Effect of mutation of a calmodulin binding site on Ca^{2+} regulation of inositol trisphosphate receptors

Xianchao ZHANG and Suresh K. JOSEPH¹

Department of Pathology, Thomas Jefferson University, Philadelphia, PA 19107, U.S.A.

Several studies have shown that calmodulin (CaM) modulates *D-myo*-inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R) channel activity and ligand binding to IP_3Rs . It has been proposed that CaM may act as the Ca^{2+} sensor for mediating Ca^{2+} inhibition of IP_3R channel activity. However, the functional role of CaM binding sites and the mechanism by which CaM regulates IP_3R activities remains unclear. Tryptophan at position 1577 of type I IP_3R has been shown to be part of a motif that is responsible for CaM binding to IP_3Rs and we have mutated this residue to alanine in the long (neuronal) and short (peripheral) SII splice variants of the type I IP_3R . CaM–Sepharose binding assays using COS-7 cell lysates confirmed that the W1577A mutant in both splice variants completely eliminated CaM binding. Functional

measurements of IP_3 -mediated $^{45}\text{Ca}^{2+}$ fluxes indicated that there was no change in the IP_3 sensitivity of the channel induced by the W1577A mutation. Such measurements also indicated that the W1577A mutants of both splice variants have a dependence on external $[\text{Ca}^{2+}]$ that was indistinguishable from the corresponding wild-types. Although subtle differences in the Ca^{2+} and CaM sensitivity of $[\text{H}^3]\text{IP}_3$ binding were noted between wild-type and mutant receptors, our data suggest that the CaM binding motif involving the W1577A locus does not play a role in Ca^{2+} regulation of IP_3R channel activity.

Key words: calcium, endoplasmic reticulum, IP_3 .

INTRODUCTION

D-myo-inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) are ubiquitous tetrameric intracellular Ca^{2+} release channels, which serve to discharge Ca^{2+} from the endoplasmic reticulum into the cytoplasm in response to agonist stimulation of cells [1]. IP_3Rs are encoded by three different genes, and different cells express different amounts of the three isoforms. In addition, the type I IP_3R isoform is subject to alternative splicing at three sites denoted as SI, SII and SIII [2–4]. Functional studies on the SI(+) and SI(–) isoforms have not revealed any differences in ligand binding or channel gating [5,6], and the functional consequences of the SIII splice site have not been investigated. The expression of the type I IP_3R containing the SII insert is restricted to neuronal cells, whereas the isoform in peripheral tissues is missing this insert [3,7]. In the present paper we refer to these variants as long and short respectively. The SII splice site encodes 40 amino acids that are flanked by many regulatory sites, including protein kinase A phosphorylation sites and binding sites for calmodulin (CaM), ATP and 12 kDa FK506-binding protein (reviewed in [1]). Hence, it has been suggested that the long and short forms may be differentially regulated by some of these factors [7–9].

The principal regulator of IP_3R function is Ca^{2+} . Both positive and negative feed-back effects of Ca^{2+} act on the type I IP_3R to generate a biphasic dependence of channel function on $[\text{Ca}^{2+}]$ (reviewed in [10]). The molecular basis of Ca^{2+} regulation of IP_3Rs is unknown. Several studies have shown that CaM can interact with IP_3Rs in a Ca^{2+} -dependent and -independent manner [11–13]. Exogenous CaM has been found to inhibit $[\text{H}^3]\text{IP}_3$ binding and to decrease the concentration of Ca^{2+} required to inhibit IP_3 -mediated Ca^{2+} release [14]. The single channel activity of the purified type I long form of the IP_3R was found to be insensitive to Ca^{2+} inhibition unless CaM was added [15]. It has therefore been proposed that CaM may act as the

Ca^{2+} sensor to confer Ca^{2+} inhibition of IP_3R channel activity [15].

