca^{2+} [45]. In preliminary studies, anandamide (in the absence of extracellular BSA) caused a concentration-dependent (10 – $30 \mu\text{M}$) release of Ca^{2+} from internal stores (results not shown). Thus compartmentalized production of an endovanilloid such as anandamide or *N*-arachidonoyl dopamine [38] may give rise to Ca^{2+} signalling events localized to specific subcellular microdomains. There is growing evidence that members of the TRP channel family are held together in large multimeric signalling complexes, which may contain other proteins and enzymes critical to the signal transduction cascade (e.g. PLC, protein kinase C and calmodulin). Furthermore, the 'signalplex' may be localized to specific subcellular domains by

PDZ-containing scaffolding proteins such as *Drosophila* protein responsible for the inactivation-no-after-potential D mutant or Na^+/H^+ -exchanger regulatory factor [5]. In this regard, Delmas et al. [46] showed that TRPC1 overexpressed in superior cervical ganglion neurons appears to be localized to a specific microdomain that includes a plasmalemmal bradykinin receptor and an ER $\text{Ins}(1,4,5)\text{P}_3$ receptor. This localization of TRPC1 appears to be important for channel activation by depletion of the internal Ca^{2+} store. In platelets, it appears that TRPC6 is localized to the plasmalemma, whereas TRPC1 is found in intracellular membrane compartments [47]. There is also growing evidence for functional heterogeneity of Ca^{2+} stores in neurons [48], but the role of TRP channels in these stores remains unknown. Compartmentalized production of anandamide or *N*-arachidonoyl dopamine along with selective localization of TRPV1 to specific ER microdomains in neurons may play an important role in 'intracellular' Ca^{2+} signalling events without involvement of plasmalemmal Ca^{2+} channels.

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