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Phosphorylation of the rat Ins(1,4,5)P_3 receptor at T930 within the coupling domain decreases its affinity to Ins(1,4,5)P_3

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The Ins(1,4,5)P_3 receptor acts as a central hub for Ca^{2+} signaling by integrating multiple signaling modalities into Ca^{2+} release from intracellular stores downstream of G-protein and tyrosine kinase-coupled receptor stimulation. As such, the Ins(1,4,5)P_3 receptor plays fundamental roles in cellular physiology. The regulation of the Ins(1,4,5)P_3 receptor is complex and involves protein-protein interactions, post-translational modifications, allosteric modulation, and regulation of its sub-cellular distribution. Phosphorylation has been implicated in the sensitization of Ins(1,4,5)P_3-dependent Ca^{2+} release observed during oocyte maturation. Here we investigate the role of phosphorylation at T-930, a residue phosphorylated specifically during meiosis. We show that a phosphomimetic mutation at T-930 of the rat Ins(1,4,5)P_3 receptor results in decreased Ins(1,4,5)P_3-dependent Ca^{2+} release and lowers the Ins(1,4,5)P_3 binding affinity of the receptor. These data, coupled to the sensitization of Ins(1,4,5)P_3-dependent Ca^{2+} release during meiosis, argue that phosphorylation within the coupling domain of the Ins(1,4,5)P_3 receptor acts in a combinatorial fashion to regulate Ins(1,4,5)P_3 receptor function.

Introduction

Ca^{2+} release from intracellular stores is mediated by two major classes of intracellular channels, ryanodine and Ins(1,4,5)P_3 receptors. Mammalian genomes encode three Ins(1,4,5)P_3 receptor genes, type 1–3, with disparate tissue distribution and molecular regulation.[1] The Ins(1,4,5)P_3 receptor is a complex signal integrator that consolidates input from multiple signaling cascades into Ca^{2+} release from intracellular stores.[2] The Ins(1,4,5)P_3 receptor (~2700 amino acids) is functionally divided into three domains: the Ins(1,4,5)P_3 binding domain, the coupling domain and the channel domain (**Fig. 1A**). The pore forming domain localizes to the C-terminal end of the protein and is composed of six membrane spanning regions and a pore forming loop between trans-membrane domains 5 and 6, followed by a short cytoplasmic tail. The Ins(1,4,5)P_3 binding domain in the N-terminus of the protein binds the ligand Ins(1,4,5)P_3, and the coupling region spans the region between the Ins(1,4,5)P_3 binding region and the channel domain.[2,3] The coupling domain transmits Ins(1,4,5)P_3 binding into gating of the channel to release Ca^{2+} from the endoplasmic reticulum (ER). In addition to Ins(1,4,5)P_3, Ca^{2+} functions as a co-agonist of the Ins(1,4,5)P_3 receptor regulating it's gating in a biphasic fashion.[4,5] The bulk of the Ins(1,4,5)P_3 receptor protein is cytoplasmic and offers multiple sites for regulation by interacting proteins, small molecules and post-translational modifications.[1,2]

The Ins(1,4,5)P_3 receptor requires both Ins(1,4,5)P_3 and Ca^{2+} as co-agonists to gate open and release Ca^{2+} from stores. However receptor function is also modulated through phosphorylation by various kinases. The type 1 Ins(1,4,5)P_3 receptor contains two canonical PKA phosphorylation sites S-1589 and S-1755.[6] PKA phosphorylation results in increased Ca^{2+} release activity.[7,8] At the single channel level PKA phosphorylation increases the probability of opening (P_o) of the Ins(1,4,5)P_3 receptor resulting in bursting activity without affecting its Ca^{2+} dependence, arguing for an increase in the apparent sensitivity to Ins(1,4,5)P_3.[8,9]

Although PKA phosphorylation is best understood, the Ins(1,4,5)P_3 receptor has been shown to be phosphorylated and modulated by other kinases, including PKC, CaMKII and non-receptor tyrosine kinases.[10-14] PKC phosphorylation of the Ins(1,4,5)P_3 receptor leads to enhanced Ins(1,4,5)P_3-dependent Ca^{2+} release[10,11]; and CaMKII phosphorylation modulates Ca^{2+} oscillations and inhibition of Ins(1,4,5)P_3-dependent Ca^{2+} release.[12,13]