Experiments using fusion proteins and deletion mutants of the type I IP_3R have established that CaM–Sepharose binds to a region proximal to the SII site located between amino acids 1564–1585 [11]. The mutation of tryptophan to alanine within this domain has been shown to completely eliminate CaM–Sepharose binding [11]. However, the functional consequences of this mutation have not been examined. In the present study we have investigated the effects of Ca^{2+} on $[\text{H}^3]\text{IP}_3$ binding and IP_3 -mediated Ca^{2+} release using the W1577A mutant of both the long and short forms of the type I IP_3R . Our experiments indicate that removal of this CaM binding site does not alter the Ca^{2+} inhibition of IP_3 -mediated Ca^{2+} fluxes in either splice variant. We conclude that the CaM binding site at amino acids 1564–1585, and the analogous site in the type I short isoform, are not involved in mediating Ca^{2+} inhibition of the IP_3R .

MATERIALS AND METHODS

Materials

ATP, dNTPs and a protease inhibitor cocktail were obtained from Roche Molecular Biochemicals (Nutley, NJ, U.S.A.). *Pfu* polymerase was purchased from Stratagene (Madison, WI, U.S.A.). Oligonucleotides were synthesized by Gibco BRL Life Technologies (Rockville, MD, U.S.A.). Trans IT-LT1 cationic lipid transfection reagent was obtained from Pan Vera Corp. (Madison, WI, U.S.A.). Protogel-stabilized acrylamide solution was from National Diagnostics (Atlanta, GA, U.S.A.). Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Amersham Life Sciences (Piscataway, NJ, U.S.A.), bovine brain CaM was from Veritas (Rockville, MD, U.S.A.) and IP_3 was from Calbiochem (La Jolla, CA, U.S.A.). Enhanced chemiluminescent substrates were obtained

Abbreviations used: CaM, calmodulin; DMEM, Dulbecco's modified Eagle's medium; HEDTA, *N*-hydroxyethyl-ethylenediamine-triacetic acid; IP_3 , *D-myo*-inositol 1,4,5-trisphosphate; IP_3R , IP_3 receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase.

¹ To whom correspondence should be addressed (e-mail Suresh.Joseph@mail.tju.edu).

from Pierce (Rockford, IL, U.S.A.). Saponin and CaM-Sepharose were purchased from Sigma (St Louis, MO, U.S.A.), and $^{45}\text{Ca}^{2+}$ and $[^3\text{H}]\text{IP}_3$ were from NEN Life Science Products (Boston, MA, U.S.A.). All other chemicals and reagents were purchased from Fisher (Pittsburgh, PA, U.S.A.) at the highest quality available.

Expression constructs

Type I long and short

The type I long and short constructs encoding a Kozac sequence and subcloned into pcDNA 3.1 have been described previously [8,16]. Both constructs retained the SI and SIII splice sites.

W1577A

Tryptophan 1577 was mutated into alanine using the Quick-change point mutation kit (Stratagene). The forward primer was designed following the manufacturer's recommendations as follows: 5'-CAGAAACAGCCATGAACGCGCGGTTATC-AGCCCGCAAC-3' (codon change is shown in bold). The reverse primer was the complementary sequence of the forward primer. PCR cycling conditions were performed according to the manufacturer's instructions, and the product was confirmed by automated sequencing (Applied Biosystems model 377; Nucleic Acid Facility, Thomas Jefferson University). It should be noted that the numbering of the IP_3R amino acids employed in the present paper corresponds to the rat cDNA [2].

Human sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase (SERCA)-2b

The cDNA encoding the human isoform of SERCA-2b in pcDNA 3.1 was a gift from Dr Jonathan Lytton (University of Calgary, Alberta, Canada) and Dr David H. MacLennan (University of Toronto, Ontario, Canada).

Cell culture and transfection

COS-7 simian virus 40-transformed African green monkey fibroblasts (A.T.C.C.; CRL 1651) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Life Technologies) in a 37 °C humidified incubator in an atmosphere of 5% CO_2 . DNAs for transfection were purified on a CsCl gradient as described elsewhere [16] or using the Qiagen plasmid Mega kit (Valencia, CA, U.S.A.). Cells were plated at a density of 1.5×10^6 cells/75 cm^2 flask and transfected in serum-free medium as described previously [17]. Cells were exposed to DNA-lipid complexes for 5 h before replacing the medium with DMEM supplemented with 10% (v/v) fetal bovine serum. Cells were processed after 48 h as described below. To transfect cells for the preparation of microsomes, 15 μg of IP_3R variant DNA and 10 μg of SERCA-2b DNA were used to transfect each 75 cm^2 flask. To prepare cells for IP_3 binding assays, 25 μg of type I IP_3R variant DNA was added to each flask.

