Discrimination of intracellular calcium store subcompartments using TRPV1 (transient receptor potential channel, vanilloid subfamily member 1) release channel activity

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The store-operated calcium-release-activated calcium current, I_{CRAC}, is a major mechanism for calcium entry into non-excitable cells. I_{CRAC} refills calcium stores and permits sustained calcium signalling. The relationship between inositol 1,4,5-trisphosphate receptor ($InsP_3R$)-containing stores and I_{CRAC} is not understood. A model of global $InsP_3R$ store depletion coupling with I_{CRAC} activation may be simplistic, since intracellular stores are heterogeneous in their release and refilling activities. Here we use a ligand-gated calcium channel, TRPV1 (transient receptor potential channel, vanilloid subfamily member 1), as a new tool to probe store heterogeneity and define intracellular calcium compartments in a mast cell line. TRPV1 has activity as an intracellular release channel but does not mediate global calcium store depletion and does not invade a store coupled with I_{CRAC}. Intracellular TRPV1 localizes to a subset of the $InsP_3R$-containing stores. TRPV1 sensitivity functionally subdivides the $InsP_3$-sensitive store, as does heterogeneity in the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase isoforms responsible for store refilling. These results provide unequivocal evidence that a specific 'CRAC store' exists within the $InsP_3$-releasable calcium stores and describe a novel methodology for manipulation of intracellular free calcium.

Key words: calcium release activated current, calcium store, transient receptor potential, vanilloid receptor.

INTRODUCTION

Controlled manipulation of intracellular calcium levels is a fundamental mechanism for information transmission [1–3]. Cells have calcium available from two primary sources, intracellular storage compartments and the extracellular milieu. In many cell types these calcium pools are intimately linked. The depletion of calcium stores may be followed by activation of plasma membrane calcium channels that respond, by an as yet unknown mechanism, to the loss of stored calcium from intracellular compartments. Calcium influx across the plasma membrane is initiated following intracellular store depletion, via a class of store-operated calcium (SOC) channels [4,5]. The best characterized of these SOC channels is the calcium-release-activated calcium current I_{CRAC} [5–8], present in non-excitable cells, although it may not be the only store-coupled conductance in this context. SOC channel activation results in sustained increases in cytosolic free calcium that permit the refilling of calcium storage compartments via calcium ATPases and the activation of calcium-dependent cellular processes, including exocytosis and transcriptional activation [2,3,9].

Intracellular calcium stores are not homogeneous. Store heterogeneity permits considerable complexity in calcium signalling, enabling local manipulation of calcium levels and accommodating multiple-response thresholds within the same cell [10], and may be important for store-dependent functional responses such as I_{CRAC} activation. It is clear that considerable complexity can be encoded in calcium responses that derive from stores that are spatially or functionally separated. Any spatial or functional subdivision of a store provides the potential for complex and specific responses to store-depleting signals. Heterogeneity of calcium stores has been documented and proposed via a variety of mechanisms. First, various distinct cellular organelles can act as calcium stores: these include compartments such as the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, nucleus and lysosomes. Secondly, there is increasing evidence that stores may be subdivided within a single organellar compartment such as the ER.

ER calcium stores are heterogeneous in terms of the distribution/activity of both release channel isoforms and refilling calcium ATPases [11–13]. We, and others, have recently shown that the response threshold of inositol trisphosphate-gated calcium stores can be set by differential localization and activity of the enzymes that metabolize inositol 1,4,5-trisphosphate ($InsP_3$) [14–16,33]. The potential for signalling specificity as an outcome of this heterogeneity is well illustrated by the activation characteristics of the SOC conductance I_{CRAC}. Depletion of $InsP_3$-responsive calcium stores activates I_{CRAC}, whether achieved directly by $InsP_3$ or by other depletion-inducing stimuli. There is considerable evidence to suggest that a model of global $InsP_3$-sensitive store-depletion coupling with I_{CRAC} activity may be simplistic. Several studies indicate that the store coupling with I_{CRAC} activation may in fact be a specialized compartment, which, albeit $InsP_3$-sensitive, can be regulated separately from

Abbreviations used: I_{CRAC}, calcium-release-activated calcium current; ER, endoplasmic reticulum; FBS, foetal bovine serum; fura 2/AM, fura 2 acetoxymethyl ester; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; MOI, multiplicity of infection; pfu, plaque-forming units; RBL, rat basophilic leukaemia; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; SOC, store-operated calcium; TRPV1, transient receptor potential channel, vanilloid subfamily member 1.

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the whole $InsP_3$-sensitive store [6,15,17,18]. Taken together, previous studies may be used to argue that, in fact, I_{CRAC} activation depends on specific depletion of a subcompartment of the $InsP_3$ receptor ($InsP_3R$)-containing store. Thus the global release response measured when treating cells with $InsP_3$-mobilizing ligands may be largely irrelevant to depletion coupling of I_{CRAC}.

The present methodologies for studying calcium store subcompartments are limited. Differential sensitivity to pharmacological depletion agents, such as the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin and $InsP_3$-mobilizing compounds, is widely used to delineate store compartments. However, the relationship between sensitivity of a store compartment to these compounds and its molecular complement of release/refilling activities is not clear. We have developed a store-depletion methodology employing a heterologously expressed, ligand-gated calcium channel and used this tool to delineate the functional store subdivisions in I_{CRAC}-containing mast cells. We have identified that the TRPV1 (transient receptor potential channel, vanilloid subfamily member 1) cation channel can function as an intracellular calcium release channel in both native and overexpression contexts. TRPV1 is a member of the transient receptor potential superfamily of ion channels [41,42]. TRPV1 has a membrane-permeant lipophilic ligand, capsaicin, and was originally identified as the cellular receptor for this lipid that mediates the burning sensation associated with exposure to chilli peppers [25,43]. Physiologically, TRPV1 responds to noxious thermal and acidic stimuli and is expressed in sensory neurons [25,43]. We have exploited the intracellular functionality of TRPV1 and its ligation by a membrane-permeant lipid to evaluate the relationships between calcium store subcompartments in a mast cell context.

MATERIALS AND METHODS

Cells and vaccinia virus transient expression system

RBL-2H3 (where RBL stand for rat basophilic leukaemia) and RBL-2H3-M_1 cells were cultured at 37 °C/5 % CO_2 in Dulbecco's modified Eagle's medium, supplemented with 15 % (v/v) foetal bovine serum (FBS) and 2 mM glutamine. Continued expression of the muscarinic acetylcholine receptor M_1 was ensured in stable transfectants by maintenance of a parental cell stock in 1 mg/ml G418 (Gibco BRL, Gaithersburg, MD, U.S.A.). Cath.a cells (A.T.C.C., Manassas, VA, U.S.A.) were maintained in a medium containing RPMI 1640, 8 % (v/v) horse serum, 4 % FBS and 2 mM glutamine at 37 °C/5 % CO_2.

