Calreticulin Modulates Capacitative Ca\(^{2+}\) Influx by Controlling the Extent of Inositol 1,4,5-Trisphosphate-induced Ca\(^{2+}\) Store Depletion*

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Calreticulin (CRT) is a highly conserved Ca\(^{2+}\)-binding protein that resides in the lumen of the endoplasmic reticulum (ER). We overexpressed CRT in Xenopus oocytes to determine how it could modulate inositol 1,4,5-trisphosphate (InsP\(_3\))-induced Ca\(^{2+}\) influx. Under conditions where it did not affect the spatially complex elevations in free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) due to InsP\(_3\)-induced Ca\(^{2+}\) release, overexpressed CRT decreased by 46% the Ca\(^{2+}\)-gated Cl\(^{-}\) current due to Ca\(^{2+}\) influx. Deletion mutants revealed that CRT requires its high capacity Ca\(^{2+}\)-gated Cl\(^{-}\) current due to Ca\(^{2+}\) influx. This functional domain was also required for CRT to attenuate the InsP\(_3\)-induced decline in the free Ca\(^{2+}\) concentration within the ER lumen ([Ca\(^{2+}\)]\(_{ER}\)), as monitored with a “chameleon” indicator. Our data suggest that by buffering [Ca\(^{2+}\)]\(_{ER}\) near resting levels, CRT may prevent InsP\(_3\) from depleting the intracellular stores sufficiently to activate Ca\(^{2+}\) influx.

Since it was first isolated a quarter of a century ago (1), the protein calreticulin (CRT)\(^3\) has been identified in a great variety of cells, implying an essential biological activity (2). Although CRT may be critical for cardiac development (3), to date, the nature of the cellular function of CRT remains poorly understood. CRT binds to steroid receptors (4, 5) and to integrins (6, 7) and thus could modulate gene transcription (8, 9) or cellular adhesion (2, 10–12). To reach these targets, however, CRT would need to be present both in the nucleus and the cytosol. Although CRT has been reported in these cellular compartments (see Refs. 2 and 10 but also see Ref. 13), the bulk of the protein clearly resides elsewhere, i.e. inside the endoplasmic reticulum (ER). Within the ER lumen, CRT may assist in the folding and assembly of glycoproteins (14–17). ER CRT may also act as a repository of readily releasable Ca\(^{2+}\), with each mole of CRT binding as many as 20–25 mol of Ca\(^{2+}\). Since Ca\(^{2+}\) release from the ER is controlled mainly by the second messenger inositol 1,4,5-trisphosphate (InsP\(_3\)), CRT emerges as a potential regulator of this ubiquitous signal transduction pathway.

The InsP\(_3\)-induced Ca\(^{2+}\) signal has two main components, the release of Ca\(^{2+}\) from the intracellular stores (InsP\(_3\)-induced Ca\(^{2+}\) release, or IICR) and the influx of Ca\(^{2+}\) across the plasma membrane (InsP\(_3\)-induced Ca\(^{2+}\) influx, or IICI). IICR starts when InsP\(_3\) binds to its intracellular receptor (InsP\(_3\)-R), a ligand-gated Ca\(^{2+}\) channel that traverses the lipid membrane surrounding the ER. The resulting discharge of ER Ca\(^{2+}\) into the cytosol is often spatially complex, with periodic focal release of Ca\(^{2+}\) actively spreading to the rest of the cell through mechanisms that remain incompletely understood (18). IICI commonly follows IICR, thereby allowing cells to recharge their internal stores. Stimulation of Ca\(^{2+}\) influx appears closely linked to the depletion of the intracellular Ca\(^{2+}\) stores, a relationship known as capacitative Ca\(^{2+}\) entry. We do not yet understand how Ca\(^{2+}\) store depletion stimulates Ca\(^{2+}\) influx. Store depletion could communicate with the plasma membrane through a diffusible messenger (19, 20), through secretion-like vesicular docking (21, 22), or through protein-protein interactions that may involve the InsP\(_3\)-R itself (23–25).