Preparation of microsomal vesicles

Microsomes containing type I IP_3R variants along with human SERCA-2b were prepared as described previously [17]. Briefly, flasks with cells ready to harvest were washed twice with PBS. Cells were then released from the flask by incubation with 8 ml of PBS containing 5 mM EDTA and 0.5% BSA for 10 min at 23 °C. Cells were washed again with PBS and then swollen in hypotonic solution [10 mM Tris (pH 7.5)/0.05 mM MgCl_2] for 10 min on ice. PMSF (0.5 mM) was added, and the cells were homogenized in a tightly fitting Dounce homogenizer (20

strokes). Cell disruption was confirmed by Trypan Blue exclusion. Cells were then brought to isotonicity by dilution in 0.5 M sucrose, 6 mM 2-mercaptoethanol, 40 μM CaCl_2 , 300 mM KCl and 10 mM Tris, pH 7.5. Homogenates were spun at 5000 g to remove debris, and the supernatant was adjusted to 0.6 M KCl. The supernatant was spun at 100000 g , and the pellet was resuspended in 0.25 M sucrose, 0.15 M KCl, 3 mM 2-mercaptoethanol and 10 mM Tris, pH 7.5, and either used fresh or stored at -80 °C in 20% (v/v) glycerol until use.

$^{45}\text{Ca}^{2+}$ -flux measurements

$^{45}\text{Ca}^{2+}$ -release measurements were carried out in a buffer containing 20 mM Tris/Hepes (pH 7.4), 120 mM KCl, 0.3 mM MgCl_2 , 0.5 mM EGTA, 0.5 mM *N*-hydroxyethyl-ethylene-diamine-triacetic acid (HEDTA), 2 mM DL-dithiothreitol, 1 mM MgATP, 10 mM phosphocreatine, 10 units/ml creatine kinase, 3.0 $\mu\text{Ci/ml}$ $^{45}\text{Ca}^{2+}$ and 2 μM Ruthenium Red. The buffer was calibrated in the absence of $^{45}\text{Ca}^{2+}$ to the desired free $[\text{Ca}^{2+}]$ using a Ca^{2+} mini-electrode. Microsomes were incubated at 30 °C in this buffer for 15 min, and 5 μg aliquots were removed in triplicate and filtered through 0.3 μm PHWP filters (Millipore, Bedford, MA, U.S.A.). Filters were subsequently washed with 10 ml of 150 mM KCl and counted in scintillant (Budget-solve; Research Products International Inc., Mount Prospect, IL, U.S.A.).

CaM-Sepharose binding assay

Cell lysates were prepared from cells transfected with type I long, type I short, W1577A long or W1577A short DNA with a lysis buffer containing 50 mM Tris/HCl (pH 7.8), 150 mM NaCl, 1% (w/v) Triton X-100, 1 mM EDTA, 0.5 mM PMSF and a 0.5 $\times$ dilution of a commercial cocktail of protease inhibitors. Lysates (75 μg of protein) were incubated with 50 μl of CaM-Sepharose [50% (v/v) slurry] in a final volume of 500 μl in a buffer containing 20 mM Tris/HCl (pH 7.4), 0.2 M NaCl, 0.2% Triton X-100 and 1 mM CaCl_2 . After rotation for 4 h at 4 °C, the beads were spun down (12000 g for 1 min), washed in the same buffer and quenched in 20 μl of SDS sample buffer containing 0.1 M Tris/HCl (pH 6.8), 2% (w/v) SDS, 1.85% (v/v) 2-mercaptoethanol, 0.25% Bromophenol Blue and 10% (v/v) glycerol. IP_3Rs were identified by immunoblotting with an antibody raised against the C-terminus of the type I IP_3R [18].

