

IP₃ receptor subtype-dependent activation of store-operated calcium entry through I_{CRAC}

Christine Peinelt¹, Andreas Beck, Mahealani K. Monteilh-Zoller, Reinhold Penner, Andrea Fleig*

Center for Biomedical Research at The Queen's Medical Center and John A. Burns School of Medicine at the University of Hawaii, Honolulu, HI 96813, USA

ARTICLE INFO

Article history:

Received 29 September 2008

Received in revised form

13 November 2008

Accepted 8 December 2008

Available online 20 January 2009

Keywords:

I_{CRAC}

IP₃ receptor

Ca²⁺ store heterogeneity

ABSTRACT

The store-operated, calcium release-activated calcium current I_{CRAC} is activated by the depletion of inositol 1,4,5-trisphosphate (IP₃)-sensitive stores. The significantly different dose–response relationships of IP₃-mediated Ca²⁺ release and CRAC channel activation indicate that I_{CRAC} is activated by a functionally, and possibly physically, distinct sub-compartment of the endoplasmic reticulum (ER), the so-called CRAC store. Vertebrate genomes contain three IP₃ receptor (IP₃R) genes and most cells express at least two subtypes, but the functional relevance of various IP₃R subtypes with respect to store-operated Ca²⁺ entry is completely unknown. We here demonstrate in avian B cells (chicken DT40) that IP₃R type II and type III participate in IP₃-induced activation of I_{CRAC}, but IP₃R type I does not. This suggests that the expression pattern of IP₃R contributes to the formation of specialized CRAC stores in B cells.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Calcium release from intracellular Ca²⁺ stores by inositol 1,4,5-trisphosphate (IP₃) represents an important mechanism for calcium (Ca²⁺) influx, since in many cell types, store depletion results in activation of store-operated CRAC channels in the plasma membrane. Interestingly, however, a significant discordance exists between IP₃-induced Ca²⁺ release and I_{CRAC} activation. While IP₃ causes considerable Ca²⁺ release in the nanomolar range, CRAC channel activation occurs only at micromolar IP₃ concentrations [1]. Such differing response thresholds may arise from a number of circumstances, including different complements of IP₃R subtypes with different affinities for IP₃, store heterogeneity with separate compartments containing specific molecular components, and/or differential localization and activity of enzymes involved in IP₃ metabolism with different parts of the endoplasmic reticulum (ER) experiencing different IP₃ concentrations [2–6]. This concept of store-heterogeneity is contentious, with some studies favoring the concept, whereas others propose a continuous calcium store [7,8]. Some of these discrepancies may be due to the cell under investigation, possibly linked to the utilization of store-operated calcium entry as a primary mechanism for signaling [9].

It seems clear by now that the ER is not the only organelle involved in Ca²⁺ storage. Mitochondria, Golgi, nucleus and lysosomes—all have been implicated in agonist-induced Ca²⁺ release [10]. Most recently, peroxisomes have been ascribed a role in Ca²⁺ storage [11], further contributing to cellular Ca²⁺ store heterogeneity. Clearly, store heterogeneity would also be critically influenced by the complement of calcium release channels within the respective organelle. In the ER for example, the activation of the dual Ca²⁺-influx and Ca²⁺-release channel transient receptor potential vanilloid type 1 (TRPV1) by capsaicin can mobilize substantial amounts of Ca²⁺ from thapsigargin-insensitive intracellular stores without resulting in I_{CRAC} activation [12]. Thus, there is evidence to suggest the presence of a functionally and morphologically separate CRAC store that contains IP₃ receptors and thapsigargin-sensitive Ca²⁺ pumps. Heterogeneity also exists within IP₃-sensitive Ca²⁺ stores, as they respond to IP₃ with different sensitivities and complex release kinetics [13]. IP₃-sensitive receptors in vertebrates are encoded by three different genes, expressed as IP₃R types I, II, and III, which can form homo- or heterotetrameric channel complexes [14–20]. The question therefore arises whether IP₃R subtype composition contributes to the formation of a distinct CRAC store.

2. Methods

2.1. Cell culture

All DT40 chicken B cell lines were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, 5% chicken serum, penicillin, streptomycin, and glutamine. DT40 cells

* Corresponding author at: Center for Biomedical Research, UHT 809, The Queen's Medical Center, Honolulu, HI 96813, USA. Tel.: +1 808 537 7931 fax: +1 808 537 7939.

E-mail address: afleig@hawaii.edu (A. Fleig).

¹ Current address: Institute for Biophysics, University Saarland, Homburg 66421, Germany.

expressing all three types of P₃ receptors, the triple P₃ receptor knock-out cell line and the cell lines expressing type I, type II or type III P₃ receptor were a gift of Dr. Kurosaki and coworkers [21].

