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IP_3 RECEPTOR SUBTYPE-DEPENDENT ACTIVATION OF STORE-OPERATED CALCIUM ENTRY THROUGH I_{CRAC}

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Abstract

The store-operated, calcium release-activated calcium current I_{CRAC} is activated by the depletion of inositol 1,4,5-trisphosphate (IP_3)-sensitive stores. The significantly different dose-response relationships of IP_3-mediated Ca^{2+} 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 IP_3-receptor (IP_3R) genes and most cells express at least two subtypes, but the functional relevance of various IP_3R subtypes with respect to store-operated Ca^{2+} entry is completely unknown. We here demonstrate in avian B cells (chicken DT40) that IP_3R type II and type III participate in IP_3-induced activation of I_{CRAC}, but IP_3R type I does not. This suggests that the expression pattern of IP_3R contributes to the formation of specialized CRAC stores in B cells.

Keywords

I_{CRAC}; IP_3 receptor; Ca^{2+} store heterogeneity

Introduction

Calcium release from intracellular Ca^{2+} stores by inositol 1,4,5-trisphosphate (IP_3) represents an important mechanism for calcium (Ca^{2+}) 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 IP_3-induced Ca^{2+} release and I_{CRAC} activation. While IP_3 causes considerable Ca^{2+} release in the nanomolar range, CRAC channel activation occurs only at micromolar IP_3 concentrations [1]. Such differing response thresholds may arise from a number of circumstances, including different complements of IP_3R subtypes with different affinities for IP_3, store heterogeneity with separate compartments containing specific molecular components, and/or differential localization and activity of enzymes involved in IP_3 metabolism with different parts of the endoplasmic reticulum (ER) experiencing different IP_3 concentrations [2-6]. This concept of store-heterogeneity is

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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 Ca^{2+} storage. Mitochondria, Golgi, nucleus and lysosomes - all have been implicated in agonist-induced Ca^{2+} release [10]. Most recently, peroxisomes have been ascribed a role in Ca^{2+} storage [11], further contributing to cellular Ca^{2+} 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 Ca^{2+}-influx and Ca^{2+}-release channel TRPV1 (Transient Receptor Potential Vallinoid type 1) by capsaicin can mobilize substantial amounts of Ca^{2+} 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 IP_3 receptors and thapsigargin-sensitive Ca^{2+} pumps. Heterogeneity also exists within IP_3-sensitive Ca^{2+} stores, as they respond to IP_3 with different sensitivities and complex release kinetics [13]. IP_3-sensitive receptors in vertebrates are encoded by three different genes, expressed as IP_3R type I, II, and III, which can form homo- or heterotetrameric channel complexes [14-20]. The question therefore arises whether IP_3R subtype composition contributes to the formation of a distinct CRAC store.

Methods

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 expressing all three types of $InsP_3$ receptors, the triple $InsP_3$ receptor knock-out cell line and the cell lines expressing type I, type II or type III $InsP_3$ receptor were a gift of Dr. Kurosaki [21].

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 (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 sec. Liquid junction potential was 10 mV. Currents were filtered at 2.9 kHz and digitized at 100 μs 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 ± S.E.M. with n determinations. Standard external solutions were as follows (in mM): 120 NaCl, 2.8 KCl, 2 $MgCl_2$, 20 $CaCl_2$, 10 HEPES, 11 glucose, pH 7.2 with NaOH, 300 mosm. In some experiments 2 μM ionomycin in external solution containing no $CaCl_2$ was applied for 2 seconds. Standard internal solutions were as follows (in mM): 120 Cs-glutamate, 8 NaCl, 10 Cs·BAPTA, 3 $MgCl_2$, 4 $CaCl_2$, 10 HEPES, pH 7.2 with CsOH, 300 mOsm. IP_3 concentration was adjusted as indicated. $[Ca^{2+}]i$ was buffered to 150 nM free $[Ca^{2+}]i$ using 10 mM Cs·BAPTA and 4 mM $CaCl_2$ as calculated with WebMaxC (http://www.stanford.edu/~cpatton/webmaxcS.htm). All chemicals were purchased from Sigma-Aldrich Co.

In combined patch-clamp and balanced Fura-2 experiments, cells were preloaded with 5 μM Fura-2-AM for 30 minutes. In subsequent whole-cell patch clamp experiments 200 μM Fura-2 was added to the standard internal solution in addition to 10 μM IP_3.