Ca^{2+} sensitivity of $[^3\text{H}]\text{IP}_3$ binding to permeabilized COS-7 cells

COS-7 cells were harvested by trypsinization. The cells were centrifuged at 150 g for 1 min and resuspended in a buffer containing 20 mM Tris/Hepes (pH 7.2), 120 mM KCl, 2 mM HEDTA and 1 mM PMSF, and then permeabilized with saponin (10 $\mu\text{g/mg}$ of protein). A 0.8 ml aliquot of the permeabilized cells was incubated with 0.8 ml of a label mix containing 20 mM Tris/Hepes (pH 7.2), 120 mM KCl, 10 nM $[^3\text{H}]\text{IP}_3$ and various amounts of CaCl_2 (final protein concentration of 1–2 mg/ml). The Ca^{2+} concentration required to generate a range of free $[\text{Ca}^{2+}]$ was measured using a Ca^{2+} mini-electrode. After incubation on ice for 5 min, triplicate 0.5 ml samples were vacuum-filtered through A/E glass-fibre filters (Gelman Science, Ann Arbor, MI, U.S.A.), and the filters were washed with 10 ml of buffer containing 50 mM Tris/HCl (pH 7.8), 1 mM EDTA and 1 mg/ml BSA. The radioactivity on the filters was counted in Budget Solve scintillation fluid. IC_{50} values were calculated by fitting the data to a four parameter logistic equation using Sigma-Plot (Jandel Scientific) as described previously [8].

RESULTS

Site-directed mutagenesis was used to construct the W1577A mutant in both the long and short forms of the type I IP_3R . Both wild-type and mutant receptors were transiently expressed in COS-7 cells, and lysates were incubated with CaM–Sepharose to assess the effectiveness of the mutation (Figure 1A). In agreement with previous studies [11], the mutant form of the type I long

Figure 1 Characteristics of W1577A mutants of long and short type I IP_3Rs

(A) The ability of W1577A mutants (MUT) of long and short type I IP_3Rs to bind to CaM–Sepharose in comparison with their wild-type (WT) counterparts. The binding of lysates to CaM–Sepharose was carried out as described in the Materials and methods section, and is shown in the top panel. The bottom panel shows an immunoblot of 20 μg of the cell lysates, which indicates that wild-type and mutant receptors were expressed at equal levels in these experiments. Immunoblotting was carried out with an antibody directed against the C-terminus of the type I IP_3R (CT-1). The data are representative of three experiments. (B and C) The effect of different concentrations of IP_3 on microsomal $^{45}\text{Ca}^{2+}$ uptake measured at a free $[\text{Ca}^{2+}]$ of 20 nM. The inhibitory effect on the rate of $^{45}\text{Ca}^{2+}$ flux was measured at each IP_3 concentration and the data are expressed as percentages of the maximum effect observed for the wild-type constructs using 10 μM IP_3 . The values for the percentage inhibition of $^{45}\text{Ca}^{2+}$ flux under these conditions was $74.7 \pm 0.8\%$ and $79.1 \pm 2.8\%$ for the long and short forms respectively. Closed circles represent data collected from wild-type long (B) and short (C) type I IP_3Rs . Open circles represent data collected from W1577A mutant long (B) and short (C) type I IP_3Rs . Data shown in (B and C) are presented as means $\pm$ S.E.M. for three individual experiments, with each assay performed in triplicate.