A recombinant vaccinia virus containing the TRPV1 cDNA was prepared as follows. The TRPV1 cDNA was amplified by PCR and subcloned into the pSCF4 vector (A. M. Scharenberg, Department of Pediatrics and Immunology, University of Washington, Seattle, WA, U.S.A.), which confers a FLAG epitope tag at the extreme N-terminus of the expressed protein. This construct was verified by sequencing and used to prepare recombinant vaccinia as described previously. Recombinant viruses were subjected to two rounds of lac-z-based selection to remove contaminating wild-type viruses. The recombinant virus was amplified in a tk^- cell line and purified by high-speed centrifugation across a sucrose bed. Multiplicity of infection (MOI) was established by a plaque formation assay in tk^- cells. Several viral clones were tested for their ability to confer TRPV1 expression; no significant difference between clones was observed. The control virus, pSCGβ, contains an antisense version of the heterotrimeric G protein β subunit, does not drive production of a protein and is our standard control infection. Before use, virus stocks [MOI = (2–3) $\times 10^9$ pfu (plaque-forming units)/ml] were sonicated (Misonix XL, 50 % power setting) for two bursts of 30 s to disperse virus aggregates. Adherent monolayers of RBL cells were grown to 50–70 % confluence under standard conditions. The culture medium was removed and replaced with Dulbecco's modified Eagle's medium/10 % FBS containing pSCFTRPV1 or pSCGβ at 3–10 pfu/cell. Typical time course of infection was 8 h (calcium assay and patch-clamp analysis) or 16–18 h (expression analysis).

Immunoprecipitation and Western-blot analysis

RBL-2H3-M_1 or Cath.a cells were removed from the culture and washed twice in complete PBS. Cell lysis was then performed in 0.5 ml/1 $\times 10^7$ cells of a buffer containing (in mM) NaCl 75, NaF 20, iodoacetamide 10, phenylmethylsulphonylchloride 1, Hepes 50 (pH 7.4) and 0.5 % (v/v) Triton X-100 for 30 min on ice. Nuclei were pelleted by centrifugation and lysates were transferred to a tube containing 2 μg/sample of pre-coupled anti-FLAG M_2 antibody (Sigma) or control pre-coupled IgG. Endogenous TRPV1 immunoprecipitations were performed using 4 μg/point anti-TRPV1 (Chemicon International, Temecula, CA, U.S.A.). Immunoprecipitations were incubated with rotation at 4 °C for 90 min. Immunocomplexes were washed three times in 1 ml lysis buffer and then boiled for 8 min in 50 μl of a standard reducing sample buffer. Captured proteins were resolved by SDS/PAGE (10 % gel) and transferred to PVDF membrane.

Western-blot analysis of immunocomplexes used a 5 % (w/v) BSA blocking solution followed by 1 h incubation in 2 μg/ml anti-FLAG M_2 or anti-TRPV1 (Chemicon International) in PBS/0.1 % BSA/0.05 % (v/v) Tween 20. Membranes were washed as mentioned above before exposure for 30 min to 1:10000 dilution of goat anti-mouse or donkey anti-rabbit conjugated to horseradish peroxidase (in PBS/0.1 % BSA/0.05 % Tween 20) (Amersham). After further washing of the membrane, immunocomplexes were visualized by ECL®.

Electrophysiology

For patch-clamp experiments, cover-slips were transferred to the recording chamber and kept in a standard modified Ringer's solution of the following composition (in mM): NaCl 145, KCl 2.8, CsCl 10, $CaCl_2$ 10, $MgCl_2$ 2, glucose 10, Hepes/NaOH 10 (pH 7.2). For fura 2 acetoxymethyl ester (fura 2/AM) measurements, the external calcium concentration was reduced to 1 mM. Intracellular pipette-filling solutions contained (in mM): Cs-glutamate 145, NaCl 8, $MgCl_2$ 1, Cs-BAPTA [bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid] 10 (pH 7.2) adjusted with CsOH. Capsaicin was used at 1 μM, carbachol at 100 μM and thapsigargin at 1 μM (Sigma). The agonists were dissolved in the standard extracellular solution containing 10 mM calcium in patch-clamp experiments and 1 mM calcium for fura 2 measurements. In experiments where agonists were applied under calcium-free conditions, the extracellular solution was kept nominally calcium-free (i.e. no $CaCl_2$ was added).

Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21–25 °C. High-resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9; HEKA Electronik, Lambrecht, Germany). Sylgard-coated patch pipettes had resistances between 2 and 4 MΩ after filling with the standard intracellular solution. Immediately after the establishment of the whole-cell configuration, voltage ramps of 50 ms duration spanning the voltage range of -100 to $+100$ mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 400–800 s.

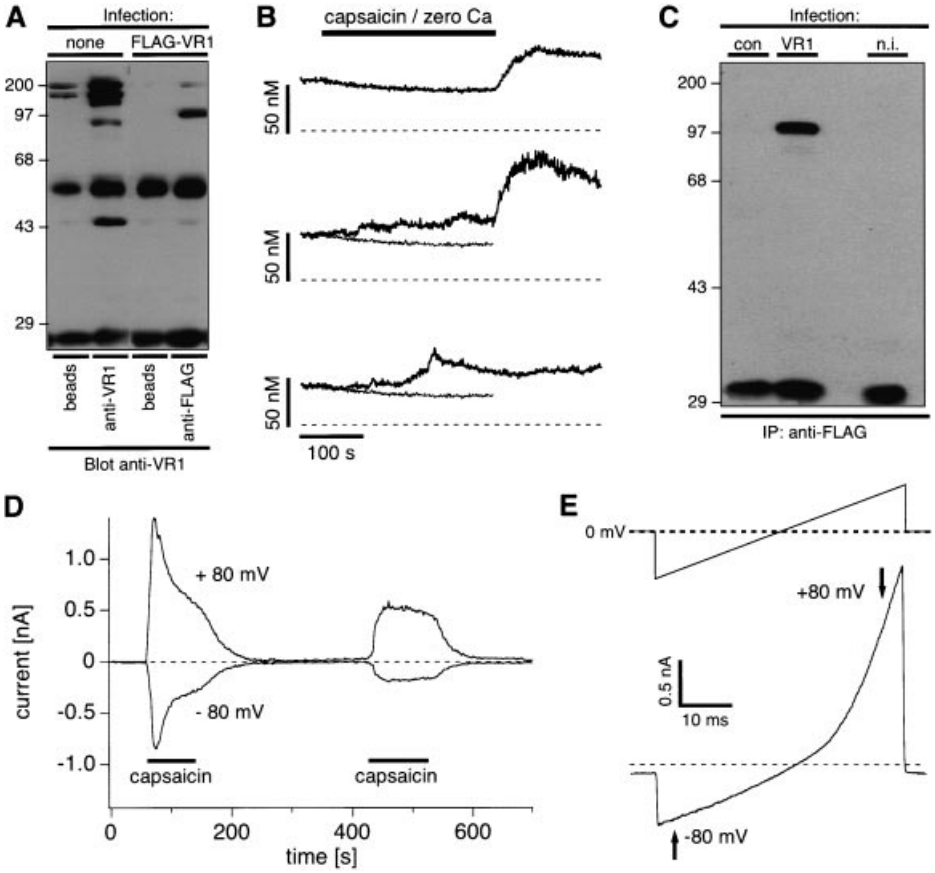

Figure 1 Cath.a cells (A) express endogenous TRPV1 protein and (B) exhibit TRPV1-mediated calcium release and influx responses; heterologous expression of TRPV1 in RBL cells (C) results in the expression of a capsaicin-activated cation channel (D) and displays an *I–V* relationship (E)

