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Inositol 1,4,5-trisphosphate receptor determines intracellular Ca^{2+} concentration in *Trypanosoma cruzi* throughout its life cycle

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Regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is vital for eukaryotic organisms. Recently, we identified a Ca^{2+} channel ($TcIP_3R$) associated with intracellular Ca^{2+} stores in *Trypanosoma cruzi*, the parasitic protist that causes Chagas disease. In this study, we measured $[Ca^{2+}]_i$ during the parasite life cycle and determined whether $TcIP_3R$ is involved in the observed variations. Parasites expressing R-GECO1, a red fluorescent, genetically encoded Ca^{2+} indicator for optical imaging that fluoresces when bound to Ca^{2+}, were produced. Using these R-GECO1-expressing parasites to measure $[Ca^{2+}]_i$, we found that the $[Ca^{2+}]_i$ in epimastigotes was significantly higher than that in trypomastigotes and lower than that in amastigotes, and we observed a positive correlation between *$TcIP_3R$* mRNA expression and $[Ca^{2+}]_i$ during the parasite life cycle both *in vitro* and *in vivo*. We also generated R-GECO1-expressing parasites with $TcIP_3R$ expression levels that were approximately 65% of wild-type (wt) levels (SKO parasites), and $[Ca^{2+}]_i$ in the wt and SKO parasites was compared. The $[Ca^{2+}]_i$ in SKO parasites was reduced to approximately 50–65% of that in wt parasites. These results show that $TcIP_3R$ is the determinant of $[Ca^{2+}]_i$ in *T. cruzi*. Since Ca^{2+} signaling is vital for these parasites, $TcIP_3R$ is a promising drug target for Chagas disease.

Calcium ion (Ca^{2+}) is the most important and versatile intracellular messenger in eukaryotes [1]. Ca^{2+} signaling regulates various biological process, including secretion, fertilization, cell growth, and cell death [2]; thus, the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is vital. In mammals, $[Ca^{2+}]_i$ is regulated by several factors, including Ca^{2+} influx into the cytosol through voltage-gated Ca^{2+} channels (VGCCs), receptor-operated Ca^{2+} channels (ROCs), and store-opened Ca^{2+} channels (SOCs); buffering of Ca^{2+} with plasma membrane and cytosolic proteins; accumulation of Ca^{2+} in intracellular Ca^{2+} stores through the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA); and efflux of Ca^{2+} from stores through Ca^{2+} channels, such as inositol 1,4,5-trisphosphate receptors (IP_3Rs), and ryanodine receptors (RyRs) [3].

Abbreviations
$[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3Rs, inositol 1,4,5-trisphosphate receptors; R-GECO1, red fluorescent, genetically encoded Ca^{2+} indicator for optical imaging; SKO, single-knockout; $TcIP_3R$, IP_3R homolog in *T. cruzi*.

Trypanosoma cruzi is the parasitic protist that causes Chagas disease in Latin America. At present, only two drugs are available for Chagas disease (benznidazole and nifurtimox), and these often induce severe side effects and are effective for only the acute phase of the disease. Since no practical drug or vaccine for Chagas disease is available, new treatments are greatly needed [4]. The life cycle of the parasite comprises two phases, the insect and mammalian phases [5]. In the insect vector (the reduviid bug), the epimastigote replicates and transforms into a metacyclic trypomastigote (metacyclogenesis). A nonproliferating metacyclic trypomastigote invades a mammalian host, and is then transformed into an amastigote inside a wide variety of nucleated cells. The intracellular amastigote multiplies by binary fission, and is then transformed back into a trypomastigote, which is released into the circulation after host cell disruption.