Ins(1,4,5)P_3-dependent Ca^{2+} release is also modulated during the cell cycle with the most dramatic example at fertilization. Ca^{2+} release through Ins(1,4,5)P_3 receptors is essential for egg activation and for mediating the egg to embryo transition in vertebrates.[15-17] In Xenopus oocytes, Ins(1,4,5)P_3-dependent Ca^{2+} release is sensitized during meiosis, and this sensitization is essential to generate the fertilization-specific Ca^{2+} transient that is required for egg activation at fertilization.[18-20] In addition, we

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have recently shown that remodeling of the endoplasmic reticulum during oocyte meiosis contributes to Ins(1,4,5)P_3 receptor sensitization through a process termed "geometric sensitization"[21]. Geometric sensitization is due to ER remodeling during meiosis resulting in the formation of large ER patches enriched in Ins(1,4,5)P_3 receptors. Ins(1,4,5)P_3 receptors that localize to these ER patches display increased sensitivity to Ins(1,4,5)P_3 as compared with their counterparts in the reticular ER.[21]

We have also argued that phosphorylation by cell cycle kinases is involved in Ins(1,4,5)P_3 receptor sensitization during meiosis.[22] Xenopus oocytes express the SII- variant of the type 1 Ins(1,4,5)P_3 receptor, and the receptor is phosphorylated at both PKA sites throughout oocyte maturation.[22] During meiosis the Ins(1,4,5)P_3 receptor is specifically phosphorylated on three additional residues T931, T1136 and T1145.[22] Both T931 and T1136 localize to consensus sites for proline-directed kinases such as MAPK and Cdk1. Furthermore, activation of the MAPK cascade or Cdk1 sensitizes Ins(1,4,5)P_3-dependent Ca^{2+} release in *Xenopus* oocytes.[22] In fact phosphorylation of T1136 requires Cdk1 activity.[22] All three residues, T-931, T-1136 and S-1145, localize within the coupling domain and correspond to T-930, T-1140 and S-1152 of the rat SI, SII, SIII- isoform. The Ins(1,4,5)P_3 receptor is also phosphorylated during mitosis.[23,24]

Here we test the role of phosphorylation at T-930 of the rat Ins(1,4,5)P_3 receptor (SI, SII, SIII- splice variant). We focus on T-930 because it is specifically phosphorylated during meiosis and because the sequence surrounding this residue is conserved among vertebrates (**Fig. 1A**). We show that a phosphomimetic mutation at T-930 (T930E) inhibits Ca^{2+} release through the Ins(1,4,5)P_3 receptor. Furthermore, Ins(1,4,5)P_3-binding affinity of the T930E mutant is significantly decreased compared with the wild-type or T930A mutant. These data show that Ins(1,4,5)P_3 receptor phosphorylation at T-930, a site within the coupling domain, decreases Ins(1,4,5)P_3 binding affinity of the receptor resulting in decreased Ins(1,4,5)P_3-dependent Ca^{2+} release.

Results

To investigate the role of phosphorylation at T-930 on Ins(1,4,5)P_3 receptor function, we engineered two mutants one with an alanine substitution at T-930 (T930A), and the other with a phosphomimetic mutation where the threonine is replaced by a glutamic acid (T930E). We chose glutamic acid as its side chain closely resembles that of a phosphorylated negatively charged threonine side chain. As such the T930E mutant is expected to mimic the behavior of the Ins(1,4,5)P_3 receptor phosphorylated at T-930. In contrast, the alanine substitution at T930 serves as a control and provides a mutant that is not phosphorylatable at this residue. Furthermore, it also controls for the structural need for a threonine at this position.

The ubiquitous distribution of the Ins(1,4,5)P_3 receptor makes it challenging to study the effect of different mutants on Ins(1,4,5)P_3 receptor function because of the background signal due to endogenous Ins(1,4,5)P_3 receptors in most cells. Therefore to study the functional consequences of phosphorylation at T-930, we used the DT40 chicken lymphocyte cell line because of the existence of a DT40 line (3KO), where all three Ins(1,4,5)P_3 receptor genes (type 1, 2, and 3) have been knocked out. This provides a clean background to analyze the function of different expressed Ins(1,4,5)P_3 receptor mutants. Using the 3KO line as the parental line, we generated stable DT40 cell lines expressing the wild-type rat Ins(1,4,5)P_3 receptor, the T930A or the T930E mutants. Following the establishment of the cell lines we amplified and sequence confirmed that they are in fact expressing the relevant Ins(1,4,5)P_3 receptor mutant.

