Functional Consequences of Phosphomimetic Mutations at Key cAMP-dependent Protein Kinase Phosphorylation Sites in the Type 1 Inositol 1,4,5-Trisphosphate Receptor*

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Regulation of Ca2+ release through inositol 1,4,5-trisphosphate receptors (InsP3R) has important consequences for defining the particular spatio-temporal properties of intracellular Ca2+ signals. In this study, regulation of Ca2+ release by phosphorylation of type 1 InsP3R (InsP3R-1) was investigated by constructing “phosphomimetic” charge mutations in the functionally important phosphorylation sites of both the S2+ and S2– InsP3R-1 splice variants. Ca2+ release was investigated following expression in Dt-40 3ko cells devoid of endogenous InsP3R. In cells expressing either the S1755E/S1755A or S1589E/S1755E S2– InsP3R-1, InsP3-induced Ca2+ release was markedly enhanced compared with nonphosphorylatable S2+ S1755A and S2– S1589A/S1755A mutants. Ca2+ release through the S2– S1589E/S1755E InsP3R-1 was enhanced 8-fold over wild type and 50-fold when compared with the nonphosphorylatable S2– S1589A/S1755A mutant. In cells expressing S2– InsP3R-1 with single mutations in either S17589E or S1755E, the sensitivity of Ca2+ release was enhanced 3-fold; sensitivity was midway between the wild type and the double glutamate mutation. Paradoxically, forskolin treatment of cells expressing either single Ser/ Glu mutation failed to further enhance Ca2+ release. The sensitivity of Ca2+ release in cells expressing S2+ S1755E InsP3R-1 was comparable with the sensitivity of S2– S1589E/S1755E InsP3R-1. In contrast, mutation of S2+ S1589E InsP3R-1 resulted in a receptor with comparable sensitivity to wild type cells. Expression of S2– S1589E/S1755E InsP3R-1 resulted in robust Ca2+ oscillations when cells were stimulated with concentrations of α-IgM antibody that were threshold for stimulation in S2– wild type InsP3R-1-expressing cells. However, at higher concentrations of α-IgM antibody, Ca2+ oscillations of a similar period and magnitude were initiated in cells expressing either wild type or S2– phosphomimetic mutations. Thus, regulation by phosphorylation of the functional sensitivity of InsP3R-1 appears to define the threshold at which oscillations are initiated but not the frequency or amplitude of the signal when established.

Inositol 1,4,5-trisphosphate receptors are intracellular ion channels that function to couple the activation of cell surface receptors for neurotransmitters, hormones, and growth factors to the initiation of intracellular Ca2+ release (1). Three genes have been cloned that encode distinct proteins of a molecular mass of ~300 kDa, named the type 1 InsP3R-1,1 type 2 (InsP3R-2), and type 3 (InsP3R-3) InsP3Rs (2–4). In addition, multiple receptor proteins with distinct tissue distributions are produced by alternate splicing of the type 1 receptor gene (5, 6). Most cells express multiple isoforms of InsP3R (7). Furthermore, the expression level and complement of receptors differ in individual tissues, and this together with regulation of the activity of the channel is thought to be a major determinant of the rich diversity of Ca2+ signaling events observed in cells (7, 8).

The functional channel is formed co-translationally by the tetrameric association of four individual receptor subunits (9, 10). Each subunit has a binding site for InsP3 toward the N terminus formed by a cluster of positively charged amino acids thought to coordinate the negatively charged phosphatase groups of InsP3 (11, 12). The C terminus of each subunit is postulated to span intracellular membranes six times and forms a single cation-selective pore (13, 14). In addition, this region signals retention of the protein to the endoplasmic reticulum (15, 16). Although the InsP3-binding pocket and channel pore are highly conserved among InsP3R family members, the intervening sequence between the binding region and pore is more divergent and consists of the so-called “regulatory and coupling” or “modulatory” domain. This region, consisting of ~1600 amino acids, is thought to be important in modulating the Ca2+ release properties of the InsP3R. Indeed, Ca2+ release through the InsP3R is markedly influenced by many factors, most importantly by Ca2+ itself (17). InsP3R activity is also influenced through interaction with numerous factors such as proteins, adenosine nucleotides, and phosphorylation and in particular by cyclic nucleotide-dependent kinases (8).

Two protein kinase A (PKA) consensus sites (RRX XS) at Ser-1589 and Ser-1755 are present in the InsP3R-1 (5, 18), and the most recent studies suggest that phosphorylation of these sites results in a marked enhancement of Ca2+ release (19–22). Most interestingly, these sites are conserved through evolution.

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1 The abbreviations used are: InsP3R-1, inositol 1,4,5-trisphosphate receptor type 1; [Ca2+]i, intracellular calcium concentration; InsP3, inositol 1,4,5-trisphosphate; CCh, carbachol (carbachol); PKA, cAMP-dependent protein kinase; PKG, cGMP dependent protein kinase; 8-Br cGMP, 8-bromoguanosine-3’-5’ cyclic monophosphophate; ci-IP/PM, caged isopropylidene inositol trisphosphate; WT, wild type; cm-IP3, caged methoxymethylene inositol triphosphate.
from Drosophila to humans in InsP3R-1, but corresponding sites are not present in either the InsP3R-2 or InsP3R-3. It should be noted, however, that other regions, which are presently not defined, appear to function as PKA phosphorylation sites in InsP3R-2 and InsP3R-3. Several reports have demonstrated biochemically that both Ser-1589 and Ser-1755 can be phosphorylated in InsP3R-1 (18, 23, 24); however, these studies have not provided a consensus as to which sites are important physiologically in tissue that expresses either of the two major splice variants of InsP3R-1.