2.2. Electrophysiology

Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21–25 °C. High-resolution current recordings were acquired using the EPC-9 patch-clamp amplifier (HEKA). Voltage ramps of 50 ms duration spanning a range of –150 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 300 s. Liquid junction potential was 10 mV. Currents were filtered at 2.9 kHz and digitized at 100 μ s intervals. Extracting the current amplitude at –130 mV from individual ramp current records assessed the low-resolution temporal development of currents. Where applicable, statistical errors of averaged data are given as means \pm S.E.M. with *n* determinations. Standard external solutions were as follows (in mM): 120 NaCl, 2.8 KCl, 2 MgCl₂, 20 CaCl₂, 10 HEPES, 11 glucose, pH 7.2 with NaOH, 300 mOsm. In some experiments 2 μ M ionomycin in external solution containing no CaCl₂ was applied for 2 s. Standard internal solutions were as follows (in mM): 120 Cs-glutamate, 8 NaCl, 10 Cs-BAPTA, 3 MgCl₂, 4 CaCl₂, 10 HEPES, pH 7.2 with CsOH, 300 mOsm. IP₃ concentration was adjusted as indicated. [Ca²⁺]_i was buffered to 150 nM free [Ca²⁺]_i using 10 mM Cs-BAPTA and 4 mM CaCl₂ as calculated with WebMaxC (<http://www.stanford.edu/~cpatton/webmaxc.htm>). All chemicals were purchased from Sigma–Aldrich Co.

In combined patch-clamp and balanced Fura-2 experiments, cells were preloaded with 5 μ M Fura-2-AM for 30 min. In subsequent whole-cell patch clamp experiments 200 μ M Fura-2 was added to the standard internal solution in addition to 10 μ M IP₃.

3. Results

To identify whether differential expression of IP₃R occurs in CRAC stores, we used wild-type DT40 chicken B-lymphocytes expressing all three IP₃R types as well as genetically altered DT40 cells that lack all IP₃R or express only one of the three IP₃R subtypes [21,22]. We first assessed the sensitivity of I_{CRAC} to intracellular IP₃ in wild-type (wt) DT40 cells using whole-cell patch-clamp recording. Experiments were performed in a standard extracellular NaCl-based saline containing 20 mM CaCl₂ and cells were perfused with a standard Cs-glutamate-based intracellular solution, where intracellular free Ca²⁺ ([Ca²⁺]_i) was clamped to 150 nM using a mixture of 10 mM Cs-BAPTA and 4 mM CaCl₂. At this [Ca²⁺]_i, activation of I_{CRAC} by passive store depletion is prevented and optimal sensitivity of IP₃Rs to IP₃ is ensured [23–26]. Data were acquired using a voltage ramp of 50 ms length that spanned from –150 to +100 mV and was applied every 2 s. Adding 1 μ M IP₃ to the pipette solution caused the development of a current that plateaued at 200 s (Fig. 1A) and exhibited the inward rectification typical for I_{CRAC} ([27]; Fig. 1B). A further increase in IP₃ concentrations beyond 1 μ M did not result in larger currents and reducing IP₃ to 500 nM was insufficient to activate CRAC currents (Fig. 1C). This all-or-none behavior of I_{CRAC} in response to IP₃ is consistent with previous investigations performed in mast cells [1,28]. To obtain the activation time constant, the averaged current development of CRAC was fitted with a single exponential function. The activation time constants for all concentrations of IP₃ that activated I_{CRAC} were ~60 s. The average current amplitudes measured at 200 s into the experiment were plotted against their respective IP₃ concentrations and fitted with a dose–response curve. The resulting fit rendered a half-maximal effective concentration (EC₅₀) of 670 nM with a Hill coefficient fixed to 12. The EC₅₀ did not change significantly when