$[Ca^{2+}]_i$ regulation is vital for *T. cruzi*, and the molecular mechanisms of $[Ca^{2+}]_i$ regulation in the parasite are thought to be quite different from those in mammalian cells [6]. In fact, no homologs of the typical Ca^{2+} transporters—ROCs, SOCs, or Na^+/Ca^{2+} exchangers, have been detected in Trypanosomes. The results of a proteome analysis of *T. brucei* suggested that a putative VGCC is localized to the flagellum [7]. Two homologs of plasma membrane Ca^{2+} ATPase (PMCA) have also been reported; one is localized on the plasma membrane, and the other is localized to the acidocalcisome of *T. brucei* [8]. In addition, a SERCA has been shown to be localized to the ER of *T brucei* like mammalian cells [9]. However, no RyR homologs have been reported. Recently, we identified an IP_3R homolog in *T. cruzi* ($TcIP_3R$), and showed that it is mainly localized to the ER. When the expression level of $TcIP_3R$ is reduced to less than one-half of that in wild-type (wt) cells, the parasite cannot grow [10]. Therefore, $TcIP_3R$ may be a promising drug target [11]. We also previously showed that $TcIP_3R$ regulates parasite growth, transformation, infectivity, and virulence in mammalian hosts, indicating that $TcIP_3R$ is an important regulator of the parasite life cycle [10,12]. In fact, experiments using classical Ca^{2+} indicators, such as Fura-2, showed that Ca^{2+} signaling is important for host cell invasion [10,13,14] as well as proliferation and transformation [15].

In this paper, we reported the successful preparation of parasites expressing R-GECO1 (a red fluorescent, genetically encoded Ca^{2+} indicator for optical imaging), which is a green fluorescent protein (GFP) variant that fluoresces only upon binding to Ca^{2+} [16]. It has recently been reported that other parasites including *Plasmodium falciparum* and *Toxoplasma gondii* that express genetically encoded Ca^{2+} indicators are useful for investigating Ca^{2+} signaling in the parasite [17,18]. Importantly, our findings revealed that analysis of *T. cruzi* expressing R-GECO1 revealed that the $[Ca^{2+}]_i$ in the parasite changes significantly during its life cycle, and that $TcIP_3R$ is the determinant of $[Ca^{2+}]_i$ in *T. cruzi*.

Materials and methods

Plasmid construction

The *R-GECO1* gene was amplified by PCR from the pCMV-R-GECO1 plasmid vector (Addgene, plasmid 45494) using specific primers (forward: 5′-CACCATGGTCGACCTTCACGTCGTA-3′ and reverse: 5′-CTACTTCGCTGTCATCATTTGTAC-3′; the CACC sequence required for directional cloning in pENTR/D-TOPO is underlined) and KOD-Plus Neo (TOYOBO Co., Ltd, Osaka, Japan). The PCR-amplified gene was inserted into pENTR/D-TOPO (Life Technologies, Rockville, MD, USA). The resultant plasmid, pENTR/*R-GECO1*, was converted to a pTREX vector [19,20], which contains a neomycin resistance gene as the selection marker, and was modified by the Gateway Vector Conversion System (pTREX(neo^R)-GW; Life Technologies) using the Gateway recombination system, to generate pTREX (neo^R)-GW/*R-GECO1*.

The puromycin resistance gene was amplified by PCR using pBApo-CMV Pur DNA plasmid vector (Clontech Laboratories, Inc., Mountain View, CA, USA) as the template with the specific primers (forward: 5′-ATGACCGAGTACAAGCCCAC-3′ and reverse: 5′-TCAGGCACCGGGCTTGC-3′). To remove the neomycin resistance gene from pTREX (neo^R)-GW/*R-GECO1*, we used PCR with pTREX (neo^R)-GW/*R-GECO1* as the template and the primers (forward: 5′-GGGGATCGATCCGGAACAA-3′ and reverse: 5′-ATTGGCTGCAGGGTCGCT-3′). These two PCR fragments were ligated with DNA Ligation Kit Ver. 2.1 (Takara Bio Inc., Shiga, Japan), to generate pTREX (pur^R)-GW/*R-GECO1*.