A recent study (19) from our laboratory has elucidated the particular sites that are functionally important for the phosphoregulation of the two major splice variants of the InsP3R-1. These studies were performed by using mutational analysis by substituting alanine for the serine residues present at individual putative phosphorylation sites. Although phosphorylation of both neuronal (S2+) and peripheral (S2−) forms of InsP3R-1 by PKA resulted in enhanced Ca2+ release, mutational analysis indicated that only phosphorylation of Ser-1755 was functionally important in the neuronal S2+ InsP3R-1. In contrast, both Ser-1589 and Ser-1755 appeared to be phosphorylated and significant in the peripheral S2− form of InsP3R-1. In addition, although the S2+ form of the receptor was subject to direct phosphoregulation by cGMP-dependent protein kinase (PKG), the S2− form was not influenced by activation of PKG. These data represent one of the few major differences reported for the regulation of the two major splice variants of the InsP3R-1.

In the present study, we have constructed charge mutations, substituting glutamate residues for the serine residues in the functionally important phosphorylation sites in both S2− and S2+ variants of the InsP3R-1. These mutations are predicted to mimic phosphorylation and to allow the assessment of the functional effects of phosphorylation of InsP3R-1. Most importantly, the Ca2+ release properties of phosphomimetic mutations are predicted to be essentially independent of cell type-specific factors, including the expression of accessory proteins such as protein A-kinase anchoring proteins. These effects should also be unambiguously specific to InsP3R and thus independent of confounding PKA effects on other Ca2+ handling machinery. This latter consideration has historically plagued the functional assessment of PKA phosphorylation of InsP3R. These mutations have allowed us to define the relative sensitivity of Ca2+ release of the phosphomimetic mutations and to confirm which sites are important in each splice variant. The present study has also addressed whether phosphorylation of individual sites is permissive or additive in each splice variant, and we have investigated the consequences of phosphorylating InsP3R-1 on Ca2+ oscillations, the physiological pattern of Ca2+ signaling in nonelectrically excitable cells.

MATERIALS AND METHODS

The acetoxymethyl esters of Fura-2 and Fluo-4 were purchased from Molecular Probes (Eugene, OR). Cell-permeable cyclic nucleotides and forskolin were purchased from Bioworld (Plymouth Meeting, PA). All other chemicals were purchased from Sigma. The Dt-40 cells lacking InsP3R (Dt-40 3ko) were kindly provided by Dr. Kurosaki (Kansai Medical University, Japan) and were maintained as described previously (25–27).

Production of Mutations—The rat S2+ InsP3R-1 in the expression plasmid pRES-GFP was digested with the restriction endonuclease Sall. The overhang created by digestion was blunted by using T4 polymerase. An EcoRI linker was then ligated onto the blunted ends of the construct. The entire receptor DNA was excised from the plasmid using EcoRI and ligated into the plasmid MXT-1. The region containing the S2− splice variant and potential PKA phosphorylation sites was excised from its backbone in pCDNA 3.1+ by RsrII and KasI and ligated into the InsP3R construct in MXT-1. The potential PKA phosphorylation sites Ser-1589 and Ser-1755 were mutated, individually in both splice variants and together in the S2− splice variant, to alanines or glutamates by using sequential PCR mutagenesis. The outside primers used for the mutagenesis reaction flanked the restriction sites RsrII and KasI. Following mutation, the resulting fragments were cut with RsrII and KasI and inserted into the InsP3R-1 backbone at the corresponding sites. The mutations were confirmed by Big Dye fluorescent sequencing. Mutated receptor DNAs were excised from MXT-1 by using EcoRI and ligated into the mammalian expression vector pGW (provided by Dr. David Yue, The Johns Hopkins University). Orientation was confirmed by using restriction enzyme digestion. Mutants were named based on the splice variant, either S2+ or S2− followed by the amino acid present at position 1589 and 1755. Thus, a mutation in S2+ InsP3R-1 S1755E is designated “S2+ SE” and in InsP3R-1 S1755A is designated “S2+ SA”. Similarly, an S2− InsP3R-1 with mutations in both S1589E and S1755E is designated “S2− EE”. The numbering of residues is based on the full-length rat InsP3R-1.

Transfection of Dt-40 Cells—Dt-40 cells lacking all three InsP3R receptor subtypes were transfected by using electroporation at 350 V and 950 microfarads (4-mm gap cuvette). 2 × 106 cells were co-transfected with 25 μg of the InsP3R-1 cDNA, 25 μg of the muscarinic type 3 (m3R) receptor DNA, and 4 μg of the red fluorescent protein plasmid pHChRed1-N1 (Clontech). Cells were incubated with DNA in 500 μl of Opti-MEM media (Invitrogen) on ice for 10 min. The cell/DNA mixture was electroporated, incubated on ice for 30 min, increased to 5 ml with Opti-MEM, and placed in a 5% CO2 incubator at 39 °C for 5 h. The cells were then centrifuged and resuspended in 12 ml of complete RPMI media (Invitrogen). Transfection efficiency was typically ~20%. Experiments were performed within 32 h of transfection.

Transfection of HEK-293 Cells and Assessment of Phosphorylation of S2− InsP3R-1—HEK-293 cells were plated onto 25-cm2 culture flasks and allowed to grow to near-confluency. Cells were transfected with 5 μg of each S2− InsP3R-1 DNA construct by using the LipofectAMINE reagent (Invitrogen) as per the manufacturer’s instructions. The following day, batches of cells were treated in the presence or absence of 20 μM forskolin for 10 min, aspirated from flasks, lysed, and immunoprecipitated with a polyclonal α-InsP3R-1 antibody that recognizes amino acids 2731–2749 of InsP3R-1. Immunoprecipitates were separated on 5% SDS gels transferred to nitrocellulose and then probed with either the α-InsP3R-1 antibody or a polyclonal antibody that recognizes the carboxyl terminus of the receptor (28) (α-phospho-S2EE) provided by Dr. S. Snyder. Blots that were probed with α-phospho-Ser-1755 were stripped and reprobed with the α-InsP3R-1 antisera to confirm the presence and relative quantity of the InsP3R-1.

