The acidocalcisome inositol-1,4,5-trisphosphate receptor of Trypanosoma brucei is stimulated by luminal polyphosphate hydrolysis products

Acidocalcisomes are acidic calcium stores rich in polyphosphate (polyP) and are present in trypanosomes and also in a diverse range of other organisms. Ca$^{2+}$ is released from these organelles through a channel, inositol 1,4,5-trisphosphate receptor (TbIP$_3$R), which is essential for growth and infectivity of the parasite Trypanosoma brucei. However, the mechanism by which TbIP$_3$R controls Ca$^{2+}$ release is unclear. In this work, we expressed TbIP$_3$R in a chicken B lymphocyte cell line in which the genes for all three vertebrate IP$_3$Rs were stably ablated (DT40−3KO). We show that IP$_3$-mediated Ca$^{2+}$ release depends on Ca$^{2+}$ but not on ATP concentration and is inhibited by heparin, caffeine, and 2-aminoethoxydiphenyl borate (2-APB). Excised patch clamp recordings from nuclear membranes of DT40 cells expressing only TbIP$_3$R disclosed that luminal inorganic orthophosphate (P$_i$) or pyrophosphate (PP$_i$), and neutral or alkaline pH can stimulate IP$_3$-generated currents. In contrast, polyP or acidic pH did not induce these currents, and nuclear membranes obtained from cells expressing rat IP$_3$R were unresponsive to polyP or its hydrolysis products. Our results are consistent with the notion that polyP hydrolysis products within acidocalcisomes or alkalization of their luminal pH activate TbIP$_3$R and Ca$^{2+}$ release. We conclude that TbIP$_3$R is well-adapted to its role as the major Ca$^{2+}$ release channel of acidocalcisomes in T. brucei.

Inositol-1,4,5-trisphosphate receptors (IP$_3$Rs) are intracellular Ca$^{2+}$ channels mostly found in the endoplasmic reticulum (ER) of animal cells (1). When plasma membrane receptors are stimulated, activation of a phospholipase C results in IP$_3$ formation and opening of these channels leading to a rise in cytosolic Ca$^{2+}$ (2). IP$_3$ and Ca$^{2+}$ function as co-agonists of IP$_3$Rs, and Ca$^{2+}$ release from an IP$_3$R leads to opening of its neighbors and, consequently, IP$_3$-regulated Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) with the eventual generation of Ca$^{2+}$ waves (3). The IP$_3$R has been considered essential for Ca$^{2+}$ signaling in animals and for the regulation of a variety of processes including gene expression, signal initiation, contraction, secretion, proliferation, fertilization, development, and cell death (4). Constitutive IP$_3$-mediated Ca$^{2+}$ transfer to mitochondria is essential for maintaining cellular bioenergetics (5).

Trypanosomes, like Trypanosoma brucei, which causes African trypanosomiasis or sleeping sickness, and Trypanosoma cruzi, the agent of Chagas’ disease, belong to the eukaryotic supergroup Excavata (6) and possess a very peculiar acidic calcium store, the acidocalcisome (7, 8). Acidocalcisomes are rich in polyphosphate (polyP), a polymer of orthophosphate linked by high-energy phosphoanhydride bonds, and found in a diverse range of organisms (9). We have shown that the IP$_3$R of T. brucei is localized to acidocalcisomes rather than to the ER and is maximally activated at high IP$_3$ concentrations (10−20 μM) (10, 11). The acidocalcisome localization was also confirmed in T. cruzi (12). We also demonstrated that Ca$^{2+}$ signaling through the TbIP$_3$R has roles in parasite growth in vitro and in vivo (10). The relevance and essentiality of the IP$_3$R for growth in vitro and in vivo and for differentiation were also demonstrated in T. cruzi (13).

Orthologs to IP$_3$Rs have been reported in protists of five of the supergroups in which eukaryotes have been divided (6) (Amoebozoa (14), Archaeplastida (15), Ophistokonta (16, 17), Chromalveolate (18), and Excavata (10, 12, 13, 19)). However, their functional roles in Ca$^{2+}$ release have been studied only in Paramecium tetraurelia (18), T. brucei (10), T. cruzi (13), and Capsaspora owczarzaki (17). The results support the early emergence of this Ca$^{2+}$ signaling pathway, preceding the origin of these supergroups, and fundamental differences with that present in animal cells.

Several proteins are known to interact with different IP$_3$R mammalian isoforms and to modify their activity (4). Most of them interact with the cytosolic portion of the IP$_3$R. However, some proteins, like the Ca$^{2+}$ storage proteins chromogranin A and B, have been shown to interact with the luminal portion of the IP$_3$R (4). Acidocalcisomes apparently have few luminal proteins but they do have polyP, which has been shown to modulate the function of ion channels (20).