Results

To identify whether differential expression of IP_3R occurs in CRAC stores, we used wild-type DT40 chicken B-lymphocytes expressing all three IP_3R types as well as genetically altered DT40 cells that lack all IP_3R or express only one of the three IP_3R subtypes [21,22]. We first assessed the sensitivity of I_{CRAC} to intracellular IP_3 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 $CaCl_2$ and cells were perfused with a standard Cs-based intracellular solution, where intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was clamped to 150 nM using a mixture of 10 mM Cs-BAPTA and 4 mM $CaCl_2$. At this $[Ca^{2+}]_i$, activation of I_{CRAC} by passive store depletion is prevented and optimal sensitivity of IP_3Rs to IP_3 is ensured [23-26]. Data were acquired using a voltage ramp of 50 ms length that spanned from -150 mV to +100 mV and was applied every 2 s. Adding 1 μM IP_3 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 IP_3 concentrations beyond 1 μM did not result in larger currents and reducing IP_3 to 500 nM was insufficient to activate CRAC currents (Fig. 1C). This all-or-none behavior of I_{CRAC} in response to IP_3 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 IP_3 that activated I_{CRAC} were ~60 s. The average current amplitudes measured at 200 s into the experiment were plotted against their respective IP_3 concentrations and fitted with a dose-response curve. The resulting fit rendered a half-maximal effective concentration (EC_{50}) of 670 nM with a Hill coefficient fixed to 12. The EC_{50} did not change significantly when 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 IP_3-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 IP_3R subtype expression.

DT40 cells lacking all three types of IP_3 receptors (T0) fail to respond to IP_3-producing stimuli, but exhibit store-operated Ca^{2+} entry when emptying stores with thapsigargin [29]. It is reasonable to assume that in the absence of any IP_3 receptors, store-operated recruitment of I_{CRAC} via the second messenger IP_3 should be abolished, provided that the functionality of CRAC currents is not affected by the absence of IP_3Rs. To test this, we perfused T0 cells with IP_3, and neither 1 μM nor 20 μM IP_3 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 μM IP_3, which failed to activate I_{CRAC}, and induced IP_3-independent store-depletion by applying the Ca^{2+} ionophore ionomycin. A brief exposure of cells to 2 μM ionomycin for 2 s applied from the outside of the cell in a Ca^{2+}-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 IP_3Rs only prevents IP_3-induced activation of I_{CRAC}, but does not prevent its recruitment by IP_3-independent store-depletion, consistent with observations made with thapsigargin [29].

To determine whether all IP_3Rs or only specific subtypes are coupled to I_{CRAC} activation we studied DT40 cells in which all but one specific IP_3R subtype were knocked out [21], resulting in cells expressing either IP_3R type I (T1), type II (T2) or type III (T3). To confirm that IP_3-induced Ca^{2+}-release occurs in these cells, we conducted combined patch- and balanced Fura-2 experiments. Fura-2-AM loaded wild-type, T1, T2 or T3 DT40 cells were subsequently perfused with 10 μM IP_3 and 200 μM Fura-2 after whole-cell break-in in the absence of extracellular Ca^{2+}. Consistent with previous observations [22], T1, T2 and T3 cells were

capable of causing Ca^{2+} release that was almost identical to wild type DT40 expressing all three IP_3R subtypes (Fig. 2C). These cells were then tested for IP_3-induced activation of CRAC channels. At 1 μM IP_3, 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 IP_3 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 Ca^{2+} from intracellular stores [22], this IP_3-sensitive store does not appear to couple to CRAC channel activation.

In marked contrast to T1 DT40, challenging T2 DT40 with 1 μM intracellular IP_3 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 IP_3, we completed a dose-response curve for IP_3 in T2 DT40 cells. Interestingly, while 20 μM IP_3 did not cause larger CRAC currents than 1 μM IP_3, full activation of I_{CRAC} was already achieved at 200 nM of IP_3 (Fig. 3C). At 10 nM IP_3, 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 IP_3 concentrations resulted in a dose-response curve with an EC_{50} 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 5-fold more sensitive to intracellular IP_3. These results establish that Type II IP_3R 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 IP_3R, but possibly contains the Type III subtype.