Digital Imaging of [Ca2+]i—Transfected Dt-40 3ko cells were washed once in a HEPES-buffered physiological saline solution (HEPES-PSS) containing (in mM) 5.5 glucose, 137 NaCl, 0.56 MgCl2, 4.7 KCl, 1 Na2HPO4, 10 HEPES (pH 7.4), 1.2 CaCl2, and 1% w/v bovine serum albumin. Cells were then resuspended in bovine serum albumin HEPES-PSS with 1 μM Fura-2 (AM), placed on a 15-mm glass coverslip in a low volume perfusion chamber, and allowed to adhere for 30 min at room temperature. Cells were perfused continuously for 10 min with HEPES-PSS before experimentation to allow complete Fura-2 de-esterification of the cell. A glass pipette for imaging was then chosen that provided a wide range of transfection efficiency based upon the intensity of red fluorescence emitted when excited at 560 nm. Individual cells that had emission gray levels between 1500 and 2500 were subsequently chosen to standardize expression levels. [Ca2+]i imaging was performed essentially as described previously by using an inverted epifluorescence Nikon microscope with a 40× oil immersion objective lens (numerical aperture, 1.3) (19). Cells were excited alternately with light at 340 and 380 ± 10 nm bandpass filters (Chroma, Rockingham, VT) using a monochrometer (TILL Photonics, Pleasanton, CA). Fluorescence images were captured and digitized with a digital camera driven by TILL Photonics software. Images were captured every 2 s with an exposure of 2 ms and 4 by 4 binning. 340/380 ratio images were calculated online and averaged immediately before disk.

Flash Photolysis—Transfected cells were simultaneously loaded with the visible wavelength indicator Fluoro-4 and a cell-permeable form of caged inositol trisphosphate (ci-IP3/PM) for 30 min. ci-IP3/PM is a homologue of cm-IP3/PM. The 2- and 3-hydroxyls of the inositol ring are protected by an isopropyliengroup in ci-IP3/PM and are protected by a methoxymethylene group in cm-IP3/PM (29). Like cm-IP3/PM, ci-IP3/PM diffuses across cell membranes, and the PM group is hydrolyzed by cellular esterases, and Ca2+ release can be induced upon photocaging as if ci-IP3, is liberated from the cage and acts in a similar fashion to InsP3 at InsP3R.2 A further period of ~30 min was allowed for de-esterification of both dye and cage. Cells were illuminated at 488 ±

2 W.-H. Li, unpublished data.
Phosphoregulation of S2\textsuperscript{+} / InsP\textsubscript{3}R-1

10 nm and fluorescence collected through a 525 ± 25-nm bandpass filter and captured using the Till Photonics imaging suite. These traces are displayed as \( \Delta F/F_0 \), where \( F \) is the recorded fluorescence, and \( F_0 \) is the mean of the initial 10 sequential frames. Photolytic release was performed as described previously by using a pulsed xenon arc lamp (Till Photonics). A high intensity (0.5–5 ms duration; 80 J) discharge of UV light (360 ± 7.5 nm) was reflected onto the plane of focus by using a DM400 dichroic mirror and Nikon 40× oil immersion objective, 1.3 NA.

Frequency Distribution of Ca\textsuperscript{2+} Oscillations—Cells transfected with WT and mutant S2 isomers were stimulated with varying concentrations of an α-IgM antibody (Southern Biotechnology Associates, Inc., Birmingham, AL). Infrequent Ca\textsuperscript{2+} oscillations were produced presumably through B cell receptor cross-linking, activation of phospholipase C-γ, and subsequent production of InsP\textsubscript{3}. The frequency of Ca\textsuperscript{2+} oscillations was determined by selecting individual peaks that displayed an increase in ratio units greater than 0.05 and are listed as frequency in milliHertz (number of oscillations in 1000 s).

Concentration-Response Relationships—Normalized \( \Delta F \) concentration-response relations were fit with the following logistic Equation 1,

\[
\Delta F = \frac{1}{1 + (C/E_{50})^{1/Slope}}
\]

where \( \Delta F \) is the change in fluorescence normalized to the maximal response; \( C \) is agent concentration; \( E_{50} \) is the concentration where the response is half of maximum, and \( Slope \) is a slope factor related to the Hill coefficient.

Statistical Analysis—The effects of treatment were determined by normalizing the peak change in fluorescence ratio by stimulation following forskolin or 8-Br-cGMP exposure to that of stimulation in control cells. The Hill coefficient.

RESULTS AND DISCUSSION

Phosphomimetic Mutations in Functionally Important Phosphorylation Sites in Both S2\textsuperscript{+} and S2– InsP\textsubscript{3}R-1 Result in Enhanced Ca\textsuperscript{2+} Release—Phosphorylation of proteins results in the addition of net negative charge to the phosphoacceptor residue. In the case of PKA phosphoregulation, the functional effects of phosphorylation are thought to occur as the negative charge added to the serine or (less frequently) threonine residues neutralizes the positive charge of basic arginine or lysine residues present upstream in the classical RBX(S/T) consensus motif (30). This charge neutralization, in turn, is thought to result in a conformational change in the protein. A common approach employed to investigate the functional effects of phosphorylation is to construct phosphomimetic mutations whereby glutamic or aspartic acid residues are substituted at the phosphoacceptor site (S1, 32). The rationale for this strategy is that the negatively charged side chain of the substituted acidic amino acid will mimic, to an extent, the addition of a phosphate moiety to the protein.