(**A**) Cath.a cells were infected (right two lanes) or not infected (left two lanes), for 16 h with 5 pfu/cell vaccinia-TRPV1 (FLAG–VR1). Lysates from 10^7 Cath.a cells per lane were immunoprecipitated with Ig-coupled beads, or antibodies specific to TRPV1 (anti-VR1) or the FLAG epitope tag. Consistent 55 and 26 kDa bands represent heavy and light immunoglobulin chains respectively. Identity of the 43 kDa band is unknown; it is probably a proteolytic fragment of TRPV1. High-molecular-mass bands can be removed by extensive pre-clearing with Ig-coupled beads, although a 200 kDa species remains, probably representing a TRPV1 glycoform. FLAG-TRPV1 appears size-shifted relative to the endogenous 97 kDa TRPV1 protein. (**B**) Responses of fura 2/AM-loaded Cath.a cells to the application of nominally calcium-free external solution supplemented with 2 μM capsaicin (black bar). Upper panel: average responses of 5 cells where application of capsaicin in nominally calcium-free medium (0 Ca) did not elicit detectable calcium release. Readmission of calcium resulted in an off-response, probably consisting of influx through slowly deactivating TRPV1 channels in the plasma membrane. Middle panel: average responses of 4 cells where application of capsaicin resulted in an increase in the intracellular calcium levels, indicating release of stored intracellular calcium. In these cells, readmission of calcium also elicited an off-response. Since removal of calcium actually shifted the baseline to lower cytosolic calcium levels (see upper panel), data from the cells without a release response were superimposed here to highlight the actual magnitude of calcium release in responding cells (grey line). Lower panel: average responses of 5 cells where application of capsaicin did elicit calcium release but readmission of calcium had no significant effect on calcium levels. The grey line has the same meaning as in the upper panel. (**C**) RBL cells were not infected (n.i.) or infected with 5 pfu/cell control pSCGβ (con)- or pSCflTRPV1 (TRPV1)-containing vaccinia viruses (16 h). Anti-FLAG-reactive proteins were immunoprecipitated and resolved by SDS/PAGE. Immunocomplexes were transferred to PVDF membrane and visualized by anti-FLAG Western-blot analysis. TRPV1 is present as a prominent, 97 kDa, FLAG-immunoreactive species. (**D**) Average current amplitudes of three individual experiments plotted against time. Extracellular application of 1 μM capsaicin activates large inward and outward membrane currents in pSCflTRPV1-infected RBL-2H3-M_1 cells. High-resolution current recordings were acquired every 2 s by applying voltage ramps (-100 to $+100$ mV) of 50 ms duration. (**E**) Isolated high-resolution data trace obtained after 80 s of the experiment. *I–V* relationship exhibits outward rectification and $E_{rev} = 14.8$ mV $\pm$ 1.1 (means $\pm$ S.D., $n = 3$), both typical for TRPV1 and indicating calcium permeability. Control pSCGβ-infected cells did not exhibit capsaicin-dependent currents.

All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions. Currents were filtered at 2.3 kHz and digitized at 100 μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. For analysis, the very first ramps before application of capsaicin or carbachol were digitally filtered at 2 kHz, pooled and used for leak-subtraction of all subsequent current records. The low-resolution temporal development of currents at a given potential was extracted from the leak-corrected individual ramp current records by measuring the current amplitudes at voltages of -80 or $+80$ mV.

Calcium measurements

The cytosolic calcium concentration was monitored at a rate of 5 Hz with a photomultiplier-based system using a monochromatic light source tuned to excite fura 2 fluorescence at 360 and 390 nm for 20 ms each. Emission was detected at 450–550 nm with a photomultiplier whose analogue signals were sampled and processed by the X-Chart software package (HEKA Electronik). Fluorescence ratios were translated into free intracellular calcium concentration based on calibration parameters derived from patch-clamp experiments with calibrated calcium concentrations. Ester loading of intact cells was performed by incubating cells for

45–60 min in the modified Ringer's solution (1 mM extracellular calcium), supplemented with 5 μM fura 2/AM. Local perfusion of individual cells with capsaicin, carbachol or thapsigargin was achieved with a wide-tipped, pressure-controlled application pipette (3 μm diameter).

RESULTS

TRPV1 mediates intracellular calcium release activity in endogenous and heterologous systems

Dorsal root ganglion-derived neurons that express TRPV1 exhibit capsaicin-induced calcium release from an undefined intracellular calcium store [19,20]. We have noted that TRPV1 acts as both an intracellular calcium release channel and a plasma membrane influx channel in a catecholaminergic neuronal cell line which is a native context for this conductance [21–24]. Figure 1(A) shows Western-blot analysis of TRPV1 expression in Cath.a cells. This expression is concomitant with both intracellular and plasma membrane localization of TRPV1, as evidenced by the calcium release and influx responses exhibited by Cath.a cells treated with capsaicin (Figure 1B). TRPV1 is an attractive potential tool in the dissection of intracellular calcium responses, since the protein is probably present in intracellular compartments as part of its biosynthesis and trafficking and has a lipophilic, membrane-permeant ligand, capsaicin [25].

We established a heterologous expression system for TRPV1 in the RBL cell background, a mast cell line in which SOC responses have been studied extensively [15,26–28]. We used a variant RBL line with stable expression of the muscarinic M_1 receptor, permitting generation of large, rapid $InsP_3$ transients via carbachol application. We constructed a recombinant vaccinia virus, pSCflTRPV1, containing the TRPV1 cDNA fused to an N-terminal FLAG epitope tag. Typically, vaccinia-driven expression systems allow the expression of relatively large amounts of heterologous protein in 75–95% of the cell population. RBL-2H3-M_1 cells were infected with either pSCflTRPV1 or a control virus, pSCGβ. Figure 1(C) shows that infection with pSCflTRPV1, but not the control virus, results in the expression of a 97 kDa protein with an immunoreactive FLAG epitope.

We tested whether the heterologously expressed 97 kDa species represented a functional capsaicin receptor using electrophysiological techniques. TRPV1 introduction into RBL-2H3-M_1 cells resulted in the presence of a distinctive cation current. Figures 1(D) and 1(E) show the results of high-resolution current recordings obtained in the whole-cell patch-clamp configuration. Application of 1 μM capsaicin induced large inward and outward membrane currents in TRPV1-infected (but not control) cells. Under the ionic conditions used, inward currents are carried by Ca^{2+} and Na^+, whereas the outward portion is carried by Cs^+ ions. Significant outward rectification is observed for the TRPV1-mediated currents, presumably resulting from decreased inward currents at negative potentials where calcium permeation occurs. This would tend to decrease the total inward (Ca^{2+}/Na^+) current by acting as a permeation blocker, or by causing a calcium-dependent alteration in the TRPV1 open probability [25]. Outward rectification is accompanied by a positive reversal potential [$E_{rev} = 14.8 \pm 1.1$ mV (means $\pm$ S.D., $n = 3$)], indicating a significant calcium permeability of TRPV1 cation currents. In the presence of extracellular calcium, TRPV1-mediated cation currents rapidly desensitize in the RBL-2H3 system, and remain suppressed during repeated applications of capsaicin.