Even though Ca\(^{2+}\) storage was the first function ascribed to CRT (1), we do not yet know what role, if any, CRT plays in the regulation of the InsP\(_3\)-induced Ca\(^{2+}\) signal. Whereas gene knock-out experiments indicate that CRT is not essential for IICR (11), overexpression of CRT has been associated with a decrease in the magnitude of IICI in mammalian cell lines (26–28). These results have prompted the hypothesis that CRT reduces capacitative Ca\(^{2+}\) entry by buffering the free Ca\(^{2+}\) concentration within the ER lumen ([Ca\(^{2+}\)]\(_{ER}\)) above the levels required to trigger capacitative Ca\(^{2+}\) influx. In this work, our objective was to put this hypothesis to a rigorous test by directly measuring [Ca\(^{2+}\)]\(_{ER}\) in a widely used model cell, the Xenopus oocyte. Our data indicate that CRT requires its high capacity Ca\(^{2+}\)-binding domain both to attenuate the InsP\(_3\)-induced decrease in [Ca\(^{2+}\)]\(_{ER}\) and to reduce the elevations of [Ca\(^{2+}\)]\(_{ER}\), due to IICI. The ability of CRT to buffer [Ca\(^{2+}\)]\(_{ER}\) may thus be functionally relevant to the InsP\(_3\)-induced Ca\(^{2+}\) signal.

EXPERIMENTAL PROCEDURES

CRT and CRT Deletion Mutant Expression Vectors—We inserted the CRT cDNA cloned from the HL60 human leukemia cell line (29) between the PsI and SalI sites of the pMT3 plasmid vector (30). We used the polymerase chain reaction (PCR) to build CRT mutants lacking functional domains. To remove the C-domain (CRT-ΔC), we amplified...
of the N and P domains of CRT (amino acids 2–315) and added a KDEL ER retrieval sequence at the C terminus (primers 1 and 2). The PCR fragment was digested with MluI and EcoRI and inserted between those sites in a modified pMT3. For the construct lacking the P-domain (CRT-3P), we amplified the N-domain (amino acids 2–197, primers 1 and 2) and the C-domain (amino acids 316–401, primers 4 and 5) separately. The PCR fragments were then respectively digested with MluI/SpeI and with SpeI/EcoRI and ligated between the MluI and EcoRI sites of pMT3. Primers sequence were as follows, from 5’ to 3’: primer 1, GTCAACGCTTGCTCATCAGGTGCGGC; primer 2, CCGAATTCTCAGAGATCTGACCGACGCAAGGCAATGGTATCT; primer 3, CCGACTATTTCTCAAGAGAGCAGG; primer 4, GTCACTAGTTGCTGGCTGCGGCCGCTG; primer 5, CCGAATTCTCAGGCAGTTCTCACACAGAGAGCCAGGGAG.

cDNA-directed Protein Expression and Western Blotting—We prepared stage V–VI oocytes from albino Xenopus laevis and injected the DNA constructs into the nucleus as described previously (31, 32). Oocytes were microinjected with 4 nmol of either pMT3 (18) or pMT3-CRT (20) in a volume of 20 nl. When compared with controls (1st lane), CRT bands facing the closed arrow) was increased in cells microinjected with pMT3-CRT (2nd lane). Note the appearance of new bands (open arrow) in cells microinjected with pMT3-CRT-3P (doublet in 3rd lane) or pMT3-CRT-3C (4th lane).

CALCIUM IMAGING—We performed calcium imaging using a Hitachi 3700 electron microscope (Hitachi Ltd., Tokyo) as described previously (32).

RESULTS AND DISCUSSION

Overexpression of CRT in Xenopus Oocytes—On a Western blot of microsomal proteins from which we have previously purified CRT, the ER luminal protein calnexin is stained (33). To perform CRT overexpression in the oocyte system, oocytes were injected (40) with either 250 nl of pure CRT expression construct (40) and analyzed as described previously (32). CRT is overexpressed within the ER lumen.