Cell culture

Epimastigotes of the *T. cruzi* Tulahuen strain were cultured as previously described [21]. The mammalian stages of the parasites were maintained in HeLa cells or 3T3-Swiss albino cells (Health Science Research Resources Bank, Tokyo, Japan), and trypomastigotes were collected from subcultures of infected 3T3-Swiss albino cells by centrifugation as previously described [22]. Metacyclogenesis was performed as previously described [23]. Quantitative real-time RT-PCR analysis was performed as previously described [10]. 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), which is a cell-impermeant Ca^{2+} chelator and reduces the levels of extracellular Ca^{2+}, and 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic Acid, tetraacetoxymethyl ester (BAPTA-AM), which is a cell-permeant Ca^{2+} chelator thereby reduces

$[Ca^{2+}]_i$, were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), and an IP_3R inhibitor, 2-aminoethoxydiphenyl borate (2-APB), was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Expression of R-GECO1 in *T. cruzi*

A total of 1×10^7 epimastigotes were resuspended in Amaxa Basic® Parasite Nucleofector Kit 2 solution (Lonza, Köln, Germany). The resuspended wt or $TcIP_3R$ SKO parasites [10] were mixed with 10 μg of pTREX (neo^R)/*R-GECO1* or pTREX (pur^R)/*R-GECO1*, respectively, and then electroporated with an Amaxa Nucleofector Device (Lonza) using program U-033. Stable wt transformants expressing R-GECO1 were selected by incubating the cells for 30 days in LIT medium containing 0.5 $mg{\cdot}mL^{-1}$ G418, and then clonal derivatives were isolated by limiting dilution. The stable SKO transformants expressing R-GECO1 were selected by incubating the cells for 30 days in LIT medium containing 0.25 $mg{\cdot}mL^{-1}$ G418 and 3 $\mu g{\cdot}mL^{-1}$ puromycin. Amastigotes or trypomastigotes stably expressing R-GECO1 were transformed from the epimastigotes expressing R-GECO1 using the methods described above.

Detection of $[Ca^{2+}]_i$ by fluorescence microscopy

Fluorescence images of the parasites were acquired using a fluorescent microscope (Axio Imager M2; Carl Zeiss Co. Ltd., Oberkochen, Germany) or a laser confocal microscope (Nikon A1R, Nikon Co. Ltd., Tokyo, Japan). After acquiring fluorescence images in normal culture medium, the maximal fluorescence signal (F_{max}) of R-GECO1 in individual parasites was determined by treating them with high Ca^{2+} solution (10 mM) and ionomycin (0.26 mM; Nacalai Tesque, Kyoto, Japan), which increase the cytosolic Ca^{2+} content of parasites so as to saturate R-GECO1 with Ca^{2+}. To convert fluorescence intensity to Ca^{2+} concentration, the following formula was used:

$$[Ca^{2+}]_i^n = K_d^n \times (F - F_{MIN})/(F_{MAX} - F)$$

where K_d is the dissociation constant for R-GECO1 (482 nM); n is the Hill coefficient of R-GECO1 (2.0); F is the fluorescence intensity in each parasite; F_{MAX} is the maximal fluorescence intensity of R-GECO1 in the parasite (see above); and F_{MIN} is the minimal intensity of R-GECO1 calculated using the ratio change value obtained by Zhao *et al.* (= 1/16 of F_{MAX}).

Statistical analysis

Statistical analysis was performed with SIGMA PLOT ver. 12 software (Systat Software, Inc., San Jose, CA, USA) using one-way ANOVA or Student's *t*-test.

Results and Discussion

The $[Ca^{2+}]_i$ changes significantly during the life cycle of *T. cruzi*

To investigate the changes in the $[Ca^{2+}]_i$ in *T. cruzi* during its life cycle, parasites expressing R-GECO1 were prepared. The *R-GECO1* gene was cloned in the *T. cruzi* pTREX expression vector [19], in which *R-GECO1* is expressed under the ribosomal promoter; therefore, R-GECO1 was constitutively expressed throughout the parasite life cycle. We at first investigated whether the fluorescence signal in the parasites expressing R-GECO1 was reduced after treatment with a cell-permeant Ca^{2+} chelator BAPTA-AM. After replacement of the parasite medium with PBS, BAPTA-AM (final concentration 100 μM) was added to the PBS to reduce Ca^{2+}, and $[Ca^{2+}]_i$ was measured after 3 min (Fig. 1A). Treatment of the parasites with BAPTA-AM significantly reduced the parasite $[Ca^{2+}]_i$, indicating that R-GECO1 works as a Ca^{2+} indicator in the parasites. We also found that the parasites were killed by the treatment.