As in the endogenous Cath.a cell context, capsaicin application to RBL-2H3-M_1 cells resulted in the presence of two distinct fura 2 (calcium flux) responses. We performed single-cell calcium measurements in pSCflTRPV1-infected RBL-2H3-M_1 cells loaded with fura 2, in the presence and absence of external calcium. The experimental set-up used a wide-tipped application pipette to puff external solution over a single cell. This solution may contain calcium or be nominally calcium-free. Thus for the duration of the application, the external environment of the cell under examination can be controlled and maintained nominally free of calcium. Cessation of application results in the re-admission of external calcium from the surrounding bath sol-

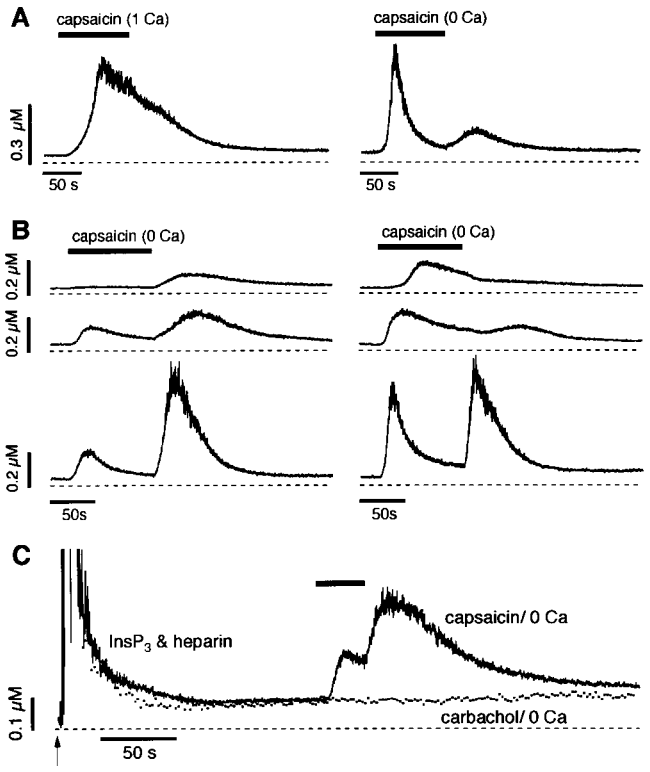

Figure 2 (A) Capsaicin elicits both influx and release responses in TRPV1-expressing cells; (B) diversity of capsaicin-induced release responses in TRPV1-expressing cells; (C) TRPV1-induced calcium release is not an $InsP_3$-mediated process

(**A**) RBL-2H3-M_1 cells were infected with pSCflTRPV1 and loaded with fura 2. Cells were treated with 1 μM capsaicin for the times indicated by the bars and single-cell measurements of changes in intracellular free calcium levels were taken. Left panel: standard extracellular bath solution contained 1 mM Ca^{2+} (1 Ca); capsaicin treatment in the presence of external calcium elicits a rapid increase in intracellular calcium which slowly decays to baseline. Right panel: capsaicin applied in nominally calcium-free medium (0 Ca) elicits a rapid transient increase in the intracellular calcium levels, indicating release of stored intracellular calcium. Readmission of calcium results in an off-response, probably consisting of influx through slowly deactivating TRPV1 channels in the plasma membrane. (**B**) After infection with pSCflTRPV1, fura 2-loaded RBL-2H3-M_1 cells were treated with 1 μM capsaicin for the times indicated by the bars and single-cell measurements of changes in intracellular free calcium levels were taken in the absence of external calcium. Six traces from individual cells present examples of the diverse responses to capsaicin present in a cell population. (**C**) RBL-2H3-M_1 cells expressing TRPV1 were patch-clamped in the whole-cell configuration and continuously perfused with standard intracellular solution supplemented with 200 μM fura 2, 20 μM $InsP_3$ and 0.1 μg/ml heparin. The arrow indicates time of whole-cell establishment, when $InsP_3$ induced a large calcium transient and sustained influx via store operation of I_{CRAC}. Solid trace ($n = 6$): after 3 min, cells were locally superfused with 1 μM capsaicin in nominally calcium-free solution (indicated by black bar), eliciting calcium release via TRPV1. Broken trace ($n = 4$): after 3 min, cells were locally superfused with 100 μM carbachol in nominally calcium-free solution (indicated by black bar). This trace is shifted -139 nM for display purposes. Note that $InsP_3$/heparin perfusion prevents carbachol induction of release responses, indicating that $InsP_3$Rs are fully ligated/blocked. TRPV1-mediated release responses are therefore unlikely to involve $InsP_3$ or $InsP_3$R.

ution. The results in Figure 2(A) show that, in the presence of extracellular calcium (left panel), a typically rapid response to capsaicin is observed which slowly desensitizes. In the absence of extracellular calcium, accomplished by the application of capsaicin in a zero calcium solution (right panel), a significant calcium signal in response to capsaicin application is also observed, indicating that TRPV1 is mediating the release of calcium from an intracellular store. After depletion of this store, calcium re-admission leads to an off-response (right panel), where plasma membrane TRPV1 channels can mediate an influx of calcium, whereas the cation channels slowly deactivate. Moreover, since store depletion in RBL-2H3-M_1 cells can couple with I_{CRAC} activation [27,28], we reasoned that this smaller membrane conductance might be a component of the off-response observed.

We noted considerable diversity in the single-cell responses to capsaicin. Some cells exhibited both release and influx components, whereas others displayed either component of the calcium response in isolation (see Figure 2B). Both release and influx responses are sensitive to capsazepine (results not shown), a competitive inhibitor of capsaicin binding to TRPV1. A similar degree of variability was observed throughout the time course of protein expression and no correlation was observed between the magnitude of release and influx responses. A similar variation exists for both thapsigargin- and $InsP_3$-mediated store depletion [6], and is a predictable feature of intact cell fura 2 measurements where the membrane potential of individual cells is not being clamped. These non-clamped conditions apply in all the intact cell calcium measurement experiments of the present study.

TRPV1 release responses do not involve $InsP_3Rs$

Our results suggest that TRPV1 is a physiologically occurring calcium release channel, analogous in function to the $InsP_3$ and ryanodine receptors. Calcium release activity for capsaicin has been noted in primary dorsal root ganglia neurons [19], but has not been definitively attributed to TRPV1. We established that TRPV1 release activity is not mediated through TRPV1 accessing a conventional calcium release pathway (i.e. through the generation of $InsP_3$). These results are shown in Figure 2(C). High concentrations of $InsP_3$ and heparin were supplied to the cytosol of a TRPV1-expressing RBL-2H3-M_1 cell via the patch pipette. As shown in the control trace (broken line), this perfusion throughout the time course of the experiment ensures that $InsP_3Rs$ are fully ligated/blocked [6,29]. Initial application of $InsP_3$ caused release from $InsP_3$-sensitive stores, which then refilled in the presence of external calcium. However, the continued perfusion of high doses of $InsP_3$ and heparin ensures that any further release responses observed cannot be a result of $InsP_3$ interaction with its receptor. Nevertheless, application of capsaicin causes a large release transient, which under the conditions of $InsP_3$/heparin perfusion, cannot be attributed to any $InsP_3$ interaction with $InsP_3R$ release channels (Figure 2).