Western blot analysis shows that the three different cell lines express the Ins(1,4,5)P_3 receptor at equivalent levels, whereas no immuno-reactivity is detected in the 3KO line (**Fig. 1B**). The growth rate of the different DT40 cell lines were equivalent, arguing that expression of the different mutants did not have a dramatic effect on cell survival or ability to replicate (**Fig. 1C**). Finally, both resting Ca^{2+} levels and Ca^{2+} store content were similar among the different cell lines (**Fig. 1D**), showing that expression of the T930E or T930A mutant does not alter Ca^{2+} homeostasis significantly at rest.

To test the sensitivity of the different Ins(1,4,5)P_3 receptor mutants, we developed an assay to measure Ca^{2+} release from intracellular stores in situ in response to an Ins(1,4,5)P_3 dose response. For these experiments DT40 cells were loaded with caged-Ins(1,4,5)P_3 and Ins(1,4,5)P_3 was uncaged for different durations as indicated in **Figure 2**. Cells expressing wild-type (WT) Ins(1,4,5)P_3 receptor respond with an exponential increase in Ca^{2+} release along the Ins(1,4,5)P_3 uncaging dose response (**Fig. 2**). This assay provides a direct test of Ins(1,4,5)P_3 receptor function because Ins(1,4,5)P_3 produced following uncaging binds to and gates the Ins(1,4,5)P_3 receptor without invoking additional intermediaries.

Surprisingly in cells expressing the T930A mutant Ins(1,4,5)P_3-dependent Ca^{2+} release is sensitized compared with the wild-type receptor (**Fig. 2**). The T930A mutant receptor responds with increased Ca^{2+} release at lower Ins(1,4,5)P_3 concentrations although the maximum amount of Ca^{2+} release is comparable to the wild-type receptor (**Fig. 2**). This sensitization is unlikely to be due to higher expression levels of the T930A mutant as compared with WT, because the different cell lines express equivalent levels of Ins(1,4,5)P_3 receptors (**Fig. 1**) and because the maximal Ca^{2+} release is similar between the two cell lines (**Fig. 2**).

In contrast, cells expressing the T930E mutant exhibit the opposite phenotype of decreased Ca^{2+} release in response to Ins(1,4,5)P_3 uncaging (**Fig. 2**). Interestingly, even at high levels of Ins(1,4,5)P_3 these cells release significantly smaller levels of Ca^{2+} (**Fig. 2**). This is not due to decreased store Ca^{2+} content, since T930E expressing cells have a similar store Ca^{2+} load as cells expressing the wild-type receptor or the T930A mutant (**Fig. 1D**). In contrast cells expressing the T930A mutant or the wild-type receptor produce significantly higher Ca^{2+} release levels at long uncaging durations (**Fig. 2**). Because the T930E cell line expresses equivalent amount of Ins(1,4,5)P_3 receptors, these results argue that either the affinity of the T930E mutant to Ins(1,4,5)P_3 is low or that gating/permeation or Ca^{2+} dependence of the receptor are affected by the T930E mutation leading to short lived or small Ca^{2+} release.