To characterize the TbIP$_3$R we used chicken B lymphocytes in which the genes for all three vertebrate IP$_3$Rs have been stably eliminated (DT40−3KO) (21). We previously stably trans-
fected these cells with the gene encoding TbIP$_3$R or the rat IP$_3$R1 (RnIP$_3$R1), used as positive control (10). Heterologous expression of the TbIP$_3$R and RnIP$_3$R1 was confirmed by immunofluorescence analysis, which showed ER localization, and by Western blot analysis (10). Here, we report that opening of the TbIP$_3$R expressed in DT40–3KO cells is stimulated by the luminal polyP hydrolysis products, P$_i$ and PP$_i$, or by alkalinization.

**Results**

**TbIP$_3$R expression in DT40 cells and regulation by Ca$^{2+}$ and ATP**

In mammalian cells, low intracellular free calcium ([Ca$^{2+}$]$_i$) potentiates whereas high [Ca$^{2+}$]$_i$, inhibits channel activity. The mechanism of this regulation is not yet clear (17), but it has been shown that a conserved glutamate residue (Glu-2100) in the regulatory domain of mammalian IP$_3$R is important to determine its Ca$^{2+}$ sensitivity (22). TbIP$_3$R contains a conserved glutamate residue homologous to the mammalian Glu-2100 (Glu-2405) (Fig. 1). To investigate whether TbIP$_3$R is regulated by Ca$^{2+}$, we trapped a low-affinity Ca$^{2+}$ indicator (Mag-Fluo4) within the ER to measure luminal free [Ca$^{2+}$] in saponin-permeabilized DT40–3KO–TbIP$_3$R cells. Addition of MgATP stimulated Ca$^{2+}$ uptake until a steady-state Ca$^{2+}$ loading was reached (Fig. 2, A and B). Ca$^{2+}$ release was induced by addition of IP$_3$ in the presence of either 50 nM or 1 μM Ca$^{2+}$ and either low (10 μM) or high (100 μM) IP$_3$. Fig. 2C shows that the rate of TbIP$_3$R-mediated Ca$^{2+}$ release is higher at 1 μM Ca$^{2+}$ than at 50 nM Ca$^{2+}$: at either low (1 μM) or high (100 μM) IP$_3$, which is consistent with previous findings using DT40–3KO cells expressing RnIP3RI (17). Fig. 2C shows the quantification of the changes observed in four experiments.

Animal IP$_3$Rs are regulated by ATP, which binds to a glycine-rich motif (GXXGXXG), also known as the Walker motif (1, 23, 24). RnIP$_3$R1 contains two Walker motifs, named ATPA and ATPB, whereas TbIP$_3$R does not contain sequences corresponding to either ATPB or ATBC in the regulatory domain although it contains a Walker motif (GGLGNEGL) at the N-terminal region of the protein (suppressor domain) with similarity to the ATPA motif (GGLGLGGL) of RnIP$_3$R1 (Fig. 1). Accordingly, although Ca$^{2+}$ loading affects the response to IP$_3$ (Fig. 3, A and B) the rate of Ca$^{2+}$ release was not significantly increased by increasing the ATP concentration from 0.3 to 3 mM (Fig. 3C). In this regard, physiological concentrations of ATP in trypanosomes are in the range of 1–3 mM (25, 26). The rate of Ca$^{2+}$ release by IP$_3$ was inhibited by previous addition of the IP$_3$R inhibitors 2-aminoethoxydiphenyl borate (2-APB) and heparin and (Fig. 3D).

**Electrophysiological characterization of the TbIP$_3$R**

To characterize the electrophysiological properties of the TbIP$_3$R we used nuclear patch clamp recordings. This is because the nuclear envelope is continuous with the ER membrane and channels that are normally expressed within ER membranes can pass into the outer nuclear envelope, permitting the recording of their activity from patches of nuclear membrane in a near-physiological situation (27).