Depletion of calcium stores using TRPV1 does not activate I_{CRAC}

I_{CRAC} activation is a key event in mast cell activation that well illustrates the potential for store heterogeneity to add complexity to cellular signalling pathways, since I_{CRAC} activation is thought to be dependent on a distinct store subset. We examined whether the capsaicin-releasable store compartment could cause I_{CRAC} activation. The outcome of this experiment would then indicate an important and specific molecular feature of the CRAC store, namely the presence or absence of TRPV1 release channels.

We performed whole-cell current recordings of RBL-2H3-M_1 cells expressing TRPV1. We applied independently either capsaicin, to stimulate TRPV1, or extracellular carbachol, which

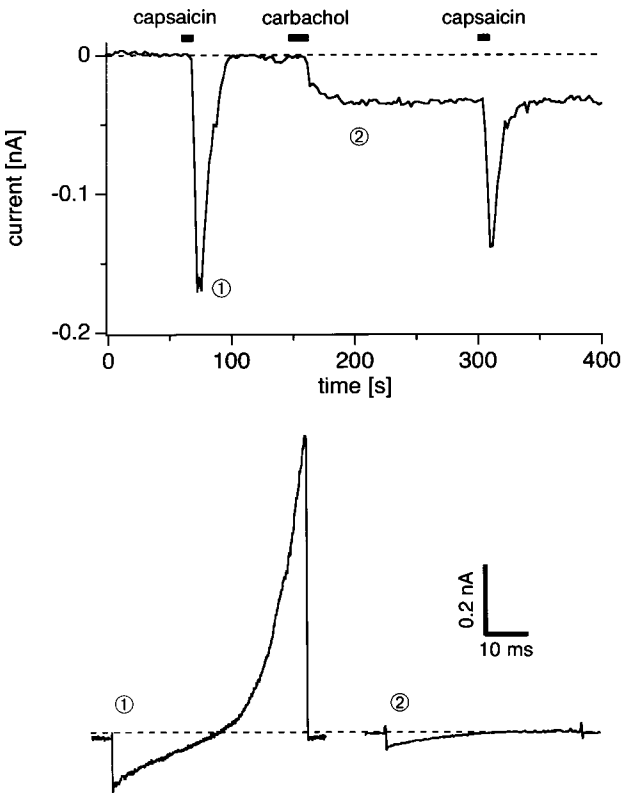

Figure 3 Depletion of intracellular calcium stores through TRPV1 does not activate I_{CRAC}

Upper panel: low-resolution time course of inward currents measured at −80 mV and plotted against time. Inward current amplitudes were derived from high-resolution current records (lower panel) acquired every 2 s by applying voltage ramps (−100 to +100 mV) of 50 ms duration. The first capsaicin application evoked a transient increase in inward current that returned to baseline after termination of capsaicin application. The cell was stimulated with carbachol, eliciting the sustained inward current I_{CRAC}. While I_{CRAC} was in effect, a second application of capsaicin again transiently activated TRPV1 cation channels, and the TRPV1-mediated current returned to a new baseline corresponding to the sustained I_{CRAC}. The I_{CRAC} current level was neither enhanced nor diminished by TRPV1. Lower panel: high-resolution current recordings taken at 80 and 200 s of the experiment, demonstrating cation current with an *I–V* relationship typical of TRPV1 channels (80 s) and a smaller inward-rectifying current with positive reversal potential, the characteristic signature of I_{CRAC} (200 s). Data are representative of 3 cells treated with this method and > 40 cells were also analysed where capsaicin did not induce I_{CRAC}.

causes $InsP_3$ production following activation of the M_1 receptor. Our prediction was that if the capsaicin-induced influx contained an I_{CRAC} component, this would be apparent immediately as a capsaicin-induced activation of I_{CRAC} or reflected as an alteration in the sustained I_{CRAC} that normally follows application of carbachol. These results are shown in Figure 3, which documents a representative trace from three cells treated using this method. Application of capsaicin resulted in the induction of a large inward current, which returned to baseline shortly after the termination of capsaicin exposure. We then developed I_{CRAC} by the application of carbachol, generating a sustained calcium current with the classical signature of I_{CRAC}. Re-application of capsaicin again elicited the TRPV1-mediated current, which also returned to a new baseline. This baseline corresponded to the sustained component of I_{CRAC}, which was neither diminished nor enhanced by the depletion of capsaicin-sensitive stores. A 10-fold increase in the applied [capsaicin] also had no effect on I_{CRAC} (results not shown), indicating that this is not a dose effect, but rather a marked delineation between the CRAC-operating cal-

cium store and the TRPV1-containing compartment. These results demonstrate unequivocally, using a functional rather than pharmacological depletion method, that calcium release does not automatically couple with CRAC activity.

TRPV1 sensitivity functionally subdivides the $InsP_3$-responsive intracellular calcium store

The results presented so far show that TRPV1 is present and functional in two discrete locations, at the plasma membrane and in an intracellular calcium store. TRPV1-mediated calcium release does not couple with I_{CRAC} activity. We hypothesized that this inability to activate I_{CRAC} reflected a separate location for the TRPV1 compartment, either completely distinct from the $InsP_3$R-containing store or in an $InsP_3$-sensitive store that does not couple with I_{CRAC}. We therefore investigated the relationship between TRPV1-containing stores and compartments that were sensitive to depletion by $InsP_3$.

We established a saturating dose for the depletion of capsaicin-sensitive calcium stores. Figure 4(A) shows that in a protocol where TRPV1-expressing RBL-2H3-M_1 cells were sequentially treated with 10 and 20 μM capsaicin, the first application effectively saturated the TRPV1 release channels. We then employed this saturating capsaicin dose, and the $InsP_3$-mobilizing M_1 receptor ligand carbachol to analyse the relationship between the $InsP_3$-sensitive calcium store and the TRPV1-containing compartment.

In Figure 4(B), we serially applied carbachol and capsaicin to RBL-2H3-M_1 cells expressing TRPV1. No refilling of calcium stores from extracellular calcium was permitted during the time course of exposure to capsaicin or carbachol. After depletion of intracellular stores via carbachol, TRPV1 activation by capsaicin can no longer elicit a significant calcium release response. Readmission of extracellular calcium results in a large off-response, presumably a composite signal derived from both capsaicin-gated plasma membrane channels and $InsP_3$-mediated activation of store-operated I_{CRAC}. These results indicate that most of the capsaicin-responsive stored calcium is in a compartment that may also be depleted via $InsP_3$Rs.

These results suggest that, in fact, TRPV1 proteins do reside in an $InsP_3$R-containing calcium store. The question which arises immediately is whether TRPV1 activation can deplete the entire $InsP_3$-sensitive store. This was tested by the reverse sequence of agonist application. Figure 4(C) illustrates the outcome of this experiment and demonstrates that a large carbachol-induced release persists in cells treated with capsaicin. Thus the capsaicin-sensitive compartment is a functional subdivision of the total $InsP_3$-responsive store with two potential release activities present, the $InsP_3$R and TRPV1.