To exclude variations in signal intensity among the parasite clones, a clonal derivative was isolated and used for the experiments. Figure 1B shows the R-GECO1 signal in the epimastigote, trypomastigote, and amastigote. Although the signal was detected throughout the cytoplasm of the parasites at all stages, the signal intensity was quite different among the different life cycle stages, and the maximal fluorescence intensities of R-GECO1 obtained in the presence of 260 μM ionomycin and 10 mM Ca^{2+} were similar. The $[Ca^{2+}]_i$ was calculated from the R-GECO1 signal intensity and compared among the three stages, as described in the Methods section (Fig. 1C). The $[Ca^{2+}]_i$ was clearly higher in amastigotes (579 ± 204 nM) and epimastigotes (327 ± 44 nM) than in trypomastigotes (85 ± 39 nM). These results indicated that the $[Ca^{2+}]_i$ changes significantly during the progression of the parasite life cycle. Ca^{2+} oscillation was not detected at any stage.

Live cell imaging revealed changes in the $[Ca^{2+}]_i$ of *T. cruzi* parasitizing host cells

We further investigated the changes in the $[Ca^{2+}]_i$ of parasites during intracellular growth. 3T3-Swiss cells were infected with R-GECO1-expressing trypomastigotes, and then the growth of one parasite was monitored under a fluorescent microscope (Fig. 2A). We successfully monitored them until the parasite divided three times within a host cell. The results showed that the $[Ca^{2+}]_i$ in amastigotes did not change significantly after division.