Overexpression of CRT on a Western blot of microsomal proteins from which we have previously purified CRT, the ER luminal protein calnexin is stained (33). We injected either with pMT3 alone or with pMT3-CRT in oocytes. We then performed nuclear run-on assays using cell-free nuclear extracts from control cells, but much brighter (Fig. 2). We observed a similar fluorescence pattern to that of control cells, but much brighter (Fig. 2). We observed a similar fluorescence pattern to that of control cells, but much brighter (Fig. 2).
current reflects \([Ca^{2+}]\), just beneath the plasma membrane (48, 49), where it is most likely to be affected by \(Ca^{2+}\) influx. The assay integrates the submembranous \([Ca^{2+}]\), changes across the entire surface of the plasma membrane, thereby maximizing our ability to detect changes in the magnitude of \(Ca^{2+}\) influx. In control cells, microinjection of \(InsP_3\) (10 \(\text{mM}\) in the pipette) causes a biphasic response; there is a short initial increase in \([Ca^{2+}]\), followed by a slow increase (Fig. 4A). The fast initial component of the response, which is not affected by the removal of extracellular \(Ca^{2+}\), is due to the release of \(Ca^{2+}\) from the intracellular stores (50, 51). In contrast, the slow component of the response can be inhibited by decreasing the extracellular \(Ca^{2+}\) concentration or by adding inorganic \(Ca^{2+}\) channel blockers (\(Mn^{2+}\), \(Ni^{2+}\), and \(La^{3+}\)) to the bath and thus depends on \(Ca^{2+}\) influx (50–52). Although the magnitude of the \(Cl^-\) currents due to IICR was similar to that of control cells (272 ± 28 \(nA\) in \(CRT\) versus 247 ± 23 \(nA\) in controls, \(n = 31\) pairs of cells), the \(Ca^{2+}\) influx-dependent \(Cl^-\) current was reduced almost in half in cells overexpressing \(CRT\) (77 ± 15 \(nA\) versus 143 ± 19 \(nA\) in controls, \(n = 31\) matched pairs of cells, one of which is shown in Fig. 4, \(p < 0.001\)). These results, which are consistent with those obtained in mammalian cell lines (26, 27), indicate that \(CRT\) reduces the rise in \([Ca^{2+}]\), due to \(Ca^{2+}\) influx. Direct measurements of whole cell current due to \(Ca^{2+}\) or \(Ba^{2+}\) entry have indicated that the \(Ca^{2+}\)-gated \(Cl^-\) current assay has a high sensitivity for measuring the magnitude of \(Ca^{2+}\) influx (22, 43). Thus, our results suggest that \(CRT\) overexpression reduces the rate at which extracellular \(Ca^{2+}\) enters the cell.

**CRT Attenuates the \(InsP_3\)-induced Decline in \([Ca^{2+}]_{\text{ER}}\)**—In the context of capacitative \(Ca^{2+}\) entry, \(CRT\) could reduce \(IICR\) by buffering \([Ca^{2+}]_{\text{ER}}\) to levels above those required to stimulate \(Ca^{2+}\) influx. Because neither the precise value of \([Ca^{2+}]_{\text{ER}}\) nor the degree to which \(CRT\) contributes to the overall \(Ca^{2+}\)-binding properties of the ER luminal milieu is presently known, we could not confidently predict if/how altered \(CRT\) levels affect \([Ca^{2+}]_{\text{ER}}\). To test this hypothesis, we therefore monitored \([Ca^{2+}]_{\text{ER}}\) directly with an \(ER\)-targeted FRET indicator, chameleon 3er (3er) (44). At base line, the 535/485 fluorescence emission ratios in cells co-expressing 3er and \(CRT\) were similar to the ratios found in cells expressing 3er alone (1.08 ± 0.07 versus 1.09 ± 0.07, \(n = 16\) matched cell pairs). Because the 3er indicator saturates at \([Ca^{2+}]\) in excess of 100 \(\muM\) (44), we repeated the experiments, this time expressing a lower affinity indicator, 4er, that could report \([Ca^{2+}]_{\text{ER}}\) between 10^{-4} and 10^{-2} \(M\). The 535/485 ratios in cells co-expressing 4er and \(CRT\) were also similar to the ratios found in control cells (0.79 ± 0.04 versus 0.81 ± 0.05, \(n = 6\) matched cell pairs).
These data indicate that overexpressed CRT does not measurably change the resting $[\text{Ca}^{2+}]_{\text{ER}}$.

When microinjected with InsP$_3$ ($10^{-4}$ M in the pipette), the control oocyte showed a reversible decrease in the 535/485 fluorescence emission ratio of 3er, indicating a decrease in $[\text{Ca}^{2+}]_{\text{ER}}$. B, peak decline in the 535/485 ratio of 3er due to an injection of InsP$_3$ is more pronounced in cells expressing 3er alone (closed circles on the left) than in cells co-expressing 3er and CRT (open circles on the right). Base-line 535/485 ratio was normalized to 1.