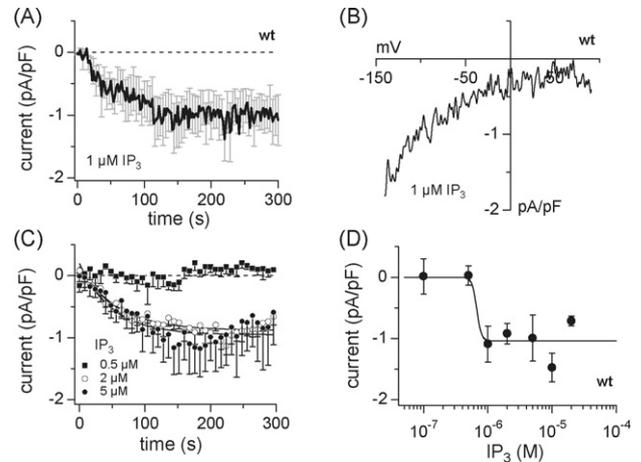


Fig. 1. Non-linear activation of I_{CRAC} by IP₃ in wild-type DT40 B cells. (A) Average CRAC currents induced by 1 μ M IP₃ in wild-type DT40 cells expressing all three types of IP₃ receptors (*n* = 5). Error bars indicated S.E.M. Currents were acquired using a 50 ms voltage ramp spanning from –150 to +100 mV applied at 2 s intervals. Current sizes were extracted at –130 mV, normalized to cell size, averaged and plotted versus time. Currents were leak-corrected by subtracting averages of the first one to three ramp measurements after whole-cell establishment from subsequent current records. The intracellular calcium concentration was clamped to 150 nM (10 mM Cs-BAPTA and 4 mM CaCl₂). (B) Average current–voltage (*I/V*) relationship of CRAC currents extracted from representative DT40 cells shown in panel A at 300 s into the experiment (*n* = 3). (C) Average time-course of CRAC currents induced by 500 nM IP₃ (filled squares, *n* = 3), 2 μ M IP₃ (open circles, *n* = 5) and 5 μ M IP₃ (filled circles, *n* = 5). The calcium concentration was clamped to 150 nM (10 mM Cs-BAPTA and 4 mM CaCl₂) to prevent passive depletion of intracellular calcium stores. Data were fitted using the single exponential function $I_{norm}(t) = I_{total} \times \exp(-t/\tau) + \text{amplitude}$. (D) Average I_{CRAC} amplitude assessed at 200 s and plotted against IP₃ concentration. A dose–response fit to the data yielded an EC₅₀ of 670 nM and a Hill coefficient of 12.

increasing the values for the Hill coefficient between 12 and 30, thus the Hill coefficient value is unlikely to reflect any mechanistic process in this case but allows a phenomenological description of the data. These data show that IP₃-induced activation of I_{CRAC} in wild-type DT40 cells proceeds in a highly non-linear fashion, indicating that similar to RBL mast cells [1,28], B cells might express a functionally distinct CRAC store. This was further explored in DT40 cells with individual IP₃R subtype expression.

DT40 cells lacking all three types of IP₃ receptors (T0) fail to respond to IP₃-producing stimuli, but exhibit store-operated Ca²⁺ entry when emptying stores with thapsigargin [29]. It is reasonable to assume that in the absence of any IP₃ receptors, store-operated recruitment of I_{CRAC} via the second messenger IP₃ should be abolished, provided that the functionality of CRAC currents is not affected by the absence of IP₃Rs. To test this, we perfused T0 cells with IP₃, and neither 1 μ M nor 20 μ M IP₃ were able to activate any measurable CRAC currents (Fig. 2A). To ascertain that store-operated calcium influx via CRAC channels was functional in T0 cells, we perfused T0 DT40 cells with 1 μ M IP₃, which failed to activate I_{CRAC}, and induced IP₃-independent store-depletion by applying the Ca²⁺ ionophore ionomycin. A brief exposure of cells to 2 μ M ionomycin for 2 s applied from the outside of the cell in a Ca²⁺-free solution reliably activated I_{CRAC} with a time constant of 22 s (Fig. 2A). Here, CRAC current density reached 1.7 pA/pF and showed an *I/V* relationship typical for I_{CRAC} (Fig. 2B). These data indicate that the absence of IP₃Rs only prevents IP₃-induced activation of I_{CRAC}, but does not prevent its recruitment by IP₃-independent store-depletion, consistent with observations made with thapsigargin [29].

To determine whether all IP₃Rs or only specific subtypes are coupled to I_{CRAC} activation we studied DT40 cells in which all but one specific IP₃R subtype were knocked out [21], resulting in cells expressing either IP₃R type I (T1), type II (T2) or type III