We tested the above hypothesis in T3 DT40 cells by establishing a dose-response curve of IP_3-induced I_{CRAC} activation. Here, current recruitment at 1 μM IP_3 was significantly slower than either in wt or T2 cells, with an estimated time constantof ~150 s. Increasing intracellular IP_3 concentration to 10 μM gave rise to larger CRAC currents, but could not be further augmented by 20 μM IP_3 (Fig. 3D). In addition, 10 μM and 20 μM IP_3 caused faster I_{CRAC} recruitment with identical time constants (□ = 58 s) that approached the kinetics of wt and T2 cells. The current voltage relationships of IP_3-activated currents in T3 DT40 cells were typical for CRAC currents (Fig. 3E). A dose-response fit to these data yielded an EC_{50} 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 IP_3R 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.

Discussion

Our results show that IP_3R type II and type III participate in IP_3-induced activation of I_{CRAC} in DT40 B cells, but IP_3R type I does not. This suggests that the expression pattern of IP_3R contributes to the formation of specialized CRAC stores in B cells.

DT40 cells express all three IP_3R 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 was comparable within a factor of two among DT40 clones expressing different IP_3R, suggesting that expression levels of functional channel proteins are

very similar for all three subtypes. Measurements of cytosolic Ca^{2+} signals in intact cells expressing individual IP_3R isoforms and stimulated through B cell receptors (BCR) reveal distinct $[Ca^{2+}]i$ signals in the form of monophasic transients (Types I and III) or repetitive oscillations (Type II). Moreover, measurements of luminal Ca^{2+} levels of intracellular stores in permeabilized cells and functional characterization in planar lipid bilayers have established that individual IP_3 receptor isoforms have different sensitivities to IP_3, 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 EC_{50}'s of 130 nM and 720 nM, respectively. While T1 DT40 cells do not develop CRAC currents in response to IP_3 and hence elude the functional assay employed here, these cells do produce IP_3-induced calcium release (Fig. 2C and [22]). This confirms that all IP_3 receptors can release Ca^{2+} from stores, but that they may give rise to distinct Ca^{2+} signals, possibly shaped by differential sensitivity to IP_3, different Ca^{2+} release and uptake activities of multiple stores, and/or specific regulatory feedback mechanisms exerted by ATP and or $[Ca^{2+}]i$ itself [22,30].

The data presented in the present study have important implications for a number of cell biological questions regarding the expression of IP_3R isoforms and Ca^{2+} store heterogeneity. They demonstrate IP_3 receptor 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 IP_3-sensitive isoform, can fully account for CRAC currents in DT40 cells. Since both isoforms give rise to very similar Ca^{2+} signals [22] and have similar rates of release, it would seem that the bulk of Ca^{2+} release and store depletion may not be the determinant factor for mediating CRAC current activation. Instead, the release of Ca^{2+} from and the depletion of a specialized store that may not contribute significantly to the overall Ca^{2+} release response appears to be responsible for store-operated Ca^{2+} entry. This is entirely compatible with previous work in mast cells demonstrating that low concentrations of IP_3 release almost all Ca^{2+} from intracellular stores without activating CRAC [1]. However, a subsequent increase in IP_3 concentration can trigger CRAC currents without significant additional Ca^{2+} release. The relevance of type II and III IP_3R subtypes for Ca^{2+} release and Ca^{2+} entry has recently been demonstrated for pancreatic acinar cells [31]. Acinar cells isolated from knockout mice lacking these two IP_3Rs cannot respond to IP_3-coupled receptor stimulation via carbachol. This loss in phenotype is consistent with our result that IP_3R type II and III are required for CRAC activation and Ca^{2+} influx in DT40 cells. However, the complete absence of agonist-induced Ca^{2+} release observed in acinar cells from double knockout mice would prevent CRAC activation by itself, especially since ionomycin-stimulation confirms intact Ca^{2+} storage in these mice [31]. Nevertheless, the involvement of IP_3R type II and III in the differentiation of granule cell precursors after postnatal day 12 implicates that receptor-initiated Ca^{2+} signaling is fundamentally perturbed in the double knockout mice [32].