Nuclei were isolated from DT40–3KO cells stably expressing TbIP$_3$R or RnIP$_3$R1 as described under “Experimental procedures,” and recordings were obtained from excised nuclear patches using symmetrical 140 mM K$^+$ as the charge carrier, rather than Ca$^{2+}$, to increase the single channel conductance (γ) and prevent feedback regulation by permeating cations. The holding potential was maintained at +40 mV unless stated otherwise. Recordings were only continued if channel activity was stable. Under these conditions, channel current amplitude following pulses to negative and positive potentials were voltage-dependent. Fig. 4A shows a representative example of channel activity recorded with 10 μM IP$_3$ in the patch pipette following pulses from −60 mV to +60 mV in DT40–3KO expressing TbIP$_3$R. In many patches (−50%) we observed the presence of two or more active channels which may indicate TbIP$_3$R clustering (28, 29). Typically, channel activity was sustained over prolonged periods of time. Fig. 4B shows the current versus voltage (I-V) relationship for this channel generated from the peak K$^+$ conductance at each potential in Fig. 4A. The I-V relationship behaved as a linear conductance and thus in symmetrical 140 mM K$^+$, the I-V relationship was nearly linear with an extrapolated reversal potential at 0 mV (Fig. 4B). The conductance of TbIP$_3$R was typically in the range of 150–350 pS with dominant conductance of 200–220 pS, indicating the presence of different states (30, 31). We chose IP$_3$ channels with one stable overtone conductance to study how different conditions could modulate Ca$^{2+}$ release. In our experiments, no currents were detected in the nuclear envelope from DT40–TbIP$_3$R cells when IP$_3$ was omitted from the patch pipette ($n > 100$, meaning in more than 100 successful patches with gigaseal formation) (Fig. 4C). Acidic (Fig. 4D; n > 100), but not alkaline (Fig. 4E), pH also abolished the currents generated by IP$_3$. Data analysis of the currents detected at different pH levels show significant changes in frequency and open probability, as well as in total power, at acidic pH, as compared with neutral (pH 7.4) or alkaline (pH 8.0) pH (Fig. 4F). In agreement with these results, alkalinization of acidic compartments by NH$_4$Cl resulted in rapid Ca$^{2+}$ release, which was completed by addition of ionomycin (Fig. 4, G and H). Ionomycin is not effective in releasing Ca$^{2+}$ from acidic compartments (32) but alkalinization of the acidoCalcisomes by addition of NH$_4$Cl allowed its release (8). Alkalinization of acidoCalcisomes by the combination of nigericin–ionomycin also rapidly released Ca$^{2+}$ (Fig. 4, I and J).

The high-affinity agonist adenosphostin A (in place of IP$_3$) stimulated the TbIP$_3$R activity at 1 μM (Fig. 5A), as previously investigated by fluorescence determination of Ca$^{2+}$ release in permeabilized DT40–3KO cells expressing TbIP$_3$R (10). The competitive antagonist heparin (400 μg/ml, n > 100) abolished (Fig. 5B), whereas 2-APB had only partial inhibitory effect on, TbIP$_3$R conductance (100 μM; Figs. 5C and 3H) (33), and high concentrations of the membrane-permeable caffeine (70 mM in the bath solution; Fig. 5D) had a potent inhibitory effect on the channel conductance generated by IP$_3$ addition.

**Effect of luminal polyP and its hydrolysis products**

AcidoCalcisomes are rich in P$_i$, PP$_i$, and short- and long-chain polyphosphate (polyP) (9). Interestingly, polyP has been shown to activate transient receptor potential (TRP) channels of the
melastatin family (TRPM8) (20). We therefore investigated the
effect of luminal polyP and its hydrolysis products, Pi and PPi,i n
nuclear patches of DT40–3KO cells expressing TbIP3Ro r
RnIP3R1.

Fig. 6 shows representative current recordings obtained from
excised nuclear patches of DT40–3KO cells expressing TbIP3R
at a holding potential of +20 mV. When either Pi (Fig. 6A) or
PPi (Fig. 6C) was added to the bath solution (corresponding to

IP3 receptor stimulated by orthophosphate and pyrophosphate
the luminal phase of acidocalcisomes) there was a significant increase in the channel conductance, as confirmed by the data analyses of four independent experiments shown in Fig. 6, B and D, respectively. In contrast, addition of polyP3 decreased the frequency and the total power/min (Fig. 6, E and F), whereas addition of polyP100 had no significant effect on TbIP3R conductance (Fig. 7, A and B).

In contrast to these results, when the current recordings of nuclear patches of DT40–3KO cells expressing RnIP3R were analyzed (Figs. 7, C and D, and 8), neither Pp, nor PP, polyP3 or polyP100 had any effect on this channel conductance.

**Discussion**

Here we report the expression and electrophysiological properties of the *T. brucei* IP3R expressed in DT40 cells devoid of the three vertebrate IP3Rs (DT40–3KO). TbIP3R has similarities and differences with vertebrate IP3Rs. As occurs with the vertebrate orthologs, TbIP3R has a very conserved C-terminal, and pore (GGGVGD) regions and has a glutamate residue (calcium sensor) in the regulatory domain whose mutation in mammalian IP3R alters Ca2+ sensitivity (22) (Fig. 1). Accordingly, TbIP3R is stimulated by Ca2+. It is also inhibited by heparin and caffeine and partially by 2-APB and stimulated by ATP. Here we report the expression and electrophysiological properties of *T. brucei* IP3R expressed in DT40 cells devoid of the three vertebrate IP3Rs (DT40–3KO). TbIP3R has similarities and differences with vertebrate IP3Rs. As occurs with the vertebrate orthologs, TbIP3R has a very conserved C-terminal, and pore (GGGVGD) regions and has a glutamate residue (calcium sensor) in the regulatory domain whose mutation in

**Figure 6. Regulation of TbIP3R by ATP.** A and B, DT40–3KO cells expressing TbIP3R were loaded with Mag-Fluo4, permeabilized, and incubated with 3 mM ATP to prefill the intracellular Ca2+ stores. A and B, where indicated, 10 (black traces) or 100 (red traces) μM IP3 was added to cells incubated with 50 nM (A) or 1 μM (B) Ca2+. Fluorescence was normalized to the initial fluorescence intensity prior to addition of IP3. C, rates of Ca2+ release in A and B, values are mean ± S.E. *, p < 0.05; n = 4; Student’s t test.