Figure 2 Ca^{2+} sensitivity of IP_3 -mediated Ca^{2+} release in wild-type and W1577A mutant IP_3Rs

Microsomes were prepared from COS-7 cells co-transfected with SERCA-2b and either wild-type (WT) or W1577A forms of the long type I (A) or short type I (B) IP_3Rs . The uptake of $^{45}\text{Ca}^{2+}$ was measured over a 15 min period at 30 $^{\circ}\text{C}$ in an oxalate-containing medium as described previously [17]. The percentage inhibition of this uptake in the presence of 10 μM IP_3 was measured at buffered free $[\text{Ca}^{2+}]$ covering a range from 80 nM to 10 μM . Each measurement was made in triplicate and the data shown represent the means $\pm$ S.E.M. for three independent experiments.

isoform was completely inactive in CaM–Sepharose binding assays when compared with the wild-type. This was also the case for the corresponding mutation in the type I short isoform. Immunoblot analysis of cell lysates indicated that wild-type and mutant receptors were expressed at approximately equal levels in these experiments (Figure 1A). In order to determine if the W1577A mutation caused gross alterations in IP_3R protein, we assessed the functional activity of the receptors using a $^{45}\text{Ca}^{2+}$ -flux assay in which microsomes were prepared from COS-7 cells transfected with both the IP_3R and SERCA-2b. Under these assay conditions we have shown that the flux assay measures the activity of only the recombinant IP_3Rs [17]. Figures 1(B) and 1(C) show that the IP_3 dose–response curves for the wild-type and W1577A forms of the type I long and short forms were almost identical, with EC_{50} values in the range of 47–55 nM. Thus the data show that the W1577A mutation in both type I IP_3R splice variants abolishes the binding of IP_3Rs to CaM–Sepharose without causing a gross alteration in the ability of IP_3 to modulate channel function.

In order to examine if the W1577A mutations had altered the Ca^{2+} regulation of IP_3Rs we carried out the microsomal flux assays over a range of free $[\text{Ca}^{2+}]$ from 80 nM to 10 μM using a maximum concentration of 10 μM IP_3 . Under these conditions, the type I long IP_3R was inhibited by increases of $[\text{Ca}^{2+}]$ above 1 μM (Figure 2A). By comparison, the type I short isoform was

Figure 3 Effect of Ca^{2+} on $[\text{H}]\text{IP}_3$ binding to permeabilized COS-7 cells transfected with wild-type or W1577A IP_3Rs

Binding of $[\text{H}]\text{IP}_3$ was measured to saponin-permeabilized COS-7 cells transfected with the indicated constructs and incubated at different buffered free $[\text{Ca}^{2+}]$. The values are presented as percentages of $[\text{H}]\text{IP}_3$ binding measured in the absence of Ca^{2+} . The absolute values for the amount of $[\text{H}]\text{IP}_3$ bound (pmol/mg) for wild-type (WT) long, mutant long, wild-type short and mutant short were 1.42 ± 0.23 , 0.97 ± 0.09 , 1.02 ± 0.08 and 0.98 ± 0.02 respectively. The endogenous level of binding in mock-transfected cells was 0.072 ± 0.011 pmol/mg of protein [8]. The data shown are presented as means $\pm$ S.E.M. for three experiments, with each binding assay performed in triplicate. (A) Comparison of long wild-type (●) and long W1577A mutant (○) type I IP_3Rs . (B) Comparison of the short wild-type (●) and short W1577A mutant (○) type I IP_3Rs . The IC_{50} values given in the text were calculated by fitting the data to a four parameter logistic equation as described in the Materials and methods section.

inhibited by $[\text{Ca}^{2+}]$ above 200 nM (Figure 2B). The enhanced Ca^{2+} sensitivity of the short splice variant has been observed previously [17]. The W1577A mutants did not show any significant difference in their Ca^{2+} sensitivity when compared with the corresponding wild-type isoforms, suggesting that this site does not play a role in mediating Ca^{2+} inhibition of IP_3R function. The lack of a difference in the Ca^{2+} sensitivity of the wild-type and W1577A mutants was also observed when $^{45}\text{Ca}^{2+}$ flux was measured using a sub-saturating IP_3 concentration of 100 nM (results not shown). The effect of exogenously added CaM (10–30 μM) was tested in the flux assay for both wild-type and mutant constructs. However, no significant effect of added CaM on IP_3 responses was observed (results not shown).