The specialized store that mediates the IP_3-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 IP_3 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 IP_3 receptors poses a significant challenge, as it would require the combination of immunofluorescent labeling of IP_3 receptor isoforms with highly specific antibodies for chicken IP_3R, fluorimetric determination of Ca^{2+} release, and electrophysiological recording of CRAC currents. However, multiphoton excitation imaging of heterologously expressed and epitope-tagged IP_3R Type III in DT40 cells lacking all three isoforms has revealed that this isoform is expressed throughout the ER, but also in some small non-ER areas just underneath or in the plasma membrane [33]. If the overex-pressed protein distributes identically as the

native protein, these areas might represent IP_3 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 IP_3R, but specifically expresses IP_3R Type III. The IP_3R Type II appears to have partial access to the CRAC store, since it can recruit partial I_{CRAC} even at lower concentrations of IP_3 than either wt or T3 cells. While this confirms the notion that IP_3R subtypes have different sensitivities to IP_3, 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 IP_3 in wild-type cells represents a mixture of the non-linear recruitment seen with type II receptor involvement, but the overall IP_3-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 IP_3-induced calcium signaling.

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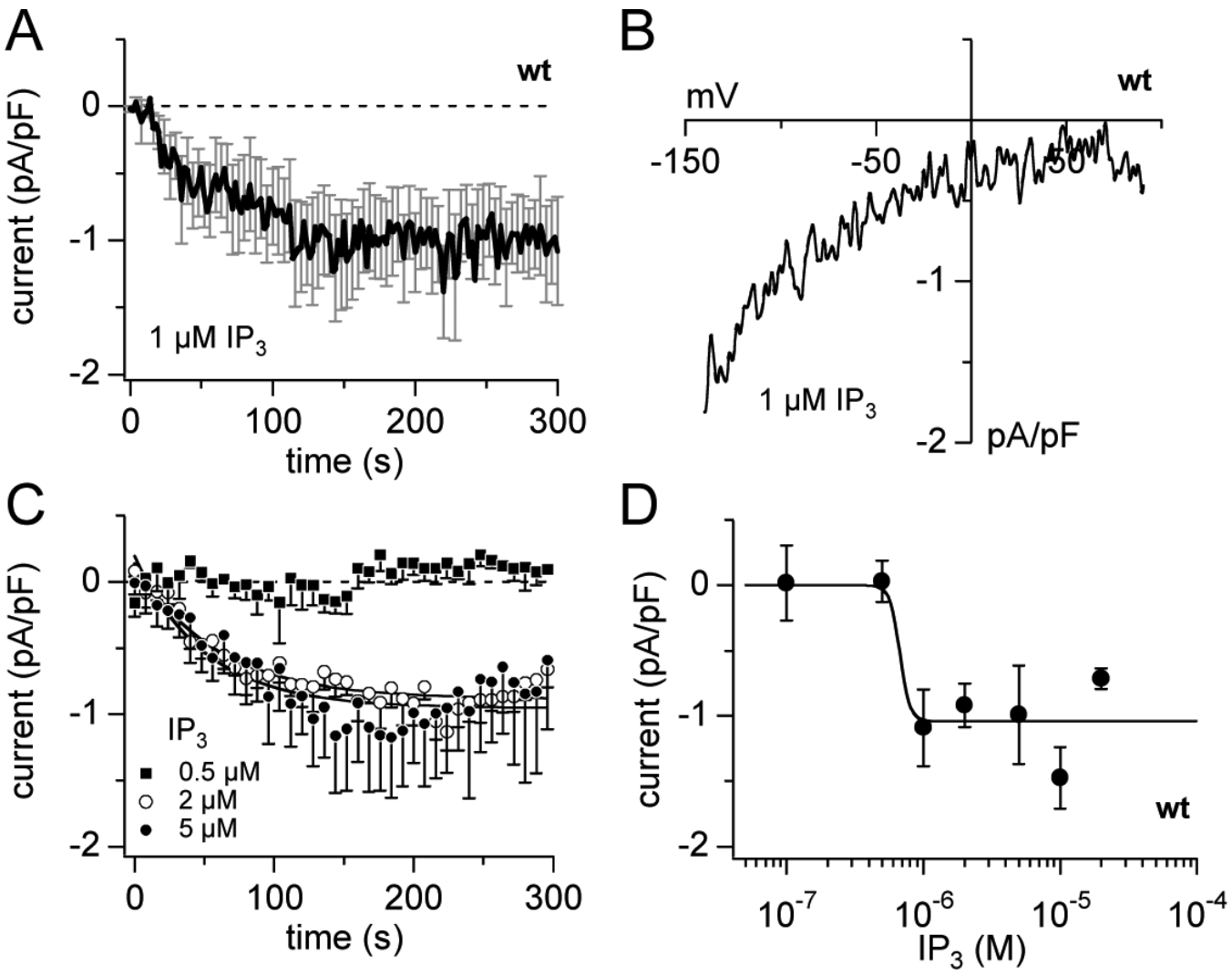