**Figure 7. Regulation of TbIP3R by Ca2+.** DT40–3KO cells expressing TbIP3R were loaded with Mag-Fluo4, permeabilized, and incubated with 3.0 mM ATP to prefill the intracellular Ca2+ stores. A and B, where indicated, 10 (black traces) or 100 (red traces) μM IP3 was added to cells incubated with 50 nM (A) or 1 μM (B) Ca2+. Fluorescence was normalized to the initial fluorescence intensity prior to addition of IP3. C, rates of Ca2+ release in A and B, values are mean ± S.E. *, p < 0.05; n = 4; Student’s t test.

**Figure 8. Regulation of TbIP3R by Ca2+.** DT40–3KO cells expressing TbIP3R were loaded with Mag-Fluo4, permeabilized, and incubated with 3.0 mM ATP to prefill the intracellular Ca2+ stores. A and B, where indicated, 10 (black traces) or 100 (red traces) μM IP3 was added to cells incubated with 50 nM (A) or 1 μM (B) Ca2+. Fluorescence was normalized to the initial fluorescence intensity prior to addition of IP3. C, rates of Ca2+ release in A and B, values are mean ± S.E. *, p < 0.05; n = 4; Student’s t test.
adenophostin A. In contrast to the vertebrate orthologs TbIP3R localizes to the acidocalcisomes (10, 11) instead of to the ER, and it has only 5 of the 10 basic residues that were proposed to form a binding pocket to accommodate the negatively charged IP3 (34) (Fig. 1A); it does not have ATP-binding domains in the regulatory domain but instead has such a domain in the N-terminal region of the protein and is stimulated by the luminal polyP hydrolysis products, P_i and PP_i, and inhibited by acidic pH.

Numerous regulatory proteins interact with the cytosolic portion of the mammalian IP_3Rs but only a few have been described to interact with their luminal portions (4). Among them the Ca^{2+} storage proteins chromogranin A (CGA) and B (CGB), which interact with all three IP_3R types (35–38), and the
ER protein 44 (Erp44) (39), which interacts with the IP₃R type I. Chromogranins, which are located in secretory granules, increase IP₃R activity at acidic pH (40, 41), whereas Erp44 inhibits IP₃R opening (39).

X-ray microanalysis of acidocalcisomes of different species has revealed very low or no sulfur content in their lumen, suggesting the absence of proteins or a very low protein content (9). However, the luminal region has very high concentrations of polyP, which have been calculated to reach molar levels (9). Interestingly, early ³¹P NMR studies of isolated acidocalcisomes from T. brucei (42) found that the average chain length of polyP was 3.39, and we observed that polyP₃ has an inhibitory effect on TbIP₃R conductance. The results suggest that this conductance is stimulated when polyP is hydrolyzed to P₃ and PP_i. Acidocalcisomes of T. brucei possess a vacuolar soluble pyrophosphatase (TbVSP) that is able to hydrolyze polyP in the presence of Zn²⁺ at an optimal pH of 6.5, more alkaline than that of acidocalcisome pH of 5.0 – 5.5 (43). Alkalization of the acidocalcisomal TbIP₃R would result in polyP hydrolysis with accumulation of P₃ and PP_i, which also activate the channel and would explain our Ca²⁺ release results.

The stimulation of the acidocalcisomal TbIP₃R by alkalization and polyP hydrolysis products has physiological relevance when trypanosomes are submitted to osmotic stress. Under the very acidic conditions of resting acidocalcisomes (9) polyP is in its polymerized state and the channel is closed. When trypanosomes are submitted to hypo-osmotic stress it has been reported that, as a result of amino acid catabolism, there is an increase in ammonia (NH₃) that has been proposed to be sequestered as ammonium (NH₄⁺) in acidocalcisomes, leading to their alkalization (45). Alkalization would lead to activation of the vacuolar soluble pyrophosphatase (43) with generation of polyP hydrolysis products leading to channel opening and Ca²⁺ release. PolyP and Ca²⁺ signaling have been shown to be important for the regulatory volume recovery that follows (46, 47). In this regard, trypanosomes are exposed to drastic changes in osmolality when circulating in the blood of their mammalian hosts. They must be able to resist up to 1400 mosm when passing through the renal medulla and then return to the much lower osmotic environment of the general circulation (48).