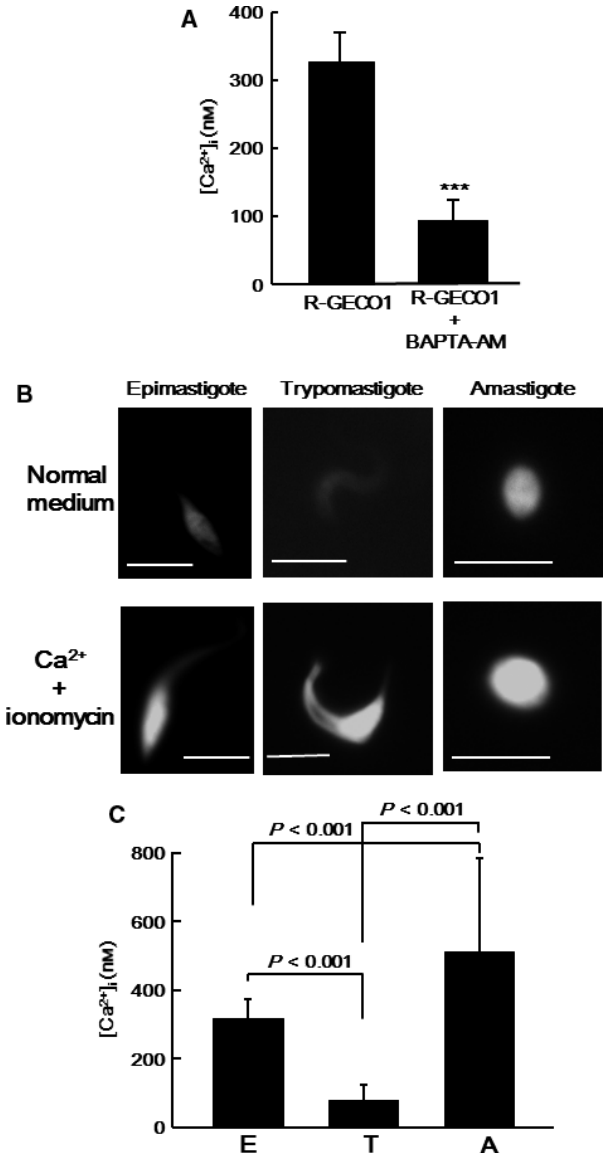

Fig. 1. Changes in *T. cruzi* $[Ca^{2+}]_i$ during its life cycle. (A) The fluorescence intensity of epimastigotes expressing R-GECO1 was measured after treatment with 100 μM BAPTA-AM for 3 min. The $[Ca^{2+}]_i$ were calculated from the fluorescence intensity and compared to that in untreated epimastigotes. The fluorescence intensity of 20 parasites for each condition was measured. Statistical analysis between the two groups was performed using Student's *t*-test. ***$P < 0.001$. (B) Typical images of a clonal derivative of *T. cruzi* expressing R-GECO1 in normal culture medium (top) and in high Ca^{2+} medium with ionomycin (bottom), including an epimastigote, trypomastigote, and amastigote, are shown. *Bar*, 5 μm. (C) The $[Ca^{2+}]_i$ in epimastigotes (E), trypomastigotes (T), and amastigotes (A), as measured by fluorescence intensity, was compared. The fluorescence intensity of 20 parasites was measured for each stage. Statistical analysis between the groups was performed using one-way ANOVA and Tukey's Test.

Amastigotes divide several times within host cells, transform into trypomastigotes, and then lyse the host cells. We investigated whether the $[Ca^{2+}]_i$ in trypomastigotes parasitizing host cells was decreased similar to that observed in tissue culture-derived trypomastigotes (Fig. 2B, Movie S1). The results showed that the $[Ca^{2+}]_i$ in trypomastigotes within host cells (Fig. 2B, arrowhead in the right panel) was significantly lower than that in amastigotes (Fig. 2B, arrow in the right panel).

These results indicate that amastigotes are able to maintain $[Ca^{2+}]_i$ even in environments where the Ca^{2+} concentration is very low, such as the cytosol of host cells, and that the $[Ca^{2+}]_i$ in the trypomastigote when inside host cells is significantly lower than that in the amastigote $[Ca^{2+}]_i$.

TcIP$_3$R is the determinant of $[Ca^{2+}]_i$ in *T. cruzi*

Previously, we reported that *TcIP$_3$R* mRNA expression varied significantly among the parasite life cycle stages [10]. Here, we investigated a possible correlation between *TcIP$_3$R* mRNA expression and the $[Ca^{2+}]_i$ of the parasite (Fig. 3A). There was a positive correlation between the parasite $[Ca^{2+}]_i$ at each life stage and the *TcIP$_3$R* mRNA expression level ($R^2 = 0.66$). These results suggest that TcIP$_3$R is important for the regulation of $[Ca^{2+}]_i$ in *T. cruzi*.

To investigate whether TcIP$_3$R is involved in the regulation of $[Ca^{2+}]_i$ in *T. cruzi*, the level of *TcIP$_3$R* was reduced in parasites expressing R-GECO1, and the $[Ca^{2+}]_i$ in wt and mutant parasites was measured (Fig. 3B). We previously found three *TcIP$_3$R* genes in the genome of the *T. cruzi* Tulahuen strain, and we prepared single-knockout (SKO) parasites, in which one of the *TcIP$_3$R* genes was disrupted by homologous recombination [10]. We observed the specific disruption of only one *TcIP$_3$R* gene by Southern blot analysis and an approximately 35% reduction in TcIP$_3$R expression levels in the SKO parasites, and these parasites show various phenotypes, such as inhibition of epimastigote growth [10]. Since the knockout cassette used to prepare the SKO parasites contained a neomycin resistance gene, the transformants were selected with G418. Then, the *R-GECO1* gene was cloned into an expression plasmid vector for *T. cruzi* containing a puromycin resistance gene (pTREX(pur^R)), and then this plasmid was transfected into the SKO parasites. SKO parasites expressing R-GECO1 were selected in culture medium containing G418 and puromycin. For the control, wt Tulahuen strain parasites were transfected with pTREX(pur^R)/*R-GECO1* and selected in