To investigate the consequences of InsP\textsubscript{3}R-1 phosphorylation, we analyzed in Dt-40 3ko cells the Ca\textsuperscript{2+} release properties of phosphomimetic mutations in the functionally important sites in both S2– and S2+ InsP\textsubscript{3}R-1. This cell line provides the only known InsP\textsubscript{3}R null background (26, 27). In initial experiments, a comparison was made between the sensitivity of Ca\textsuperscript{2+} release by InsP\textsubscript{3}R-1 phosphomimetic mutations versus non-phosphorylatable alanine mutations at the sites we reported previously (19) to be relevant. Dt-40 3ko cells were transfected with DNA encoding HcRed to facilitate identification of transfected cells and either S2– S1589E/S1755E InsP\textsubscript{3}R-1 (S2– EE) or S2– S1589A/S1755A (S2– AA). Ca\textsuperscript{2+} release was monitored following flash photolysis of ci-IP\textsubscript{3}, a cell-permeable form of caged InsP\textsubscript{3}. This experimental paradigm provides a relatively direct assessment of the effects of InsP\textsubscript{3}R-1 phosphorylation on the Ca\textsuperscript{2+} release process. The amount of ci-IP\textsubscript{3} photo-released was controlled by varying the duration of the UV flash discharge (0.5–5 ms). No increase in [Ca\textsuperscript{2+}], was observed following the longest UV discharge in cells either not loaded with ci-IP\textsubscript{3}/PM or not expressing HcRed. Fig. 1, B and C, shows traces from typical experiments in individual Dt-40 3ko cells expressing S2– EE or S2– AA (Fig. 1, B and C, respectively, and pooled data in Fig. 1F). In cells expressing S2– EE, Ca\textsuperscript{2+} release as defined by a >0.05% \( \Delta F/F_0 \) increase in initial fluo-4 fluorescence was observed in ~60% of cells when exposed to UV discharge for 0.5 ms. Subsequent exposure to UV light for 1.25 ms elicited a more robust increase in [Ca\textsuperscript{2+}], in all cells. Finally, photo-release following a 5-ms flash, in general evoked a further increase in the magnitude of Ca\textsuperscript{2+} release. In contrast, an elevation of intracellular Ca\textsuperscript{2+} was never observed under identical conditions following a 0.5-ms UV flash in cells expressing S2– AA. A significant Ca\textsuperscript{2+} release was only observed in ~50% of cells following a 1.25-ms flash, and the majority of cells only responded to the longest uncaging duration, albeit with a smaller maximum magnitude than in S2– EE-expressing cells exposed to the same stimulus.

A similar pattern of sensitivity was observed in Dt-40 3ko cells expressing S2+: S1755E (S2– SE) and S2– S1755A (S2– SA) as shown in Fig. 1, D and E, respectively, and pooled data in Fig. 1G. Cells expressing S2– SE responded more robustly to photo-release of ci-IP\textsubscript{3} than cells expressing S2– SA exposed to an identical stimulus. These data indicate that serine to glutamate mutations at the functionally important phosphorylation sites in both splice variants of InsP\textsubscript{3}R-1 are “phosphomimetic,” i.e. charge mutations mimic phosphorylation in that these constructs display an apparent increased functional sensitivity to InsP\textsubscript{3}.

Sensitivity of S2– InsP\textsubscript{3}R-1 and Phosphorylation Site Mutants—Experiments were next performed to determine the relative sensitivity of Ca\textsuperscript{2+} release through the phosphomimetic mutations of the S2– InsP\textsubscript{3}R-1 with respect to the wild type and phosphorylation-deficient mutants. Dt-40 3ko cells were transfected with cDNAs encoding m3R, HcRed together with the InsP\textsubscript{3}R-1 construct of interest. Stimulation with the muscarinic agonist CCh results in robust increases in [Ca\textsuperscript{2+}], in transfected cells through the G\textsubscript{q/11}-coupled stimulation of phospholipase C-β and subsequent formation of InsP\textsubscript{3}. The magnitude of the initial peak provides a good estimation of the extent of Ca\textsuperscript{2+} release as this parameter in Dt-40 cells, like many cells, is essentially independent of Ca\textsuperscript{2+} influx. Furthermore, in Dt-40 cells the [Ca\textsuperscript{2+}], response to stimulation with CCh does not appreciably desensitize, and thus the effects of multiple concentrations of agonist can be assessed in a single cell. Individual HcRed-expressing cells were stimulated with increasing concentrations of CCh (1 nM to 1 μM) for 60 s followed by a 5-min wash between applications of agonist. In each case, these experiments were performed on multiple cells, expressing a narrow range of HcRed fluorescence and from multiple batches of transfected cells to minimize variation because of expression level of m3R and InsP\textsubscript{3}R-1. Concentration-response relationships were generated by normalizing each initial peak to the maximum response in the individual cell and subsequently averaging the pool of cells expressing a particular construct. Fig. 2A shows a typical example of this experimental procedure performed on Dt-40 3ko cells expressing S2– EE. A significant increase in Ca\textsuperscript{2+} was detected in the majority of S2– EE-expressing cells following stimulation with 1 nM CCh, and the peak response occurred following stimulation between 10 and 50 nM CCh. In contrast, as shown in Fig. 2, B and C, Ca\textsuperscript{2+} release following CCh stimulation in the wild type S2– InsP\textsubscript{3}R-1 or S2– AA was considerably less sensitive to CCh stimulation. Analysis of the pooled data indicated that the magnitude of the peak response to any of these constructs was
not significantly different (maximum peak Δ response: S2− WT = 0.9 ± 0.1 ratio units; S2− AA = 0.67 ±0.1 ratio units, and S2− EE = 1.13 ± 0.1 ratio units), suggesting that the efficacy of Ca²⁺ release was essentially unaltered. However, when an estimate of the relative sensitivity was made by fitting the normalized concentration-response relationships for each construct (Fig. 2D), CCh-induced Ca²⁺ release in the S2− EE mutant (EC₅₀ 4.3 ± 1.2 nM CCh) was 7.5-fold more sensitive when compared with WT S2− InsP₃R-1 (EC₅₀ = 32.6 ± 7.5 nM) and some 50-fold more sensitive than the nonphosphorylatable S2− AA mutant (EC₅₀ = 229.5 ± 14.6 nM). These data provide strong evidence that the S2− EE construct is more sensitive to stimulation by InsP₃ and present evidence that phosphorylation of the InsP₃R-1 results in marked regulation of channel
Phosphoregulation of S2⁺/⁻ InsP₃R-1

