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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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.³⁻⁵ 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 its gating in a biphasic fashion.³⁻⁵ 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.⁶ 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$^{2+}$ release¹⁰,¹¹; 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...
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 S1, 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.
Our results further show that phosphorylation at T930 leads to a significant decrease in Ins(1,4,5)P₃ binding affinity.

**Discussion**

Using stable DT40 cell lines expressing different Ins(1,4,5)P₃ receptor mutants we show that phosphorylation of the type 1 receptor at T930 is likely to decrease Ins(1,4,5)P₃-binding affinity. As discussed above the Ins(1,4,5)P₃ receptor is targeted by various kinases and in most cases phosphorylation results in increased sensitization of Ins(1,4,5)P₃-dependent Ca²⁺ release. In contrast, a phosphomimetic mutation at T930 results in decreased Ins(1,4,5)P₃ binding affinity and reduced Ins(1,4,5)P₃-dependent Ca²⁺ release. The T930 residue localizes within the coupling domain and is distant from the Ins(1,4,5)P₃ binding domain in the linear sequence of the Ins(1,4,5)P₃ receptor. This brings into question the ability of phosphorylation at this site to modulate Ins(1,4,5)P₃ binding affinity. However, the available structure of the Ins(1,4,5)P₃ receptor obtained from cryo-EM studies shows that the coupling domain lies below the
Ins(1,4,5)P₃ binding domain in the 3-D structure of the receptor.²⁵ Hence phosphorylation at T-930 could modulate Ins(1,4,5)P₃-binding if this residue interacts with the Ins(1,4,5)P₃-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)P₃-binding domain thus modulating its ability to bind Ins(1,4,5)P₃. One argument supporting this possibility is the sensitization of Ins(1,4,5)P₃-dependent Ca²⁺ 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)P₃ receptor sensitivity.

The Ins(1,4,5)P₃ receptor is phosphorylated at T930 during meiosis. Furthermore, Ins(1,4,5)P₃-dependent Ca²⁺ 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)P₃ binding affinity and as such reduces the sensitivity of Ins(1,4,5)P₃-dependent Ca²⁺ release. This is quite interesting as it implicates cross talk among different phosphorylation sites within the Ins(1,4,5)P₃ receptor to define its biological function. During meiosis the Xenopus Ins(1,4,5)P₃ 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)P₃-dependent Ca²⁺ 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)P₃ 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)P₃ binding affinity and the function of the Ins(1,4,5)P₃ receptor.

Materials and Methods

Clones and cell culture. Plasmid pcDNA3 containing the rat Ins(1,4,5)P₃ receptor (pcDNA3 rIP3R) was a kind gift from Suress Joseph. Mutations in the coding region of the Ins(1,4,5)P₃ 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 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)P₃ receptor isoforms are deleted. Stable cell lines were established using G418 selection.
Western blot analysis. Lysates from -5 x 10^7 DT40 cells expressing wild-type tIP3R, 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% Hamsterin 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 mM KC1, 1 mM 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 microtube and placed on ice. The pellet was suspended in 3 ml of E buffer and centrifuged again at 1000xg. The supernatants were combined and centrifuged at 2000xg for 15 min. The supernatants were 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. The resulting supernatant was then centrifuged at 20,000xg for 1 h. The supernatants were centrifuged at 1000xg for 15 min at 4°C and the resulting supernatant was combined and centrifuged at 2000xg 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 μM [3H]Ins(1,4,5)P_3 (5nM [3H]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.

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