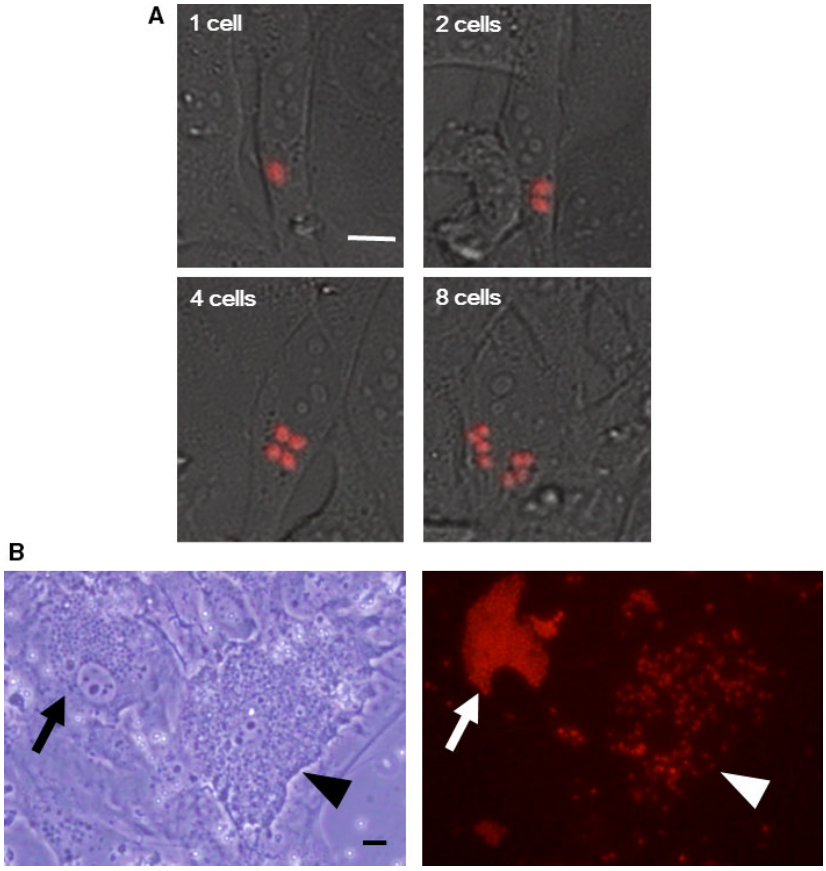

Fig. 2. Changes in *T. cruzi* $[Ca^{2+}]_i$ within host cells. (A) 3T3-Swiss albino cells were infected with R-GECO1-expressing trypomastigotes (red), and imaged 84 h after infection. The movement of an amastigote was recorded using real-time confocal microscopy with 40× dry objective lens (Nikon AIR). The time interval of the serial images was 15 min. The amastigotes (one cell) that divided once (two cells), twice (four cells), and three times (eight cells) are shown. *Bar*, 5 μm. (B) A bright-field image of cells that are heavily infected with R-GECO1-expressing amastigotes (arrow) and trypomastigotes (arrowhead) is shown (left). A representative microscopic image was obtained with an inverted microscope (IX72; Olympus, Tokyo, Japan). A video of the same field is available as Movie S1. Note that the trypomastigotes in the host cell move intensely. A fluorescent image of the same field is also shown (right). *Bar*, 10 μm.

culture medium containing puromycin. Since the expression level of R-GECO1 among the parasite clones might vary, the fluorescence intensity in the parasites was randomly measured without cloning. The fluorescence intensity in the SKO parasites was significantly lower than that in the wt parasites. Importantly, the $TcIP_3R$ expression level in the SKO parasites was reduced to approximately 65% of wt levels [10], and the R-GECO1 signal in SKO parasites was reduced to approximately 50% of wt levels.