Fig. 2. InsP₃ sensitivity of S2⁻ InsP₃R-1 and phosphomimetic mutations. Concentration-response relationships for CCh-induced Ca²⁺ signals were examined in Dt-40 3ko cells expressing m3 receptors and InsP₃R-1 constructs. A, cells transfected with S2⁻ EE were stimulated with increasing concentrations of CCh as indicated (n = 9 cells). A similar paradigm was performed for cells expressing S2⁻ WT (n = 10) (B) and for cells expressing S2⁻ AA constructs (n = 9) (C). The magnitude of the initial peak (as an indicator of Ca²⁺ release) for each response was normalized to the maximum response in each cell. The pooled data and the fit that describes each concentration-response relationship for each construct is shown in D and illustrates that the sensitivity of Ca²⁺ release was greatest in S2⁻ EE followed by S2⁻ WT and S2⁻ AA.

function. This profound regulation could be expected to have major consequences for calcium signaling events in peripheral tissue such as liver, tests, and smooth muscle which express the S2⁻ InsP₃R-1 (5, 6). The observation that S2⁻ AA is relatively less sensitive than S2⁻ WT is consistent with the possibility that a proportion of the wild type receptor is constitutively phosphorylated in Dt-40 cells, thus contributing to the intermediate sensitivity of the wild type S2⁻ InsP₃R-1 relative to the phosphomimetic S2⁻ InsP₃R-1 receptor.

Although we have demonstrated that each site can be phosphorylated, it is not definitively known whether the functional effects of phosphorylating individual sites are independent and additive or alternatively if the full effect is seen following phosphorylation of an individual site. Thus, experiments were next performed to assess the sensitivity of single phosphomimetic mutations within each phosphorylation site. Concentration-response relationships for CCh-induced Ca²⁺ release were constructed for Dt-40 3ko cells expressing either S2⁻ S1755E InsP₃R-1 (S2⁻ SE) or S2⁻ S1589E InsP₃R-1 (S2⁻ ES). The data were analyzed as described previously for Fig. 2D. Fig. 3A shows the fit for the normalized concentration-response relationship for S2⁻ SE and S2⁻ ES and for comparison also shows the fit for S2⁻ EE and S2⁻ WT (Fig. 3A, dotted lines; data from Fig. 2D). Once again the maximal initial peak responses in either mutant were not significantly altered from wild type (S2⁻ ES = 0.54 ± 0.1 Δ ratio units; S2⁻ SE = 0.78 ± 0.1 Δ ratio units); however, the sensitivity of each of these mutants was significantly shifted, such that the EC₅₀ for CCh-induced Ca²⁺ release was enhanced ~3-fold over the response in wild type for either mutant (EC₅₀ for CCh-induced release: S2⁻ ES = 12.4 ± 0.5 nM; S2⁻ SE = 13.5 ± 1 nM). These data are summarized in Table I.

The enhanced apparent sensitivity of individual phosphomimetic mutants was essentially equal and intermediate between the sensitivity of the S2⁻ WT and S2⁻ EE mutations. These data are consistent with phosphorylation of individual sites being functionally additive. To address this possibility directly, experiments were performed evaluating the effects of activating endogenous PKA in cells expressing S2⁻ SE or S2⁻ ES to mimic prior phosphorylation of an individual site. As shown in Fig. 3B, and reported previously, activation of PKA by incubation with forskolin results in a dramatic potentiation of Ca²⁺ release in Dt-40 3ko cells expressing S2⁻ WT (19). Most surprisingly, although the sensitivity to CCh was enhanced, as evidenced by the low concentration of CCh necessary to evoke threshold Ca²⁺ release, no potentiation of Ca²⁺ release following forskolin incubation was observed in Dt-40 3ko cells expressing either S2⁻ ES (Fig. 3C; pooled data in Fig. 3F), S2⁻ SE (Fig. 3D; pooled data in Fig. 3F), or S2⁻ EE (Fig. 3E; pooled data in Fig. 3F). Thus, although each phosphorylation site in S2⁻ InsP₃R-1 can be phosphorylated and mimicking phosphorylation of both sites leads to a receptor with enhanced sensitivity relative to phosphorylation of an individual site, phosphorylation of both sites in situ does not appear to occur and therefore is not functionally additive. Although these data may seem paradoxical, a possible explanation, consistent with all the observations is that the initial phosphorylation of either Ser-1589 or Ser-1755 leads to a conformational change in the receptor that now precludes the phosphorylation of the additional site. To test this hypothesis, experiments were performed to determine the phosphorylation state of the various mutants after raising cAMP levels. These experiments were performed in HEK-293 cells because of the high transfection efficiency and low endogenous levels of InsP₃R-1 (18, 33). HEK-293 cells were transfected with the constructs as indicated in Fig. 4, incubated in the presence or absence of forskolin for 10 min, then pelleted, and lysed. Following incubation of the lysates with α-InsP₃R-1 antibody, the immune complexes were captured and separated on SDS gels, and the phosphorylation status of Ser-1755 was determined by Western blotting with an antibody that specifically recognizes this phosphorylated residue in InsP₃R-1 (28). As shown in Fig. 4A, wild type S2⁻ InsP₃R-1 was robustly phosphorylated after forskolin incuba-
tion, whereas no phosphorylation was detected in wild type S2– AA or untransfected HEK-293 cells. Similarly, in Fig. 4B, a marked increase in phosphorylation could be detected in S2– InsP₃R-1, whereas no phosphorylation could be detected in cells transfected with S2– EE, S2– SE or, most importantly, the S2– ES construct. These data are strongly supportive of the contention that the initial phosphorylation of one site precludes further phosphorylation at the additional residue as