The ability of CRT to maintain $[\text{Ca}^{2+}]_{\text{ER}}$ near resting levels un couples Ca$^{2+}$ store depletion from intracellular InsP$_3$ levels in a novel way; unlike the Ca$^{2+}$ ATPase inhibitor thapsigargin, which depletes the stores at basal InsP$_3$ levels, overexpressed CRT prevented store depletion despite high InsP$_3$ levels. Recent evidence suggests that the role played by the InsP$_3$R in Ca$^{2+}$ influx extends beyond simply lowering $[\text{Ca}^{2+}]_{\text{ER}}$ (25). In this context, our direct measurements of $[\text{Ca}^{2+}]_{\text{ER}}$ served as a reminder that store depletion is required to stimulate Ca$^{2+}$ influx and that elevated InsP$_3$, by itself, is not sufficient.

**Effect of CRT on IICR**—The simplest mechanism by which CRT could prevent the InsP$_3$-induced decrease in $[\text{Ca}^{2+}]_{\text{ER}}$ would be to inhibit IICR. However, our data suggested that this was not the case with saturating concentrations of InsP$_3$ in the cells where CRT had decreased the Ca$^{2+}$ influx-related Cl$^-$ current, there was no change in the initial Cl$^-$ currents due to IICR. Yet, in oocytes, CRT was previously reported to inhibit the Ca$^{2+}$ waves triggered by sub-maximal concentrations of Ins(1,4,5)P$_3$ (53). To test the effect of CRT on the spatial aspects of IICR under experimental conditions where it could reduce Ca$^{2+}$ influx, we microinjected sub-maximal InsP$_3$ concentrations into oocytes loaded with fluorescent Ca$^{2+}$ indicators and visualized the resulting Ca$^{2+}$ waves with a confocal microscope (46, 54). As shown in Fig. 6A, overexpression of CRT did not affect the amplitude, the velocity, or the frequency of the repetitive Cu$^{2+}$ waves. To determine whether an increase in CRT levels changes the rate at which Cu$^{2+}$ is released from intracellular stores, we examined the fluorescence intensity along a narrow linear path running perpendicular to the wave front; by multiplying the average slope of the increasing fluorescence values at the wave front ($\delta F/\delta t$ distance) by the instantaneous speed of the wave ($\delta$ distance$/\delta$ time), we obtained the rate at which [Cu$^{2+}$] rises at the wave front ($\delta F/\delta t$ time) (32).

The rate of rise in fluorescence intensity over base line at the wave front was $38 \pm 4\%$ s$^{-1}$ for control cells and 36 $\pm 2\%$ s$^{-1}$ for CRT-expressing cells (19 wave pairs were randomly chosen from 8 CRT/control cell pairs) suggesting a similar rate of Ca$^{2+}$ release at the wave front (Fig. 6A). Although our results that CRT expression did not change the amplitude of the Ca$^{2+}$ waves agree with those of Camacho and Lechleiter (53), we did not find that CRT decreases Ca$^{2+}$ waves frequency. Many reasons could explain this difference. 1) We studied our cells 2–3 days after DNA injection, whereas Camacho’s group studied theirs after 5–7 days. Although we studied our cells at a time when CRT was already expressed maximally and could reduce Ca$^{2+}$ influx, we may have missed a time-dependent effect of CRT on the Ca$^{2+}$ waves. 2) We studied only those cells that had proven exogenous protein expression and that had preserved plasma membrane electrical resistance. Camacho
and Lechleiter (53) did not screen individual cells for protein overexpression or for plasma membrane electrical integrity. Thus, the reported decrease in the frequency of Ca$^{2+}$ waves could not be unequivocally ascribed to an increase in CRT levels and may have been restricted to cells that were electrically leaky. 3) Most of the experimental data reported by Camacho and Lechleiter (53) were obtained in cells co-expressing the Ca$^{2+}$-ATPase SERCA2b. In a later publication, the same group (55) reported that CRT–ΔC, which had inhibited the Ca$^{2+}$ waves in cells co-expressing SERCA2b, had no effect on the Ca$^{2+}$ waves in cells co-expressing SERCAAs lacking an ER-luminal glycosylation site. Thus, their results may mainly reflect an interplay between CRT and SERCA2b (55). Our results suggest that under conditions where an increased amount of CRT within the ER can reduce the InsP$\sub{3}$-induced decline in [Ca$^{2+}$]$_{\text{ER}}$, CRT does not materially affect the mechanisms that initiate and propagate Ca$^{2+}$ waves. Thus, CRT does not appear to diminish the InsP$\sub{3}$-induced decline in [Ca$^{2+}$]$_{\text{ER}}$ by inhibiting ICR.