Next, we investigated whether Ca^{2+} influx from the extracellular fluid or efflux to the extracellular fluid is important for maintenance of $[Ca^{2+}]_i$ (Fig. 3C). Excessive amounts of $CaCl_2$ or BAPTA, a noncell-permeable Ca^{2+} chelator, was added to the cultivation medium of epimastigotes expressing R-GECO1, and the $[Ca^{2+}]_i$ in treated parasites was compared to that in untreated parasites after 2 h. No increase was detected in parasites treated with $CaCl_2$ compared to that in untreated parasites. When the Ca^{2+} in the culture medium was chelated by the addition of BAPTA, we speculated that $[Ca^{2+}]_i$ might be reduced by PMCA function. However, the $[Ca^{2+}]_i$ in parasites treated with BAPTA was not reduced when compared with that in untreated parasites. These results indicate that *T. cruzi* do not constitutively import or export Ca^{2+}. Therefore, Ca^{2+} released from intracellular Ca^{2+} store(s) into the cytosol by $TcIP_3R$ should be effectively returned to the store(s) by SERCA [24,25].

In animal cells, $[Ca^{2+}]_i$ are kept at low concentrations (~ 100 nM) in the absence of extracellular stimuli [26]. Phosphoinositide phospholipase C (PI-PLC) is activated in response to signals from cell surface receptors, and it catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate IP_3, which activates IP_3R and transiently increases $[Ca^{2+}]_i$ [27]. Our present data indicate that $[Ca^{2+}]_i$ in epimastigotes and amastigotes is constitutively high. Recently, it has been reported that *Trypanosoma brucei* PI-PLC may be constitutively activated [28]. Furthermore, the molecular properties of *T. cruzi* PI-PLC have been reported to be similar to that of *T. brucei* PI-PLC [29] (e.g., plasma membrane localization). Together, these findings suggest that constitutive activation of *T. cruzi* PI-PLC might maintain high $[Ca^{2+}]_i$ through constitutive $TcIP_3R$ activation.

According to the cell boundary theorem, $[Ca^{2+}]_i$ is determined by the balance between Ca^{2+} influx and efflux, and Ca^{2+} release via IP_3R does not result in