**Fig. 3. Functional sensitivity of single phosphomimetic mutations in S2–InsP₃R-1.**

A, concentration-response relationships were generated exactly as shown in Fig. 2D and as described under “Experimental Procedures.” Cells expressing either S2– SE (n = 6) or S2– ES (n = 5) displayed intermediate sensitivity between S2– WT and S2– EE constructs. Dotted lines indicate S2– WT and S2– EE for comparison (data from Fig. 2D). B, threshold CCh-stimulated Ca²⁺ release is markedly potentiated by activation of PKA following forskolin treatment in cells expressing S2– WT. In contrast, no potentiation of threshold CCh-stimulated Ca²⁺ release is observed in cells expressing S2– SE in D, S2– ES in C, or S2– EE in E. Pooled data are shown in F; potentiation by forskolin is only seen in S2– WT. Numbers in parentheses indicate the number of analyzed cells.
TABLE I
Summary of the effects of phosphorylation by PKA or PKG of the S2+/S2− splice variants of InsP3R-1 and phosphoregulatory mutations at Ser-1589 and Ser-1755 expressed in D40 3 knock out cells

| Amino acid at 1589/1755 | S2+ InsP3R-1 | S2− InsP3R-1 |
|------------------------|--------------|--------------|
| CCh EC50 ( μM) | Ca2+ release after PKA/PKG activation | CCh EC50 ( μM) | Ca2+ release after PKA/PKG activation |
| Change from WT | | Change from WT | |
| Wild-type SS | 32.1 ± 4.2 | Enhanced/enhanced | 32.6 ± 7.5 | Enhanced/no change |
| AS | NT | Enhanced/enhanced | NT | Enhanced/no change |
| SA | 116.3 ± 3.0 | No change/no change | NT | No change/no change |
| AA | NT | NT/NT | 229.5 ± 14.6 | No change/no change |
| SE | 3.6 ± 0.2 | No change/no change | 13.5 ± 1.1 | No change/NT |
| EE | 23.7 ± 1.2 | Enhanced/enhanced | 12.4 ± 0.6 | No change/NT |
| FU | NT | NT/NT | 4.3 ± 1.2 | No change/NT |

Fig. 4. Phosphorylation of S2− InsP3R-1 mutants. The phosphorylation status of S2− InsP3R-1 constructs was assessed as described under “Materials and Methods.” A, incubation with 20 μM forskolin results in phosphorylation of Ser-1555 in S2− InsP3R-1. No phosphorylation is seen in S2− AA-transfected cells or untransfected HEK-293 cells (unt). B, forskolin treatment results in phosphorylation of Ser-1755 in S2− InsP3R-1 but not S2− EE, S2−, or S2− SE. The lack of phosphorylation of Ser-1755 in the S2− ES mutant is interpreted as indicating that mimicking phosphorylation of Ser-1589 by phosphomimetic substitution precludes the phosphorylation of Ser-1755 consistent with the functional data shown in Fig. 3. IP, immunoprecipitation; WB, Western blot.

predicted by the functional data and indicate that physiologically only phosphorylation of a single residue is functionally relevant in the S2− splice variant of InsP3R-1.

Sensitivity of S2+ InsP3R-1 and Phosphorylation Site Mutants—The sensitivity of Ca2+ release via the neuronal S2+ InsP3R-1 was next assessed by using a similar experimental paradigm to that used for the S2− form of the receptor. Although biochemically both Ser-1558 and Ser-1755 are equally susceptible to phosphorylation by PKA in S2+ InsP3R-1 (18), only Ser-1755 appears to be functionally relevant in terms of modulating Ca2+ release (19). Fig. 5A shows the fits for the normalized concentration-response relationships for CCh-induced Ca2+ release in cells expressing S2+ WT, phosphomimetic S2+ SE, and S2+ SA. The apparent sensitivity of CCh-induced Ca2+ release in cells expressing S2+ WT was essentially identical to S2− WT InsP3R-1 (EC50 for CCh-induced Ca2+ release; S2+ InsP3R-1 = 32.1 ± 4.2 nM versus S2− 32.6 ± 7.5 nM). These data are in agreement with the published literature that indicates that InsP3 binding and InsP3-induced calcium release is identical in the two major splice variants of the InsP3-R1 (34–36) and is therefore supportive of our contention that the initial CCh-stimulated [Ca2+]i peak is a good indicator of InsP3-R1 function. In a similar fashion to the S2− InsP3R-1, mutation of Ser-1589 to either alanine or glutamic acid did not significantly alter the maximal initial peak upon CCh stimulation (maximum peak Δ response: S2+ WT = 0.61 ± 0.1 Δ ratio units; S2+ SE = 0.59 ± 0.1 Δ ratio units; S2+ SA = 0.84 ± 0.2 Δ ratio units) but did, however, significantly affect the apparent sensitivity of Ca2+ release as shown in Fig. 5A. The S2+ SE mutant exhibited a similar EC50 for CCh-induced Ca2+ release as the S2− EE mutation (EC50; S2+ SE = 3.6 ± 0.2 nm CCh; S2− EE = 4.3 nm), being 9-fold more sensitive than S2+ WT and 32-fold more sensitive than the nonphosphorylatable S2+ SA mutation (EC50 S2+ SA = 116.3 ± 3 nm CCh). These data are summarized in Table I.