In conclusion, by analysis of the TbIP₃R localized in the nucleus of DT40 cells, we demonstrate that the channel is predominantly closed at acidic pH but that alkalization or increase in P₃ and PP_i levels in its luminal site opens it up. Therefore, the single-channel properties of TbIP₃R are well-adapted to its role as the Ca²⁺ release channel of acidocalcisomes.
**Experimental procedures**

**Chemicals and reagents**

Adenophostin A was from EMD Millipore (Billerica, MA) and 2-APB, heparin, and caffeine were purchased from Sigma. 1-(2-amino-5-methylphenoxy)ethane-1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxyn)ethane-\(N,N',N''\)-tetraacetic acid, pentaoctoxymethyl ester (Fura 2/AM) was from Molecular Probes. All other reagents were of analytical grade or were described before (10). DT40–3KO cells stably expressing \(TbIP3R\) and \(RnIP3R1\) were generated previously (10) and maintained in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum, 1% heat-inactivated chicken serum, 4 mM glutamine, 50 \(\mu\)M mercaptoethanol, 100 units/ml penicillin and 10 \(\mu\)g/ml streptomycin, and 2 mg/ml G418. Cells were grown at 39 °C in an atmosphere of 95% air and 5% CO\(_2\) and were used or passaged when they reached a density of \(10^6\) cells/ml up to 30–35 times. Phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) was added to facilitate \(IP3R\)s expression (49).

*Trypan* \(brucei\) Lister 427 strain PCF were cultivated at 28 °C in SDM-79 medium (50) supplemented with 10% heat-inactivated fetal bovine serum and hemin (7.5 mg/ml).

**Functional assays of \(IP3R\)s in DT40 cells**

Uptake of Ca\(^{2+}\) into intracellular stores of permeabilized DT40–3KO–\(TbIP3R\) or DT40–3KO–\(RnIP3R1\) cells and its release regulated by IP\(_3\), Ca\(^{2+}\), or ATP were measured using a low-affinity Ca\(^{2+}\) indicator (Mag-Fluo4) trapped within the ER as described previously (10). Confluent cells (50 ml, \(2 \times 10^6\) cells/ml) were collected by centrifugation at 600 \(\times \) g for 2 min and suspended in 3 ml Hepes-buffered saline containing 1 mg/ml BSA, 0.02% (w/v) Pluronic F127, and 20 \(\mu\)M Mag-Fluo4 AM as described (51). After incubation at 20 °C for 1 h in the dark with gentle shaking, cells were centrifuged and re-suspended in 10 ml Ca\(^{2+}\)-free cytosol-like medium (CLM) (51), containing (in mM) 140 KCl, 20 NaCl, 1 EGTA, 2 MgCl\(_2\), and 20 Pipes, pH 7.0, and 50 \(\mu\)g/ml saponin (Sigma). Cells were incubated with shaking at 37 °C for 4 min. Twenty-\(\mu\)l cells were sampled to confirm permeabilization of cells by using 0.1% Trypan Blue and the cells were centrifuged and gently resuspended in 2.5 ml Mg\(^{2+}\)-free CLM supplemented with 375 \(\mu\)M CaCl\(_2\) and 10 \(\mu\)M FCCP. Ca\(^{2+}\) uptake was initiated by adding 250 \(\mu\)l of permeabilized cells into a cuvette containing 2.25 ml Mg\(^{2+}\)-free CLM supplemented with the final concentration of Ca\(^{2+}\) and Mg\(^{2+}\)-ATP indicated in the figures. Various concentrations (1–100 \(\mu\)M) for IP\(_3\) were tested in the presence of 50 nm–1 \(\mu\)M Ca\(^{2+}\) or 300 \(\mu\)M–3 mM ATP. The free Ca\(^{2+}\) concentration of the solution was monitored continuously under constant agitation at room temperature in a F-7000 Fluorescence Spectrophotometer (Hitachi, Tokyo, Japan) with excitation at 490 nm and emission at 525 nm.
**IP$_3$** receptor stimulated by orthophosphate and pyrophosphate

**DT40 nuclei isolation**

We used a combination of osmotic and mechanical lysis for nuclei isolation (52). DT40 cells were centrifuged (600 × g for 2 min, 4 °C) and washed once with ice-cold phosphate buffer (PBS) and nuclear isolation media (NIM). PBS composition was (in mM): 137 NaCl, 2.7 KCl, 10 Na$_2$PO$_4$, 10 KH$_2$PO$_4$, pH 7.4, with NaOH. NIM composition was (in mM): 250 sucrose, 150 KCl, 3 mM β-mercaptoethanol, 10 Tris-HCl, 1 phenylmethanesulfonyl fluoride (PMSF), pH 7.5. Cells were resuspended in NIM supplemented with Roche protease inhibitor mixture (1 tablet/20 ml). One ml of cell suspension was taken into a Dounce homogenizer and gently stroked for 8–12 times; 0.2 ml of crude lysate was then transferred into the recording chamber coated with poly-L-lysine and isolated nuclei were left to attach at room temperature (+24 °C) for 5 min. This procedure was repeated every 40–60 min for 5 h.