Figure 1. Non-linear activation of I_{CRAC} by IP_3 in wild-type DT40 B cells

(A) Average CRAC currents induced by 1 μM IP_3 in wild type DT40 cells expressing all three types of IP_3 receptors (n = 5). Error bars indicated S.E.M. Currents were acquired using a 50 ms voltage ramp spanning from -150 mV 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 1-3 ramp measurements after whole-cell establishment from subsequent current records. The intracellular calcium concentration was clamped to 150 nM (10 mM BAPTA and 4 mM $CaCl_2$). **(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 IP_3 (filled squares; n = 3), 2 μM IP_3 (open circles; n = 5) and 5 μM IP_3 (filled circles; n = 5). The calcium concentration was clamped to 150 nM (10 mM BAPTA and 4 mM $CaCl_2$) to prevent passive depletion of intracellular calcium stores. Data were fitted using the single exponential function $I_{norm}(t) = I_{total} \cdot \exp(-t/\pi) + \text{Amplitude}$. **(D)** Average I_{CRAC} amplitude assessed at 200 s and plotted against IP_3 concentration. A dose-response fit to the data yielded a EC_{50} of 670 nM and a Hill coefficient of 12.

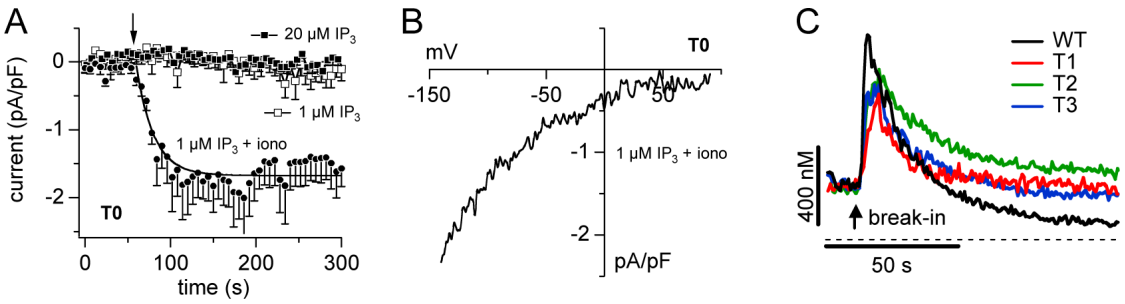

Figure 2. Ionomycin but not IP_3 activates I_{CRAC} in the absence of IP>3 receptors

(A) Average CRAC currents in T0 DT40 cells in response to 1 μM IP_3 (open squares, n = 5), 20 μM IP_3 (filled squares, n = 3) and 1 μM IP_3 plus stimulation for 2 s with 2 μM ionomycin in Ca^{2+}-free saline as indicated by the arrow (filled circles, n = 5). $[Ca^{2+}]_i$ 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 Ca^{2+} 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 IP_3 in combined patch- and balanced Fura-2 experiments (see methods). Baseline of traces was adjusted to T2 DT40 for clarity (50 nM to 130 nM). Arrow indicates time of whole-cell break-in. Cells were kept in regular Ca^{2+}-containing solution but were superfused with a Ca^{2+}-free saline before whole-cell break-in and throughout the experiment.