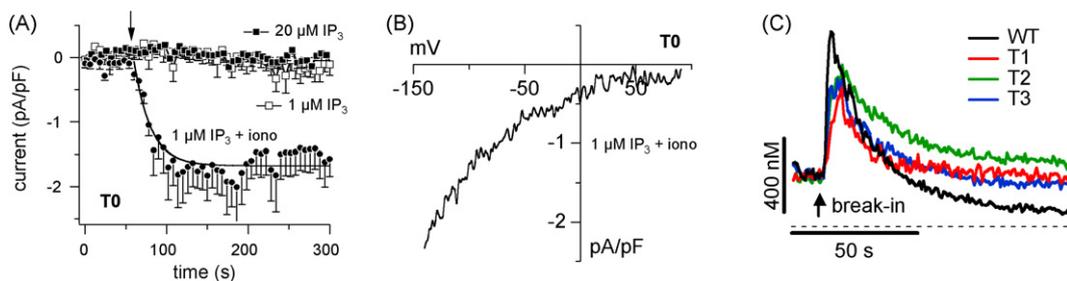


Fig. 2. Ionomycin but not IP₃ activates I_{CRAC} in the absence of IP₃ receptors. (A) Average CRAC currents in T0 DT40 cells in response to 1 μM IP₃ (open squares, *n* = 5), 20 μM IP₃ (filled squares, *n* = 3) and 1 μM IP₃ plus stimulation for 2 s with 2 μM ionomycin in Ca²⁺-free saline as indicated by the arrow (filled circles, *n* = 5). [Ca²⁺]_i was clamped to 150 nM. Data were analyzed as in Fig. 1A. (B) Average I/V curves of I_{CRAC} extracted from representative T0 cells at 300 s and obtained after application of ionomycin (*n* = 3). (C) Average Ca²⁺ release responses evoked by perfusion of wild-type (black, *n* = 5), T1 (red, *n* = 4), T2 (green, *n* = 4) and T3 (blue, *n* = 4) DT40 with 10 μM IP₃ in combined patch-clamp and balanced Fura-2 experiments (see Section 2). Baseline of traces was adjusted to T2 DT40 for clarity (50–130 nM). Arrow indicates time of whole-cell break-in. Cells were kept in regular Ca²⁺-containing solution but were superfused with a Ca²⁺-free saline before whole-cell break-in and throughout the experiment.

(T3). To confirm that IP₃-induced Ca²⁺-release occurs in these cells, we conducted combined patch-clamp and balanced Fura-2 experiments. Fura-2-AM loaded wild-type, T1, T2 or T3 DT40 cells were subsequently perfused with 10 μM IP₃ and 200 μM Fura-2 after whole-cell break-in in the absence of extracellular Ca²⁺. Consistent with previous observations [22], T1, T2 and T3 cells were capable of causing Ca²⁺ release that was almost identical to wild-type DT40 expressing all three IP₃R subtypes (Fig. 2C). These cells were then tested for IP₃-induced activation of CRAC channels. At 1 μM IP₃, which causes maximal activation of I_{CRAC} in wt DT40, T1 DT40 cells did not activate measurable CRAC currents and cells remained unresponsive even when increasing the intracellular IP₃ concentration to 20 μM (Fig. 3A). However, similar to T0 DT40 cells (Fig. 2), challenging T1 DT40 cells with ionomycin activated inwardly rectifying currents with a time constant of 51 s (Fig. 3A) and the typical current–voltage relationship of I_{CRAC} (Fig. 3B). These data suggest that, although T1 DT40 cells can release Ca²⁺ from intracellular stores (see Fig. 2C, [22]), this IP₃-sensitive store does not appear to couple to CRAC channel activation.

In marked contrast to T1 DT40, challenging T2 DT40 with 1 μM intracellular IP₃ did cause I_{CRAC} activation with a time constant comparable to wt DT40 cells (66 s) and characteristic I/V relationship (Fig. 3E). However, the overall current size was significantly reduced compared to wt cells (0.6 pA/pF compared to 1.1 pA/pF, respectively; Fig. 3C). To see whether smaller current amplitudes were due to incomplete CRAC recruitment at 1 μM IP₃, we completed a dose–response curve for IP₃ in T2 DT40 cells. Interestingly, while 20 μM IP₃ did not cause larger CRAC currents than 1 μM IP₃, full activation of I_{CRAC} was already achieved at 200 nM of IP₃ (Fig. 3C). At 10 nM IP₃, no measurable CRAC currents could be observed in T2 DT40 cells. Plotting and fitting the current amplitudes measured at 200 s into the experiment versus the respective IP₃ concentrations resulted in a dose–response curve with an EC₅₀ of 130 nM that was not significantly affected when varying Hill coefficient values between 12 and 30. Thus, I_{CRAC} activation in both wt DT40 and T2 DT40 was highly non-linear, with T2 DT40 being about fivefold more sensitive to intracellular IP₃. These results establish that type II IP₃R is located in a compartment that upon depletion can activate CRAC channels. The reduced amplitude of the response, however, suggests that some of the CRAC channels are not recruited, raising the possibility that the remaining CRAC channels are controlled by another compartment that lacks type II IP₃R, but possibly contains the type III subtype.

We tested the above hypothesis in T3 DT40 cells by establishing a dose–response curve of IP₃-induced I_{CRAC} activation. Here, current recruitment at 1 μM IP₃ was significantly slower than either in wt or T2 cells, with an estimated time constant of ~150 s. Increasing intracellular IP₃ concentration to 10 μM gave rise to larger CRAC currents, but could not be further augmented by 20 μM IP₃ (Fig. 3D).