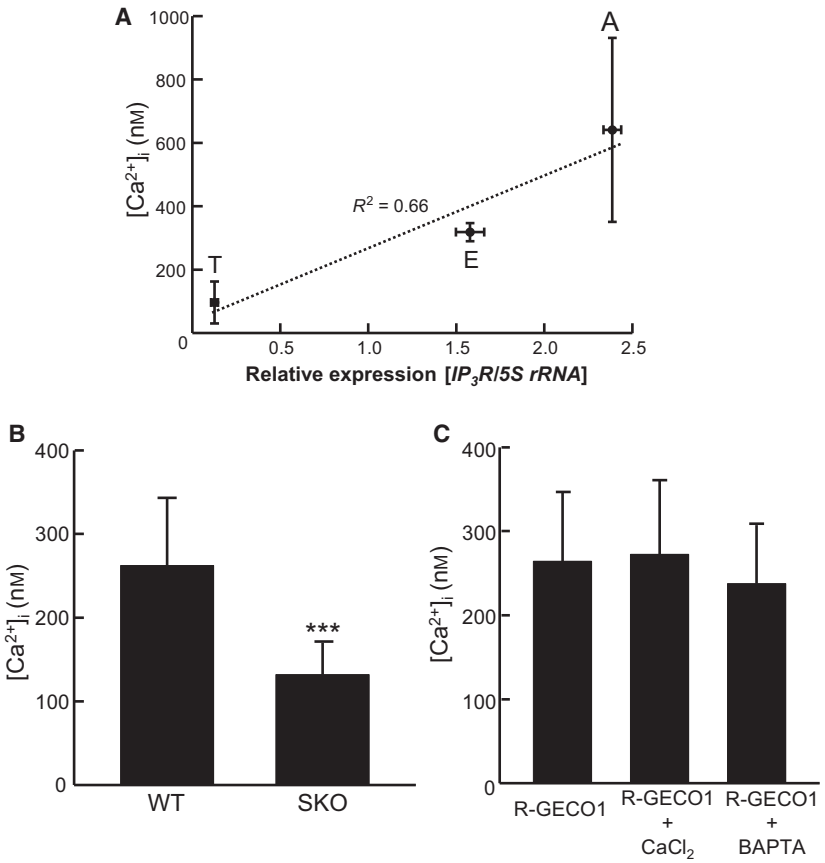

Fig. 3. Effect of Ca^{2+} chelators or reduced $TcIP_3R$ expression on *T. cruzi* $[Ca^{2+}]_i$. (A) Correlation between *TcIP$_3$R* mRNA expression level and $[Ca^{2+}]_i$ in *T. cruzi* throughout the parasite life cycle. $[Ca^{2+}]_i$ shows a linear relationship with *TcIP$_3$R* mRNA expression (R^2 = 0.66). To measure $[Ca^{2+}]_i$, the R-GECO1 fluorescence intensity of 20 parasites was measured. To measure *TcIP$_3$R* mRNA expression, quantitative real-time RT-PCR analysis of relative transcript levels was performed, and the data shown are the mean ± SD of three independent experiments. (B) $[Ca^{2+}]_i$ was compared between R-GECO1-expressing wt and SKO epimastigotes. The fluorescence intensity of 20 parasites was measured. Statistical analysis between the groups was performed using Student's *t*-test. ***$P < 0.001$. (C) R-GECO1-expressing epimastigotes were treated with 10 mM $CaCl_2$ or 10 mM BAPTA for 2 h; fluorescence was measured, and $[Ca^{2+}]_i$ was calculated and compared to that in untreated parasites. The fluorescence intensity of 20 parasites was measured.

higher $[Ca^{2+}]_i$ [30,31]. In mammalian cells, Ca^{2+} influx increases through the SOC mechanism activated by Ca^{2+} release from the ER, thereby resulting in an increase of $[Ca^{2+}]_i$ [25]. Furthermore, it might be possible that $[Ca^{2+}]_i$ in *T. cruzi* is not always increased through $TcIP_3R$ directly but the parasites have some unknown mechanism(s) to increase Ca^{2+} influx. Interestingly, since amastigotes parasitize the host cell cytoplasm, where the concentration of Ca^{2+} is much lower than the $[Ca^{2+}]_i$ in amastigotes, the parasites may not receive Ca^{2+} from outside through a SOC-like mechanism. However, how amastigotes maintain high $[Ca^{2+}]_i$ within the host cells remains unknown at present.

In conclusion, our present study revealed that basal $[Ca^{2+}]_i$ levels in *T. cruzi* are determined by the level of $TcIP_3R$ expression. Since Ca^{2+} signaling is essential for the parasite and the primary structure of $TcIP_3R$ shares low similarity with that of mammalian IP_3Rs, $TcIP_3R$, the key Ca^{2+} signaling molecule, is a promising drug target for Chagas disease.

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Author contributions

MH, MD, NK, KM, and NT designed the study. MH, MD, NK, KF, and HM did the experiments. MH, MD, NK, and TN wrote the manuscript. MH, MD, NK, KF, HM, YO, TS, TM, KM, and TN interpreted the data. All authors reviewed the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Movie S1. Changes in *T. cruzi* $[Ca^{2+}]_i$ within host cells. 3T3-Swiss albino cells were infected with R-GECO1-expressing trypomastigotes. A bright-field movie of cells that are heavily infected with R-GECO1-expressing amastigotes and trypomastigotes is shown (A). A representative microscopic movie was obtained with an inverted microscope (IX72; Olympus). Note that the trypomastigotes in the host cell move intensely. A fluorescent image of the same field is also shown (B).