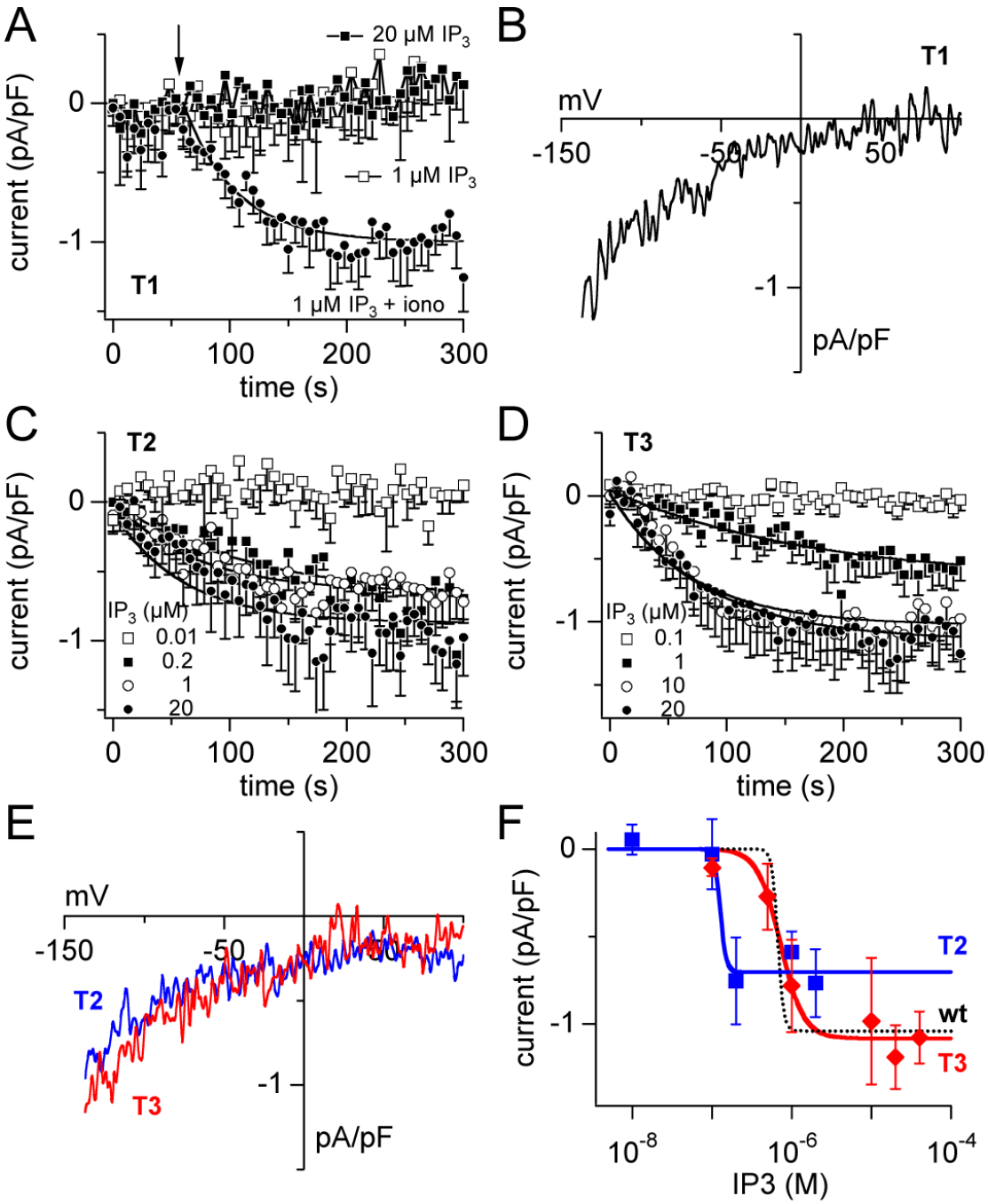

Figure 3. IP_3 receptor type I does not couple to I_{CRAC} activation

(A) Average CRAC currents in T1 DT40 cells perfused with 1 μM IP_3 (open squares, n = 6), 20 μM IP_3 (filled squares, n = 3) or 1 μM IP_3 plus stimulation for 2 s with 2 μM ionomycin in Ca^{2+}-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 IP_3 (open squares, n = 3), 200 nM IP_3 (filled squares, n = 5), 1 μM IP_3 (open circles, n = 5) or 20 μM IP_3 (filled circles, n = 8). $[Ca^{2+}]_i$ 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 IP_3 (open squares, n = 4), 1 μM IP_3 (filled squares, n = 10), 10 μM IP_3 (open circles, n = 7) and 20 μM IP_3 (filled circles, n = 8) in the presence of 150 nM $[Ca^{2+}]_i$. 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 IP_3 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 IP_3 concentration. Dose-response fits yielded EC_{50} 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.