**Patch clamp recordings**

Single channel patch clamp recordings were obtained according to Mak et al. (53). We used equilibrium solutions with K$^+$ as the charge carrier. For some initial experiments K$^+$ was replaced with Cs$^+$, which is permeable for IP$_3$ channels but not for potassium channels (1). The bath solution, unless stated, typically contains (in mM): 140 KCl, 10 Hepes, 0.1 BaPATA (tetrapotassium salt), and 51 μM CaCl$_2$ (free [Ca$^{2+}$] ~ 200 nm), pH 7.1 with KOH. The usual pipette solution composition was (in mM): 140 KCl, 10 Hepes, 0.5 BaPATA, pH 7.1, with KOH. To activate IP$_3$Rs, Na$_2$ATP (4 mM), IP$_3$ (10 μM), and free [Ca$^{2+}$] adjusted to 1 μM, as calculated with MAXCHELATOR software (Stanford University) were added to pipette solution. Ten μM niflumic acid were added to block calcium-activated chloride channels.

Intracellular recording electrodes made of borosilicate glass capillars (Harvard Apparatus, Holliston, MA) with tip resistance of ~3 megohms were pulled by Flaming/Brown Micropipette Puller (Sutter Instruments) and polished by Microforge (Narishige MF-830) to the final tip resistance of 15–25 megohms. An Axopatch 200b amplifier (Axon Instruments, Jakarta, Indonesia) was used for registration of current. Data were filtered at 1000 Hz, digitized with Digidata 5100A (Axon Instruments) at 16-bit 2-kHz resolution and analyzed offline using PClamp 10 software. After formation of gigaseal (~10 GΩ) and withdrawal of the pipette, excised inside-out configuration was established. This configuration allowed us to precisely control the solution composition at both luminal and cytoplasmic sides of the membrane. Each experiment was done at least four times with four nuclei in each different preparation. Only low-noise recordings with stable IP$_3$Rs activity were taken into consideration. The conductance used for the majority (~80%) of the recordings was 200–220 pS. The success rate was 5% meaning that we detected active IP$_3$ responsive channels in 5% of patches with good gigaseal formation (no inhibitors added). When we used inhibitors, we observed 0% of active IP$_3$ responsive channels in >100 patches with good gigaseal formation. For the calculations of mean frequency, amplitude, open probability, and total power, we selected all events occurring in 60 s of representative recordings of samples in the presence or absence of phosphate compounds.

**Cytosolic Ca$^{2+}$ determinations**

Fura 2 determinations were performed essentially as described before (8). After harvesting the cells, they were washed twice at 3000 × g for 10 min at 4 °C in buffer A, which contained (in mM): 116 NaCl, 5.4 KCl, 0.8 MgSO$_4$, 5.5 d-glucose, and 50 Hepes, pH 7.2. Cells were resuspended in loading buffer consisting of buffer A plus 1.5% sucrose and 6 μM Fura 2/AM. The suspension was incubated for 30 min in a 30 °C water bath with mild agitation. The cells were then washed twice with ice-cold buffer A to remove extracellular dye. Cells were resuspended to a final density of 10⁹ cells/ml in buffer A and were kept in ice. For fluorescence measurements, a 125 μl sample of the cell suspension was diluted into 2.5 ml of buffer A (5 × 10⁷ cells/ml final density) in a cuvette placed in a thermostatically regulated (30 °C) Hitachi F-7000 spectrofluorometer. Excitation was at 340 and 380 nm and emission was at 510 nm. The fura-2 fluorescence response to [Ca$^{2+}$], concentration was calibrated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. (54). Other experimental conditions and calibrations were as described previously (8).

**Statistical analysis**

All values are expressed as mean ± S.E., unless indicated. Differences between groups were compared using unpaired t-tests. Differences were considered statistically significant at $p < 0.05$, and n refers to the number of independent biological experiments. All statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

**Author contributions**—E. P., N. W. N., and R. D. data curation; E. P. software; E. P., N. W. N., and R. D. formal analysis; E. P., N. W. N., G. H., and R. D. validation; E. P., N. W. N., G. H., and R. D. investigation; E. P., N. W. N., G. H., and R. D. methodology; E. P. and R. D. writing—original draft; R. D. conceptualization; R. D. resources; R. D. supervision; R. D. funding acquisition; R. D. project administration; R. D. writing—review and editing.

**Acknowledgments**—We thank David Yule for the DT40–3KO cells and RnIP$_2$R1 plasmids and discussions about their use and Ciro D. Cordeiro for preliminary experiments on Ca$^{2+}$ release.