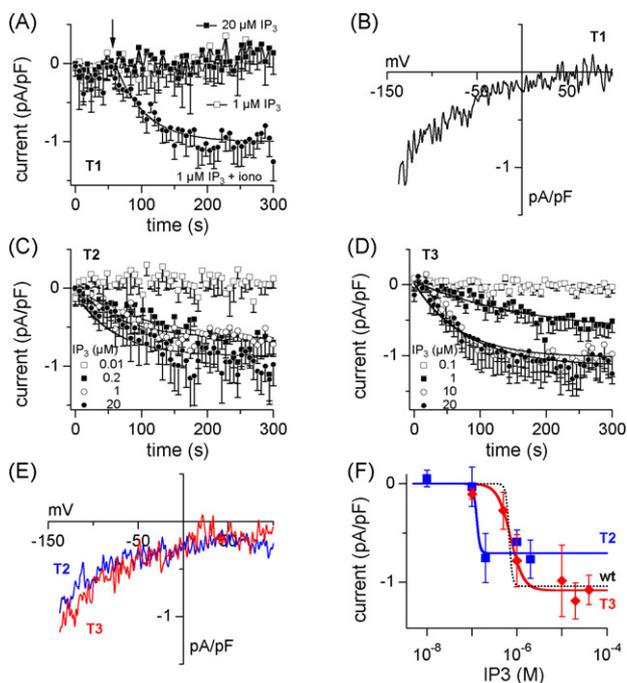


Fig. 3. IP₃ receptor type I does not couple to I_{CRAC} activation. (A) Average CRAC currents in T1 DT40 cells perfused with 1 μM IP₃ (open squares, *n* = 6), 20 μM IP₃ (filled squares, *n* = 3) or 1 μM IP₃ plus stimulation for 2 s with 2 μM ionomycin in Ca²⁺-free saline as indicated by the arrow (closed circles, *n* = 4). Intracellular calcium concentration was clamped to 150 nM. Data were analyzed as in Fig. 1A. (B) Average I/V curves of I_{CRAC} extracted from representative T1 cells at 300 s and obtained after application of ionomycin (*n* = 4). (C) Average time-course of I_{CRAC} in T2 DT40 cells perfused with 10 nM IP₃ (open squares, *n* = 3), 200 nM IP₃ (filled squares, *n* = 5), 1 μM IP₃ (open circles, *n* = 5) or 20 μM IP₃ (filled circles, *n* = 8). [Ca²⁺]_i was clamped to 150 nM. The data were fitted with a single exponential function as in Fig. 1C. (D) Average time-course of I_{CRAC} in T3 DT40 cells perfused with 100 nM IP₃ (open squares, *n* = 4), 1 μM IP₃ (filled squares, *n* = 10), 10 μM IP₃ (open circles, *n* = 7) and 20 μM IP₃ (filled circles, *n* = 8) in the presence of 150 nM [Ca²⁺]_i. Data analysis was performed as in Fig. 1A and data fit as in Fig. 1C. (E) Average I/V relationships of CRAC currents induced with 1 μM IP₃ and extracted at 300 s from representative T2 (blue, *n* = 3) and T3 cells (red, *n* = 3). (F) Average I_{CRAC} amplitude of T2 (blue) and T3 cells (red) assessed at 200 s and plotted against IP₃ concentration. Dose–response fits yielded EC₅₀ values of 130 nM (Hill = 12) for T2 cells and 720 nM (Hill = 3) for T3 cells. For comparison purposes, the dashed curve represents the dose–response fit to I_{CRAC} recruitment in wild-type DT40 cells shown in Fig. 1D.

In addition, 10 μM and 20 μM IP₃ caused faster I_{CRAC} recruitment with identical time constants (τ = 58 s) that approached the kinetics of wt and T2 cells. The current voltage relationships of IP₃-activated currents in T3 DT40 cells were typical for CRAC currents (Fig. 3E). A dose–response fit to these data yielded an EC₅₀ for I_{CRAC} activation of 720 nM (Fig. 3F), similar to wt DT40 (670 nM). The Hill coefficient

obtained in T3 DT40 cells was 3, reflecting a more graded recruitment of CRAC channels compared to wt (Fig. 3F, dashed black curve) or type II expressing DT40 (Fig. 3F, blue curve). Taken together, these results demonstrate that the type III IP₃R can recruit the entire population of CRAC channels, suggesting that it is expressed in all CRAC-competent compartments and may co-localize with the type II subtype in subset of stores.

4. Discussion

Our results show that IP₃R type II and type III participate in IP₃-induced activation of I_{CRAC} in DT40 B cells, but IP₃R type I does not. This suggests that the expression pattern of IP₃R contributes to the formation of specialized CRAC stores in B cells.