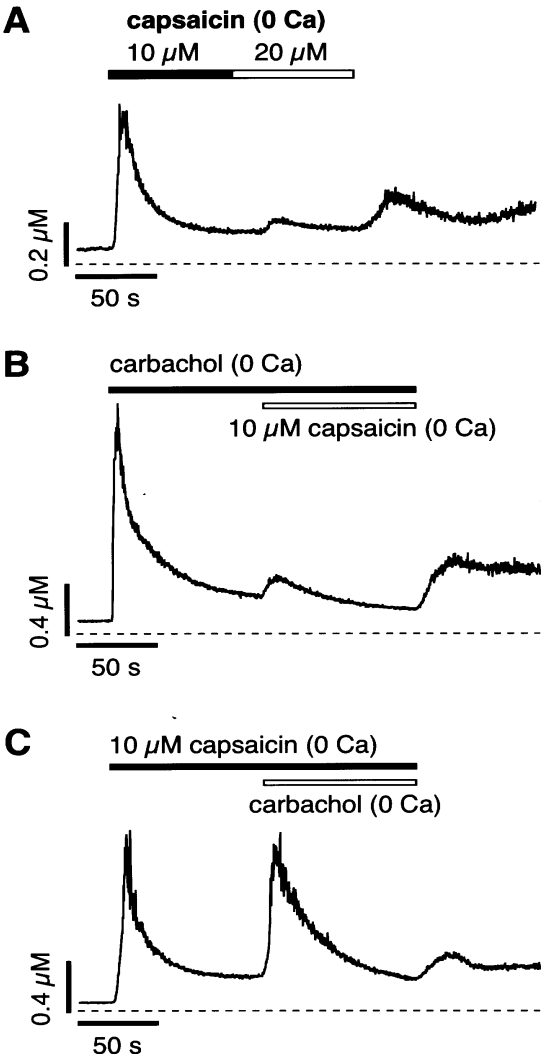

Figure 4 (A) Establishment of 10 μM capsaicin as a saturating dose for calcium release via TRPV1; (B) the capsaicin-sensitive store is encompassed by the $InsP_3$-sensitive stores; (C) the majority of the $InsP_3$-sensitive calcium store is not capsaicin-sensitive

(**A**) Average response of TRPV1-expressing RBL-2H3-M_1 cells ($n = 6$) stimulated by sequential application of 10 and 20 μM capsaicin, both in calcium-free solutions. Note that 20 μM capsaicin produced an extremely small release response, indicating that 10 μM was already a saturating concentration for release. (**B**) Average response of TRPV1-expressing RBL-2H3-M_1 cells ($n = 8$) stimulated by sequential application of carbachol (100 μM, open bar), then a saturating dose of capsaicin (10 μM, closed bar), both in calcium-free solutions. Note that capsaicin mobilized a marginal amount of calcium after pretreatment with carbachol, indicating that the carbachol-sensitive compartment encompasses most of the capsaicin-senstive store. (**C**) Average response of TRPV1-expressing RBL-2H3-M_1 cells ($n = 10$) stimulated by sequential application of capsaicin (saturating dose 10 μM) followed by 100 μM carbachol. Note the large residual carbachol response after complete depletion of TRPV1-gated calcium stores.

TRPV1 resides in a thapsigargin-insensitive calcium store with a functional refilling activity

We next addressed whether a refilling activity was present in the capsaicin-sensitive calcium store. With an initial stimulation method similar to Figure 4(B), Figure 5(A) illustrates that depleting the entire $InsP_3$-sensitive store prevents capsaicin from releasing further calcium. This first challenge is followed by a phase during which readmission of extracellular calcium is allowed to take place. Finally, the cell is subjected to a second challenge with capsaicin (in a nominally calcium-free application solution), which now triggers a substantial calcium release transient. This indicates that the capsaicin-sensitive subset of the $InsP_3$R-containing calcium store is likely to contain a calcium-ATPase activity that can replenish stored calcium after depletion.

Examination of TRPV1-mediated depletion of intracellular calcium stores has allowed us to subdivide functionally the $InsP_3$-sensitive calcium-storage compartment based on differential release activities. However, previous studies have suggested that heterogeneity in refilling activities also pervades intracellular calcium stores [30–32]. $InsP_3$R-containing stores have been shown to refill via both thapsigargin-sensitive and -insensitive calcium ATPases. We tested the sensitivity of the capsaicin-sensitive compartment to depletion mediated by the SERCA inhibitor thapsigargin.

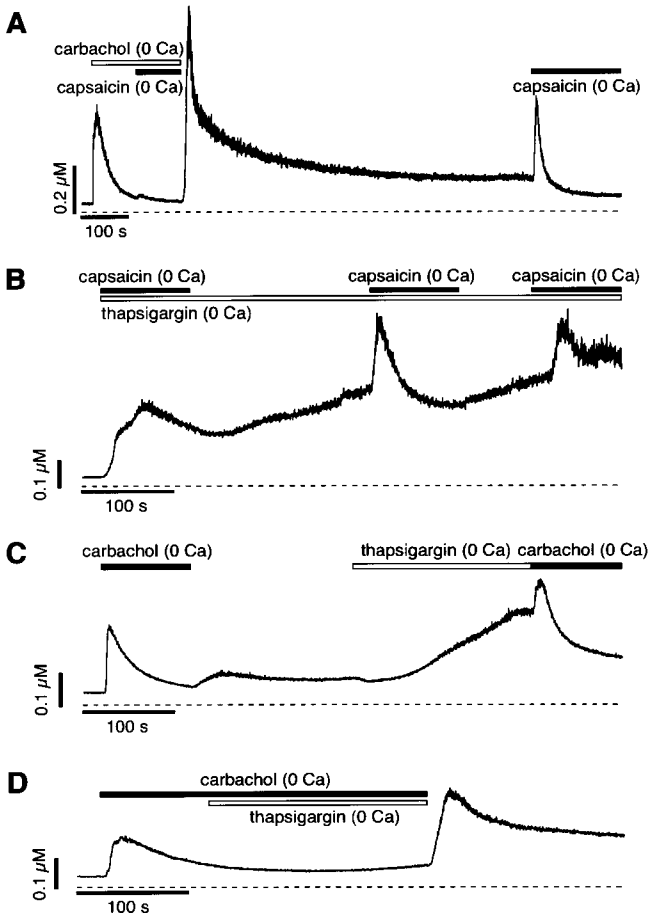

Figure 5 (A) Capsaicin-sensitive calcium stores contain a refilling activity, (B) capsaicin-sensitive calcium stores are insensitive to thapsigargin, (C) a portion of the $InsP_3$-sensitive store is also insensitive to thapsigargin, and (D) the $InsP_3$-sensitive store encompasses the thapsigargin-sensitive compartment

(**A**) Representative trace ($n = 3$) showing single TRPV1-expressing RBL-2H3-M_1 cells treated with the sequential application of carbachol and capsicin in the absence of external calcium to deplete completely the TRPV1-gated stores. At 220 s, application was terminated and external calcium readmitted, allowing store refilling. A second capsaicin application in 0 Ca solution at 1100 s resulted in a clear release transient, indicating that the TRPV1-gated compartment contains a refilling activity. (**B**) Average response of five TRPV1-expressing RBL-2H3-M_1 cells to sequential applications of 2 μM capsaicin (closed bars) in the continued presence of 1 μM thapsigargin (open bar). Throughout, the external solution was kept nominally calcium-free. Multiple applications of capsaicin elicited release, indicating that TRPV1-containing stores had refilled using a thapsigargin-insensitive calcium ATPase. (**C**) Average response of TRPV1-expressing RBL-2H3-M_1 cells to sequential application of 100 μM carbachol, 1 μM thapsigargin and, finally, a second challenge with 100 μM carbachol ($n = 14$). Both carbachol (closed bars) and thapsigargin (open bars) were applied in nominally calcium-free solutions. Note that some carbachol-induced release can occur after reaching a plateau of thapsigargin-mediated release. (**D**) Average response of cells ($n = 7$) to the application of 100 μM carbachol, followed by 1 μM thapsigargin. Both carbachol (closed bar) and thapsigargin (open bar) were applied in nominally calcium-free solutions. Note that thapsigargin could not induce release after the depletion of the $InsP_3$-sensitive compartment using carbachol.