** References

1. Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007) Inositol trisphosphate receptor Ca$^{2+}$ release channels. *Physiol. Rev.*, 87, 593–658

2. Berridge, M. J. (1987) Inositol trisphosphate and diacylglycerol: Two inextricably linked second messengers. *Annu. Rev. Biochem.*, 56, 159–193

3. Rossi, A. M., Tovey, S. C., Rahman, T., Prole, D. L., and Taylor, C. W. (2012) Analysis of IP$_3$ receptors in and out of cells. *Biochim. Biophys. Acta*, 1820, 1214–1227

4. Choe, C. U., and Ehrlich, B. E. (2006) The inositol 1,4,5-trisphosphate receptor (IP$_3$R) and its regulators: Sometimes good and sometimes bad teamwork. *Sci. STKE* 2006, re15

---

*J. Biol. Chem.* (2019) 294(27) 10628–10637 10635
23. Bezprozvanny, I. (2005) The inositol 1,4,5-trisphosphate receptors. Cell Calcium 42, 270–278 CrossRef Medline

22. Tu, H., Nosyreva, E., Miyakawa, T., Wang, Z., Mizushima, A., Iino, M., and Bezprozvanny, I. (2003) Functional and biochemical analysis of the type I inositol (1,4,5)-trisphosphate receptor calcium sensor. Biophys. J. 85, 290–299 CrossRef Medline

21. Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T., and Iino, M. (1999) Encoding of Ca2+ signals by differential expression of IP3 receptor subtypes. EMBO J. 18, 1303–1308 CrossRef Medline

20. Tu, H., Nosyreva, E., Miyakawa, T., Wang, Z., Mizushima, A., Iino, M., and Bezprozvanny, I. (2007) Functional and biochemical analysis of the type I inositol (1,4,5)-trisphosphate receptor calcium sensor. Biophys. J. 85, 290–299 CrossRef Medline

19. Prole, D. L., and Taylor, C. W. (2011) Identification of intracellular and secretory granules. J. Cell Sci. 124, 83–94 CrossRef Medline

18. Ladenburger, E. M., Kasielke, N., Wassmer, T., and Plattner, H. (2010) Dynamic clustering of IP3 receptors by IP3 retunes their regulation by IP3 and Ca2+. Nature 458, 565–569 CrossRef Medline

17. Alzayady, K. J., Sébé-Pedrós, A., Chandrasekhar, R., Wang, L., Ruiz-Trillo, I., and Taylor, C. W. (2008) Unicellular Ca2+zation by the calcium storage protein chromogranin A. J. Biol. Chem. 273, 12553–12559 CrossRef Medline

16. Cai, X. (2008) Unicellular Ca2+ signaling ‘tookit’ at the origin of metazoa. Mol. Biol. Evol. 25, 1357–1361 CrossRef Medline

15. Hashimoto, M., Enomoto, M., Morales, J., Kurabayashi, N., Sakurai, T., Hashimoto, T., Nara, T., and Mikoshiba, K. (2013) Inositol 1,4,5-trisphosphate receptor regulates replication, differentiation, infectivity and virulence of the parasitic protozoa Trypanosoma cruzi. Mol. Microbiol. 87, 1133–1150 CrossRef Medline

14. Traynor, D., Milne, J. L., Insall, R. H., and Kay, R. R. (2000) Ca2+ signalling is not required for chemotaxis in Dictyostelium. EMBO J. 19, 4846–4854 CrossRef Medline

13. Hashimoto, M., Enomoto, M., Morales, J., Kurabayashi, N., Sakurai, T., Hashimoto, T., Nara, T., and Mikoshiba, K. (2013) Inositol 1,4,5-trisphosphate receptor regulates replication, differentiation, infectivity and virulence of the parasitic protozoa Trypanosoma cruzi. Mol. Microbiol. 87, 1133–1150 CrossRef Medline

12. Lander, N., Chirriillo, M. A., Storey, M., Versesi, A. E., and Docampo, R. (2016) CRISPR/Cas9-mediated endogenous C-terminal tagging of Trypanosoma cruzi genes reveals the acidoalcalisome localization of the inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. 291, 25505–25515 CrossRef Medline

11. Huang, G., Ulrich, P. N., Storey, M., Johnson, D., Tischer, J., Tovar, J. A., Moreno, S. N., Orlando, R., and Docado, R. (2014) Proteomic analysis of the acidoalcalisome, an organelle conserved from bacteria to human cells. PLoS Pathog. 10, e1004555 CrossRef Medline

10. Huang, G., Ulrich, P. N., Storey, M., Johnson, D., Tischer, J., Tovar, J. A., Moreno, S. N., Orlando, R., and Docado, R. (2014) Proteomic analysis of the acidoalcalisome, an organelle conserved from bacteria to human cells. PLoS Pathog. 10, e1004555 CrossRef Medline

9. Docampo, R., de Souza, W., Miranda, K., Rohloff, P., and Moreno, S. N. (2005) Acidocalcisomes—conserved from bacterium to man. J. Eukaryot. Microbiol. 52, 227–233 CrossRef Medline

8. Docampo, R., Scott, D. A., Vercesi, A. E., and Moreno, S. N. (1995) Intra-cellular Ca2+ storage in acidocalcisomes of Trypanosoma cruzi. Biochem. J. 329, 290–299 CrossRef Medline

7. Vercesi, A. E., Moreno, S. N., and Docampo, R. (1994) Inorganic polyphosphate modulates TRPM8 channels. PLoS One 9, 1133–1150 CrossRef Medline