DT40 cells express all three IP₃R isoforms. Based on Northern analysis, the type I receptor far outnumbers the other two types, with type III being expressed at the lowest level [22]. However, maximal release rates were comparable within a factor of two among DT40 clones expressing different IP₃R, suggesting that expression levels of functional channel proteins are very similar for all three subtypes. Measurements of cytosolic Ca²⁺ signals in intact cells expressing individual IP₃R isoforms and stimulated through B cell receptors (BCR) reveal distinct [Ca²⁺]_i signals in the form of monophasic transients (types I and III) or repetitive oscillations (type II). Moreover, measurements of luminal Ca²⁺ levels of intracellular stores in permeabilized cells and functional characterization in planar lipid bilayers have established that individual IP₃R isoforms have different sensitivities to IP₃, with type II being most sensitive and type III being least sensitive [22,30]. Our data are entirely consistent with these findings for T2 and T3 DT40 cells with apparent EC₅₀'s of 130 and 720 nM, respectively. While T1 DT40 cells do not develop CRAC currents in response to IP₃ and hence elude the functional assay employed here, these cells do produce IP₃-induced calcium release (Fig. 2C and [22]). This confirms that all IP₃ receptors can release Ca²⁺ from stores, but that they may give rise to distinct Ca²⁺ signals, possibly shaped by differential sensitivity to IP₃, different Ca²⁺ release and uptake activities of multiple stores, and/or specific regulatory feedback mechanisms exerted by ATP and or [Ca²⁺]_i itself [22,30].

The data presented in the present study have important implications for a number of cell biological questions regarding the expression of IP₃R isoforms and Ca²⁺ store heterogeneity. They demonstrate IP₃R heterogeneity in that the most abundantly expressed type I receptor does not couple to store-operated CRAC channels, whereas the type III receptor, which is the least abundantly expressed and least IP₃-sensitive isoform, can fully account for CRAC currents in DT40 cells. Since both isoforms give rise to very similar Ca²⁺ signals [22] and have similar rates of release, it would seem that the bulk of Ca²⁺ release and store depletion may not be the determinant factor for mediating CRAC current activation. Instead, the release of Ca²⁺ from and the depletion of a specialized store that may not contribute significantly to the overall Ca²⁺ release response appears to be responsible for store-operated Ca²⁺ entry. This is entirely compatible with previous work in mast cells demonstrating that low concentrations of IP₃ release almost all Ca²⁺ from intracellular stores without activating CRAC [1]. However, a subsequent increase in IP₃ concentration can trigger CRAC currents without significant additional Ca²⁺ release. The relevance of type II and III IP₃R subtypes for Ca²⁺ release and Ca²⁺ entry has recently been demonstrated for pancreatic acinar cells [31]. Acinar cells isolated from knockout mice lacking these two IP₃R cannot respond to IP₃-coupled receptor stimulation via carbachol. This loss in phenotype is consistent with our result that IP₃R types II and III are required for CRAC activation and Ca²⁺ influx in DT40 cells. However, the complete absence of agonist-induced Ca²⁺ release

observed in acinar cells from double knockout mice would prevent CRAC activation by itself, especially since ionomycin-stimulation confirms intact Ca²⁺ storage in these mice [31]. Nevertheless, the involvement of IP₃R types II and III in the differentiation of granule cell precursors after postnatal day 12 implicates that receptor-initiated Ca²⁺ signaling is fundamentally perturbed in the double knockout mice [32].

The specialized store that mediates the IP₃-dependent activation of CRAC remains to be identified. It could be a subcompartment of the ER or a completely separate store with distinct molecular markers, although it would have to harbor IP₃ receptors of type III as well as thapsigargin-sensitive SERCA isoforms, since activation of the former and inhibition of the latter can activate CRAC. The identification of such a small store in DT40 cells expressing native IP₃R poses a significant challenge, as it would require the combination of immunofluorescent labeling of IP₃R isoforms with highly specific antibodies for chicken IP₃R, fluorimetric determination of Ca²⁺ release, and electrophysiological recording of CRAC currents. However, multiphoton excitation imaging of heterologously expressed and epitope-tagged IP₃R type III in DT40 cells lacking all three isoforms has revealed that this isoform is expressed not only throughout the ER, but also in some small non-ER areas just underneath or in the plasma membrane [33]. If the overexpressed protein distributes identically as the native protein, these areas might represent IP₃ receptors within the specialized CRAC store and future work may provide insights into its nature.