Ca^{2+} is known to inhibit IP_3 binding to the type I IP_3R [19]. In order to determine if the W1577A mutations had modified this effect we measured $[\text{H}]\text{IP}_3$ binding to permeabilized COS-7 cells transiently transfected with wild-type or mutant IP_3Rs . In previous studies we have shown that mock-transfected COS-7 cells yield a negligible signal in this binding assay [8]. In COS-7 cells transfected with the type I long isoform a progressive decrease in $[\text{H}]\text{IP}_3$ binding with increasing Ca^{2+} concentration was observed with an IC_{50} value of $0.61 \pm 0.06 \mu\text{M}$ (Figure 3A).

Figure 4 Effect of exogenous CaM on $[\text{H}]\text{IP}_3$ binding to permeabilized COS-7 cells transfected with wild-type or W1577A IP_3Rs

Cells expressing long wild-type (black bars) or W1577A mutant (white bars) type I IP_3Rs (A), or short wild-type (black bars) and W1577A mutant (white bars) type I IP_3Rs (B) were permeabilized by saponin, and the binding of $[\text{H}]\text{IP}_3$ was measured as described in the Material and methods section. CaM was added at a final concentration of 25 μM . Each binding assay was performed in triplicate and the data shown represent the means $\pm$ S.E.M. for three experiments. In each case the binding measured in the presence of CaM was expressed as a percentage of the control binding measured in the absence of exogenous CaM. Values that are statistically significant ($P < 0.05$) from the control binding are indicated by *.

Binding to W1577A type I long cells was inhibited at higher Ca^{2+} concentrations with an IC_{50} value of $2.21 \pm 0.47 \mu\text{M}$ (Figure 3A). In agreement with our previous findings [8], the type I short IP_3R was almost 10-fold less sensitive to Ca^{2+} inhibition than the type I long IP_3R (IC_{50} of $5.4 \pm 0.4 \mu\text{M}$). $[\text{H}]\text{IP}_3$ binding to the W1577A mutant of the type I short isoform showed an unusual dependence on $[\text{Ca}^{2+}]$ which suggested the presence of more than one Ca^{2+} inhibitory site. However, the data was also not well fitted by a simple two-site model of Ca^{2+} inhibition. Further analysis would require additional assumptions, such as including co-operativity between Ca^{2+} binding sites, to account for the precise shape of the binding curve. Qualitatively, it is apparent that the W1577A mutation diminishes the Ca^{2+} sensitivity of ligand binding to the long form of the type I IP_3R but has the opposite effect on the short splice variant.

Exogenous CaM has been shown to inhibit $[\text{H}]\text{IP}_3$ binding to the type I long form of the IP_3R in the absence of Ca^{2+} but not in its presence [20]. We confirmed this observation in $[\text{H}]\text{IP}_3$ binding experiments on permeabilized COS-7 cells transfected with the type I long IP_3R (Figure 4A). In these experiments the addition of 25 μM CaM produced a 35% inhibition of $[\text{H}]\text{IP}_3$ binding in the absence of Ca^{2+} , and this effect was lost when the Ca^{2+} was increased. The W1577A mutant also showed a CaM-sensitive inhibition of binding in the absence of Ca^{2+} , but, unlike the wild-type receptor, the effect persisted in the presence of Ca^{2+} .

(Figure 4A). In the absence or presence of Ca^{2+} , the effect of CaM on $[^3\text{H}]\text{IP}_3$ binding to the wild-type or W1577A short isoform was small and not statistically significant (Figure 4B).

DISCUSSION

The present study was undertaken to determine the functional consequence of inactivating the CaM binding site located between amino acid residues 1565–1586 of the type I IP_3R in the context of a proposed role for CaM in mediating the feed-back inhibition of the IP_3R channel at high $[\text{Ca}^{2+}]$ [15]. The data clearly demonstrate that both IP_3 -mediated Ca^{2+} fluxes and $[^3\text{H}]\text{IP}_3$ ligand binding retain their sensitivity to Ca^{2+} inhibition in the complete absence of this CaM binding site. We therefore conclude that this particular site does not mediate the Ca^{2+} inhibition of IP_3R function. This conclusion is further supported by the observation that the 1565–1586 CaM binding site is conserved in the type II IP_3R isoform, but studies on both native and recombinant type II IP_3R suggest that this isoform is relatively insensitive to Ca^{2+} inhibition [21,22].