In contrast to the S2+ InsP3R-1 S1755E construct, mutation of Ser-1589 to glutamate in the S2+ form of InsP3R-1 did not significantly affect the apparent sensitivity of Ca2+ release as shown in Fig. 5B. Both the maximum peak response to CCh and the sensitivity of the receptor were very similar to that of S2+ WT InsP3R-1 (maximum peak Δ response = 0.59 ± 0.2 Δ ratio units; EC50 = 23.7 ± 1.2 nm CCh). However, it is formally possible that phosphorylation of Ser-1589 is only functionally important following phosphorylation of Ser-1755 in S2+ InsP3R-1. To test this idea, experiments were performed activating PKA with forskolin in DT-40 3ko cells expressing S2+ ES or SE mutants. Treatment with forskolin resulted in a marked potentiation of CCh-induced Ca2+ release in cells expressing either S2+ WT or S2+ ES (Fig. 6. A and B, respectively, and pooled data in D) presumably as Ser-1755 was phosphorylated. Although cells expressing S2+ SE were more sensitive to CCh, no further potentiation was observed following forskolin incubation (Fig. 6C and pooled data in 6D). These data indicate that it is unlikely that phosphorylation of Ser-1589 is permissive for any functional effect of phosphorylating Ser-1755 in S2+ InsP3R-1.

Effect of PKG Activation on S2+ InsP3R-1 and Phosphomimetic Mutants—We have reported previously that PKG activation results in direct phosphorylation of only the neuronal S2+ form of InsP3R-1 (19). In addition, PKG phosphorylation of Ca2+ release only occurs by phosphorylation of Ser-1755. In contrast to these data, a recent report (18) has shown that Ser-1589 and not Ser-1755 is phosphorylated upon activation of PKG. It should be noted that these experiments were performed by using the mouse S1+/S2− splice variant of InsP3-R1, whereas our functional experiments utilized rat S1−/S2+ InsP3R-1. The species and splice variant differences aside, the reported differences are difficult to reconcile with data demonstrating that mimicking phosphorylation of Ser-1589 in S2+ InsP3R-1 does not influence Ca2+ release (Figs. 5 and 6). Despite these differences, we performed experiments to define further the phosphorylation sites important for regulation of Ca2+ release by PKG. Fig. 7A shows a representative trace from cells expressing
ing S2+ WT InsP₃R-1 thus illustrating the marked potentiation of Ca²⁺ release upon specific activation of PKG by 8-Br-cGMP. A similar striking potentiation of Ca²⁺ release was also observed in cells expressing S2+ SE (n = 6), and S2+ SA (n = 5) are shown. The fits illustrate the increased sensitivity of the S2+ SE relative to S2+ WT and S2+ SA constructs. B, the normalized concentration-response relationship for S2+ ES is shown with S2+ SE and S2+ WT (dotted lines for comparison). These data indicate that the sensitivity of S2+ ES is similar to S2+ WT.

The specific pattern of phosphorylation occurring upon stimulation of PKA or PKG could be cell type-specific as, for example, in the case of PKA as a result of the targeting through protein A-kinase anchoring proteins, as has been demonstrated recently (37) for InsP₃R. Alternatively, cell-specific effects could conceivably occur through restricted access of the kinase to its substrate. Notwithstanding the general importance of kinase targeting for efficient, localized phosphorylation, the phosphomimetic constructs used in this study reveal the intrinsic, functionally important sites in a manner independent of the particular cellular context because any targeting step is circumvented. These sites and the functional consequences of phosphoregulation are thus likely a general property of the InsP₃R-1. In addition, the particular sites in S2+ InsP₃R-1 are entirely consistent with our earlier study (19) of the sites functionally important in enhanced Ca²⁺ release following PKA or PKG phosphoregulation, and this reinforces the view that phosphorylation of S2+ InsP₃R-1 Ser-1589 has no significant role, at least in terms of Ca²⁺ release.

**Effect of InsP₃R Phosphorylation on Ca²⁺ Signaling Events**—In many nonelectrically excitable cell types, the physiological mode of Ca²⁺ signaling is through the generation of Ca²⁺ oscillations (1, 38). Moreover, it is a generally held view that the spatial and temporal properties of Ca²⁺ oscillations make an important contribution to defining the fidelity and specificity of Ca²⁺ signaling. In many current models addressing the mechanism underlying Ca²⁺ oscillations, a key feature is the regulation of Ca²⁺ release through InsP₃R. We therefore next designed experiments to assess the consequences of InsP₃R-1 phosphorylation (expressed in isolation) on the initiation and generation of Ca²⁺ oscillations.
Experiments we failed to initiate Ca\(^{2+}\)/H\(_{11001}\) oscillations with reproducible characteristics with CCh in m3R-transfected cells (data not shown). Therefore, we chose to stimulate cells with an \(\alpha\)-IgM antibody and to initiate Ca\(^{2+}\)/H\(_{11001}\) oscillations through activation of the endogenous B cell receptor, phospholipase C-\(\gamma\) activation, and the formation of InsP\(_{3}\). Dt-40 3ko cells expressing either S2\(^{\pm}\)/H\(_{11002}\) WT or S2\(^{\pm}\)/H\(_{11002}\) EE to mimic PKA phosphorylation of the InsP\(_{3}\)R-1 were stimulated with various concentrations of \(\alpha\)-IgM antibody as illustrated by the selection of representative traces in Fig. 8. Stimulation with 250 ng/ml \(\alpha\)-IgM proved to be a threshold concentration in S2\(^{\pm}\)/H\(_{11002}\) WT-expressing cells. This degree of stimulation generally resulted in a single small increase in \([Ca^{2+}]_{i}\) after a long latency (Fig. 8A, left panel, and pooled data in Fig. 8, C–E). An identical stimulus in cells expressing S2\(^{\pm}\)/H\(_{11002}\) EE, in contrast, resulted in repetitive Ca\(^{2+}\)/H\(_{11001}\) transients following a much shorter latency, consistent with the increased apparent sensitivity of the S2\(^{\pm}\)/H\(_{11002}\) EE constructs (Fig. 8A, right panel, and pooled data Fig. 8, C–E). Most interestingly, stimulation of S2\(^{-}\)/H\(_{11002}\) WT-expressing cells with 500 ng/ml \(\alpha\)-IgM an-