In summary, our data establish heterogeneity of the ER calcium store in B cells and the presence of at least a specialized CRAC store that is characterized by the lack of type I IP₃R, but specifically expresses IP₃R type III. The IP₃R type II appears to have partial access to the CRAC store, since it can recruit partial I_{CRAC} even at lower concentrations of IP₃ than either wt or T3 cells. While this confirms the notion that IP₃R subtypes have different sensitivities to IP₃, it raises the question why wt DT40 cells do not activate I_{CRAC} at the low concentrations that are effective in T2 cells. Since I_{CRAC} activation by IP₃ in wild-type cells represents a mixture of the non-linear recruitment seen with type II receptor involvement, but the overall IP₃-sensitivity observed for type III receptors, it is tempting to speculate that these two receptor types form heteromeric channels [19,20]. The exclusion of receptor type I from CRAC stores leading to the exclusive heteromerization of only two receptor subtypes within this substore, affords both heterogeneity and fine-tuning of IP₃-induced calcium signaling.

Acknowledgments

We thank T. Kurosaki for generously providing genetically modified DT40 cell lines. We thank M. Bellinger for technical assistance. Supported in part by DFG grant PE 1478/1-1 (CP), NIH grant R01-AI050200, R01-GM080555 (RP) and R01-GM070634 (AF).

References

- [1] A.B. Parekh, A. Fleig, R. Penner, The store-operated calcium current I_{CRAC}: nonlinear activation by InsP₃ and dissociation from calcium release, *Cell* 89 (1997) 973–980.
- [2] M.D. Glitsch, A.B. Parekh, Ca²⁺ store dynamics determines the pattern of activation of the store-operated Ca²⁺ current I_{CRAC} in response to InsP₃ in rat basophilic leukaemia cells, *J. Physiol.* 523 Pt 2 (2000) 283–290.
- [3] V.A. Golovina, M.P. Blaustein, Spatially and functionally distinct Ca²⁺ stores in sarcoplasmic and endoplasmic reticulum, *Science* 275 (1997) 1643–1648.
- [4] M.C. Hermosura, H. Takeuchi, A. Fleig, A.M. Riley, B.V. Potter, M. Hirata, R. Penner, InsP₄ facilitates store-operated calcium influx by inhibition of InsP₃ 5-phosphatase, *Nature* 408 (2000) 735–740.
- [5] K. Hirose, M. Iino, Heterogeneity of channel density in inositol-1,4,5-trisphosphate-sensitive Ca²⁺ stores, *Nature* 372 (1994) 791–794.
- [6] E. Rooney, J. Meldolesi, The endoplasmic reticulum in PC12 cells. Evidence for a mosaic of domains differently specialized in Ca²⁺ handling, *J. Biol. Chem.* 271 (1996) 29304–29311.