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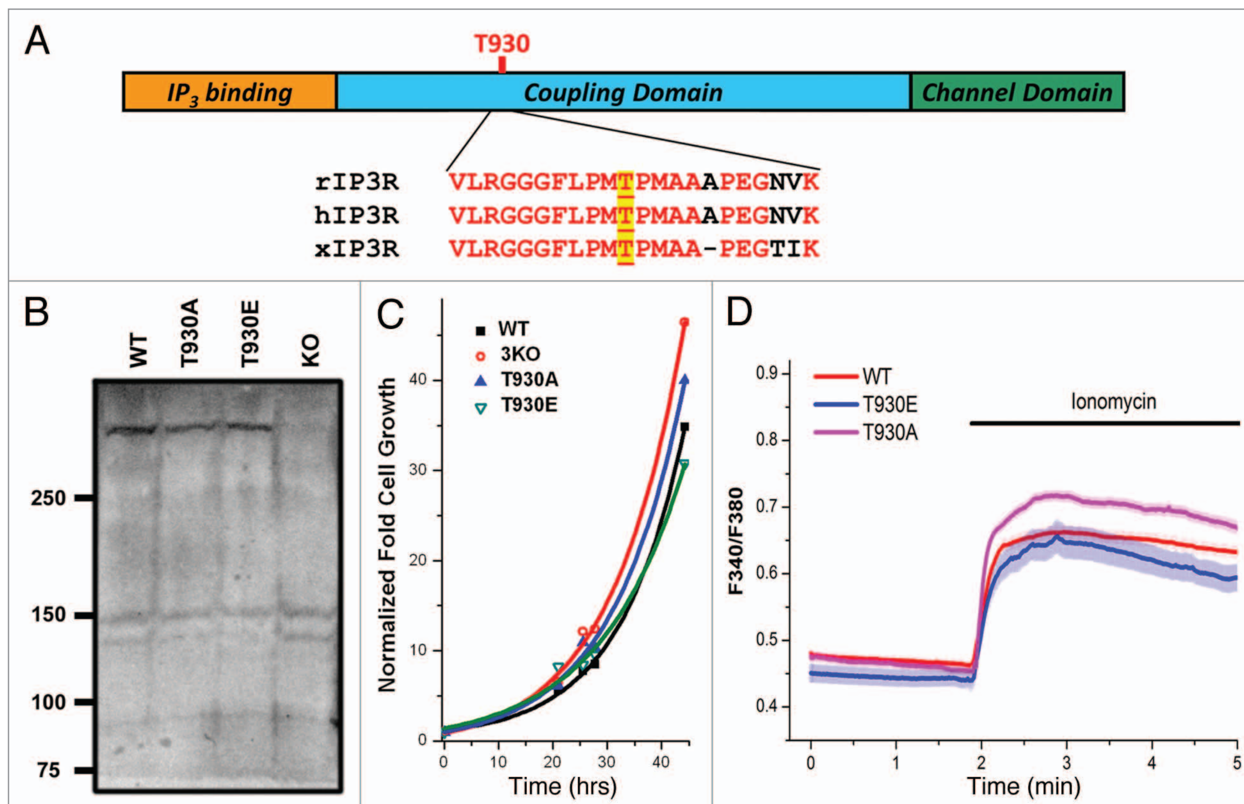

Figure 1. Generation and characterization of stable DT40 cell lines expressing Ins(1,4,5)P_3 receptor phosphorylation mutants. (**A**) Cartoon representation of Ins(1,4,5)P_3 receptor structure with sequence alignment around T-930, for the rat (rIP3R), human (hIP3R) and *Xenopus* (xIP3R) Ins(1,4,5)P_3 receptors. (**B**) Ins(1,4,5)P_3R expression levels in the different cell lines. Different cell lines were generated using the DT40 cell line where all three Ins(1,4,5)P_3R isoform are deleted (KO). The 3KO cells were electroporated with the linearized plasmid for either the T930A or T930E mutants. The plasmid carrying the mutants contains G418 resistance. Stable cell lines for each mutant were established using G418 selection. WT: DT40 cell line expressing only the wild-type Ins(1,4,5)P_3 receptor (type 1); T930A and T930E: expressing the respective mutant Ins(1,4,5)P_3Rs. (**C**) Growth rates of the different cell lines are also equivalent. The same number of cells was plated at time zero and cells were counted at different time points under equivalent culturing conditions to determine whether the expression of the different mutants affects cell growth. Although the T930E mutant grew at a slightly slower rate the difference were not significant. (**D**) Resting Ca^{2+} levels and store Ca^{2+} content were measured in DT40 cells loaded with Fura-2 and incubated in Ca^{2+}-free solution. Ionomycin was added as indicated to release Ca^{2+} from stores.

To further investigate which of these mechanisms is affected in the T930E mutant, we directly measured the Ins(1,4,5)P_3 binding affinity of the different mutants in microsomes derived from the stable DT40 cell lines (**Fig. 3**). Microsomes from brain, which is enriched in Ins(1,4,5)P_3 receptors, were used as a positive control and showed significant Ins(1,4,5)P_3-binding that was competed effectively by cold Ins(1,4,5)P_3 (**Fig. 3**). Ins(1,4,5)P_3 binding to microsomes isolated for the DT40 cell lines expressing the wild-type Ins(1,4,5)P_3 receptor showed lower binding than brain microsomes, which was also effectively competed away using cold Ins(1,4,5)P_3 (**Fig. 3**). Consistent with the Ins(1,4,5)P_3 uncaging data (**Fig. 2**), microsomes from cells expressing the T930A mutant exhibited higher Ins(1,4,5)P_3 binding affinity than their wild-type counterparts, whereas Ins(1,4,5)P_3 binding to T930E microsomes was hardly detectable and only slightly above the background binding observed in the 3KO cells (**Fig. 3**). These data show that the behavior of the T930A and T930E mutants in situ can be explained solely based on their Ins(1,4,5)P_3 binding affinity without the need to invoke alterations to Ca^{2+} dependence, gating or permeation of the receptor. Our results further show that phosphorylation at T930 leads to a significant decrease in Ins(1,4,5)P_3 binding affinity.