6. Adl, S. M., Bass, D., Lane, C. E., Lukes, J., Schoch, C. L., Smirnov, A., Agatha, S., Berney, C., Brown, M. W., Burki, F., Cardenas, P., Ceciika, I., Chistyakovka, L., Del Campo, J., Douthun, M., et al. (2019) Revisions to the classification, nomenclature, and diversity of eukaryotes. J. Eukaryot. Microbiol. 66, 4–119 CrossRef Medline

5. Cardenas, C., Miller, R. A., Smith, I., Bui, T., Molgol, J., Muller, M., Vais, H., Cheung, K. H., Yang, J., Parker, L., Thompson, C. B., Birnbaum, M. J., Hallows, K. R., and Foskett, J. K. (2010) Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca2+ transfer to mitochondria. Cell 142, 270–278 CrossRef Medline

4. Yule, D. I., Betzenhauser, M. I., and Joseph, S. K. (2010) Linking structure to function: Recent lessons from inositol 1,4,5-trisphosphate receptor mutagenesis. Cell Calcium 47, 469–479 CrossRef Medline

3. Li, F. J., Xu, Z. S., Soo, A. D., Lun, Z. R., and He, C. Y. (2017) ATP-driven and AMPK-independent autophagy in an early branching eukaryotic parasite. Autophagy 13, 715–729 CrossRef Medline

2. Galizzi, M., Bastamante, J. M., Fang, I., Miranda, K., Soares Medeiro, C. L., Tarleton, R. L., and Docado, R. (2013) Evidence for the role of vacuolar soluble pyrophosphatase and inorganic polyphosphate in Trypanosoma cruzi persistence. Mol. Microbiol. 90, 699–715 CrossRef Medline

1. Rahman, T., and Taylor, C. W. (2010) Nuclear patch-clamp recording from inositol 1,4,5-trisphosphate receptors. Methods Cell Biol. 99, 199–224 CrossRef Medline

0. Tafquf-Ur-Rahman, Skupin, A., Falcke, M., and Taylor, C. W. (2009) Clustering of InsP3 receptors by InsP3 retunes their regulation by InsP3 and Ca2+. Nature 458, 655–659 CrossRef Medline
43. Lemercier, G., Espiau, B., Ruiz, F. A., Vieira, M., Luo, S., Baltz, T., Docampo, R., and Bakalara, N. (2004) A pyrophosphatase regulating polyphosphate metabolism in acidocalcisomes is essential for Trypanosoma brucei virulence in mice. J. Biol. Chem. 279, 3420–3425 CrossRefMedline

44. Ruiz, F. A., Rodrigues, C. O., and Docampo, R. (2001) Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation, and environmental stress in Trypanosoma cruzi. J. Biol. Chem. 276, 26114–26121 CrossRefMedline

45. Rohloff, P., and Docampo, R. (2006) Ammonium production during hypo-osmotic stress leads to alkalinization of acidocalcisomes and cytosolic acidification in Trypanosoma cruzi. Mol. Biochem. Parasitol. 150, 249–255 CrossRefMedline

46. Rohloff, P., Rodrigues, C. O., and Docampo, R. (2003) Regulatory volume decrease in Trypanosoma cruzi involves amino acid efflux and changes in intracellular calcium. Mol. Biochem. Parasitol. 126, 219–230 CrossRefMedline

47. Fang, J., Ruiz, F. A., Docampo, M., Luo, S., Rodrigues, J. C., Motta, L. S., Rohloff, P., and Docampo, R. (2007) Overexpression of a Zn²⁺-sensitive soluble exopolyphosphatase from Trypanosoma cruzi depletes polyphosphate and affects osmoregulation. J. Biol. Chem. 282, 32501–32510 CrossRefMedline

48. Lang, F. (2007) Mechanisms and significance of cell volume regulation. J. Am. Coll. Nutr. 26, 613S–623S CrossRef Medline

49. Jayaraman, T., Ondriasová, E., Ondrias, K., Harnick, D. J., and Marks, A. R. (1995) The inositol 1,4,5-trisphosphate receptor is essential for T-cell receptor signaling. Proc. Natl. Acad. Sci. U.S.A. 92, 6007–6011 CrossRefMedline

50. Cunningham, I. (1977) New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. 24, 325–329 CrossRefMedline

51. Laude, A. J., Tovey, S. C., Dedos, S. G., Potter, B. V., Lummis, S. C., and Taylor, C. W. (2005) Rapid functional assays of recombinant IP₃ receptors. Cell Calcium 38, 45–51 CrossRefMedline

52. Boehning, D., Joseph, S. K., Mak, D. O., and Foskett, J. K. (2001) Single-channel recordings of recombinant inositol trisphosphate receptors in mammalian nuclear envelope. Biophys. J. 81, 117–124 CrossRefMedline

53. Mak, D. O., Vais, H., Cheung, K. H., and Foskett, J. K. (2013) Patch-clamp electrophysiology of intracellular Ca²⁺ channels. Cold Spring Harb. Protoc. 2013, 787–797 CrossRefMedline

54. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450 Medline

55. Edgar, R. C. (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797 CrossRefMedline