**Effect of CRT on Ca$^{2+}$ Uptake and/or Ca$^{2+}$ Extrusion**—CRT could blunt the InsP$\sub{3}$-induced decrease in [Ca$^{2+}$]$_{\text{ER}}$ if it accelerated the reuptake of cytosolic Ca$^{2+}$ into the ER lumen. To investigate this alternative hypothesis, we first estimated the rate at which [Ca$^{2+}$]$_{i}$ returns to baseline following the passage of a Ca$^{2+}$ wave front using an analysis similar to that described in the preceding paragraph. We found that the rate of [Ca$^{2+}$]$_{i}$ decline was not accelerated in cells expressing CRT (Fig. 6B). Next, we scanned the site of an intracellular injection of CaCl$_2$ (1 nl, 50 mM) with a single laser line (scanning rate = 500 Hz) in oocytes loaded with fluo 3. Fluorescence due to this exogenous addition of Ca$^{2+}$ returned to baseline at a slower rate in oocytes expressing CRT (Fig. 6B). To assay more specifically Ca$^{2+}$ uptake into the intracellular stores, we compared the ATP-dependent 45Ca$^{2+}$ uptake into microsomes isolated either from cells overexpressing CRT or from control cells. At 3 min, the time that the microsomes reach 50% of their total ATP-dependent 45Ca$^{2+}$ uptake (33), we found a trend toward lower 45Ca$^{2+}$ accumulation in microsomes from cells overexpressing CRT (Fig. 6B, $p < 0.06$). Together with the Ca$^{2+}$ injections
probe the relationship between Ca$^{2+}$-buffering properties of CRT and its ability to curb the InsP$_3$-induced decrease in [Ca$^{2+}$]$_{ER}$, we deleted the C-terminal domain, or C-domain (CRT-AC), which is responsible for the high Ca$^{2+}$-binding capacity of CRT (13). To serve as a control, and to investigate the alternative possibility that CRT could regulate the InsP$_3$-induced Ca$^{2+}$ signal by sensing a decrease in [Ca$^{2+}$]$_{ER}$ (53), we also made a mutant lacking the P-domain (CRT-ΔP), which contains the only high affinity Ca$^{2+}$-binding site of CRT (13). Successful expression of both deletion mutants was confirmed by Western blotting (Fig. 1, 3rd and 4th lanes).

At isotopic equilibrium, whole oocytes or microsomes extracted from oocytes expressing CRT-ΔC had $^{45}$Ca$^{2+}$-related counts that were no different from controls (respectively 103 ± 8% (25 cell pairs) and 91 ± 6% (n = 3) of controls (100%)). In contrast, oocytes or microsomes from oocytes expressing CRT-ΔP had greater $^{45}$Ca$^{2+}$ counts than did controls (respectively, 148 ± 13% (25 cell pairs, $p < 0.004$) and 174 ± 14% (n = 3, $p < 0.01$) of controls (100%)) (Fig. 7A). Thus, CRT-ΔP increased cellular and microsomal Ca$^{2+}$-binding capacity to an extent similar to that observed with wild-type CRT. These results confirm that it is the C-domain that confers on CRT the ability to bind large quantities of Ca$^{2+}$. Because this additional Ca$^{2+}$ binds to the C-domain with low affinity ($K_s$ < 2 mM), it should therefore be readily available for release into the cytosol upon opening of the InsP$_3$R. Yet, CRT overexpression did not affect the elevations in [Ca$^{2+}$]$_{ER}$ due to IICR. Thus, additional releasable Ca$^{2+}$ appears to have little impact on the magnitude of IICR in cells that already own substantial intracellular Ca$^{2+}$ reserves.