- [7] H. Mogami, K. Nakano, A.V. Tepikin, O.H. Petersen, Ca^{2+} flow via tunnels in polarized cells: recharging of apical Ca^{2+} stores by focal Ca^{2+} entry through basal membrane patch, *Cell* 88 (1997) 49–55.
- [8] M.K. Park, O.H. Petersen, A.V. Tepikin, The endoplasmic reticulum as one continuous Ca^{2+} pool: visualization of rapid Ca^{2+} movements and equilibration, *Embo J.* 19 (2000) 5729–5739.
- [9] R. Rizzuto, T. Pozzan, Microdomains of intracellular Ca^{2+} : molecular determinants and functional consequences, *Physiol. Rev.* 86 (2006) 369–408.
- [10] F. Michelangeli, O.A. Ogunbayo, L.L. Wootton, A plethora of interacting organellar Ca^{2+} stores, *Curr. Opin. Cell Biol.* 17 (2005) 135–140.
- [11] F.M. Lasorsa, P. Pinton, L. Palmieri, P. Scarcia, H. Rottensteiner, R. Rizzuto, F. Palmieri, Peroxisomes as novel players in cell calcium homeostasis, *J. Biol. Chem.* 283 (2008) 15300–15308.
- [12] H. Turner, A. Fleig, A. Stokes, J.P. Kinet, R. Penner, Discrimination of intracellular calcium store subcompartments using TRPV1 (transient receptor potential channel, vanilloid subfamily member 1) release channel activity, *Biochem. J.* 371 (2003) 341–350.
- [13] J. Meldolesi, T. Pozzan, The heterogeneity of ER Ca^{2+} stores has a key role in nonmuscle cell signaling and function, *J. Cell Biol.* 142 (1998) 1395–1398.
- [14] I. Fujino, N. Yamada, A. Miyawaki, M. Hasegawa, T. Furuichi, K. Mikoshiba, Differential expression of type 2 and type 3 inositol 1,4,5-trisphosphate receptor mRNAs in various mouse tissues: in situ hybridization study, *Cell Tissue Res.* 280 (1995) 201–210.
- [15] T. Furuichi, S. Yoshikawa, A. Miyawaki, K. Wada, N. Maeda, K. Mikoshiba, Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400, *Nature* 342 (1989) 32–38.
- [16] S.K. Joseph, The inositol triphosphate receptor family, *Cell Signal.* 8 (1996) 1–7.
- [17] S.K. Joseph, C. Lin, S. Pierson, A.P. Thomas, A.R. Maranto, Heterooligomers of type-I and type-III inositol triphosphate receptors in WB rat liver epithelial cells, *J. Biol. Chem.* 270 (1995) 23310–23316.
- [18] G.A. Mignery, T.C. Sudhof, K. Takei, P. De Camilli, Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor, *Nature* 342 (1989) 192–195.
- [19] T. Monkawa, A. Miyawaki, T. Sugiyama, H. Yoneshima, M. Yamamoto-Hino, T. Furuichi, T. Saruta, M. Hasegawa, K. Mikoshiba, Heterotetrameric complex formation of inositol 1,4,5-trisphosphate receptor subunits, *J. Biol. Chem.* 270 (1995) 14700–14704.
- [20] R.J. Wojcikiewicz, Y. He, Type I, II and III inositol 1,4,5-trisphosphate receptor co-immunoprecipitation as evidence for the existence of heterotetrameric receptor complexes, *Biochem. Biophys. Res. Commun.* 213 (1995) 334–341.
- [21] H. Sugawara, M. Kurosaki, M. Takata, T. Kurosaki, Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor, *Embo J.* 16 (1997) 3078–3088.
- [22] T. Miyakawa, A. Maeda, T. Yamazawa, K. Hirose, T. Kurosaki, M. Iino, Encoding of Ca^{2+} signals by differential expression of IP3 receptor subtypes, *Embo J.* 18 (1999) 1303–1308.
- [23] J.K. Foskett, C. White, K.H. Cheung, D.O. Mak, Inositol trisphosphate receptor Ca^{2+} release channels, *Physiol. Rev.* 87 (2007) 593–658.
- [24] R.E. Hagar, A.D. Burgstahler, M.H. Nathanson, B.E. Ehrlich, Type III InsP_3 receptor channel stays open in the presence of increased calcium, *Nature* 396 (1998) 81–84.
- [25] D.O. Mak, S. McBride, J.K. Foskett, Regulation by Ca^{2+} and inositol 1,4,5-trisphosphate (InsP_3) of single recombinant type 3 InsP_3 receptor channels. Ca^{2+} activation uniquely distinguishes types 1 and 3 InsP_3 receptors, *J. Gen. Physiol.* 117 (2001) 435–446.
- [26] J.E. Swatton, S.A. Morris, T.J. Cardy, C.W. Taylor, Type 3 inositol trisphosphate receptors in RINm5F cells are biphasically regulated by cytosolic Ca^{2+} and mediate quantal Ca^{2+} mobilization, *Biochem. J.* 344 (Pt 1) (1999) 55–60.
- [27] M. Hoth, R. Penner, Depletion of intracellular calcium stores activates a calcium current in mast cells, *Nature* 355 (1992) 353–356.
- [28] W.C. Chang, J. Di Capite, C. Nelson, A.B. Parekh, All-or-none activation of CRAC channels by agonist elicits graded responses in populations of mast cells, *J. Immunol.* 179 (2007) 5255–5263.
- [29] L.M. Broad, F.J. Braun, J.P. Lievreumont, G.S. Bird, T. Kurosaki, J.W. Putney Jr., Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry, *J. Biol. Chem.* 276 (2001) 15945–15952.
- [30] H. Tu, Z. Wang, E. Nosyreva, H. De Smedt, I. Bezprozvanny, Functional characterization of mammalian inositol 1,4,5-trisphosphate receptor isoforms, *Biophys. J.* 88 (2005) 1046–1055.
- [31] A. Futatsugi, T. Nakamura, M.K. Yamada, E. Ebisui, K. Nakamura, K. Uchida, T. Kitaguchi, H. Takahashi-Iwanaga, T. Noda, J. Aruga, K. Mikoshiba, IP3 receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism, *Science* 309 (2005) 2232–2234.
- [32] A. Futatsugi, E. Ebisui, K. Mikoshiba, Type 2 and type 3 inositol 1,4,5-trisphosphate (IP3) receptors promote the differentiation of granule cell precursors in the postnatal cerebellum, *J. Neurochem.* 105 (2008) 1153–1164.
- [33] T. Morita, A. Tanimura, A. Nezu, T. Kurosaki, Y. Tojyo, Functional analysis of the green fluorescent protein-tagged inositol 1,4,5-trisphosphate receptor type 3 in Ca^{2+} release and entry in DT40 B lymphocytes, *Biochem. J.* 382 (2004) 793–801.