It should be stressed that our results do not exclude the possibility that other CaM binding sites on the IP_3R may be important for Ca^{2+} regulation. The mutation of tryptophan at 1577 abolished all CaM–Sepharose interaction in both the long and the short type I IP_3R isoforms. This observation, originally made with the W1577A type I long mutant, led Yamada et al. [11] to propose that the 1565–1586 site in the regulatory domain may represent the sole CaM interacting site within the IP_3R . However, experimental evidence using ^{125}I -labelled CaM and scintillation proximity assays have identified additional binding sites [12,13,23]. These have been localized to the first 159 residues of the ligand binding domain and to a fusion protein (cyt11; amino acids 1499–1649) which encompasses the 1564–1586 site [23]. Furthermore, it has also been proposed that the cyt11 fusion protein may itself contain more than one CaM binding site [23]. The fact that the CaM–Sepharose binding assay appears to recognize only the 1564–1586 binding site may be a reflection of the high affinity of this site and/or may reflect restricted access of the CaM–Sepharose beads to the additional binding sites. The presence of multiple CaM binding sites within the IP_3R means that we cannot rule out the possibility that CaM exerts its inhibitory effect on IP_3R function at a binding site different from the site mutated in the present study.

A gross alteration in the function of the W1577A mutation was not apparent in either of the type I splice variants as judged by the $^{45}\text{Ca}^{2+}$ flux measurements. Nevertheless, the mutation did display subtle changes in both the effects of Ca^{2+} and CaM on $[^3\text{H}]\text{IP}_3$ binding. In the case of the type I long IP_3R , the W1577A mutation decreased the sensitivity with which Ca^{2+} inhibits $[^3\text{H}]\text{IP}_3$ binding to the receptor and diminished the ability of Ca^{2+} to reverse the inhibitory effect of CaM on ligand binding. The effects of Ca^{2+} on ligand binding are thought to be mediated by ancillary proteins or to involve the direct association of Ca^{2+} with Ca^{2+} binding sites located within the receptor [10]. The Ca^{2+} -independent binding of CaM to a site within the ligand binding domain is thought to be responsible for the effect of CaM on $[^3\text{H}]\text{IP}_3$ binding [12,13,23]. The molecular basis of both of these effects on IP_3 binding to the IP_3R are poorly understood and it is therefore difficult to provide an exact mechanistic explanation of the changes induced by the W1577A mutation. However, it is apparent that the 1564–1585 site functions to modify the interaction of CaM with the ligand binding site and to alter the sensitivity of ligand binding to $[\text{Ca}^{2+}]$.

The present study highlights some of the differences apparent in the regulatory behaviour of the two type I IP_3R splice variants.

These include differences in the Ca^{2+} sensitivity of IP_3 -mediated Ca^{2+} release (Figure 2) and $[^3\text{H}]\text{IP}_3$ binding (Figure 3), as well as differences in the effect of exogenous CaM on $[^3\text{H}]\text{IP}_3$ binding (Figure 4). These findings demonstrate that the deletion of the 40 amino acids from the SII splice site modifies the response of the IP_3R to both Ca^{2+} and to CaM. We have previously presented evidence in favour of the hypothesis that the deletion of the SII splice site may create an additional CaM binding site at the splice junction [8]. However, no residual binding to CaM–Sepharose was observed in the type I short isoform after inactivation of the 1564–1585 site. This may indicate that the original hypothesis was incorrect or that inactivation of the 1564–1585 site also inactivates adjacent CaM binding sites. Further studies to identify and mutate all the CaM binding sites within the IP_3R are required to clarify the role of CaM in the regulation of IP_3R function.

This work was supported by National Institutes of Health grant R01-DK34804. We would like to thank Dr Gyorgy Hajnoszky and Dr Darren Boehning for reading the manuscript prior to submission.

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Received 27 April 2001/21 August 2001; accepted 26 September 2001