The InsP$_3$-induced decline in the 535/485 emission ratio of 3er in control (closed bar, n = 10) and CRT-expressing oocytes (open bar, n = 12).

experiments, these last results suggest that CRT overexpression slows the rate of Ca$^{2+}$ uptake into filled stores. In contrast, our data with the Ca$^{2+}$ waves suggest that CRT does not measurably affect the rate of Ca$^{2+}$ reuptake into InsP$_3$-depleted stores. Our results are thus compatible with a previous report of CRT inhibiting the ER Ca$^{2+}$ ATPase (55) but further suggest that this inhibition can be overcome when [Ca$^{2+}$]$_{ER}$ decreases. Overall, these data do not support our alternative hypothesis that CRT reduces the InsP$_3$-induced decline in [Ca$^{2+}$]$_{ER}$ by accelerating Ca$^{2+}$ reuptake/extrusion.

**CRT Increases Cellular and Microsomal Ca$^{2+}$-Binding Capacity**—If CRT increases neither the magnitude of IICR nor the rate at which Ca$^{2+}$ is retrieved back into the ER, then CRT should therefore be readily available for release into the cytosol upon opening of the InsP$_3$R. Yet, CRT overexpression did not affect the elevations in [Ca$^{2+}$]$_{ER}$ due to IICR. Thus, additional releasable Ca$^{2+}$ appears to have little impact on the magnitude of IICR in cells that already own substantial intracellular Ca$^{2+}$ reserves.

The InsP$_3$-induced decline in the 535/485 emission ratio of 3er was smaller in cells co-expressing CRT-ΔP than it was either in cells co-expressing CRT-ΔC (2.6 ± 0.9% versus 8.3 ± 1.4% decline, respectively, n = 11 pairs, $p < 0.002$) or in control cells (7.9 ± 0.8%, n = 11) (Fig. 7B). As shown in Fig. 7C, CRT-ΔP reduced the Cl$^{-}$ currents due to Ca$^{2+}$ influx, whereas CRT-ΔC did not; neither protein changed the magnitude of the initial Cl$^{-}$ current due to intracellular Ca$^{2+}$ release. Taken together, these data indicate that CRT relies on the C-domain both to blunt the InsP$_3$-induced decline in [Ca$^{2+}$]$_{ER}$ and to reduce the [Ca$^{2+}$]$_{ER}$, elevations due to IICR.

CRT Does Not Prevent Thapsigargin-induced Store Depletion from Fully Activating Ca$^{2+}$ Influx—If CRT acts primarily as a Ca$^{2+}$ buffer to reduce Ca$^{2+}$ influx, then a prolonged depletion of the Ca$^{2+}$ stores should eventually lead to a full activation of Ca$^{2+}$ influx. To test this hypothesis, we incubated oocytes with the Ca$^{2+}$-ATPase inhibitor thapsigargin for 3 h (42), and we directly measured the resulting whole cell Ca$^{2+}$ influx current according to the protocol of Yao and Tsien (43). As the tracings in Fig. 8A exemplify, the magnitude of the thapsigargin-induced Ca$^{2+}$ influx current in cells overexpressing CRT was similar to that of controls (aggregate data for 7 cell pairs shown in Fig. 8B). Experiments with 3er-expressing oocytes confirmed that thapsigargin exposure decreases [Ca$^{2+}$]$_{ER}$ to a similar extent in control and in CRT-expressing cells (Fig. 8C). By demonstrating that store depletion can still fully stimulate Ca$^{2+}$ influx in cells overexpressing CRT, these data further support the hypothesis that CRT controls InsP$_3$-induced Ca$^{2+}$ influx by buffering [Ca$^{2+}$]$_{ER}$.

In summary, our results establish that an increase in calreticulin levels can reduce the InsP$_3$-induced decline in [Ca$^{2+}$]$_{ER}$, thereby confirming the proposal originally put forth by Pozzan and co-workers (26, 28). The experimental results also link the high Ca$^{2+}$-buffering capacity of CRT to its ability both to prevent Ca$^{2+}$ store depletion and to reduce Ca$^{2+}$ influx. Taken together, the results directly support the notion that changing
levels of CRT can alter \([\text{Ca}^{2+}]_{\text{ER}}\) within ranges that are relevant to the cellular mechanisms that control \(\text{InsP}_3\)-induced \(\text{Ca}^{2+}\) influx.

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