Discussion

Using stable DT40 cell lines expressing different Ins(1,4,5)P_3 receptor mutants we show that phosphorylation of the type 1 receptor at T930 is likely to decrease Ins(1,4,5)P_3-binding affinity. As discussed above the Ins(1,4,5)P_3 receptor is targeted by various kinases and in most cases phosphorylation results in increased sensitization of Ins(1,4,5)P_3-dependent Ca^{2+} release.[1] In contrast, a phosphomimetic mutation at T930 results in decreased Ins(1,4,5)P_3 binding affinity and reduced Ins(1,4,5)P_3-dependent Ca^{2+} release. The T930 residue localizes within the coupling domain and is distant from the Ins(1,4,5)P_3 binding domain in the linear sequence of the Ins(1,4,5)P_3 receptor. This brings into question the ability of phosphorylation at this site to modulate Ins(1,4,5)P_3 binding affinity. However, the available structure of the Ins(1,4,5)P_3 receptor obtained from cryo-EM studies shows that the coupling domain lies below the

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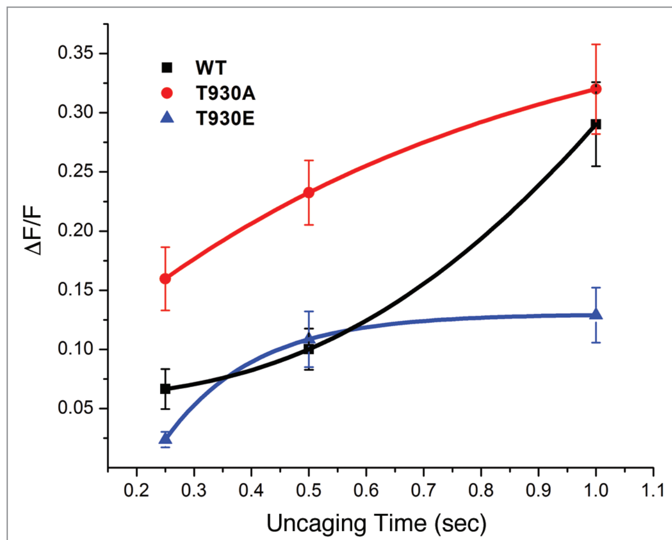

Figure 2. The T930E mutant exhibits decrease Ins(1,4,5)P_3-dependent Ca^{2+} release sensitivity. DT40 cells were loaded with a Ca^{2+} dye and caged-Ins(1,4,5)P_3 and exposed to different UV uncaging pulse durations to produce a dose response of Ins(1,4,5)P_3 intracellulary. The level of Ca^{2+} release following the uncaging pulses was used as a measure of the sensitivity of the Ins(1,4,5)P_3 receptor mutants as compared with the wild-type receptor.