Figure 5(B) shows the averaged calcium response from five TRPV1-expressing RBL-2H3-M_1 cells stimulated as follows. Capsaicin was applied repetitively while a maximal dose of 1 μM thapsigargin was continuously applied. Both stimuli were applied in external buffer containing no calcium so that during the entire experiment calcium influx was not allowed. Thus no calcium changes due to external calcium or the action of plasma membrane calcium channels are featured in the present experiment. Since thapsigargin was continually perfused on to the cells, thapsigargin-sensitive SERCA pumps were also not available to refill capsaicin-sensitive stores. As expected, initial application of capsaicin along with 1 μM thapsigargin depleted TRPV1-containing stores. Despite the continued presence of thapsigargin and the absence of calcium influx, reapplication of capsaicin caused a marked release from intracellular stores, showing that these stores must refill via a thapsigargin-independent mechanism simply by the uptake of available cytosolic calcium. We noted in some cells (and hence in the averaged results presented in Figure 5B) that calcium levels exhibited a continuous increase between applications of stimuli in calcium-free external conditions. The lack of extracellular calcium indicates that these calcium increases between stimuli cannot be attributed to influx but rather are likely to be due to a passive store depletion (leak) that is normally opposed by the SERCA pumps that we have inactivated here with thapsigargin.

We have shown that TRPV1 is present within a subset of the intracellular calcium store that may be depleted using $InsP_3$. Since the above results suggest that the TRPV1-containing store is insensitive to thapsigargin, it follows that some part of the $InsP_3$-depleted store must also be thapsigargin-insensitive. We tested this idea in the experiments represented by Figure 5(C). Here, the magnitude of release from the total $InsP_3$-sensitive store was established by the application of carbachol in the absence of extracellular calcium. Stores were then allowed to refill by readmission of external calcium. Thapsigargin-sensitive stores were then depleted, until a plateau phase was reached that indicates full depletion of any thapsigargin-sensitive compartment. At this point, carbachol was reapplied. In close agreement with the results obtained with capsaicin, we observed that carbachol elicited a significant release response following pre-exposure to thapsigargin. These results indicate that, indeed, there is a part of the total $InsP_3$-sensitive store that is not impacted by thapsigargin. We would propose that this probably corresponds to the compartment containing TRPV1. Finally, we can also show that the $InsP_3$-sensitive store encompasses the thapsigargin-sensitive compartment, since cells treated with carbachol do not responsd to subsequent application of thapsigargin (Figure 5D).

Calcium store subdivisions defined

Clearly, capsaicin-evoked influx does not contain an I_{CRAC} component. TRPV1 mediates a significant release response that does not couple with I_{CRAC} activation. These results affirm, unequivocally, the suggestion made by our group, and others, that store depletion does not automatically couple with CRAC activation. The concept of a specific 'CRAC store' arises from the result that release and influx can be dissociated. Significant release from $InsP_3$-responsive stores occurs in the absence of I_{CRAC} induction. It has been proposed that CRAC activation follows selective depletion of a store subcompartment with a high threshold for responsiveness to $InsP_3$. Clearly, TRPV1 does not functionally invade this store. Within the $InsP_3$-sensitive calcium store, there is clearly heterogeneity at the levels of release and refilling. Two subsets of stores are $InsP_3$-sensitive; one of these is also capsaicin-sensitive and thapsigargin-insensitive. The remaining $InsP_3$-responsive store refills via thapsigargin-sensitive SERCA and does not contain functional TRPV1 molecules. This encompasses a third compartment that is either functionally, or physically, delineated by its high threshold for responsiveness to $InsP_3$ [6,15]. Our results provide direct evidence for a functional compartmentalization of $InsP_3$R-containing calcium stores. A

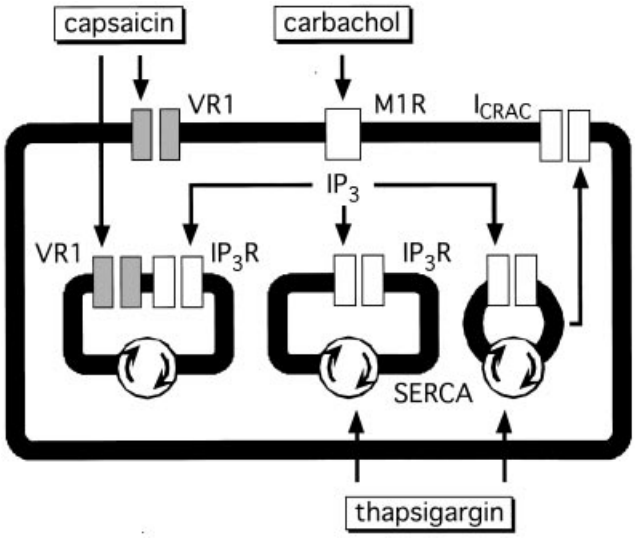

Figure 6 Store heterogeneity and coupling with the store-operated current I_{CRAC}

Model illustrating our observations that (1) TRPV1 may act as a release channel in addition to mediating influx, (2) TRPV1-mediated release occurs from a functional subset of the $InsP_3(IP_3)$-sensitive store and (3) the TRPV1-releasable store refills via a thapsigargin-insensitive SERCA, whereas the non-capsaicin-sensitive portion of the $InsP_3$ store refills via a thapsigargin-sensitive calcium ATPase. Finally, we find that the TRPV1-containing portion of the $InsP_3$-sensitive store does not couple with the depletion-activated calcium current I_{CRAC}. We postulate that since global depletion of $InsP_3$-sensitive stores is not required for I_{CRAC} activation, a small independent store, possibly physically separated from the bulk of the $InsP_3$-reactive compartment, controls I_{CRAC} activity. VR1, TRPV1; M1R, muscarinic M1 receptor.

model that is consistent with the experimental evidence from the present study is presented in Figure 6.

DISCUSSION

In the present study, we have used the capsaicin-gated calcium channel TRPV1 as a novel tool to examine heterogeneity within intracellular calcium storage compartments. We can dissect intracellular stores based on differential release activities; within the $InsP_3$-sensitive store there is a compartment that also contains functional TRPV1 molecules that mediate release. There is also heterogeneity at the level of store refilling. The TRPV1/$InsP_3R$-containing store is apparently thapsigargin-insensitive, suggesting that it refills via a SERCA pump isoform that is unaffected by this inhibitor. In contrast, the remaining $InsP_3R$-containing compartment cannot refill in the presence of thapsigargin.