**Fig. 6.** Effects of PKA phosphorylation on S2\(^{\pm}\)/H\(_{11002}\) InsP\(_{3}\)R-1 single glutamate substitution constructs. Threshold CCh-stimulated Ca\(^{2+}\)/H\(_{11001}\) release is markedly potentiated by activation of PKA following forskolin treatment in S2\(^{\pm}\)/H\(_{11002}\) WT-expressing cells (A) or S2\(^{\pm}\)/H\(_{11002}\) ES (B). C, in contrast, no potentiation of threshold CCh-stimulated Ca\(^{2+}\)/H\(_{11001}\) release is observed in cells expressing S2\(^{\pm}\)/H\(_{11002}\) SE. D, pooled data illustrate that S2\(^{\pm}\)/H\(_{11002}\) ES has properties identical to S2\(^{\pm}\)/H\(_{11002}\) WT, and thus phosphorylation of this residue is unlikely to impact Ca\(^{2+}\)/H\(_{11001}\) release. Numbers in parentheses indicate the number of analyzed cells.

**Fig. 7.** Effect of PKG activation in single phosphomimetic constructs. Threshold CCh-stimulated Ca\(^{2+}\)/H\(_{11001}\) release is markedly potentiated by activation of PKG following 8-Br-cGMP treatment in S2\(^{\pm}\)/H\(_{11002}\) WT (A) or S2\(^{\pm}\)/H\(_{11002}\) ES-expressing cells (B). C, in contrast, no potentiation of threshold CCh-stimulated Ca\(^{2+}\)/H\(_{11001}\) release is observed in S2\(^{\pm}\)/H\(_{11002}\) SE-expressing cells. D, pooled data illustrate that S2\(^{\pm}\)/H\(_{11002}\) ES has properties identical to S2\(^{\pm}\)/H\(_{11002}\) WT, and thus phosphorylation of this residue following activation of PKG is unlikely to impact Ca\(^{2+}\)/H\(_{11001}\) release. Numbers in parentheses indicate the number of analyzed cells.
Body resulted in Ca²⁺/H11001 oscillations with similar frequency to cells expressing S2/H11002 EE (Fig. 8B and pooled data C–E). However, the latency before the initiation of an increase in Ca²⁺ was significantly shorter in S2/H11002 EE-expressing cells. In addition, the magnitude of the initial transient was also significantly larger in S2/H11002 EE-expressing cells when compared with WT. Stimulation with 1 μg/ml α-IgM antibody resulted in transients that were indistinguishable in terms of latency, frequency, or initial peak magnitude in S2– EE- or S2– WT-expressing cells (pooled data Fig. 8, C–E). Thus, PKA-mediated phosphorylation, by increasing the sensitivity of the InsP₃R-1 to InsP₃, may define the threshold at which cells begin to oscillate but does not markedly influence the temporal properties of Ca²⁺ oscillations when initiated. This latter observation presumably reflects the fact that the frequency of oscillations is primarily defined by mechanisms such as Ca²⁺ feedback (39) rather than the absolute sensitivity of the InsP₃R-1 to InsP₃ within a defined range. These data are largely consistent with data from hepatocytes where PKA activation resulted in cells exhibiting a lower threshold for activation by InsP₃ infusion (40) or agonist activation (41).

These data using phosphomimetic mutations of InsP₃R-1 splice variants are in broad agreement with a number of studies that have reported increased sensitivity of InsP₃R-1 activity following phosphoregulation by PKA (19–22, 41, 42). The most important consequence of this increased sensitivity appears to be in defining the threshold where a cell will respond to a stimulus. Given the almost ubiquitous expression of various forms of InsP₃R-1, this is likely a generally important phenomenon. A number of mechanisms are plausible to explain the increased sensitivity of InsP₃-inducing Ca²⁺ release. These include modulation of InsP₃₉ binding, an idea supported by measurements of InsP₃₉ binding in hepatocytes (42) (presumably S2– InsP₃₉ and InsP₃₉–2). In these studies the apparent affinity of InsP₃₉ binding was enhanced ~2-fold at resting [Ca²⁺], and the [Ca²⁺] necessary for half-maximal stimulation of InsP₃₉ binding was reduced. In contrast, a study of recombinant S1– S2+ InsP₃₉-1 expressed in SP9 cells and reconsti-
tutted into lipid bilayers has reported a similar increase in InsP$_3$R sensitivity to InsP$_3$ but that the bell-shaped Ca$^{2+}$ sensitivity of channel opening is not altered following phosphorylation of the InsP$_3$R-1 (20). These findings suggest that modulation of the Ca$^{2+}$ sensitivity of channel activity is unlikely to account for the increased apparent sensitivity of the receptor, at least in this form of the InsP$_3$R-1. A number of alternative mechanisms for altering InsP$_3$R sensitivity are conceivable. For example, phosphorylation of the receptor could alter the gating of the channel directly. In addition, phosphorylation might secondarily modulate the receptor by regulation of the association of regulatory factors such as proteins or adenine nucleotides (43–45). Indeed, a precedent for this type of regulation exists because PKA phosphorylation has been shown to alter the association of calmodulin with the S2$^-$ form of InsP$_3$R-1 (36). It is envisioned that further studies utilizing phosphomimetic mutations of InsP$_3$R-1 will greatly facilitate elucidating the mechanism responsible for the increased sensitivity of InsP$_3$-induced Ca$^{2+}$ release via InsP$_3$R-1.

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Functional Consequences of Phosphomimetic Mutations at Key cAMP-dependent Protein Kinase Phosphorylation Sites in the Type 1 Inositol 1,4,5-Trisphosphate Receptor

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