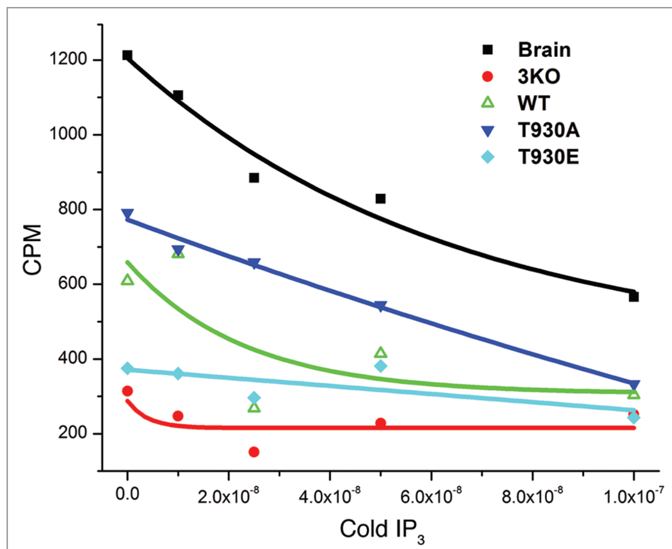

Figure 3. The T930E mutation decreases Ins(1,4,5)P_3 binding affinity. Microsomal preparations from brain as a positive control and from the different DT40 cell lines as indicated were subjected to the Ins(1,4,5)P_3 binding assay to measure the binding affinity of Ins(1,4,5)P_3 receptor mutants. Briefly lysates were allowed to bind radioactively (^3H) labeled Ins(1,4,5)P_3 and the label competed with cold Ins(1,4,5)P_3. The competitive assay provides a relative comparative measure Ins(1,4,5)P_3R affinity in the different mutants as compared with the wild type receptor (WT). The negative control was the 3KO cell line which does not express any of the three Ins(1,4,5)P_3 receptor isoforms and as such provides nonspecific background binding.

Ins(1,4,5)P_3 binding domain in the 3-D structure of the receptor.[25] Hence phosphorylation at T-930 could modulate Ins(1,4,5)P_3-binding if this residue interacts with the Ins(1,4,5)P_3-binding and/or suppressor domains. Alternatively, phosphorylation at T930 could alter the conformation of the coupling domain in close proximity of the Ins(1,4,5)P_3-binding domain thus modulating its ability to bind Ins(1,4,5)P_3. One argument supporting this possibility is the sensitization of Ins(1,4,5)P_3-dependent Ca^{2+} release observed in the cells expressing the T930A mutant. This sensitization argues that structural/sequence modifications within this region of the coupling domain affect Ins(1,4,5)P_3 receptor sensitivity.

The Ins(1,4,5)P_3 receptor is phosphorylated at T930 during meiosis. Furthermore, Ins(1,4,5)P_3-dependent Ca^{2+} release is highly sensitized during meiosis and this sensitization is critical for egg activation and the egg-to-embryo transition at fertilization. This creates a conundrum, since the phosphomimetic T930E mutation decreases Ins(1,4,5)P_3 binding affinity and as such reduces the sensitivity of Ins(1,4,5)P_3-dependent Ca^{2+} release. This is quite interesting as it implicates cross talk among different phosphorylation sites within the Ins(1,4,5)P_3 receptor to define its biological function. During meiosis the Xenopus Ins(1,4,5)P_3 receptor is also phosphorylated at two additional residues that match the consensus for proline-directed kinases, T-1136 and S-1145. So it is possible that the combinatorial phosphorylation at all three residues sensitizes Ins(1,4,5)P_3-dependent Ca^{2+} release. Another formal possibility is that the rat receptor used in this study behaves differently in response to phosphorylation at T-930 as compared with the Xenopus receptor. However, given the high degree of sequence conservation between the two receptors (89% identity) this possibility seems remote. In addition, because of the high levels of endogenous PKA activity in oocytes, the Ins(1,4,5)P_3 receptor is also phosphorylated at the consensus PKA sites during maturation.[22] Interestingly, all these residues localize within the coupling domain, arguing that combinatorial phosphorylation within this domain may differentially affect Ins(1,4,5)P_3 binding affinity and the function of the Ins(1,4,5)P_3 receptor.

Materials and Methods

Clones and cell culture. Plasmid pcDNA3 containing the rat Ins(1,4,5)P_3 receptor (pcDNA3 rIP3R) was a kind gift from Suresh Joseph. Mutations in the coding region of the Ins(1,4,5)P_3 receptor were generated using Quick Change II Site-Directed Mutagenesis kits (Strategene) to change the bases "ACT" to "GCT" in the coding DNA strand resulting in an amino acid substitution of threonine (T930) with alanine (T930A). Likewise, "ACT" was replaced with "GAA" resulting in the substitution of amino acid threonine (T930) with glutamic acid (T930E). Mutations were confirmed by sequence analysis.

To generate DT40 cell lines expressing the mutated IP3R, linearized pcDNA3 T930A and pcDNA3 T930E plasmids were electroporated into a DT40–3KO cell line where all three Ins(1,4,5)P_3 receptor isoforms are deleted. Stable cell lines were established using G418 selection.

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Western blot analysis. Lysates from ~5 x 10^7 DT40 cells expressing wild-type rIP3R, T930A, T930E, or from the 3KO cell line were separated on denaturing NuPAGE 3–8% Tris-Acetate gradient gels (Invitrogen). Primary rabbit anti-IP3R (T443) antibody and secondary goat anti-rabbit-HRP antibody (Jackson Immunoresearch) at 1:1000 and 1:7500 dilution, respectively, in 1% Hammerstein casein, 2% BSA were used for western analysis. Ins(1,4,5)P_3 receptor protein bands were detected using ECL-Plus (Amersham) detection reagent.