It is important that this heterogeneity of release and refilling activities is unlikely to be merely distributive, since there are important functional consequences for the cell. We have examined the activity of the depletion-activated calcium current, I_{CRAC}. Our results argue against a simple model where a small depletion in stored calcium across the whole $InsP_3$-sensitive compartment activates I_{CRAC}. Rather, we find that depletion of almost the entire $InsP_3R$/TRPV1-containing calcium store can occur without I_{CRAC} activation. Thus coupling with I_{CRAC} is restricted to a subset of the $InsP_3$ stores. Our results strongly argue for the presence of a specific 'CRAC store' within the $InsP_3$-sensitive compartment: the non-TRPV1 containing $InsP_3$- and thapsigargin-sensitive compartment contains or comprises the store that specifically couples with I_{CRAC}.

The existence of a specific $InsP_3$-sensitive compartment that couples with I_{CRAC} has been postulated [6,17,18]. Previous studies have defined first that the $InsP_3$-sensitive store is a subcompartment of the entire ionomycin-releasable store. Moreover, within the $InsP_3R$-containing store, distinct compartments are suggested by pharmacological data. Induction of calcium release with either different concentrations of $InsP_3$ [6] or $InsP_3R$ agonists of different affinities [17] demonstrate that all $InsP_3$ compartments are not identical with regard to depletion-activated calcium influx. Differential distribution of $InsP_3R$ isoforms and their inherently different affinities for $InsP_3$ and other agonists means that functional compartmentalization of the $InsP_3$-sensitive store has not been accomplished previously. However, those results can now be used to predict characteristics of the CRAC store, in parallel with the conclusions in the current report. Parekh and Penner [6] suggested that calcium release from the bulk of $InsP_3$-sensitive stores is accomplished by low concentrations of $InsP_3$, whereas higher levels of $InsP_3$ are required for I_{CRAC} activation. This would suggest that a high-affinity interaction or lack of metabolism of the ligand occurs in a selective environment [15,33]. Indeed, activation of influx is readily achieved if a high-affinity $InsP_3R$ agonist (adenophostin A) is used, but not if the low-affinity L-(α-glycerophospho)-D-*myo*-inositol-4,5-bisphosphate agonist is applied [17]. Thus the $InsP_3$-coupled CRAC store will probably be activated only when a threshold level of $InsP_3$ is reached. We have suggested that this threshold is set by specialization in the local populations of $InsP_3$-metabolizing enzymes [15,33]. Since there is some differential affinity of $InsP_3$ for its receptor subtypes and possibly differential subcellular localization of the isoforms, it is also possible that the CRAC store contains a non-random population of $InsP_3R$ isotypes [34,35]. Detailed analysis of $InsP_3R$ isotype locations and the localization of TRPV1 in this overexpression system may assist in identifying the CRAC store, although the former may be complicated by the reported propensity of $InsP_3Rs$ to form hetero-oligomers [36].

We can state that the CRAC store does not contain functional TRPV1 channels. Confocal analysis (results not shown) suggests that TRPV1 is distributed throughout many intracellular membranes, as would be expected for a plasma membrane-directed protein that is synthesized in the ER and then trafficked through the Golgi apparatus to the cell surface. A Golgi compartment may be a good candidate for the store that can be released via TRPV1, since TRPV1 multimers must be fully formed and a thapsigargin-insensitive Golgi compartment has been described previously [30]. It is unlikely that the CRAC store represents any of the compartments on the biosynthesis/trafficking route of TRPV1, except possibly an early, perinuclear compartment of the ER. In this region, nascent TRPV1 molecules would not have formed functional protein complexes (see below). However, several lines of evidence suggest that a compartment deep in the cytosol is unlikely to be the CRAC store. In *Xenopus* oocytes, a local depletion-activated calcium influx is induced after local depletion of sub-plasma membrane calcium stores [37]. Jaconi et al. [38] showed, also in the oocyte system, that calcium entry was constrained to an area adjacent to ER stores that had been forced into membrane proximity. Moreover, using an agent that sensitizes the $InsP_3R$ stores to $InsP_3$ in cytosol perfusion experiments, Parekh and Penner [26] showed that the site of $InsP_3$ action for I_{CRAC} activation was probably very close to the site of $InsP_3$ production in the plasma membrane. Thus we can predict that the CRAC store will be localized close to the plasma membrane, and will be separated from the main biosynthesis/trafficking route for membrane proteins such as TRPV1.

At present, a late Golgi compartment seems the best candidate for the CRAC store. Pinton et al. [30] have shown that the Golgi

apparatus is an $InsP_3$-sensitive calcium store, with heterogeneous thapsigargin-sensitive and -insensitive refilling activities. The properties of the Golgi apparatus as a whole match the required release and refilling activities for the CRAC store. However, the spatial and functional diversity of Golgi vesicles may be sufficient to encompass multiple functional stores delineated by distinct release/refilling activities, including the CRAC store and the TRPV1-gated compartment. Moreover, a late-Golgi compartment would be consistent with the presence of the membrane fusion machinery that is required by a current I_{CRAC} activation model [39,40] that involves regulated fusion of CRAC-channel-containing vesicles with the plasma membrane. There would be a certain economy if the postulated CRAC-containing vesicle were also the CRAC store, a specialized late-Golgi compartment.

Our present study uses TRPV1 as a ligand-gated tool for the manipulation of intracellular calcium levels. Although the results generated have clear implications for the biology of the I_{CRAC} channels, we should also consider new insights into the behaviour of TRPV1. In our native and heterologous cell contexts, TRPV1 mediated calcium release from an intracellular store. It is important to determine now whether physiological activators of TRPV1, including heat, low pH and cannabinoid lipids, can induce this release activity and the physiological implications of calcium available from this source. Moreover, we note that TRPV1 subunits are assembled and function early in the biosynthetic pathway, raising questions as to whether intracellular activition is a general feature of nascent ion channels if their activatory mechanism is available in that location. It is possible that the poorly characterized ER 'leak current' represents the partial activity of newly formed channel species.

Understanding of the heterogeneity of intracellular stores requires both an assay for a specialized function, such as I_{CRAC} activation, and a targetable experimental tool, such as TRPV1. We have provided evidence that a specialized CRAC store exists within the $InsP_3$-sensitive compartment and that calcium stores may be further subdivided based on their intersection with ion channel biosynthesis/trafficking routes. Information on how functional subdivision of stores arises is probably intrinsic to the protein sequences of the differentially distributed proteins. Suborganellar targeting sequences are not well understood, and are not immediately obvious in the protein players described here, such as the $InsP_3R$, SERCA and TRPV1. Thus a wealth of new information lies in genetic dissection of these molecules and future manipulation of their subcellular localization.

We thank Dr David Julius for the generous gift of TRPV1 cDNA. Murielle Koblan-Huberson and Mahealani Monteilh-Zoller provided excellent technical assistance. During the greater part of this work, H.T. was an International Prize Traveling Fellow of the Wellcome Trust. This work was supported in part by NIH R21 AI50200-01 to R.P. Members of the Laboratory of Cell Biology and Immunology at QCBR provided comments on the manuscript.

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Received 2 September 2002/5 December 2002; accepted 6 January 2003
Published as BJ Immediate Publication 6 January 2003, DOI 10.1042/BJ20021381