Ins(1,4,5)P_3 binding assay. Microsomes were prepared from rabbit cerebellum by homogenizing 1g of tissue in 12 ml of E Buffer (20 mM TRIS-HCl pH 8.3, 10 mMKCl, 1mM EDTA, 1 mM DTT, Cocktail inhibitor III (Calbiochem) and 1 mM PMSF) using a Polytron homogenizer. The lysate was centrifuged at 1000xg for 15 min at 4°C and the resulting supernatant was transferred to a microfuge tube and placed on ice. The pellet was suspended in 3 ml of E buffer and centrifuged again at 1000x g. The supernatants were combined and centrifuged at 2000xg for 15 min. The resulting supernatant was then centrifuged at 105,000xg for 30 min. The pellet containing the microsomes was suspended in E buffer and the protein concentration was determined using a BioRad protein assay.

DT40 cells were collected by centrifugation at 1,500xg for 15 min. The cell pellets were washed once in E Buffer then centrifuged at 1,500xg for 15 min. The cells were lysed with 20 strokes in a glassDounce homogenizer (Pyrex 7727-15) in 5 ml of E buffer. Cell lysates were centrifuged at 1,500xg for 15 min. The supernatants were transferred to polyallomer ultracentrifuge tubes (Beckman #326814) and centrifuged at 100,000 xg for 30 min at 4°C. The pellets were suspended in E buffer and protein concentrations were determined using a BioRad protein assay.

Ins(1,4,5)P_3 binding assays were performed using microsome preparations from rabbit cerebellum and DT40 cells expressing wild-type Ins(1,4,5)P_3 receptor, T930A, T930E, and from the 3KO DT40 cells. Each reaction contained 120 μg of brain or 1 mg of DT40 microsomes. The reaction mix contained 15 nM [^{3}H]Ins(1,4,5)P_3 (5nM [^{3}H]Ins(1,4,5)P_3/μl NET911, 0.005 mCi, 21.4Ci/mmol); various concentrations ranging from 0 to 2,000 μM of cold Ins(1,4,5)P_3 [10 mM Ins(1,4,5)P_3, Invitrogen], Ins(1,4,5)P_3 binding buffer (50 mMTRIS-HCl, pH 8.3, 1 mM EDTA, 1 mM DTT, 0.1 M KCl), EDS buffer (20 mM TRIS-HCl, pH 8.3, 20 mM NaCl, 10 mM KCl, 1mM EDTA, 1 mM DTT, 10% sucrose plus protease inhibitors) in a final volume of 500 μl. The reaction mixtures were incubated on ice for 10 min and then 5 μl of γ-globulin and 5 μl of glycogen were added and incubated for additional 5 min. The mixtures were centrifuge at 20,000x g for 15 min, the supernatants were aspirated, and the pellets were solubilized in Solubilization buffer (0.625 M TRIS-HCl, pH 7.5, 5% 2-mercaptoethanol, 2% SDS) and the amount of ^{3}H was determined using a scintillation counter.

Ins(1,4,5)P_3 uncaging n DT40 cells. Measurements of intracellular free calcium concentration ([Ca^{2+}]$_i$) were made using the fluorescent indicator Ca^{2+}-green-AM. Cells (approximately 2.5 x 10^6) were loaded for 45 min in growth medium at 40° containing 5 μM ci-IP_3, 2μM Ca^{2+}-green-AM and 0.0025% Pluronic F-127. Cells were washed three times and re-suspended at 4°C in HBSS (in mM: 137 NaCl, 5.4 KCl, 0.25 Na_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 1.0 $MgSO_4$, 4.2 $NaHCO_3$, 5.5 D-glucose, 10 Hepes pH 7.4) to a final density of 10^6 cells ml^{-1} and kept on ice for up to 1 h. Cells were then pre-warmed to 40° for 15min. Fluorescence of the stirred cell suspension was then measured by emission at 520 nm while uncaging of ci-Ins(1,4,5)P_3 was performed at 330 ± 40nm. Removal of all residual extracellular calcium was achieved by addition of 3 mM EGTA at the beginning of fluorescence measurements.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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